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Improved diagnostics and clinical applications for the
detection and control of proliferative enteropathy

by

Jeremy James Kroll

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Microbiology

Program of Study Committee:
D. L. Hank Harris, Co-major Professor
Mike Roof, Co-major Professor
Lorraine Hoffman
James Dickson
Joan Cunnick

Iowa State University
Ames, Iowa
2005

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For the Major Program
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CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

The following dissertation consists of a general introduction, a review of the literature contained within Chapter 2, three separate manuscripts, a general conclusion, and acknowledgments. The doctoral candidate, Jeremy James Kroll, is the senior author and principal investigator for all manuscripts.

Introduction

Proliferative Enteropathy (ileitis, PE) is a common intestinal disease affecting susceptible pigs raised under various management systems around the world. The disease is characterized by a thickening of the mucosal lining of the small, and sometimes large intestine (29). Other distinguishing features include proliferation of the immature epithelial cells of the intestinal crypts, forming a hyperplastic to adenoma-like mucosa (25).

Histological lesions can be confirmed as *Lawsonia*-specific by visualization of the tiny, vibrioid shaped bacteria found in the enterocytes of the terminal ileum, cecum and spiral colon, but also within macrophages located in the lamina propria between intestinal crypts, and in the mesenteric lymph nodes (5, 25, 28).

There are several different syndromes of PE. Porcine intestinal adenomatosis (PIA) is considered to be the chronic form of PE mainly affecting young growing pigs (25). Proliferative hemorrhagic enteropathy (PHE) is often seen in adult pigs and is classified as acute PE resulting in bloody diarrhea, blood clots and sudden death (25). Necrotic enteritis is less common than PIA and PHE and is found in pigs of all ages exhibiting severe
thickening of the mucosa with brownish-yellow necrotic lesions on the luminal surface (25). Recently, a new form of PE has been described in sows in which *L. intracellularis* infections are sub-clinical (no sign of disease) and persist in a “carrier” state (26).

Initially, the bacteria were referred to as “*Campylobacter-*like organisms” or “CLO” because of their similarities in morphology to *Campylobacter* species (25). Later, the intracellular bacteria were given the name Ileal Symbiont (IS) intracellularis and were identified as a distinct genus that differed from *Campylobacter* species (6). The name *Lawsonia intracellularis* was formally given to the organism in 1995 in honor of the Scottish scientist G. H. K. Lawson who discovered the bacterium (22).

*Lawsonia intracellularis* is classified as a gram-negative, microaerophilic, obligate intracellular, non-flagellated, non-spore forming, curved or S-shaped bacillus (16). Recently, a long, single, polar flagellum has been observed by electron microscopy in multiple pure culture isolates of *L. intracellularis* (17). The bacterium measures out to 1.25 to 1.75 μm long and 0.25 to 0.43 μm wide and is comprised of a trilaminar outer envelope separated from the cytoplasmic membrane by an electron-lucent zone; neither fimbriae nor spores have been observed (17). The entire genome has recently been sequenced by the University of Minnesota (7) and contains approximately 1.46 Mb.

Currently, the growth of *L. intracellularis* in cell free media or broth has not been accomplished. Therefore, successful cultivation relies on growth of this bacterium on susceptible tissue culture cells in a reduced oxygen or anaerobic atmosphere (4, 9, 11, 16, 31). Koch’s postulates were fulfilled when pure cultures of the intracellular bacterium induced PE in pigs (20).
Diagnosis of PE in pigs relies on various ante and post mortem methods that exclude conventional bacteriological and biochemical techniques as routine isolation of the bacterium in pure culture is not practical. Histopathology and silver staining of tissue sections use to be the preferred methods of diagnosis for determining microscopic lesions and morphologic features of *L. intracellularis* infections. Since then, more sensitive and specific detection techniques such as polymerase chain reaction (PCR) (1, 10, 18, 21), immunohistochemistry (IHC) (12, 19) immunofluorescence/immunoperoxidase antibody tests (IFAT/IPMA) (8, 12) and an LPS-based enzyme-linked immunosorbent assay (LPS-ELISA) (2, 15) have been developed to assist veterinarians and pig farmers in determining with better precision when to implement control and prevention measures in herds where PE is endemic.

Proliferative enteropathy is an endemic disease that is widespread among swine herds and production systems across every continent where pork production is found. It has been determined that 20% to 50% of farms are infected with *L. intracellularis* world wide (3, 26, 30, 32). The disease may be more prevalent in the USA and Northern Europe where greater than 90% and 70% of the farms, respectively, are positive for PE based on serology (26). Most recent estimated losses exceed €100 ($121 USD) per affected breeding pig which translates to an extra €0.50 ($0.61 USD) per growing pig (27). The total losses due to endemic PE on most European farms could potentially exceed €1 ($1.22 USD) per affected grower-finisher pig (27).

The challenges of isolating and maintaining *L. intracellularis* cultures *in vitro* have made antimicrobial susceptibility testing an extremely difficult task. Regardless of this fact,
in vitro evaluations of the minimum inhibitory concentration (MIC) of 20 different antimicrobial agents and the minimal bactericidal concentration (MBC) of 10 of these agents indicate a broad range of antibiotic activity against *L. intracellularis* (23, 24). Included in this list are the macrolides (i.e. erythromycin and tylosin), tetracyclines, pleuromulins (i.e. tiamulin), penicillins, and fluoroquinolones. Antibiotics that have no activity against *L. intracellularis* include the aminoglycosides and aminocyclitols (i.e. neomycin, gentamicin and apramycin) (23, 24). The preferred treatment of PHE in adult pigs is one of the following antibiotics delivered orally for 14 days in pre-mixed feed; tiamulin at 120 ppm, tylosin at 100 ppm, licomycin at 110 ppm, or chlorotetracycline at 300 ppm (25). Where PE is endemic in grower-finisher pigs, the preferred treatment is continuous in-feed medication with one of the following antibiotics in pre-mix feed; tiamulin at 50 ppm, chlortetracycline at 200 ppm, lincomycin at 110 ppm and tylosin at 100 ppm to minimize severe production losses caused by the disease (25).

Currently, an avirulent live *L. intracellularis* vaccine (Enterisol® Ileitis) was developed by Boehringer Ingelheim Vetmedica, Inc. for use in pigs 3 weeks of age or older for control and prevention of gross and microscopic lesions typical of PE after virulent challenge (13, 14).

The goals of the following studies were to identify the effectiveness of a new lyophilized, European-derived, avirulent live *L. intracellularis* vaccine in animals challenged with a virulent pure culture challenge model, to evaluate various clinical applications for effective delivery and stimulation of protective immunity against PE in susceptible age pigs, and to evaluate the level of maternal immunity and safety of
vaccination in late-term pregnant swine. Studies were also conducted to develop and validate an improved diagnostic method for detecting *L. intracellularis* exposure in vaccinated or experimentally challenged pigs.

References


relationship to *Desulfovibrio* species. International Journal of Systematic Bacteriology, **43**:533-538.


CHAPTER 2. PROLIFERATIVE ENTEROPATHY: A GLOBAL ENTERIC DISEASE OF PIGS CAUSED BY LAWSONIA INTRACELLULARIS

A paper submitted to Animal Health Research Reviews

Jeremy J. Kroll,1,2 Michael B. Roof,1 Lorraine J. Hoffman,3 James Dickson,3 and D. L. Hank Harris1

Abstract

Proliferative Enteropathy (ileitis, PE) is a common intestinal disease affecting susceptible pigs raised under various management systems around the world. Major developments in the understanding of PE and its causative agent, Lawsonia intracellularis, have occurred that have led to advances in the detection of this disease and methods to control and prevent it. Diagnostic tools have improved overall detection and early onset of PE in pigs includes various serological and molecular-based assays. Histological tests such as immunohistochemistry continue to be the gold standard in confirming Lawsonia-specific lesions in pigs post mortem. Despite extreme difficulties in isolating L. intracellularis, innovations in the cultivation and the development of pure culture challenge models, have opened doors to better characterization of the pathogenesis of PE through in vivo and in vitro L. intracellularis-host interactions. Advancements in molecular research such as the genetic sequencing of the entire Lawsonia genome have provided ways to identify various immunogens, metabolic pathways and methods for understanding the epidemiology
of this organism. The determinations of immunological responsiveness in pigs to virulent and attenuated isolates of *L. intracellularis* and identification of various immunogens have led to progress in vaccine development.

Introduction

*Lawsonia intracellularis* is an obligate intracellular bacterium causing proliferative enteropathy (PE) in many mammalian species and rodents, most notably pigs. The infection causes diarrhea, stunted growth and, in rare instances, sudden death in pigs and is one of the most economically important diseases in the swine industry worldwide (Lawson and Gebhart, 2000). The disease is characterized by a thickening of the mucosal lining of the small, and sometimes large intestine (Rowland and Lawson, 1992). Other distinguishing features include proliferation of the immature epithelial cells of the intestinal crypts, forming a hyperplastic to adenoma-like mucosa (McOrist and Gebhart, 1999). Histological lesions can be confirmed as *Lawsonia*-specific by visualization of the tiny, vibrioid shaped bacteria found in the enterocytes of the terminal ileum, cecum and spiral colon, and also within macrophages located in the lamina propria between intestinal crypts, and mesenteric lymph nodes (Frisk and Wagner, 1977; Roberts et al., 1980; McOrist and Gebhart, 1999).

There are several different syndromes of PE. Porcine intestinal adenomatosis (PIA) is considered to be the chronic form of PE mainly affecting young growing pigs (McOrist and Gebhart, 1999). Proliferative hemorrhagic enteropathy (PHE) is often seen in adult pigs and is classified as acute PE resulting in bloody diarrhea, blood clots and sudden death (McOrist and Gebhart, 1999). Necrotic enteritis is less common than PIA and PHE and is
found in pigs exhibiting severe thickening of the mucosa with brownish-yellow necrotic lesions on the luminal surface (McOrist and Gebhart, 1999). Recently, a new form of PE has been described in pigs in which *L. intracellularis* infections are sub-clinical (no sign of disease) and persist in a “carrier” state (McOrist et al., 2003).

Proliferative enteropathy is most commonly found in pigs, however, it has also been described in hamsters (Frisk et al., 1977), ferrets (Fox and Lawson, 1988), rabbits (Fox et al., 1994), foxes (Eriksen et al., 1985), dogs (Collins et al., 1983), rats (Vandenburgh et al., 1985), horses (Williams et al., 1996), sheep (Chalmers et al., 1990), deer (Drolet et al., 1996), emus (LeMarchand et al., 1995), ostriches (Cooper et al., 1997), primates (Klein et al., 1999) and guinea pigs (Elwell et al., 1981). Intestinal lesions are strikingly similar among all above mentioned species with intracellular bacteria identified as *L. intracellularis* observed in the proliferative epithelia (Lawson and Gebhart, 2000).

Despite its ubiquitous nature, *L. intracellularis* has never been identified in humans with enteric disease, even those affected with Crohn’s or other related diseases such as colon cancer (McOrist et al., 2003). Therefore, *Lawsonia*-specific PE is not considered to be a zoonotic disease (McOrist et al., 2003).

**Etiology**

Proliferative enteropathy has been described as an important enteric disease that has affected pigs for the past 50 years. Characteristic lesions of PE found in pigs were first described by Beister and Schwarte in 1931. It wasn’t until the 1970’s when the presence of intracellular bacteria was found within proliferating crypt cells in cases of PHE in pigs
(Rowland et al., 1973). A variety of Campylobacter species having morphologically similar features to L. intracellularis have been isolated from lesions of PE. Those include Campylobacter mucosalis (Rowland and Lawson, 1974; Love et al., 1977), C. hyointestinalis (Gebhart et al., 1983), C. jejuni and C. coli (Ericksen et al., 1990).

Despite the routine recovery of the above mentioned Campylobacter species in proliferative lesions, none of these organisms specifically cause PE or colonize intracellularly under experimental conditions (Kashiwazaki et al., 1971; McCartney et al., 1984; Boosinger et al., 1985; Alderton et al., 1992). It wasn’t until Lawson et al. (1985) inoculated rabbits with an extract containing intracellular bacteria from an intestinal lesion that did not contain Campylobacter, that a new and novel intracellular bacterium was discovered. Convalescent serum containing antibodies from inoculated rabbits did not react to various isolates of Campylobacter but reacted to intracellular bacteria in formalin-fixed sections of PE-affected intestines (Lawson et al., 1985). Progress in cultivation of this organism ensued and Koch’s postulates were fulfilled when pure cultures of the intracellular bacterium were shown to cause PE in pigs (McOrist et al., 1993). Initially, the bacteria were referred to as “Campylobacter-like organisms or CLO” because of their similarities in morphology to Campylobacter species (McOrist and Gebhart, 1999). Later, the intracellular bacteria were given the name Ileal Symbiont (IS) intracellularis and were identified as a distinct genus that differed from Campylobacter species (Gebhart et al., 1993). The name Lawsonia intracellularis was formally given to the organism in 1995 in honor of the Scottish scientist G.H.K. Lawson as the primary discoverer of the bacterium (McOrist et al., 1995a).
Figure 1. An electron micrograph of *Lawsonia intracellularis* in pure culture. Arrow indicates a single polar flagella. Bar = 10 μm

*Lawsonia intracellularis* is a member of the delta division of Proteobacteria (Gebhart et al., 1993) and is taxonomically distinct from other intracellular pathogens (McOrist et al., 1995a). DNA sequences of the 16S ribosomal RNA gene from *L. intracellularis* were found to be closely related to *Bilophila wadsworthia* (Sapico et al., 1994) and the sulfate-reducing proteobacterium, *Desulfovibrio desulfuricans* (Gebhart et al., 1993) with 92% and 91% homology respectively. *Lawsonia intracellularis* is classified as a gram-negative, microaerophilic, obligate intracellular, non-flagellated, non-spore forming, curved or S-shaped bacillus (Lawson et al., 1993). Recently, a long, single, polar flagellum has been observed by electron microscopy in multiple pure culture isolates of *L. intracellularis*; see Figure 1 (Lawson and Gebhart, 2000). The bacterium measures 1.25 to 1.75 um long and 0.25 to 0.43 um wide comprising of a trilaminar outer envelope separated from the cytoplasmic membrane by an electron-lucent zone; neither fimbriae nor
spores have been observed (Lawson and Gebhart, 2000). The entire genome has recently been sequenced by the University of Minnesota (Gebhart and Kapur, 2003) and contains approximately 1.46 Mb.

Isolation and Cultivation of *L. intracellularis*

Isolation and cultivation of an obligate intracellular organism is one of the most daunting tasks in bacteriology. *Lawsonia intracellularis* is no exception. Currently, the growth of *L. intracellularis* in cell free media or broth has not been accomplished. Therefore, successful cultivation relies on growth of this bacterium on susceptible eukaryotic tissue culture cells including rat intestinal cells (IEC-18) (Lawson et al., 1993), human fetal intestinal cells (Int 407) (Lawson et al., 1993), rat colonic adenocarcinoma cells (Lawson et al., 1993), pig kidney cells (PK-15) (Lawson et al., 1993), piglet intestinal epithelial cells (IPEC-J2) (McOrist et al., 1995a), GPC-16 cells (Stills, 1991), and mouse fibroblast cells (McCoy) (Knittel and Roof, 1999). Cultivation techniques include culturing *L. intracellularis* with adherent (Lawson et al., 1993; McOrist et al., 1995a; Collins et al., 1996) or suspension (Knittel and Roof, 1998) tissue culture cells at reduced oxygen atmospheres, preferably an anaerobic environment, at 37°C for 5 to 7 days post inoculation. Adherent cultures can be propagated in tissue culture flasks (T-flasks) of various volumes (25cm² to 150cm²) and require incubation in humidified chambers such as anaerobic gas jars or modified incubators containing 80 – 90% N₂, 4 – 10% CO₂ and 0 – 10% O₂ (Lawson et al., 1993; McOrist et al., 1995a; Collins et al., 1996). In contrast, suspension cultures do not need specialized growth chambers and are propagated in spinner flasks or bioreactors of
various sizes (250 ml to 300 L) that regulate the temperature, gas mix, pH, and agitation automatically (Knittel and Roof, 1998). This method (Knittel and Roof, 1998) has allowed the potential growth of large-scale cultures for use in the production of vaccine and diagnostic reagents and has also been used for the attenuation of a Danish isolate of *L. intracellularis* as a potential vaccine candidate (Kroll et al., 2004a).

The preferred media for growing *Lawsonia*-susceptible tissue culture cells is Dulbecco’s Modified Eagles Media (DMEM) with bovine serum at concentrations of 5-10% (Lawson et al., 1993; Knittel and Roof, 1998; Guedes and Gebhart, 2003b). Tissue cultures are usually infected with 10% v/v of inoculum containing *L. intracellularis* and are monitored daily by taking a representative sample (supernatant containing sloughed off or suspended cells and bacteria) and staining them with *Lawsonia*-specific monoclonal antibodies (McOrist et al., 1987; Guedes and Gebhart 2003c) followed by various secondary staining techniques including immunofluorescence (Lawson et al., 1993; Knittel et al., 1996), immunogold (Collins et al., 1996) or immunoperoxidase assays (Lawson et al., 1993; Guedes et al., 2002a). Cultures are monitored for increases in percent cell infections and are typically harvested, passed or used for inoculating animals when they reach 80% to 100% infectivity.

The conventional method for isolating *L. intracellularis* from infected tissue was developed by Lawson et al. (1993). This method requires homogenization of infected areas of the intestinal mucosa and subsequent treatment with 1% trypsin in phosphate-buffered saline (0.1M, pH 7.4). The mucosal homogenates are passed through a series of filters (200-mesh stainless steel, Whatman glass fiber filter, 1.2 μm, 0.8 μm and 0.65 μm syringe
filters) to make a filtrate containing *L. intracellularis* and other intestinal organisms and then, stored in a sucrose potassium glutamate solution with 10% FBS at -70°C (Lawson et al., 1993). The filtrates are used to inoculate partially confluent cell monolayers which are incubated with media containing *Lawsonia*-resistant antibiotics that inhibit growth of confounding bacteria and fungi (Lawson et al., 1993). Co-cultivation of *L. intracellularis* with tissue culture cells continue until high levels of the bacteria is achieved in pure culture (Lawson et al., 1993).

**Disease Reproduction**

The development of *in vitro* cultivation methods as described above has provided the means for fulfilling Koch’s postulates for PE in pigs. Germ-free pigs developed PE from crude intestinal filtrates containing *L. intracellularis* and other enteric bacteria (McOrist and Lawson, 1989) whereas those only exposed to pure cultures of *L. intracellularis* failed to develop disease (McOrist et al., 1993). Additionally, gnotobiotic pigs inoculated with *Lawsonia*-containing gut homogenates developed intestinal lesions typical of PE (McOrist et al., 1994a). These observations strongly suggest that intestinal flora influences the development of PE by modifying or supporting the ability of *L. intracellularis* to colonize the intestinal tract (Smith and Lawson, 2001). However, the roles of commensal bacterial and other enteric pathogens potentially present during a *Lawsonia* infection are still undefined (Smith and Lawson, 2001).

Proliferative enteropathy can be reproduced by challenging pigs with *L. intracellularis* using pure culture or intestinal mucosa homogenates from previously
infected pigs. The advantages of a pure culture challenge are that an infective dose can be quantified for stringent control of a more defined and consistent reproduction of PE in pigs (Kroll et al., 2004a). Pure culture inoculums contain no confounding effects due to potentially pathogenic intestinal bacteria or viruses (Guedes and Gebhart, 2003b). The disadvantage is the difficulties involved in the isolation and cultivation of \textit{L. intracellularis} \textit{in vitro} (Guedes and Gebhart, 2003b). The advantages of an intestinal mucosa homogenate challenge are that it’s relatively easy to produce by scraping the \textit{Lawsonia}-infected ileal mucosa from PE previously affected pigs and then, administering the scrapings immediately to naïve susceptible pigs. Also, it has proven to be successful over the years in effectively reproducing all forms of PE in pigs and hamsters (Lomax et al., 1982a and 1982b; Mapother et al., 1987; McOrist et al., 1993). The disadvantage is that mucosal homogenates contain other microflora and potentially pathogenic organisms (bacteria, viruses and fungi) that confound the effects of a \textit{Lawsonia}-only challenge. Mucosal homogenates are not easily quantified for determining the proper infectious dose and these enumeration methods have been unable to differentiate between live vs. dead \textit{L. intracellularis} organisms (Guedes and Gebhart, 2003b). A direct comparison of both \textit{L. intracellularis} challenge models was done by Guedes and Gebhart (2002c and 2003b) which demonstrated that reproduction of clinical signs and lesions typical of PE was similar in both models.
Gut homogenate challenge model

The earliest experiments designed to reproduce PE in pigs were only successful when the orally administered inocula contained fresh, unadulterated *L. intracellularis* derived directly from affected pigs (Roberts et al., 1977; Lomax et al., 1982a and 1982b; Mapother et al., 1987; McOrist and Lawson, 1989). This model is commonly used because it can be rapidly produced with minimal effort or technical skill. Until recently, gut homogenate or mucosal derived *Lawsonia* challenges had a history of providing a severe challenge in pigs that may not have correlated to typical PE found in nature. Guedes and Gebhart (2003a) reported the use of a refined gut homogenate challenge model using lower concentrations of semi-quantified *L. intracellularis* in the challenge inoculum. In this study, 10-fold dilutions of a highly virulent gut homogenate challenge resulted in disease reproduction that closely resembled field outbreaks. The lowest dose administered (approximately $5.4 \times 10^8$ *Lawsonia*/dose) induced infection in susceptible pigs indicating a low infectious dose is sufficient to reproduce field-type clinical symptoms of PE. These results were consistent with studies in which pigs challenged with approximately $10^7$ to $10^8$ *Lawsonia* organisms per dose developed moderate to severe diarrhea beginning 2 weeks post inoculation (Collins et al., 2001). To date, a minimal infectious dose has not been determined.

Pure culture challenge model

The *L. intracellularis* pure culture challenge model was originally developed by McOrist and colleagues (1993) in order to demonstrate reproduction of PE in pigs when
orally inoculated with *Lawsonia*-infected enterocytes. In this study, 4 pigs were challenged with $10^6$ *Lawsonia* organisms per dose and developed subclinical PE. There were no clinical signs, but the animals had gross and microscopic (IHC) lesions consistent with the presence of *L. intracellularis*.

Gross and IHC lesions typical of acute PE were induced in dexamethasone treated and untreated pigs using a pure culture *L. intracellularis* isolate (Joens et al., 1997). This study showed that stress induced by dexamethasone had no effect on the development of intestinal lesions (Joens et al., 1997). A pure culture *L. intracellularis* isolate N343 has been successfully used in multiple controlled challenge exposure studies for determining the efficacy of Denagard™ (tiamulin hydrogen fumarate) when given orally to pigs in the feed or drinking water (McOrist et al., 1996b; Schwartz et al., 1999; Walter et al., 2000a and 2000b). Additionally, virulent *L. intracellularis* pure culture challenge exposure studies have been performed to establish vaccine efficacy against PE (Kroll et al., 2004a). Uses of pure culture *L. intracellularis* challenge models have provided valuable information on the transmission of the organism within pig herds (Smith and McOrist, 1997; Jordan et al., 2004). This model is used in limited fashion because of the extreme difficulties in the isolation and cultivation of *L. intracellularis* in the laboratory.

**Pathogenesis**

Exposing pigs to *L. intracellularis* or to diseased mucosa containing these intracellular bacteria can reproduce PE. In typical oral challenge exposure studies of weaned pigs (3-4 weeks-old) with a standard inoculum of $10^8$ *L. intracellularis* bacteria,
numerous intracellular bacteria can be visualized in the developing proliferative small and large intestines and feces one to three weeks following inoculation with a peak of infection and lesions 3 to 4 weeks after challenge (McOrist et al., 1996a). In most of these pigs, intestinal infection, proliferative lesions and excretion persists for approximately 4 weeks, but in some exposed pigs, excretion may persist for at least 10 weeks (Smith and McOrist, 1997). At the peak of infection, moderate diarrhea and histologic lesions of PE are usually observed in 50% and 100% of the animals challenged with this inoculum (McOrist et al., 1996a; McOrist et al., 1997b). Infection and lesions in the large intestine (colon and cecum) generally start to occur a week or two after small intestinal infection, following an oral challenge (McOrist et al., 1996a). Naïve pigs of a wide age range (neonates to grower-finishers) are susceptible to oral challenge (McOrist and Gebhart, 1999).

Proliferative enteropathy initially develops as a progressive proliferation of immature epithelial cells, following invasion of the intracellular *Lawsonia* bacteria. In most cases, no significant inflammatory reaction occurs and the bacteria remain in the epithelium at this stage (McOrist et al., 1996a). In severe cases of PE, *L. intracellularis* can also be observed in the mesenteric lymph node and tonsils, but these appear to be secondary sites of infection (Jensen et al., 2000b). *In vivo* and *in vitro* studies have elucidated some of the early events in bacteria-cell interaction (Lawson et al., 1993; McOrist et al., 1995c). Bacteria associate closely with the cell membrane and then quickly enter the enterocytes via an entry vacuole (McOrist et al., 1995c). Specific adhesins or receptors have not been identified but attachment and entry appear to require specific bacterium-host cell interaction (McOrist et al., 1997c). The genomic sequence of *L. intracellularis* indicates that it may
possess a type III secretion system. This outer membrane protein complex commonly found in gram negative bacterial pathogens may assist the bacterium during cellular invasion, evasion of the host’s immune system and could be a mechanism for inducing cellular proliferation. The entry of *Lawsonia* bacteria into cells is dependent on host cell activity, but not necessarily bacterial viability, possibly indicating a type of induced phagocytosis like receptor mediated endocytosis (Lawson et al., 1993). Actin rearrangement is important for entry of obligate intracellular bacteria and may be a key component of entry for *L. intracellularis* (Lawson et al., 1995). *Lawsonia* gene sequence analysis has revealed several flagellar genes that are involved in producing a single polar flagellum which may also play a role in entry of the host cell (Nuntaprasert et al., 2004). Morphological associations of *L. intracellularis* and small pits or vesicles of the cell membrane were observed immediately upon entry (McOrist et al., 1995c). These events are similar to the association between *Chlamydia trachomatis* and *C. psittaci* entry and clathrin-coated pits (Reynolds and Pearce, 1991). Other factors may be involved as experiments using cytochalasin D (blocks cytoskeleton rearrangements) indicate that there may also be a non-actin dependent pathway utilized by *L. intracellularis* for successful host cell invasion (Lawson et al., 1995). The entry vacuole rapidly breaks down (within 3 hours) and *L. intracellularis* flourish and multiply (not membrane-bound) within the apical cytoplasm (McOrist et al., 1995c). This mechanism would explain how *L. intracellularis* escapes proteolytic degradation due to endosome-lysosome fusion. Many other species of intracellular bacteria including *Shigella, Listeria*, and some *Rickettsia* species also escape into the cytoplasm and avoid the damaging effects of phagolysosomal fusion. Escape from
the endosome is mediated by producing membrane-damaging cytolytic enzymes or toxins such as phospholipase or listeriolysin (Ewing et al., 1978; Gaillard et al., 1987; Sansonetti 1992; Todd et al., 1981). *Lawsonia intracellularis* exhibits cytolytic (hemolytic) activity in vitro through expression of a novel hemolysin, *Lawsonia* hemolysin A (LhyA), which may be one of the main virulence factors involved in intracellular vacuole escape (Smith, 2001). Following vacuolar escape, the bacterial-host cell relationship observed in vitro is similar to that found in animals (McOrist et al., 1995c). Typically, *Lawsonia* bacteria located in the apical cytoplasm do not localize to any cell structures except for some association with the cell mitochondria (McOrist et al., 1995c) and the rough endoplasmic reticulum (Jansi et al., 1994).

Intracellular multiplication and cell to cell spread of *L. intracellularis* was revealed when co-cultivation experiments identified cells infected with bacteria continue to divide and spread the bacteria into newly developed daughter cells (Lawson et al., 1993). Additional evidence suggests that actively dividing cells promote bacterial propagation better than non-dividing, mature cells (Lawson et al., 1993; McOrist et al., 1995c). Experiments using cycloheximide or colchicine to stop eukaryotic cell division also inhibited *L. intracellularis* growth (Lawson et al., 1995). Furthermore, growth promotion of *L. intracellularis* was better in rapidly growing enterocyte cultures than confluent monolayers (Lawson et al., 1993). *Lawsonia* proliferation in the host is benefited by actively dividing enterocytes that facilitates bacterial expansion through continued enterocyte replication and migration, and mediates spread of the bacteria throughout the epithelium (Smith and Lawson, 2001).
Cell proliferation in PE only cases (presence of the disease in the host uncomplicated by other confounding pathogens) when cells are infected by *L. intracellularis* only and in cases where islands of hyperplasia occur amidst normal epithelium, only infected cells are proliferative (McOrist et al., 1996a). Infection experiments in hamsters indicate crypt cells start to divide at an increased rate (up to four-fold) two days after bacterial infection (Jansi et al., 1994). The stimulatory effect of the bacteria on host cell division does not persist once the lesion becomes fully developed despite the constant presence of *L. intracellularis* (McOrist et al., 1996a). The mechanism whereby *L. intracellularis* prevents cell maturation (enterocyte replication, migration upwards to the top of intestinal crypts and sloughing off into the lumen) is not known. However, *Lawsonia*-infected cells continue to undergo mitosis and proliferation within those pockets of infected cells, and form hyperplastic crypts which prevent cell sloughing and regeneration of healthy, mature enterocytes (McOrist et al., 1996a). *Lawsonia intracellularis*-infected intestinal crypts can become enormously elongated and often branched (McOrist and Gebhart, 1999). Loss of protein and amino acids into the intestinal lumen and reduced nutrient absorption by the intestinal mucosa are the likely causes of the reduction in weight gain and feed conversion efficiency seen in pigs affected with chronic uncomplicated proliferative enteropathy lesions (McOrist and Gebhart, 1999).

Clinical Signs

Clinical cases of PIA, the chronic form of proliferative enteropathy, are observed most commonly in the post-weaned pigs between 6 and 20 weeks of age (McOrist and Gebhart, 1999). The predominant signs of PIA include anorexia, diarrhea, and poor growth
which persist for a period of weeks (Lawson and Gebhart, 2000). Diarrhea may occur only when significant lesions are present (McOrist and Gebhart, 1999), which makes this form of PE very difficult to detect in clinically healthy pigs. In PIA endemic herds, pigs will exhibit normal feed intake, but will fail to sustain normal growth (McOrist and Gebhart, 1999). Severely infected pigs are often associated with varying degrees of thickening in the mucosal lining or necrotic lesions of the small intestine commonly described as “hose pipe” or “garden hose” gut (Rowland and Lawson, 1992).

Cases of PHE occur more commonly in young adult pigs between the ages of 4-12 months (McOrist and Gebhart, 1999). Black tarry feces are the first visible clinical sign commonly followed by a loose, red-tinged, watery stool (McOrist and Gebhart, 1999). However, some of the pigs die without fecal abnormality (McOrist and Gebhart, 1999). It has been estimated that half of the pigs affected with PHE will die and the other half recover over a short period of time without visible signs of reduced weight or changes of body condition (Roland and Lawson, 1992).

Subclinical PE may be the most common disease among growing pigs, but the syndrome is rarely recognized because there are no observable clinical indications in pigs with *Lawsonia*-specific subclinical enteritis. This variation of PE closely resembling PIA is defined as active *L. intracellularis* infection with the presence of microscopic and/or gross lesions resulting in reduced productivity (average daily gain with +/- feed efficiency). This occurs often in the absence of observable clinical signs of disease such as PE-associated mortality, diarrhea or other symptoms consistent with PE morbidity. Evidence of a subclinical *L. intracellularis* infection may or may not be detected by serological or PCR
methods as pigs may be colonized but not severe enough to induce shedding or seroconversion (Guedes, 2004).

With increasing incidence of other enteric diseases displaying similar clinical symptoms to the various forms of PE (hemorrhagic bowel syndrome, Coliobacillosis, Porcine circovirus type 2, transmissible gastroenteritis virus, rotavirus, Salmonellosis, and swine dysentery), it is important to differentiate among all of the common factors by performing a thorough post mortem examination and implementing proper diagnostic evaluations.

Lesions

Histopathologic lesions common to all forms of PE are characterized by the adenomatous proliferation of the epithelium in the crypts of the small intestine and in mucosal glands of the large intestine, and by the presence of curved intracellular bacteria within these enterocytes (Rowland and Lawson, 1974). The crypts are elongated, enlarged and lined with crowded immature epithelial cells with mitotic events (McOrist and Gebhart, 1999). Goblet cells are absent from the affected epithelium and the infiltration of inflammatory cells are not a common characteristic of PE (Rowland and Lawson, 1992). The proliferating epithelial cells contain intracytoplasmic, slender, curved, rod-shaped bacteria (Ward and Winkelman, 1990).

Proliferative hemorrhagic enteropathy

The acute and most severe form of PE is considered to be proliferative hemorrhagic enteropathy (PHE) and typically affects the terminal ileum and colon. This form of PE is
most often associated with young adult pigs 4-12 months old (McOrist and Gebhart, 1999) and commonly found in high health herds when replacement gilts and boars have been introduced into a new farm site. Clinical manifestations of PHE include extended and thickened intestines with serosal edema and a severely proliferated mucosa (McOrist and Gebhart, 1999). The lumen contains either fresh blood or a solid cast of blood and fibrin clots as seen in Figure 2 (Ward and Winkelman, 1990). However, focal points of bleeding, ulcerations or erosions are not observed (McOrist and Gebhart, 1999). Proliferative hemorrhagic enteropathy is differentiated from hemorrhagic bowel syndrome (HBS) in which there is no abnormal crypt proliferation associated with HBS and the hemorrhage occurs throughout all layers of the intestinal wall (Knittel, 1999).

Figure 2. Above: A gross lesion of the ileum containing fibrinous blood clots indicative of porcine hemorrhagic enteropathy (PHE) caused by *Lawsonia intracellularis*. Below: The normal appearance of an ileum from a healthy pig
Porcine intestinal adenomatosis

The chronic and most common form of PE is considered to be porcine intestinal adenomatosis (PIA). This form of PE is commonly found in actively growing pigs from late nursery to late finishing stages and affects the terminal 50 cm of ileum and the upper third of the proximal colon (McOrist and Gebhart, 1999). Lesions in PIA consist of intestinal mucosa thickening by epithelial proliferation but relatively free from inflammation or only mildly inflamed on the mucosal surface as seen in Figure 3 (Knittel, 1999). Histologically, the mucosa is enlarged with branching crypts lined with immature epithelial cells (McOrist and Gebhart, 1999). Mitotic figures are evident throughout the crypt while goblet cells are nonexistent (McOrist and Gebhart, 1999). Intracellular bacteria

Figure 3. A gross lesion of the ileum containing mild to moderate thickening of the intestinal mucosa indicative of porcine intestinal adenomatosis (PIA) caused by *Lawsonia intracellularis*
are a common feature residing in the apical cytoplasm of the affected epithelial cells (McOrist and Gebhart, 1999).

Necrotic enteritis

Considered a result of end-stage PIA, necrotic enteritis involves deep coagulative necrosis of the adenomatous mucosa (Rowland 1978). Yellowish-gray lesions are evident on the surface of the mucosal lining in the terminal portion of the ileum (Rowland and Lawson, 1992), see Figure 4. Severe thickening of the ileum in these cases have given the disease a unique characteristic of PE called “hose pipe or garden hose” gut (Rowland and Lawson, 1992).

Figure 4. A gross lesion of the ileum containing severe thickening and hemorrhaging of the intestinal mucosa, necrotic ulcerations and evidence of a fibrinous cast indicative of necrotic enteritis (NE) caused by *Lawsonia intracellularis*
Diagnosis

For many years, diagnosis of PE in pigs was speculative as clinical symptoms such as diarrhea or gross and microscopic examination of the intestines were the only way to determine if pigs were affected with the disease. With the advent of sensitive and specific diagnostic methods, new strides have been made to identify *L. intracellularis* specific infections and the prevalence of PE in pig herds. These methods have assisted veterinarians and pig producers in determining with better precision when to implement control and prevention measures in herds where PE is endemic. A summary of the various diagnostic techniques available for detecting *L. intracellularis* exposure in animals are listed in Table 1.

Histopathology and immunohistochemistry

Proliferative enteropathy is often diagnosed *post mortem* by the characteristic gross pathology associated with *L. intracellularis* infections. However, confirmation of PE through histopathological analysis is necessary in order to ensure proper diagnosis.

Hematoxylin and eosin (H&E) staining of tissue sections exhibiting severe PE identifies proliferative changes in the enterocytes of the intestines (Rowland, 1978). Warthin Starry (WS) silver stain allows the detection curved, rod-shaped, intracellular bacteria in histologic sections, but this staining technique is non-specific and has limitations when applied to necrotic or autolytic samples (Ward and Winkelman, 1990; Rowland and Lawson, 1992; Jensen et al., 1997). A modified Ziehl-Neelsen stain on mucosal and fecal smears or fixed infected eukaryotic cell samples of *L. intracellularis* provides a non-specific, simple
<table>
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<tr>
<th>Diagnostic technique</th>
<th>Detection method</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Reference</th>
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<tr>
<td>H&amp;E</td>
<td>Crypt hyperplasia, cell abnormalities</td>
<td>Confirms cell abnormalities with bacterial involvement in tissues</td>
<td>Post mortem diagnosis only</td>
<td>Rowland, 1978</td>
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</table>
| Warthin Starry Silver Stain | Identification of intracellular organisms | Rapid detection of bacterial involvement in tissues | • Non-specific  
• Post mortem diagnosis only | Ward and Winkelman, 1990  
Rowland and Lawson, 1992  
Jensen et al., 1997  
Knittel et al., 1997  
Guedes et al., 2003c |
| IHC/IFA              | *Lawsonia*-specific immunofluorescence or immunoperoxidase staining | • Highly sensitive and specific  
• Requires a monoclonal antibody | Post mortem diagnosis only | Gebhart et al., 1991 and 1993  
Jones et al., 1993a and 1993c  
Cooper et al., 1996 and 1997  
Elder et al., 1997  
Lindecrona et al., 2002 |
| PCR                  | *Lawsonia*-specific DNA sequences | • Ante mortem diagnosis  
• Highly specific  
• Detects active colonization and shedding  
• Detection of multiple pathogens in one test  
• Real time detection | • Less sensitive  
• Possible inactivation of test reagents  
• Possible cross-contamination of samples  
• Possibility of false negatives | Gebhart et al., 1991 and 1993  
Jones et al., 1993a and 1993c  
Cooper et al., 1996 and 1997  
Elder et al., 1997  
Lindecrona et al., 2002 |
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<th>Diagnostic technique</th>
<th>Detection method</th>
<th>Advantages</th>
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<tr>
<td>IFAT</td>
<td>serum IgG</td>
<td>• Ante mortem diagnosis</td>
<td>Requires manual determination of results</td>
<td>Knittel et al., 1998</td>
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<td></td>
<td>• Highly sensitive and specific</td>
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<tr>
<td>IPMA</td>
<td>serum IgG</td>
<td>• Ante mortem diagnosis</td>
<td>Requires manual determination of results</td>
<td>Guedes et al., 2002a</td>
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<tr>
<td></td>
<td></td>
<td>• Highly sensitive and specific</td>
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<tr>
<td>LPS-ELISA</td>
<td>serum IgG</td>
<td>• Ante mortem diagnosis</td>
<td>• Possible batch-to-batch variation</td>
<td>Kroll et al., 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Highly sensitive and specific</td>
<td>• Possible lab to lab variation</td>
<td>Boesen et al., 2005</td>
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<tr>
<td></td>
<td></td>
<td>• Automated determination of results</td>
<td>• Not commercially available</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>• Rapid detection</td>
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<td></td>
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<td>• High through-put testing</td>
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confirmatory test as the intracellular bacteria stain red within the cytoplasm of infected cells (Ward and Winkelman, 1990; Rowland and Lawson, 1992). Immunohistochemistry (IHC) and immunofluorescence antibody (IFA) procedures have made it possible for specific detection of *L. intracellularis* within tissue sections affected with various forms of PE (Knittel et al., 1997). With the use of *Lawsonia*-specific monoclonal antibodies, the bacteria can be identified in fecal smears of cases involving high levels of actively shedding *L. intracellularis* or in fixed tissue sections from various sources (McOrist et al., 1987; Guedes et al., 2003c). A comparative study revealed that the IHC (86.8%) stain was much more sensitive than H&E (36.8%) and WS (50%) staining methods for detecting *L. intracellularis* in PE-affected tissue samples (Guedes et al., 2002b). In addition, there was a high correlation (82.5%) between the IHC results and the presence of macroscopic lesions 4 weeks after infection (Guedes and Gebhart, 2002b). These results are consistent with previous reports that a histological staining technique involving the use of an anti-*Lawsonia* monoclonal antibody was highly specific and more sensitive in detecting *L. intracellularis* infections than other non-specific histological stains (McOrist et al., 1987; Jones et al., 1993b; Huerta et al., 2003). Using IHC, *L. intracellularis* positive antigen can be detected even in cases of severe necrosis in which the mucosa is completely destroyed, or during recovery stage when the bacterial antigen is found only in the cytoplasm of mononuclear cells in the lamina propria (Guedes and Gebhart, 2002b).
Serology

Early accounts of the first serology test for detecting *Lawsonia*-specific IgM and IgA antibodies in naturally exposed or experimentally infected pigs were from Lawson et al. (1988). In these studies, an indirect fluorescent antibody test (IFAT) was used to determine that IgM and, to a lesser extent, IgA was the predominant antibody in early *L. intracellularis* infections in pigs. Antibodies were detected for 8 weeks post inoculation. Holyoake et al. (1994) employed an enzyme-linked immunosorbent assay (ELISA) using Percoll purified whole cell *L. intracellularis* from PHE affected pig tissue as the primary antigen in the test system. This ELISA was able to detect *Lawsonia*-specific IgG in field-exposed pigs as early as 3 weeks of age, and represented the first recorded detection of maternally acquired antibodies. However, results indicated the antibody titers were low and variable among inoculated and uninoculated test groups and could not be differentiated statistically (Holyoake et al., 1994).

An IFAT test modified to detect IgG instead of IgM or IgA was developed using pure culture derived *L. intracellularis* (Knittel et al., 1998). This assay incorporated the use of *Lawsonia* antigen grown in pure culture that is then stained using serum from test pigs as the primary antibody and fluorescein isothiocyanate (FITC) labeled anti-swine IgG (heavy and light chains) as the secondary antibody (Knittel et al., 1998). Results from 3 experiments indicated that the IFAT was more sensitive (90%) and highly specific (96%) compared to PCR (47% and 100% respectively) in detecting *L. intracellularis* in pigs challenged with pure culture 21-28 days after inoculation (Knittel et al., 1998). Maternal antibodies were detected by the IFAT up to 5 and 6 weeks of age in these evaluations. The
IFAT has proven to be an accurate and reliable ante mortem tool for detecting the incidence of *L. intracellularis* in experimental and naturally exposed pigs around the world (Mortimer et al., 2000; Fourchon and Chouet, 2000; Dunser et al., 2000; Bakker et al., 2000; Just et al., 2001; Guedes et al., 2002b).

An immunoperoxidase monolayer antibody assay (IPMA) was developed to provide an alternative to the immunofluorescence-based assay for detecting seroconversion to *L. intracellularis* in pigs (Guedes et al., 2002a). This assay is very similar in design and utility as the IFAT except for the following: the secondary antibody is an anti-swine IgG peroxidase-labeled conjugate (vs. FITC), the methods by which sample preparations observed are different (IPMA= light microscopy; IFAT= UV microscopy), and the IPMA requires one more step than the IFAT (incubation with H$_2$O$_2$ to remove endogenous peroxidase) (Guedes et al., 2002a). The sensitivity (88.9%) and specificity (100%) of this assay in detecting anti-*Lawsonia* antibodies in pigs was also comparable to the IFAT (Guedes et al., 2002a). Both assays were compared in parallel in a controlled challenge exposure study in which serum was tested on days 7, 14, 21 and 28 after oral inoculation with intestinal homogenate from PHE infected pigs (Guedes et al., 2002a). Results showed that the percentage of agreement between the IFAT and IPMA was 98.6% (Guedes et al., 2002a). The serology results agreed in all samples tested except on days 0 and 7 in which one control animal (*Lawsonia*-negative) was positive by the IPMA, but negative by IFAT (Guedes et al., 2002a). Therefore, either assay can be used to effectively detect *L. intracellularis* exposure in pigs.
Various authors have attempted to develop ELISA methods that would be as reliable in detecting anti-*Lawsonia* antibodies in sera as the previously described assays. An ELISA would be the desired test method for detecting exposure to *L. intracellularis* in pigs because it allows for high throughput sample testing, automated reporting of test results and an unbiased determination of a *Lawsonia*-positive or negative sample. An ELISA (Watarai et al., 2004) was developed using synthetically produced peptides of the *L. intracellularis* outer surface antigen, LsaA (McCluskey et al., 2002). This ELISA was able to distinguish between rabbits naturally infected with *L. intracellularis* from those that were *Lawsonia*-naïve (Watarai et al., 2004). These results indicated that a single antigenic molecule may be used as the primary antigen in an indirect ELISA format for successfully detecting anti-*Lawsonia* IgG in rabbit serum (Watarai et al., 2004). Recently, an ELISA was developed for experimentally detecting anti-*Lawsonia* IgG in pigs that were previously inoculated with virulent pure culture *L. intracellularis* (Kroll et al., 2005b). In this test system, an indirect ELISA format using the lipopolysaccharide (LPS) component of a virulent *L. intracellularis* pure culture isolate as the primary antigen was successful in detecting anti-*Lawsonia* IgG antibodies in pigs. Compared to the IFAT, the LPS-based ELISA detected significantly (p<0.05) more anti-*Lawsonia* IgG positive pigs after vaccination and challenge on days 21, 28, 35 and 42 of the study (Kroll et al., 2005b). The sensitivity (99.5%) and specificity (100%) of this assay was slightly higher than the IFAT suggesting that this test method may be better at detecting early onset of *L. intracellularis* exposure in pigs regardless of isolate type (vaccine or wild-type) (Kroll et al., 2005b). Others have used similar technology in a capture-antibody or sandwich ELISA format in which *L. intracellularis* LPS was
immobilized with a monoclonal antibody to the bottom of each well in a microtiter plate (Boesen et al., 2005a and 2005b). In these studies, 36 out of 37 (97%) experimentally infected pigs tested positive while 31 out of 31 (100%) weaned pigs 12 weeks of age and 30 out of 31 (97%) weaned pigs 20 weeks of age tested positive for anti-Lawsonia IgG antibodies in naturally infected herds. The reported specificity (99.3%) and sensitivity (98%) of the sandwich ELISA is similar to the indirect LPS-based ELISA and higher than the IFAT and the IPMA assays (Boesen et al., 2005b). The percentage of agreement between the IFAT and ELISA was found to be 98.8% which is similar to the agreement found between the IFAT and IPMA (98.6%) (Boesen et al., 2005b).

Polymerase chain reaction

Polymerase Chain Reaction (PCR) is a sensitive, molecular based DNA detection tool that can detect low levels of microbial pathogens in various biological specimens, especially intracellular organisms, which may be difficult to recover using conventional isolation methods and/or detect by microscopy or immunodiagnostic tests. This highly sensitive, pathogen-specific and rapid method has been used successfully for detection of L. intracellularis in feces and mucosal samples (Gebhart et al., 1993; Jones et al., 1993a and 1993d) as well as in various tissues including ileum, cecum, colon (Jones et al., 1993b), tonsil (Jensen and Svensmark, 2000), liver and lymph nodes (Jensen et al., 2000). Primers for the specific unique sequences of DNA based on 16S rDNA found to be conserved among all L. intracellularis organisms were developed (Gebhart et al., 1991 and 1993, Jones et al., 1993a; Jones et al., 1993c; Cooper, 1996; Cooper et al., 1997). Two sets of
primers; an external primer set that amplifies a 319 base pair (bp) fragment of DNA and an internal primer set that amplifies a 255-bp fragment have been used in nested PCR assays for detecting as little as $10^3$ *L. intracellularis* organisms within a gram of feces (Jones et al., 1993b). This nested PCR method was optimized by enhancing procedures for extracting genomic DNA from pig feces thus achieving a minimal detection limit of $2 \times 10^2$ *L. intracellularis* bacteria per gram of feces (Moller et al., 1998b). Still, others have found the sensitivity of this assay to be approximately $10^1$ *L. intracellularis* bacteria per gram of feces (Chang et al., 1997).

Several authors have evaluated PCR for detection of *L. intracellularis* in the feces of experimentally challenged and field exposed pigs (Jones et al., 1993b; McOrist et al., 1994b; Knittel et al., 1998). McOrist et al. (1994b) suggested that PCR will only detect positives in feces when pigs have active lesions and *L. intracellularis* is present in high numbers. However, PCR on pig feces have been used to demonstrate that pigs shed *L. intracellularis* in the presence or absence of clinical signs or gross lesions of PE (Knittel et al., 1997; Jordan et al., 1999; Jordan et al., 2004). The PCR method has been a useful diagnostic tool to identify the prevalence of *L. intracellularis* among many different pig production systems around the world (Dunser et al., 2000; Vestergaard et al., 2004; Lofstedt et al., 2004; Plawinska et al., 2004; Keita et al., 2004; Wendt et al., 2004; Tomanova and Smola 2004; Suto et al., 2004).

Polymerase chain reaction can be used to identify pigs that are actively shedding the organism, but it can not detect *Lawsonia*-colonized pigs that are not shedding the organism (Jordan et al., 1999). Due to intermittent shedding commonly found in subclinical or
chronically infected pigs, false negative results can occur when using PCR on pig feces (Jordan et al., 1999; Knittel et al., 1997). Factors that might inhibit successful PCR and contribute to false negative results are molecules naturally found in feces that inactivate DNA polymerase, degrade or capture nucleic acids or interfere with cell lysis during the extraction process (Lantz et al., 2000; Jacobson et al., 2003a). The use of a internal control or “mimic” DNA molecule that is amplified by the same primers as those used for *Lawsonia*-specific DNA would validate the PCR reaction and indicate false-negative results in clinical specimens (Jacobson et al., 2003a). Jacobson et al. (2003a) successfully developed a mimic molecule consisting of a human β-actin molecule that upon amplification results in a larger size (596-bp) DNA fragment than the *Lawsonia*-specific amplicon (319-bp). Due to the size of fragment and the source of mimic DNA (human), this mimic is expected not to competitively exclude the target DNA or non-specifically bind to DNA of bacterial or pig origin. In a recent study, the sensitivity of *Lawsonia*-spiked tissue samples was $10^1$ to $10^2$ mimic molecules per reaction tube and $10^2$ to $10^3$ mimic molecules per reaction tube in fecal samples (Jacobson et al., 2004). Still, some PCR inhibitors may exist and interfere with the DNA polymerase since the results improved by the use of enzymes (i.e. rTth and Tli polymerases) known to be less sensitive to inhibition (Al-Soud and Radstrom, 2001).

Multiplex PCR assays were developed for simultaneous detection and identification of *Serpulina (Brachyspira) hyodysenteriae*, *Salmonella* sp. and *L. intracellularis* in pig feces (Elder et al., 1997; La et al., 2004; Zmudzki et al., 2004). A one-step PCR assay was developed to detect a *Lawsonia*-specific 210-bp DNA fragment in clinical specimens (Suh
et al., 2000). This method was found to be highly specific in detecting *Lawsonia*-only DNA in crude intestinal samples (no reaction to swine genomic DNA and other enteric bacterial pathogens) and found to be more sensitive than conventional PCR (Suh et al., 2000).

Researchers have developed a 5’ nuclease assay where the PCR-product amplified by two specific primers based on the 16S rRNA gene of *L. intracellularis* and then, detected by fluorescence (Lindecrona et al., 2002). Out of 204 clinical samples, 111 (54%) samples tested positive for *Lawsonia*-specific DNA compared to 98 (48%) samples by IHC making it just as sensitive as IHC in detecting *L. intracellularis* in pig feces (Lindecrona et al., 2002). The detection limits were determined to be approximately $4 \times 10^4$ *L. intracellularis* bacteria per gram of feces (Lindecrona et al., 2002). Others have designed a real-time PCR method for rapid detection and quantification of *Lawsonia* DNA in high throughput situations (Beckler et al., 2003). Of the 45 known positive samples, 31 were PCR positive by conventional PCR and 36 were positive by real-time PCR, showing a 69% and 80% sensitivity, respectively (Beckler et al., 2003). Real-time PCR methods like those mentioned above are more attractive than conventional PCR because they allow for immediate confirmation of a *Lawsonia*-positive within a sample and provide the option of quantification. In addition, real-time PCR may be done with pure cultures, mucosal homogenates or feces and eliminates the need to visualize PCR products via gel electrophoresis leading to a reduction of time, labor and inherent problems of cross-contamination. More studies are necessary to optimize real-time PCR technology for the detection and quantification of *L. intracellularis* in pigs.
Other diagnostic techniques

Prior to the development of PCR, a DNA hybridization technique with an *L. intracellularis* specific DNA probe was used to detect *Lawsonia*-specific genomic DNA in the feces of experimentally infected pigs (Jones et al., 1993d). DNA was extracted from fecal samples and bound to a nylon membrane, then probed with a digoxigenin-labeled *Lawsonia* DNA probe (Gebhart et al., 1991). Another hybridization technique using *Lawsonia* DNA hybridized *in situ* to tissue samples taken from PHE and PLA cases confirmed that sequence similarities are evident in *L. intracellularis* from both forms of PE (Gebhart et al., 1991).

An enzyme-linked oligosorbent assay (ELOSA) was developed to specifically identify 328-bp PCR amplified *L. intracellularis* DNA in clinical samples (Zhang et al., 2000). Positive test results involved a signal greater than or equal to the optical density of 0.375 at 450 nm wavelength after hybridization of biotin-labeled PCR products with an amine-modified *Lawsonia*-specific internal oligonucleotide capture probe immobilized in 96-well microtiter plates forming an avidin-biotin-peroxidase complex (Zhang et al., 2000).

An immunological method using immunomagnetic separation and ATP bioluminescence was developed for the detection of *L. intracellularis* in fecal samples (Watarai et al., 2005). Magnetic beads coated with an anti-*Lawsonia* LsaA antibody were used to capture whole cell *L. intracellularis* in fecal samples from infected rabbits. The beads containing captured *L. intracellularis* were treated to release ATP and assayed to determine the amount of ATP in each sample. Results from these experiments revealed ATP concentrations higher for anti-LsaA antibody coated magnetic beads exposed to fecal
samples from infected rabbits than those exposed to fecal samples from uninfected rabbits (Watarai et al., 2005). This method could be useful as an alternative to PCR for the detection of active \textit{L. intracellularis} infections in animals.

\textbf{Epidemiology and Economics}

Proliferative enteropathy is an endemic disease that is widespread among swine herds and production systems across every continent where pork production can be found. Previous estimates indicated that PE in growing pigs resulted in direct financial losses of $3 to $11 US dollars (USD) per pig in the United Kingdom (McOrist et al., 1997a). In the late 1980’s and 1990’s, annual costs to global pig production were estimated at $20 million USD in the United States (Mapother et al., 1987), $3-6.5 million USD in the United Kingdom (McOrist et al., 1997a) and $25 dollars per sow in Australia (Cutler and Gardner, 1989). However, these estimates reflect pigs having clinical symptoms of PE or growing pigs with chronic PIA (McOrist 2005). This leads to an underestimation of the overall economic impact of PE that includes clinical (chronic and acute PE) and subclinical (presence of intestinal lesions but without clear diarrhea or weight loss) cases in adult and growing pigs (McOrist 2005). Most recent estimated losses exceed €100 ($121 USD) per affected breeding pig which translates to an extra €0.50 ($0.61 USD) per growing pig (McOrist 2005). The total losses due to endemic PE on most European farms could potentially exceed €1 ($1.22 USD) per affected grower-finisher pig (McOrist 2005).

Many factors contribute to the prevalence and economic impact of \textit{L. intracellularis} on pig farms including age, breed, diet, herd health status, use of antibiotic, vaccines,
disinfectants and differences in management and production systems. Other contributing factors having strong influence in maintaining PE in pig herds are the transmission and survival of \textit{L. intracellularis} in the environment. However, the understanding of the true nature of the epidemiology and economics of PE on pig farms is largely contingent upon the availability and extensive use of more refined and reliable \textit{ante mortem} diagnostic assays. Rates of exposure or prevalence of \textit{L. intracellularis} in swine herds have been determined through serological techniques such as immunoflourescence antibody test (IFAT) (Knittel et al., 1998) and the immunoperoxidase monolayer assay (IPMA) (Guedes et al., 2002a). Polymerase Chain Reaction (PCR) (Jones et al., 1993a) methodology has proven to be a reliable molecular diagnostic tool for specifically detecting \textit{Lawsonia} DNA in pig feces. Serology can provide historical information on exposure to the bacteria while PCR are measures of actual infection (Guedes 2004). Another option for tracking \textit{L. intracellularis} isolates from different geographical origins is a PCR-based molecular tool that detects variable number tandem repeats (VNTR) within the \textit{Lawsonia} genome (Beckler et al., 2004). Gebhart and colleagues have demonstrated that this assay can be used to screen \textit{Lawsonia}-infected tissues and can discriminate between isolates based on the number of highly variable tandem repeat among 4 different loci (Beckler et al., 2004). High discrimination of isolates into genotypic subtypes was possible between outbreaks of PE on geographically distinct pig farms and between animal species including pigs, horses, hamsters, ferrets, ostrich and spider monkey (Weber et al., 2004). However, this test can not differentiate among high and low \textit{in vitro} and \textit{in vivo} passages of the same \textit{Lawsonia} isolate or between a vaccine isolate and its parent form (Weber et al., 2004). The VNTR
profile consisting of non-essential, intergenic DNA repeats within 4 different loci remains stable over time even when manipulated through biological processes (Weber et al., 2004). Therefore, the VNTR assay has been proposed to be an efficient research tool for tracking different genotypic subtypes of *L. intracellularis* among animal species and the possibility of determining their epidemiological relatedness (Weber et al., 2004).

Prevalence of disease

It has been determined that pigs on 20% to 50% of farms worldwide are infected with *L. intracellularis* (Stege et al., 2000; Chouet et al., 2003; McOrist et al., 2003; Suto et al., 2004). The disease may be more prevalent in the USA (96%) and Northern Europe (70-90%) compared to Southern Europe (50-70%) where higher percentages of pigs are positive for PE based on serological prevalence data (McOrist et al., 2003). A longitudinal study of a natural *L. intracellularis* infection in five large Danish pig herds revealed that the bacterium was present in all herds, and 75% of pigs examined by PCR were actively infected (Stege et al., 2004). In a cross-sectional study of 8 growing swine herds (weaning to 24 weeks of age) and 3 breeding herds in the Midwestern United States, *L. intracellularis* exposure was observed by IPMA in 75% of growing herds and 78% of replacement gilt herds (Marsteller et al., 2003).

Prevalence data depicting the true incidence of *L. intracellularis*-specific disease on farms can be skewed high or low depending on sampling times and the diagnostic assay used (PCR, IFAT and IPMA). Previous field and controlled challenge exposure studies have shown that pigs were fecal PCR positive 1 to 2 weeks before they were IPMA positive.
to *L. intracellularis* (Guedes et al., 2002b; Guedes and Gebhart, 2003a). Whereas, other studies have revealed seroconversion to *L. intracellularis* before shedding was evident via PCR (Knittel et al., 1998; Kroll et al., 2004a). Guedes (2004) reported that pigs are probably in the early stages of infection or have not had time to mount a detectable humoral immune response when they are PCR positive, but serologically negative for *L. intracellularis*. Likewise, when pigs are PCR negative, but positive for antibodies, this may indicate previous exposure or lack of sensitivity in the PCR assay to detect *L. intracellularis* (Guedes, 2004). These observations stress the need for evaluating *L. intracellularis* prevalence on farms using both PCR and serology when possible (Guedes, 2004).

White breed hybrid stock seem to develop PE more readily than Duroc-cross pigs (McOrist et al., 2003). Limited contact between sows and their offspring (segregated early weaning systems) and the movement of pigs in large groups (all in/all out systems) have influenced the dynamics of PE on farms (Bronsvoort et al., 2001). Disease transmission is reduced early in life with consequent susceptibility to PE if introduced to them at a later developmental stage (McOrist et al., 2003). *Lawsonia*-specific infections typically reach high levels in grower pigs at about 8 weeks of age and remain active throughout the finishing and breeding stages (Chouet et al., 2003).

Modes of transmission

Transmission of *L. intracellularis* from pig to pig is efficiently accomplished by the fecal-oral route through high levels of bacteria in pig feces. The environment of many pig
farms contains a sustained level of *L. intracellularis*, which allows the reintroduction of infection to new groups of pigs at various ages (McOrist et al., 2003). Previous reports revealed that pig to pig contact contributed greatly to the transmission of acute PHE among breeding stock and recently weaned or adult pigs (Rowland and Rowntree, 1972; Love et al., 1977). A controlled challenge exposure study revealed that sentinel pigs became infected when housed in contact with pigs inoculated with a relatively low dose of pure culture *L. intracellularis* (Jordan et al., 2004) confirming that pig feces is the main source of new infections in susceptible swine (Guedes, 2004). Transmission of *L. intracellularis* from sows to offspring is influenced by the age and parity of the sow (Mauch and Bilkei, 2004). In this evaluation, sera from 99 healthy, late pregnant, gilts and 98 sows of parity 3 to 5 were screened by IFAT for anti-*Lawsonia* antibodies (Mauch and Bilkei, 2004). The antibodies detected in the offspring of gilts showed strong seropositivity to *L. intracellularis* from 5 to 26 weeks of age whereas offspring from parity 3 to 5 sows showed lower IFA values which declined more rapidly (Mauch and Bilkei, 2004). These results indicated that older, seropositive sows either do not excrete sufficient organisms to induce detectable seroconversion or protect their offspring passively with maternal antibodies (Bronsvoort et al., 2001; Barna and Bilkei, 2003). Therefore, recently infected gilts and low-parity sows are the primary source of *L. intracellularis* infection for their piglets (Mauch and Bilkei, 2004). Partial protection against long term infection and reinfection may be present in offspring of seropositive gilts (Mauch and Bilkei, 2004). However, the longer lasting seropositivity of the offspring of naïve gilts suggests reinfection to *L. intracellularis* (Mauch and Bilkei, 2004). Other possible mechanisms of
*L. intracellularis* transmission include fomites (rubber boots, coveralls, etc.) and biological vectors such as mice, birds and insects, and should be considered for future evaluations (Guedes 2004).

Persistence

Persistence of *L. intracellularis* can be classified into two categories; environmental and within the host animal. Environmental persistence consists of the ability of *L. intracellularis* to remain alive and stable for long periods of time among the various conditions found in the field. Persistence within the host animal consists of the ability of *L. intracellularis* to successfully colonize and multiply while evading the host’s immune response to infection over a period of time. Environmental survival of *L. intracellularis* in pig confinements is a key factor to the reinfection among swine herds. However, due to the difficulty of isolating *L. intracellularis* from feces or PE-infected intestines, viability studies to determine the resistance of this organism to various environmental and antimicrobial stresses are limited. In one study, Collins et al. (2000) investigated the rates of intestinal colonization of *L. intracellularis* in pigs after oral inoculation with feces from *Lawsonia*-positive pigs. The *Lawsonia*-infected feces had been stored at various temperatures (between 5-15°C) for up to 2 weeks and were found to be infectious when given to naïve pigs based on PCR and histological results.

A recent study revealed that pigs can intermittently shed *L. intracellularis* for a period of 12 weeks after experimental inoculation (Guedes and Gebhart, 2003c). These results demonstrated the capability of long term colonization and survival of...
L. intracellularis in the host animal (Guedes and Gebhart, 2003c). Pigs that may carry and frequently shed the pathogen without presentation of clinical symptoms for long periods of time are considered to be subclinically infected with PE (Jacobson et al., 2003b). A study comparing the clinical, morphological and microbial findings in animals from good and poor performance herds found that clinically healthy pigs were often infected by L. intracellularis (PCR +) and had poor growth performance compared to uninfected herds (Jacobson et al., 2003b).

Immune Responses and Immunity to L. intracellularis

The etiological characteristics of L. intracellularis, an obligate intracellular mucosal pathogen that is transmitted by the fecal-oral route, suggests that a protective immune response against L. intracellularis infections would involve aspects of humoral, mucosal and cell-mediated immunity. Many authors have identified specific host immune reactions to natural and experimental L. intracellularis exposure while some have identified possible mechanisms of protective immunity against PE in pigs.

Humoral immunity

The presence of serum antibodies against an intracellular bacterium was first described in studies conducted in hamsters where convalescent serum collected from those with severe lesions react with the intracellular bacteria embedded within affected tissue sections (Jacoby, 1978). An immunoassay was developed to detect serum IgA and IgM antibodies against L. intracellularis, but was found to detect antibodies only in pigs with severe lesions (Lawson et al., 1988). The anti-Lawsonia antibodies detected in growing
pigs were predominantly IgM and were short lived (Lawson et al., 1988). Holyoake et al. (1994) developed an ELISA that was able to detect a weak anti-\textit{Lawsonia} IgG antibody response in experimentally challenged pigs around 2 to 3 weeks post inoculation. Results in younger pigs from other studies revealed passively acquired IgG antibody at 3 weeks of age and then appeared to seroconvert between 7 and 24 weeks of age (Holyoake et al., 1994). An immunofluorescent antibody test proved to be more sensitive and specific in detecting IgG levels in pig sera (Knittel et al., 1998). In these experiments, the majority of pigs (90\%) seroconverted to an experimental challenge with a virulent pure culture \textit{L. intracellularis} by 3 weeks post challenge (Knittel et al., 1998). The duration of antibody detection was not determined in these controlled exposure studies since study termination was typically around 3 weeks post challenge. In a comparison study, serological responses and duration of antibody detection was evaluated among pigs receiving virulent and avirulent (vaccine) isolates of \textit{L. intracellularis} (Guedes and Gebhart, 2003a). Pigs challenged with virulent \textit{L. intracellularis} elicited a \textit{Lawsonia}-specific serum IgG response 2 weeks after inoculation and remained detectable by IPMA up to 13 weeks post challenge (Guedes and Gebhart, 2003a). Pigs challenged with an avirulent \textit{L. intracellularis} elicited a delayed \textit{Lawsonia}-specific IgG response that was not detected until 5 weeks post challenge (Guedes and Gebhart, 2003a). A delay in a serum antibody response and lower peak IgG titers (3280 and 480 for virulent and avirulent \textit{L. intracellularis}-exposed pigs respectively) may have been the result of host responses to 2 different antigen types (virulent and avirulent), differences in quantity of \textit{L. intracellularis} within each challenge or due to
reinfection because of the higher rates and duration of shedding in the virulent

*L. intracellularis* challenge group (Guedes and Gebhart, 2003a).

Previous studies involving the evaluation of an avirulent live *L. intracellularis* vaccine in pigs have demonstrated that protective immunity against PE does not rely on an efficient and robust humoral immune response (Kroll et al., 2004a). In these experiments, pigs did not have detectable anti-*Lawsonia* IgG serum antibodies using the IFAT as the detection assay up to 5 weeks post vaccination, but were significantly protected against a virulent *L. intracellularis* challenge exposure (Kroll et al., 2004a). These results were consistent with previous assessments that anti-*Lawsonia* serum IgG levels are not expected to correlate with protection because *L. intracellularis* is an obligate intracellular organism that resides in the cytoplasm of enterocytes (Guedes and Gebhart, 2003a). Local mucosal IgA levels and cell-mediated immune responses are probably more involved with protection against infection, therefore oral or intranasally delivered modified-live vaccines against *L. intracellularis* are preferred over parentally administered vaccines (Guedes and Gebhart, 2003a).

Maternally derived IgG, IgA and IgM antibodies specific for *L. intracellularis* may be important against early infections in recently weaned piglets. Results from a controlled challenge study revealed that the presence of IFAT-detectable IgG antibodies in piglets up to 5-6 weeks of age conferred significant maternal protection against a virulent *L. intracellularis* challenge exposure (Kroll et al., 2005a). High levels of IgA and IgG and to a lesser extent, IgM were found in the colostrum of hyperimmune sows indicating passive transfer of *Lawsonia*-specific maternal antibodies to piglets during the first few days.
of life (Kroll et al., 2005a). Seropositivity of gilts resulted in protective maternal immunity for up to 3 weeks when their piglets were challenged with virulent *L. intracellularis* (Bilkei, 1996). It is likely that piglets having maternal antibody protection shed *L. intracellularis* for a shorter period of time compared to piglets born to *Lawsonia*-naïve gilts (Gebhart and Guedes, 2001). However, effective length of protection provided by maternal immunity for pigs has not yet been established (Gebhart and Guedes, 2001).

A strong positive association between the *Lawsonia* seropositivity of grower-finisher pigs and the serological status of their dams was determined by Bronsvoort et al. (2001). The positive *Lawsonia* serological status of the sows was associated with lower anti-*Lawsonia* IgG antibodies in their offspring as a result of lack of exposure or passive immunity to the organism (Winkelman, 1996). In a controlled challenge exposure study, seropositivity in offspring of IFAT-negative gilts was highest 3 weeks (84%) post challenge and declined gradually to only 10% at 24 weeks post challenge (Barna and Bilkei, 2003). At the same time, the offspring of IFAT-positive gilts showed lower and faster-decaying seroprevalence in which only 32% were IFAT positive at 3 weeks post challenge and no anti-*Lawsonia* antibodies were detected by 15 weeks post challenge (Barna and Bilkei, 2003). These evaluations suggest that maternally derived, passive immunity may give partial protection against *L. intracellularis* infections to the offspring of seropositive gilts.

Passive immunization in pigs using chicken egg yolk antibodies has been evaluated as an alternative method for prevention of enteric disease (Winkelman et al., 2004). Large quantities of *Lawsonia*-specific antibodies secreted into the yolk of eggs from chickens hyperimmunized with purified, whole cell *L. intracellularis* antigen can be subsequently
harvested and fed to pigs. In this study, chicken anti-\textit{Lawsonia} antibody titers were detected up to 1:1,920 in hen sera at 4 weeks after the first immunization while egg yolk antibody titers were higher at 1:1000 to 1:10,000 (Winkelman et al., 2004). Pigs that received 2 kg of chicken anti-\textit{Lawsonia} antibodies had significant (p<0.05) increases in ADG (25%) and average daily field intake (27%) compared to pigs that received a placebo after virulent challenge with a mucosal homogenate containing \textit{L. intracellularis} (Winkelman et al., 2004). However, no statistical differences were noted among treatment groups regarding clinical symptoms, fecal shedding (PCR), gross and microscopic (IHC) lesions (Winkelman et al., 2004).

Mucosal immunity

Intestinal sections from pigs affected with PIA and PHE revealed immense accumulations of IgA in the apical cytoplasm of proliferating enterocytes (Lawson et al., 1979). These accumulations of IgA were also evident in the Peyer’s patches (Lawson et al., 1979) and in the cytoplasm of plasma cells underlying severe proliferative lesions (Holyoake, 1993). In other studies, IgA antibodies were detected in macrophages and cell debris of the crypt lumen in PHE cases (McOrist et al., 1992). However, in all of these reports, the detection of IgA may not have been a specific response to \textit{L. intracellularis}.

A study was conducted to observe the progression of an \textit{L. intracellularis} infection through the course of the disease and evaluate the production of \textit{Lawsonia}-specific mucosal secretory IgA (Guedes and Gebhart, 2002c). Immunoglobulin A titers (1:4) in intestinal gavages of infected pigs were first detected by day 15 post challenge by a modified IPMA
test. Detectable titers were evident up to day 29 challenge with IgA titers ranging from 1:4 to 1:16 in the affected pig gut (Guedes and Gebhart, 2002c). Positive IHC staining for *L. intracellularis*, microscopic lesions and crypt hyperplasia was evident up to day 29 post challenge thus correlating with the production and subsequent detection of IgA in the affected intestines (Guedes and Gebhart, 2002c). Further studies are warranted for evaluating the level of protective immunity and the IgA response against an *L. intracellularis* infection in pigs.

Cell-mediated immunity

The inflammatory response in pigs to PE is minimal which is indicative of a well developed lamina propria (Rowland and Lawson, 1974). In previous observations, accumulations of eosinophils were observed throughout the mucosa of pigs in the early stages of hemorrhage associated with PHE (Love and Love, 1979). Various immunocytological evaluations involving intestinal sections from pigs affected with PE indicated that the initial cell mediated immune response included a mild infiltration of cytotoxic and suppressor T cells, macrophages and B lymphocytes carrying MHC Class II motifs (McOrist et al., 1992). Pigs 12 to 16 weeks of age affected with PHE revealed moderate infiltration of mononuclear lymphoid cells and polymorphonuclear leukocytes in the lamina propria and in the dome area of the Peyer’s patches (McOrist et al., 1992). Only a mild infiltration of CD8+ and CD25+ T cells were found in the lamina propria during cases of PIA, a slightly greater infiltration of these cells along with lamina propria IgM-positive B cells were found in hemorrhagic lesions of PHE affected pigs (McOrist et al., 1992).
However, MacIntyre et al. (2003) demonstrated in a controlled challenge exposure study an association between the presence of *L. intracellularis* and reduced T cell and B cell numbers in affected pigs. These authors have indicated an inverse correlation between the level of *L. intracellularis* infection and lymphocyte populations; T cells decrease as *L. intracellularis* infection increases, thus indicating an immunosuppressive mechanism of this bacterium during pathogenesis. Despite an apparent suppression of immune responses, antigen-dependent lymphocyte mitogenicity of peripheral blood mononuclear cells from *Lawsonia* infected pigs compared to non-infected pigs has demonstrated specificity of lymphocyte responses to *L. intracellularis* (McOrist et al., 1992 and 1993).

Other studies have provided further evidence of a cell mediated response in pigs exposed to virulent and vaccine isolates of *L. intracellularis* (Guedes and Gebhart, 2003a). Peripheral blood mononuclear lymphocytes were harvested weekly from pigs on days 9 to 91 post inoculation and levels of IFN-γ were determined by an ELISPOT assay (Guedes and Gebhart, 2003a). *Lawsonia*-induced, IFN-γ secreting T cell responses became detectable at day 9 post-inoculation in pigs receiving $1.76 \times 10^8$ *L. intracellularis/ml* of virulent challenge and at day 28 post inoculation in pigs receiving $5.3 \times 10^5$ *L. intracellularis/ml* of avirulent challenge (Guedes and Gebhart, 2003a). Pigs that were inoculated with the vaccine isolate showed a delayed and lower cell-mediated immune response when compared to pigs that received a virulent *L. intracellularis* challenge (Guedes and Gebhart, 2003a). However, differences in cell-mediated response among treatment groups may be explained similarly to those mentioned above regarding the humoral response in this study (Guedes and Gebhart, 2003a). Regardless of these differences, both virulent and avirulent
isolates of *L. intracellularis* induce detectable cell-mediated immunity that lasted for at least 13 weeks in some animals (Guedes and Gebhart, 2003a). Significant roles for IFN-γ have been shown for other intracellular pathogens and from previous studies using a mouse *L. intracellularis* challenge model (Smith et al., 2000). Results from these experiments revealed that IFN-γ receptor knock out mice were substantially more susceptible and had higher levels of *L. intracellularis* infection and lesion development compared to wild-type mice (Smith et al. 2000). Interferon gamma, a Th1 cytokine, is involved in directing immune responses towards a cell-mediated response when host cells are insulted by intracellular pathogens (Smith et al. 2000). Natural infections with *L. intracellularis* may stimulate IFN-γ-secreting lymphocytes similar to those in experimental exposure studies and that these may be involved in natural clearance of infection (Smith et al. 2000).

In a *L. intracellularis* challenge model comparison study, pigs were given intradermal injections of different concentrations of *L. intracellularis* antigen (formalin-fixed whole cell [10⁷/ml to 10⁹/ml], sonicated fractions [25 μg/ml to 250 μg/ml] or outer membrane proteins [7.5 μg/ml to 75 μg/ml]), 20 days after virulent challenge exposure (Guedes and Gebhart, 2002c). Delayed-type hypersensitivity (DTH) reactions involving the detection of skin reactions (reddening and swelling) and IFN-γ were evaluated 24 h and 48 h post inoculation (Guedes and Gebhart, 2002c). This type of DTH typically occurs within 48 to 72 hours in a sensitized host and activates antigen-specific T cells to secrete cytokines that mediate the hypersensitivity reaction (Roitt et al., 1998). Results showed that *Lawsonia*-challenged pigs from both treatment groups (pure culture vs. mucosal homogenate) showed a dose dependent DTH reaction to the formalin-fixed, whole cell
*L. intracellularis* preparations that was more evident 24 hours after injection (Guedes and Gebhart, 2002c). Further studies are necessary for determining *Lawsonia*-specific induction of immunological memory and its duration and how it correlates to protective immunity in pigs.

**Control and Prevention Measures**

Several risk factors have been previously defined that predispose pigs to *L. intracellularis* infections and PE. Various control and prevention measures such as *Lawsonia*-susceptible antimicrobials have been implemented in pig production systems around the world and have been successful in reducing or controlling *L. intracellularis* infections and PE. However, increasing global pressures to reduce or eliminate the routine use of antibiotics as growth promoters in food producing animals have forced farmers, producers and veterinarians to rethink how to effectively control enteric diseases such as PE. Changes in daily diets, the use of vaccines and disinfectants have shown promise as viable alternatives for reducing and eliminating *L. intracellularis* in pigs without the continuous use of antimicrobials.

**Diet**

Different feeding strategies have been investigated to determine their level of influence on *L. intracellularis* infections in pigs (Boesen et al., 2004). Three experimental trials involving 144 weaned pigs were fed five different diets all derived from a standard diet based on wheat and barley as the primary carbohydrate source and soybean as the primary protein source (Boesen et al., 2004). Experimental diets among the 5 treatment
groups consisted of standard diets with the following modifications; 1) fine ground and pelleted, 2) coarse ground and non-pelleted, 3) fermented liquid feed, 4) 1.8% formic acid and 5) 2.4% lactic acid. The effects of fermenting, acidifying and grinding the feed on *L. intracellularis* colonization and the development of PE after experimental challenge with a gut homogenate containing a Danish isolate of *L. intracellularis* were investigated (Boesen et al., 2004). The mean duration of fecal shedding of *L. intracellularis* was significantly (p<0.05) lower in pigs consuming a fermented liquid standard diet compared to pigs fed a non-fermented standard diet (Boesen et al., 2004). All treatment groups had lower average daily weight gains compared to the non-infected controls. Histopathological examinations were uneventful as only a few pigs revealed *Lawsonia* antigen in the surface epithelium and surrounding macrophages (Boesen et al., 2004). These results along with decreasing PCR positives at the time of necropsy (4-5 weeks post challenge) suggest that pigs were recovering from *L. intracellularis* infections as the peak infection period for this organism is generally 3-4 weeks post exposure (McOrist et al., 1996a, Jensen et al., 1997). The authors concluded that the fermented liquid diet delayed the excretion of *L. intracellularis* and pigs fed a diet supplemented with 2.4% lactic acid showed signs of limited pathological lesions when grossly examined at 4 weeks post challenge (Boesen et al., 2004).

Disinfectants

Since *L. intracellularis* is effectively transmitted from pig to pig in the feces, reducing cross-contamination between pig groups would help reduce levels of infection and incidence of PE. Contaminated feces can be transported on dirty boots, clothing and
equipment therefore; simple biosecurity measures such as use of disinfectants should be implemented (Guedes, 2004). Previous ex vivo evaluations of various antimicrobial disinfectants and their effects on L. intracellularis found the bacteria to be highly susceptible to 3% cetrimide (quaternary ammonium), mildly susceptible to 1% providone-iodine and resistant to 1% potassium peroxymonosulfate or a 0.33% phenolic mixture (Collins et al., 2000). Currently, no scientific studies have been published that effectively demonstrates the killing rates of these and various other disinfectants on L. intracellularis in the field under various production systems. Such evaluations are extremely difficult to conduct due to the inherent difficulties of isolating obligate intracellular organisms. Future studies are warranted to identify disinfectants that effectively reduce or eliminate L. intracellularis in various housing conditions, pig manure and fomites such as boots, gloves and clothing. Also, it would be helpful to understand the frequency of which anti-Lawsonia disinfectants should be applied in typical production settings as well as defining the duration of time for effective microbial killing under various environmental conditions.

Antibiotics

The challenges of isolating and maintaining L. intracellularis cultures in vitro have made antimicrobial susceptibility testing an extremely difficult task. Regardless of this fact, in vitro evaluations of the minimum inhibitory concentration (MIC) of 20 different antimicrobial agents and the minimal bactericidal concentration (MBC) of 10 of these agents indicate a broad range of antibiotic activity against L. intracellularis (McOrist et al., 1995b; McOrist and Gebhart, 1995). Included in this list are the microlides (i.e. erythromycin and tylosin), tetracyclines, pleuromulins (i.e. tiamulin), penicillins, and
fluoroquinolones (McOrist et al., 1995b; McOrist and Gebhart, 1995). Antibiotics that have no activity against *L. intracellularis* include the aminoglycosides and aminocyclitols (i.e. neomycin, gentamicin and apramycin) (McOrist et al., 1995b; McOrist and Gebhart, 1995).

Advancements in *L. intracellularis* pure culture challenge models and diagnostic detection systems have enabled in vivo antibiotic sensitivity trials to evaluate numerous medication protocols for effective treatment and prevention of PE. Oral administration of tiamulin in weaned pigs at 50 ppm (water) from 2 to 21 days pre and post challenge or at 150 ppm (feed) from 7 to 21 days pre and post challenge was effective at preventing microscopic lesion development and reduced clinical symptoms after a virulent pure culture *L. intracellularis* challenge (McOrist et al., 1996b). In another study, tiamulin administered at an in-feed inclusion rate of 35 g/ton or 50 g/ton significantly (p<0.05) prevented the development of gross lesions in the ileum, significantly (p<0.05) reduced the prevalence and severity of microscopic lesions and significantly (p<0.05) reduced fecal shedding of *L. intracellularis* compared to the non-medicated, control group (Schwartz et al., 1999). This study also identified significant (p<0.05) reductions in seroconversion to *L. intracellularis* in medicated pigs even though these pigs were shedding the organism in their feces during the early infection period (Schwartz et al., 1999).

Oral administration of tylosin phosphate at 100ppm or 40ppm in feed 4 days pre-challenge up to 20 days post challenge followed by 40 or 20ppm for an additional 12 days prevented *Lawsonia*-specific microscopic lesions (McOrist et al., 1997b). Tylosin given to weaned pigs at 100ppm in feed 7 days after challenge with a virulent pure culture *L. intracellularis* prevented lesion development (McOrist et al., 1997b). Marstellar et al.
(2000) demonstrated effective treatment and control of PE through marked reductions in diarrhea, quicker resolve of gross lesions in the ileum and significantly (p<0.05) decreasing microscopic lesions when administering Tylan 200 by intramuscular injection.

Chlorotetracycline (CTC) administered to 4 week old pigs at 300 ppm and 600 ppm from 4 days pre challenge to 21 days post challenge prevented the development of gross and microscopic lesions of PE (McOrist et al., 1999). Weaned pigs treated with 500 ppm of CTC for 10 days followed by 100 ppm for 10 days at 2 weeks post inoculation with an oral dose of PHE-affected gut homogenate when diarrhea was evident revealed significantly (p<0.05) less microscopic lesions of PE (Winkelman et al., 1997). Treatment with 100 ppm of CTC in feed at 2 weeks post inoculation with a severe gut homogenate failed to reduce the frequency of microscopic lesion development compared to non-medicated control pigs (Winkelman et al., 1997). However, lesions were numerically less in medicated pigs at 3 and 4 weeks post challenge (Winkelman et al., 1997).

Studies conducted by Shultz et al. (1997) revealed that continuous in-feed medication of bacitracin methylene disalicylate and chlortetracycline for 14 days post virulent *L. intracellularis* challenge prevented clinical symptoms of PE. Chlortetracycline at 110 ppm, 220 ppm and 440 ppm in feed was effective against development of PE, however Bacitracin methylene disalicylate alone will not prevent *L. intracellularis* infections and was added at 33ppm to reduce the levels of confounding bacterial pathogens.

Lincomycin administered in feed at 44 ppm and 110 ppm for 21 consecutive days beginning after the onset of clinical symptoms was effective in reducing diarrhea while increasing ADG and feed conversion efficiency after a virulent *L. intracellularis* gut
homogenate challenge (Winkelman et al., 2002). Lincomycin was effective at significantly (p<0.05) reducing PE-specific mortality at 110 ppm only (Winkelman et al., 2002). However, lincomycin at both concentrations was unable to prevent histological lesions of PE in this study. Lincomycin given to pigs at 200 ppm in feed from 7 to 21 days post challenge with a virulent gut homogenate containing *L. intracellularis* effectively controlled clinical symptoms and reduced the severity of microscopic lesion development (Winkelman et al., 1998). However, these results indicated overall microscopic lesion development was not significantly reduced in medicated pigs compared to non-medicated control pigs (Winkelman et al., 1998). Medicated pigs showed reduced levels but not elimination of fecal shedding 2 to 4 weeks post challenge (Winkelman et al., 1998).

Increases in average daily weight gains and average daily feed intake was evident in pigs receiving 44 ppm/88 ppm/132 ppm and 88 ppm/132 ppm of lincospectin (lincomycin and spectinomycin) in feed of 3-4 week old pigs after a virulent pure culture challenge of *L. intracellularis* (McOrist et al., 2000). In another study, treatment with 125 ppm or 250 ppm of doxycycline for 14 days in 3-4 week old weaned pigs had beneficial effects in terms of reductions in diarrhea, prevalence of *L. intracellularis* in intestinal tissue (PCR or histological analysis) and increases in growth performance compared to non-medicated control pigs (Kyriakis et al., 2002a).

Josamycine, belonging to the therapeutic class of macrolides, revealed beneficial effects against PE under field conditions when administered to weaned pigs 3-4 weeks of age (Kyriakis et al., 2002b). Inclusion levels of 36 mg/kg and 50 mg/kg of feed reduced diarrhea, prevalence of *L. intracellularis* in the intestine (PCR and histological analysis)
while enhancing growth performance in medicated pigs compared to non-medicated, control pigs (Kyriakis et al., 2002b).

Several studies have been conducted to evaluate the effectiveness of valnemulin hydrochloride (1.45 to 3.75 kg/mg of body weight for 7 to 21 consecutive days post exposure) for the control of PE after a mucosal gut homogenate containing virulent *L. intracellularis* (Winkelman et al., 2000a), when given simultaneously during an experimental *L. intracellularis* challenge (Winkelman et al., 2000b) and during naturally occurring outbreaks of PE in Denmark (Haugegaard et al., 2000). Results from these studies have shown that valnemulin hydrochloride was effective in the control of PE at 25 ppm to 50 ppm in the feed by reducing clinical symptoms (diarrhea) and significantly (p<0.05) improving weight gain during the treatment period.

The most common treatment of PHE in adult pigs is tiamulin at 120 ppm, tylosin at 100 ppm, licomycin at 110 ppm, or chlorotetracycline at 300 ppm for 14 days delivered orally in pre-mixed feed (McOrist and Gebhart, 1999). Where PE is endemic in grower-finisher pigs, the preferred treatment is continuous in-feed medication with tiamulin at 50 ppm, chlortetracycline at 200 ppm, lincomycin at 110 ppm and tylosin at 100 ppm to minimize severe production losses caused by the disease (McOrist and Gebhart, 1999).

Vaccines

Immunological control of intracellular pathogens commonly involves cell mediated responses and is likely a primary factor in the control and prevention of *L. intracellularis* whether stimulation comes from inactivated, avirulent live, sub-unit or other vaccine types. Regardless of which approach is taken in the development, an effective vaccine must
protect against multiple strains of the pathogen. Presently, *L. intracellularis* is considered a monotypic, single strain organism with no known antigenic variation among various isolates around the world (McOrist et al., 2003).

Multiple genomic and proteomic evaluations over the years have confirmed that *L. intracellularis* is monotypic. Western blots of 6 different antigenic outer membrane proteins (OMPs) of 77, 69, 54, 42, 36 and 18 kDa have reacted similarly to monoclonal antibodies and convalescent serum from pigs previously exposed to virulent *L. intracellularis* (McOrist et al., 1987; Guedes and Gebhart, 2003c). Additionally, these antigenic profiles are conserved among 6 different laboratory isolates of *L. intracellularis* of differing host sources and geographical origins (Guedes and Gebhart, 2003c). Monoclonal antibodies generated against *Lawsonia* surface antigens, LsaA and LPS, have been used as the primary detection antibodies in histological assays which have been successful in detecting various field isolates from affected pig tissues and other animals (Smith and Lawson, 2001; Boesen et al., 2005a). Primer sequences based on the highly conserved 16s rDNA gene of *L. intracellularis* used in the standard PCR protocol for detecting *Lawsonia* DNA in feces and tissue detected all evaluated isolates of US and European origins (Knittel et al., 1996). Furthermore, these isolates shared the same morphological features and growth characteristics *in vitro* (Knittel et al., 1996). Japanese researchers have reported that genetic sequence of 3 potential virulence factors or antigens (superoxide dismutase, LsaA and a 50 kDa OMP) derived from a Japanese porcine isolate of *L. intracellularis* show >99% homology to a UK isolate NCTC 12657 (Koyama et al., 2004). These results suggest high genetic similarity among various isolates of *L. intracellularis*. 
The completion of the *L. intracellularis* genome sequence project will allow researchers to identify gene sequences involved in pathogenicity that could be viable targets for sub-unit vaccine approaches (Gebhart and Kapur, 2003). A partial DNA library was developed for obtaining clones for production of material for taxonomic, diagnostic and pathogenesis studies in addition to identifying potential protective antigens (Dale et al., 1998). Thus far, no current information has been reported defining potential immunogens as possible vaccine candidates.

Attenuated live vaccines have been the only approach proven successful for developing protective immunity towards intracellular pathogens such as *Brucella spp* and *Chlamydia spp.* (Su et al., 2000; Morrison et al., 2002; Ko and Splitter, 2003). Killed or subunit vaccine prototypes against intracellular agents (*Chlamydia* and *Brucella*) have been futile (Shaw et al., 2002; Ko and Splitter, 2003). Currently, an avirulent live *L. intracellularis* vaccine (Enterisol® ileitis) was developed by Boehringer Ingelheim Vetmedica, Inc. for use in pigs 3 weeks of age or older for control and prevention of gross and microscopic lesions typical of PE after virulent challenge (Knittel and Roof, 1999; Kroll et al., 2004a). This vaccine has been reported to stimulate both humoral and cell-mediated immunity in pigs however, a direct correlation regarding the quantification of these immune response types to the level of protection have not been established (Kroll et al., 2005b; Guedes and Gebhart, 2003a). The *L. intracellularis* avirulent live vaccine was easily and effectively administered orally through the drinking water and provided significant (p<0.05) reductions in the prevalence and severity of gross and microscopic (IHC) lesions, colonization (PCR / IHC), fecal shedding (PCR) while significantly (p<0.05)
improving average daily weight gains in pigs challenged with a virulent pure culture challenge (Kroll et al., 2004a). Onset and duration of immunity of this vaccine appears to be 3 to 4 weeks and ≥ 22 weeks post vaccination respectively (Kroll et al., 2004b). Various global field trials evaluating the efficacy of Enterisol® Ileitis have revealed significant (p<0.05) benefits in growth performance and reductions in % mortality against a natural L. intracellularis exposure in pigs (Sick et al., 2002; Keita et al., 2004; Kolb et al., 2004). Safety of Enterisol® Ileitis has been described in pregnant sows (Kroll et al., 2005a) and in studies consisting of inoculating pigs with repeat doses and an overdose, administration in one week old piglets and demonstrating a lack of reversion to virulence (Kroll et al., 2004b).

Conclusions

Proliferative enteropathy, an enteric disease of pigs and several other animal species, includes many different syndromes, all of which are caused by a monotypic obligate intracellular organism, Lawsonia intracellularis. Hallmarks of the disease include severe thickening of the mucosal epithelia of the small and sometimes the large intestines. This lesion is commonly illustrated as a garden hose because of the thick corrugated appearance of the affected tissue. Crypt hyperplasia and absence of goblet cells are due to unregulated proliferation caused by L. intracellularis infections.

The intracellular bacteria can be cultivated in adherent or suspension co-culturing eukaryotic cell systems under reduced oxygen or anaerobic conditions. Advancements in the growth and propagation of L. intracellularis within controlled suspension systems have
allowed scientists to develop pure culture challenge models. These models provide a basis for better understanding of the pathogenesis of PE as well as the genotypic and phenotypic characteristics of the bacterium. Models also allow researchers to define processes for attenuated live vaccine development, and to develop new and improved diagnostic techniques.

*Lawsonia*-specific diagnostic methods have improved to levels where veterinarians and producers can now accurately and consistently determine the onset of exposure and prevalence of *L. intracellularis* in swine farms. Diagnosis of PE in pigs is no longer restricted to post mortem analysis. Molecular-based techniques like real-time PCR and serological tools like IFAT, IPMA and ELISAs have increased our knowledge of disease prevalence and transmission among swine farms while speeding up the process of receiving high quality results in a timely fashion.

Proliferative enteropathy is endemic among swine farms all over the world having significant impact on growth performance resulting in major financial losses for producers. Various antibiotics are commercially available that have significant activity against *L. intracellularis* and aid in the control and prevention of PE. Recently, an avirulent live *L. intracellularis* vaccine was made available to the global swine market for use as an alternative tool or in conjunction with antibiotics through prescribed strategies for effective control and prevention of PE.

Despite all of the significant advancements made in the understanding of *L. intracellularis* and PE, much remains to be resolved regarding pathogenesis, identification of metabolic and virulence characteristics, and immune responses due to wild
type and vaccine exposure in pigs. Future research should focus on bioinformatics and the utilization of the genome sequence for identifying and characterizing important immunogens, enhancing molecular techniques for epidemiological research and improving growth in vitro.

Acknowledgements

The authors recognize the invaluable scientific contributions of their collaborators; Drs. Steven McOrist, Connie J. Gebhart, Roberto M. C. Guedes, David G. E. Smith, Patricia K. Holyoake, Alison M. Collins, Kristian Moller and Henriette Toft-Boesen and the research scientists and student interns who worked on L. intracellularis projects; Drs. J. Daemmgen, P. Hayes, K. Elbers and Mr. P. Utley, Ms. S. Gannon and Ms. K. Utley.

References


Elder RO, Duhamel GE, Mathiesen MR, Erickson ED, Gebhart CJ and Oberst RD (1997). Multiplex polymerase chain reaction for simultaneous detection of 


Guedes RMC and Gebhart CJ (2003a). Onset and duration of fecal shedding, cell-mediated and humoral immune responses in pigs after challenge with a pathogenic isolate or an attenuated vaccine strain of *Lawsonia intracellularis*. *Veterinary Microbiology* **91**: 135-145.


Jacobson M, Englund S and Ballagi-Pordany A (2003a). The use of a mimic to detect polymerase chain reaction-inhibitory factors in feces examined for the presence of


Rowland AC and Rowntree PGM (1972). A haemorrhagic bowel syndrome associated with intestinal adenomatosis in the pig. *Veterinary Record* 91: 235-241


Shaw J, Grund V, Durling L, Crane D, Caldwell HD (2002). Dendritic cells pulsed with a recombinant chlamydial major outer membrane protein antigen elicit a CD4+ type 2
rather than type 1 immune response that is not protective. *Infection and Immunity* **70**: 1097-1105.


Tomanova K and Smola J (2004). Use of a nested PCR for detection of *Lawsonia intracellularis* in swine and the number of infected farms in the Czech Republic.
Proceedings of the 18th International Pig Veterinary Society Congress, Hamburg, Germany. pp319.


Weber NL, Beckler DC, Kapur V and Gebhart CJ (2004). Variable number tandem repeat analysis for the differentiation of *Lawsonia intracellularis* from various animal species. A poster presented internally to the faculty and staff at the University of Minnesota, Minneapolis-St. Paul, Minnesota, USA.


CHAPTER 3. EVALUATION OF PROTECTIVE IMMUNITY IN PIGS FOLLOWING ORAL ADMINISTRATION OF AN AVIRULENT LIVE VACCINE OF LAWSONIA INTRACELLULARIS

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Jeremy J. Kroll, BS; Michael B. Roof, PhD; Steven McOrist, BVSc, PhD

Abstract

Objective

To evaluate the efficacy of an orally administered avirulent live vaccine to protect pigs against challenge exposure with virulent heterologous Lawsonia intracellularis.

Animals

One hundred eight weaned 3-week-old pigs (35 in experiment 1 and 73 in experiment 2).

Procedure

Two experiments were conducted. On day 0, vaccinates were orally administered vaccine via drench or in drinking water, whereas challenge-control pigs were administered a placebo. On day 21, pigs were challenge exposed with a virulent heterologous isolate of L. intracellularis. Clinical observations, weights, seroconversion, and fecal excretion of

2 Department of Biological Research and Development, Boehringer Ingelheim Vetmedica Inc, 2501 N Loop Dr, Ames, IA 50010
3 Address correspondence to Mr. Kroll.
4 Department of Clinical Development, QAF Meats, Bunge, Australia, United Kingdom.
*L. intracellularis* were measured until day 42. At study termination, pigs were euthanatized and examined for *L. intracellularis*-specific lesion development of the ileum and colon.

Results

Pigs receiving a single dose of vaccine were protected when challenge exposed with virulent *L. intracellularis* (at least $10^{7.7} \text{ TCID}_{50}/\text{dose}$). In experiment 1, vaccinates had significantly less fecal excretion (47% and 40% for days 35 and 42, respectively), compared to challenge-control pigs. In experiment 2, vaccinates had significantly less fecal excretion (50% and 58% for days 35 and 42, respectively), compared to challenge-control pigs. Significant reductions in lesion development were evident in the ileum of vaccinated pigs (70% and 56% at day 42 for experiments 1 and 2, respectively), compared to challenge-control pigs.

Conclusions and clinical relevance

Oral administration by drench or via drinking water of an avirulent live vaccine against *L. intracellularis* resulted in substantial protection against proliferative enteropathy among vaccinates and offers a better way to reduce stress of pigs during vaccine administration.

Introduction

Proliferative enteropathy is a common enteric disease of pigs following weaning that is caused by the obligate intracellular bacterium, *Lawsonia intracellularis*. The characteristic pathologic feature of the disease in all species is proliferation of immature
epithelial cells in the crypts of the ileum, large intestine, or both, leading to macroscopic thickening of the mucosa.\textsuperscript{3} \textit{Lawsonia} organisms are invariably found in the apical cytoplasm of these proliferative enterocytes.\textsuperscript{2,3} Genome and protein analysis of this bacterium indicates a remarkably homogenous single strain, suggesting that this agent only recently evolved as a result of a large genetic shift from its ancestors in the Desulfovibrio family.\textsuperscript{1,4} Clinical signs in affected pigs after weaning can include diarrhea and uneven weight gain.\textsuperscript{5} In pigs >12 weeks old, there can be a more acute clinical form of the disease, with the proliferative lesions accompanied by rapid onset of diffuse hemorrhage from the affected mucosa, leading to melena and sudden death.\textsuperscript{5} Estimates of the annual economic losses attributable to the clinical and subclinical effects of this disease are approximately $100 million for the US swine industry alone.\textsuperscript{6}

\textit{In vitro} culturing and the primary causative role of \textit{L. intracellularis} in all forms of the disease in pigs was established in 1993.\textsuperscript{2,7} Before that time, an on-farm trial of a formalin-killed bacterin developed against a common secondary agent, \textit{Campylobacter mucosalis}, resulted in equivocal results.\textsuperscript{8} It was concluded that an orally administered avirulent vaccine was the formulation most likely to be effective against \textit{L. intracellularis}, which is an obligate intracellular pathogen. Uptake of \textit{L. intracellularis} by intestinal mucosal macrophages, specific humoral responses, and antigen-related mitogenesis of porcine lymphocytes all are evident following oral infection.\textsuperscript{9-12} Pigs exposed orally to raw \textit{L. intracellularis}-infected fecal material were judged to have developed protective immunity in on-farm studies.\textsuperscript{2,4} Also, protective immunity to infection by other pathogenic intracellular bacteria, such as \textit{Brucella} spp or \textit{Chlamydia} spp, has only been documented
following appropriate delivery of whole live-attenuated bacteria. Killed or subunit vaccines developed against these agents have been far less successful, despite numerous attempts.

In the study reported here, we evaluated the efficacy of an avirulent live vaccine developed by use of *L. intracellularis* (isolate B3903) and various methods of oral administration followed by challenge exposure with virulent heterologous *L. intracellularis*. Clinical evaluation of this vaccine was performed in 2 experiments: the first was to ensure proof of concept of the efficacy of the vaccine and the second to establish equivalence for the various methods of oral administration to ensure it could be practical and acceptable to the pork industry for use in the vaccination of pigs.

**Materials and Methods**

**Animals**

Weaned 3-week-old pigs (*n* = 108) were used in the study. All pigs were confirmed to be negative for *L. intracellularis* by use of a polymerase chain reaction (PCR) assay conducted on fecal samples as well as analysis of serum samples prior to inoculation. Both experiments were conducted in accordance with established guidelines for good clinical practices.

**Experimental protocol**

Two experiments were conducted. Both experiments were conducted by use of a double-blind approach in which the group identity for each pig was not disclosed to the
study monitor or investigators until processing of samples, laboratory testing, compilation of data, and statistical analysis were completed.

Preparation of the vaccine

*Lawsonia intracellularis* isolate B3903 was originally isolated by use of routine coculture methods\(^6\) from the ileum of a sow in Denmark in 1998. That sow had acute hemorrhagic proliferative enteropathy, as confirmed by the use of routine histologic techniques and immunohistochemical (IHC) staining methods. The vaccine used in both experiments was manufactured commercially, placed in sterile 100-mL glass bottles, lyophilized, and stored at 4°C. The master seed of the vaccine was tested independently at 3 laboratory sites\(^{a,b,c}\) by use of routine cell culture and bacteriologic methods to ensure that it did not contain other adventitious viruses or bacteria.

Preparation of challenge-exposure inoculum

A pure culture of virulent heterologous *L. intracellularis* used as the challenge isolate in both experiments was acquired from a 12-week-old pig from the United States that was affected by acute hemorrhagic proliferative enteropathy. This challenge isolate (*L. intracellularis* N101494) was extracted from the severely infected ileum of the pig and cocultured by use of methods described elsewhere.\(^6\) Prior to the day of challenge exposure (day 21 of each experiment), routine tissue culture and bacteriologic methods\(^{2,6,17}\) were used

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\(^a\) Boehringer Ingelheim Vetmedica Inc, St Joseph, MO.
\(^b\) Q-one Biotech Ltd, Glasgow, Scotland.
\(^c\) Center for Veterinary Biologics Laboratory, Ames, IA.
to propagate the challenge isolate in vitro. On the day of challenge exposure, each active culture was harvested and pooled with frozen-thawed concentrated (10X to 20X) stocks of low-passage L. intracellularis N101494. Each challenge inoculum was administered within 1 hour after preparation.

Bacterial quantification

Quantification of the vaccine and challenge inoculum N101494 was accomplished by performing a TCID50 endpoint assay on a representative 1-mL aliquot. The vaccine was reconstituted with sterile water and serially diluted ten-fold in Dulbecco modified Eagle’s medium fortified with Ham’s F12 with heat-inactivated 5% newborn bovine serum. The total of live L. intracellularis (TCID50/dose) was calculated by use of the Reed-Muench method. Vaccinated pigs received a dose (10^4.9 TCID50) of the vaccine L. intracellularis (isolate B3903). Pigs in challenge-exposed groups each received a dose (10^7.7 TCID50) of a pure culture of virulent heterologous L. intracellularis N101494.

Experiment 1 (proof of concept)

The objective of the first experiment was to determine the efficacy of an orally administered avirulent live vaccine for L. intracellularis (isolate B3903) in pigs challenged with a virulent heterologous isolate. Thirty-five weaned 3-week-old pigs with negative results when tested for L. intracellularis were randomly allocated to 3 groups (15 vaccinates; 10 unvaccinated challenge-control pigs; and 10 unvaccinated, unchallenge-

\[ \text{d} \] DMEM F12, JRH Biosciences, Lenexa, KS.
\[ \text{e} \] Newborn Bovine Serum, JRH Biosciences, Lenexa, KS.
exposed, negative-control pigs). To avoid cross-contamination, each group was housed in a separate room but with the same conditions (temperature, ventilation, and pen size). Pigs in each room were housed in the same pen. On day 0 of the experiment, each of the 15 vaccinates was orally administered a single 2-mL dose of commercially available *L. intracellularis* vaccine in accordance with label instructions. Each dose was applied directly to the caudal portion of the oral cavity of each pig by use of a sterile plastic 10-mL syringe. Also on day 0, challenge-control and negative-control pigs were each orally administered a 2-ml dose consisting of uninfected tissue culture cells suspended in growth medium.

On day 21, vaccinates and challenge-control pigs were challenge exposed with a single dose of virulent heterologous *L. intracellularis* isolate N101494 containing $10^{7.7}$ TCID$_{50}$; challenge exposure was achieved by use of oral gavage. The remaining 10 negative-control pigs did not receive vaccine and were not challenge exposed during the experiment.

All pigs were examined daily to monitor clinical signs (including diarrhea), behavior, and body condition. Mean clinical score of 1 to 4 was calculated for each group on each day (1, clinically normal; 4, severe illness). An indirect fluorescence antibody test was conducted on serum extracted from blood samples that were collected weekly from each pig from days 0 to 42 of the experiment; results of the antibody test were used to determine rate of seroconversion to *L. intracellularis*. Fecal samples were collected weekly

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1 Enterisol® Ileitis, Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO.
from days 0 to 42; these samples were analyzed with a PCR assay that used
*L. intracellularis*-primers and methods described elsewhere. The *L. intracellularis*-specific primers used in this PCR assay cannot differentiate between various
*L. intracellularis* isolates. Extraction and purification of bacterial DNA from fecal samples were conducted by use of a rapid DNA extraction kit.

Weight measurements were obtained on days 0, 21, and 42 to determine mean daily weight gain of each group of pigs. Mean initial weights were uniform among all groups (variation of <0.2 kg/pig). Weight gains were calculated for 2 distinct phases of the experiment. The first phase (days 0 to 21) represented the period between inoculation and challenge exposure and was used to evaluate immediate effects of inoculation with the vaccine. The second phase (days 21 to 42) represented the period between challenge exposure and end of the experiment and was used to evaluate the effect of challenge exposure with a virulent heterologous organism.

On day 42 of the experiment, all pigs in all 3 groups were euthanatized. Postmortem examinations were performed to determine the extent of macroscopic and microscopic lesions in the ileum and colon of the pigs. Macroscopic lesions in the ileum or colon were scored on the basis of the severity of mucosal thickness (1, normal; 2, mild thickening; 3, moderate thickening-inflammation; and 4, severe thickening-inflammation-mucosal hemorrhaging or necrosis).

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* IsoQuick DNA extraction kit, Orca Research Inc., Bothell, WA.
Samples of ileum and colon (2 to 4 cm in length), tonsils, and mesenteric lymph nodes were collected, fixed by immersion in buffered formalin, and processed for detection of microscopic lesions. This included staining with H&E and IHC staining by use of specific *L. intracellularis* monoclonal antibodies. Immunohistochemical analysis by use of *L. intracellularis*-specific monoclonal antibodies is currently considered the criterion-referenced test for assessment of the actual infection status of a pig. Microscopic lesions in IHC-stained tissues were scored separately on the basis of severity of *L. intracellularis*-specific cell proliferation (0, normal; 1, mild-focal; 2, moderate-diffuse; and 3, severe-diffuse). Mean scores for microscopic lesions and the frequency of lesions detected in the affected tissues were calculated for group comparisons.

In addition, 6 to 10 cm of fresh tissue samples from the ileum, colon, tonsils, and mesenteric lymph nodes were collected from each pig. Each fresh sample was subjected to extraction of bacterial DNA and the *Lawsonia*-specific PCR assay described previously to determine the extent of possible tissue colonization.

**Experiment 2 (bioequivalence of oral administration)**

The objective of this experiment was to evaluate the efficacy of orally administering the avirulent live vaccine (*L. intracellularis* isolate B3903) by 2 methods, followed by challenge exposure with virulent heterologous organisms. Seventy-three weaned 3-week-old pigs with negative results when tested for *L. intracellularis* were randomly allocated to 4 groups (20 vaccinates with vaccine orally administered via water delivery, 20 vaccinates with vaccine orally administered via a drench, 20 unvaccinated challenge-control pigs, and
13 unvaccinated, unchallenged, strict-control pigs). Housing and measures to ensure biosecurity for each group were similar to those described for experiment 1.

On day 0, pigs in the water-delivery vaccinate group were orally vaccinated by ingesting the vaccine that was added to the drinking water. The reconstituted vaccine was added to a volume of water that we estimated would be consumed by 3-week-old pigs within a 4-hour time period (i.e., 2 L of vaccine-containing water in a clean trough); it was the sole water source available to the pigs. The amount of vaccine added to the group’s drinking water was calculated to deliver 1 dose/pig. These pigs were not subjected to withholding of water prior to this vaccination process.

Pigs vaccinated orally via drench were each administered a single 2-mL dose of the vaccine. Each dose was applied directly to the caudal portion of the oral cavity of each pig by use of a plastic 10-mL syringe with a plastic tip canula. Pigs in the challenge-control group were administered 2 mL of the tissue culture growth medium directly to the caudal portion of the oral cavity. Pigs designated as strict-control pigs were not administered vaccine or tissue culture growth medium and thus were unvaccinated not challenge exposed during the experiment.

On day 21, both vaccinate groups and the challenge-control group was challenge exposed via oral gavage with a single dose of a virulent heterologous culture of *L. intracellularis* isolate N101494, as described for experiment 1. Collection of samples and clinical monitoring were conducted as for experiment 1. On day 42, pigs were euthanatized and postmortem examinations were conducted. All examinations were conducted in the same manner as for experiment 1, except for the scoring system for
macroscopic lesions. In experiment 2, this system for scoring macroscopic lesions was modified to increase scrutiny among degrees of lesion severity as follows: 0, normal; 1, mild thickening; 2, moderate thickening-inflammation; 3, severe thickening-inflammation; 4, severe thickening-inflammation-edema-mucosal hemorrhaging; and 5, necrosis. Mean scores for macroscopic lesions were calculated for group comparisons.

Statistical analysis

All key variables were analyzed statistically. Macroscopic lesions, microscopic lesions, clinical scores, and body weight gains were analyzed by use of a 1-way ANOVA or Kruskal-Wallis test; whereas tissue colonization (PCR assay), fecal shedding (PCR assay), and seroconversion were analyzed by use of $\chi^2$ or Monte-Carlo tests.\(^{24, 25}\) For experiment 1, the primary indicator of vaccine efficacy was considered to be a significant ($P<0.05$) reduction in the prevalence and severity of macroscopic and microscopic lesions in the ileum and colon. This was used to document the degree of protection achieved for administration of the vaccine. For experiment 2, the primary indicator used to document efficacy of the 2 administration methods was a significant reduction ($P<0.05$) in prevalence and severity of macroscopic and microscopic lesions in the ileum and colon.

Results

Safety of the vaccine and administration procedures

We did not detect adverse reactions attributable to the vaccine or vaccination procedure among any vaccinated pigs throughout the study.
Experiment 1

Analysis of clinical observations did not reveal significant differences between vaccinates and unvaccinated challenge-control pigs for diarrhea, behavior, and body condition. Mean initial weight for vaccinates, challenge-control pigs, and negative-control pigs was 8.2, 8.0, and 8.0 kg/pig, respectively, whereas mean final weight for vaccinates, challenge-control pigs, and negative-control pigs was 32.9, 31.0, and 32.5 kg/pig, respectively. Average daily weight gain did not differ significantly among any of the groups during the first 21 days of each experiment. After challenge exposure (i.e., days 21 to 42), average daily weight gain did not differ significantly between vaccinates (0.73 kg/d) and negative-control pigs (0.74 kg/d); however, the challenge-control pigs had a significantly (P<0.05) lower average daily weight gain (0.66 kg/d), compared with values for the vaccinates and negative-control pigs.

During the period before challenge exposure (days 0 to 21), *Lawsonia*-specific PCR analysis of fecal samples revealed that none of the groups had positive results for *L. intracellularis* (Figure 1). Initial PCR detection of *L. intracellularis* in the feces of pigs in any of the groups was on day 28 of the study (data not shown). On day 35 (14 days after challenge exposure), a significantly (P<0.05) lower proportion of vaccinated pigs (5/15, 33%) had PCR-positive fecal samples, compared with the proportion in the challenge-control group (8/10, 80%). On day 42 (termination of experiment), a significantly (P<0.05) higher proportion of pigs in the challenge-control group shed *L. intracellularis* (4/10, 40%), compared with the proportion for the vaccinated group (0/15, 0%).
Figure 1. Fecal shedding of *Lawsonia intracellularis* in pigs orally vaccinated with an avirulent live vaccine and unvaccinated challenge-control pigs that were challenge exposed 21 days later with heterologous *L. intracellularis* during experiment 1. Fecal shedding was determined on the basis of positive results for a polymerase chain reaction assay. Fifteen pigs were vaccinated and challenged exposed (triangle), 10 pigs were unvaccinated and challenge exposed (square), and 10 pigs were unvaccinated and not challenge exposed (diamond). Day 0 is the day of oral administration of vaccine or culture medium.

Figure 2. Seroconversion to *L. intracellularis* in pigs orally vaccinated with an avirulent live vaccine and unvaccinated challenge-control pigs that were challenge exposed 21 days later with heterologous *L. intracellularis* during experiment 1. Seroconversion was determined on the basis of results for an indirect fluorescence antibody assay. Fifteen pigs were vaccinated and challenge-exposed (square), 10 pigs were unvaccinated and challenge exposed (triangle), and 10 pigs were unvaccinated and not challenge-exposed (diamond). Day 0 is the day of oral administration of vaccine or culture medium.
The pattern of seroconversion during the course of the experiments was similar to the rates of fecal shedding among vaccinates and challenge-control pigs (Figure 2). All pigs were seronegative on the basis of analysis of indirect fluorescence antibody testing of sera obtained during the 3 weeks before and on the day of challenge exposure (i.e., days 0 to 21). On day 35 (14 days after challenge exposure), 4 of 15 (27%) vaccinated pigs were seropositive for *L. intracellularis*, whereas only 1 of 10 (10%) pigs were seropositive in the unvaccinated challenge-control group. On day 42 (termination of experiment), 6 of 15 (40%) vaccinates were seropositive, whereas 8 of 10 (80%) challenge-control pigs were seropositive. We did not detect significant differences in the pattern of seroconversion among treatment groups at any time period throughout the experiment.

*Lawsonia*-specific PCR analysis of various tissues revealed a higher proportion of mesenteric lymph nodes with positive results in the challenge-control group (3/10, 30%), compared with the vaccinates (0/15, 0%). Tissues obtained from the tonsils of all pigs were PCR-negative for *L. intracellularis* DNA during postmortem examination. Positive results by use of the PCR were less frequently found in tissues of the ileum and colon obtained from vaccinates (2/15 [13%] and 1/15 [7%], respectively), compared with results for ileum and colon obtained from challenge-control pigs (4/10 [40%] and 3/10 [30%], respectively).

Evaluation of the variables used to determine primary efficacy revealed that the mean scores for macroscopic lesions were significantly lower for the ileum (P<0.001) and colon (P<0.05) in the vaccinates (1.5 and 1.0, respectively) than in the unvaccinated challenge-control group (3.6 and 2.0, respectively). Mean score for macroscopic lesions of the ileum and colon in the vaccinates was similar to the score in the unvaccinated,
unchallenged negative-control group (Table 1). The IHC evaluation of ileum and colon tissues revealed a significantly (P<0.001) higher number of *Lawsonia*-specific lesions in the challenge-control group (mean score for ileum, 2.4; mean score for colon, 1.5), compared with the vaccinates (mean score for ileum, 0.4; mean score for colon, 0.0). Mean score for microscopic lesions of the ileum and colon in vaccinates was similar to the mean score of those tissues in the unchallenged negative-control group.

Experiment 2

Analysis of clinical observations did not reveal significant differences among vaccinates (orally administered via drench or delivery in water supply) and challenge-control pigs during the experiment. Mean initial weight for vaccinates via drench, vaccinates via water supply, challenge-control pigs, and negative-control pigs was 5.5, 5.5, 5.4, and 5.4 kg/pig, whereas mean final weight for each group was 26.7, 26.0, 24.9, and 27.3 kg/pig. Daily weight gains were numerically higher for pigs vaccinated via drench but not significantly different from values for the challenge-control pigs during the experiment. However, during days 21 to 42, average daily weight gain of the challenge-control group (0.49 kg/pig) was significantly (P<0.05) lower than that of the water-delivery vaccinates (0.55 kg/pig).

Fecal shedding of *L. intracellularis* became detectable by use of the PCR assay on day 28 (7 days after challenge exposure) in 4 of 20 (20%) challenge-control pigs. On day 35 of the experiment, significantly (P<0.05) more PCR-positive pigs were evident in the challenge-control group (14/20, 70%) than in the water-delivery and drench vaccinates.
Table 1. Mean ± SE scores for macroscopic and microscopic lesions in samples of ileum and colon obtained from 3 groups* of pigs in experiment 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Pigs per group (n)</th>
<th>Group identification</th>
<th>†Average macroscopic lesion scores (ileum)</th>
<th>†Average macroscopic lesion scores (colon)</th>
<th>‡Average microscopic lesion scores (ileum)</th>
<th>‡Average microscopic lesion scores (colon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>Vaccinates</td>
<td>1.5 (±0.19)a</td>
<td>1.0 (±0.0)a</td>
<td>0.4 (±0.19)a</td>
<td>0.0 (±0.0)a</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>Challenge Controls</td>
<td>3.6 (±0.22)b</td>
<td>2.0 (±0.39)b</td>
<td>2.4 (±0.34)b</td>
<td>1.5 (±0.43)b</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>Negative Controls</td>
<td>1.1 (±0.1)a</td>
<td>1.0 (±0.0)a</td>
<td>0.0 (±0.0)a</td>
<td>0.0 (±0.0)a</td>
</tr>
</tbody>
</table>

* Groups consisted of pigs vaccinated by oral administration of an avirulent live vaccine of \textit{Lawsonia intracellularis} on day 0 and challenge exposed on day 21 with virulent heterologous \textit{L. intracellularis} (vaccinates), pigs that were orally administered culture medium on day 0 and challenge exposed on day 21 with virulent heterologous \textit{L. intracellularis} (challenge-control pigs), and pigs that were orally administered culture medium on day 0 and were not challenge exposed (negative-control pigs).

†Macroscopic lesions were scored on a scale from 1 (normal) to 4 (severe thickening).

‡Microscopic lesions were scored on a scale from 0 (normal) to 3 (severe-diffuse).

\textsuperscript{a,b} Like letters indicate no significant difference (p>0.05).
(2/20 [10%] and 6/20 [30%], respectively). On day 42, significantly (P<0.05) more PCR-positive pigs were again evident in the challenge-control group (15/20, 75%) than in the water-delivery and drench vaccinates (2/20 [10%] and 4/20 [20%], respectively).

All pigs were seronegative on the basis of analysis of serum samples obtained during the 3 weeks before and at the time of challenge exposure. Seroconversion first became apparent on day 28 of the experiment. At that time, 4/20 (20%) pigs in the drench-vaccinate group were seropositive, whereas anti-Lawsonia IgG antibodies were not detected in the serum samples obtained from any other group, including the challenge-control group. On day 35, the proportion of seropositive pigs was 3 of 20 (15%) for water-delivery vaccinates, 9 of 20 (45%) for drench vaccinates, 4 of 20 (20%) for challenge-control pigs, and 0 of 13 (0%) for unvaccinated, unexposed negative-control pigs. On day 42, the proportion of seropositive pigs was 4 of 20 (20%) for water-delivery vaccinates, 10 of 20 (50%) for drench vaccinates, 12 of 20 (60%) for challenge-control pigs, and 0 of 13 (0%) for unvaccinated, unchallenged negative-control pigs. On days 35 and 42, there were no significant differences among both groups of vaccinates and challenge-control pigs with regard to the proportion of seropositive pigs.

Lawsonia-specific PCR testing for tissue colonization yielded negative results for the tonsils and mesenteric lymph nodes. On day 42, the proportion of ileum samples with positive results when tested by use of the Lawsonia-specific PCR assay was 0 of 20 (0%) for water-delivery vaccinates, 2 of 20 (10%) for drench vaccinates, 4 of 20 (20%) for challenge-control pigs, and 0 of 13 (0%) for unvaccinated, unchallenged negative-control pigs. On day 42, the proportion of colon tissues with positive results when tested by use of
the *Lawsonia*-specific PCR assay was 1 of 20 (5%) for water-delivery vaccinates, 4 of 20 (20%) for drench vaccinates, 4 of 20 (20%) for challenge-control pigs, and 0 of 13 (0%) for unvaccinated, unchallenged negative-control pigs. No significant differences among groups were detected for *L. intracellularis* tissue colonization by use of the PCR assay.

Evaluation of the primary variables used to determine vaccine efficacy revealed that in the challenge-control group, mean scores for macroscopic lesions was significantly (P<0.05) higher (i.e., lesions were more severe) in the ileum (2.55) and colon (0.75) than in those tissues for either the water-delivery (0.2 and 0.2, respectively) or drench (0.6 and 0.2, respectively) vaccinates. Similarly, the proportion of samples with positive results for the *Lawsonia*-specific IHC analysis and the mean scores for microscopic lesions in the ileum was significantly (P<0.05) lower in both vaccinate groups, compared with values for the challenge-control pigs (Table 2). Mean scores for microscopic lesions in the colon were significantly (P<0.05) reduced in both vaccinates groups (water delivery, 0.15; drench, 0.25), compared with the mean score for the challenge-control group (0.7).

**Discussion**

Results of these experiments document that oral administration of an avirulent live vaccine against *L. intracellularis* by direct drench or via drinking water to young pigs provides substantial amounts of protection against subsequent challenge exposure with virulent heterologous *L. intracellularis*. This was confirmed by a significant (P<0.05) reduction in development of lesions as indicated by the prevalence and severity of
Table 2. Mean ± SE scores for microscopic lesions and percentage of tissues with positive results for immunohistochemical (IHC) analysis by use of *Lawsonia*-specific monoclonal antibodies in samples of ileum and colon obtained from 4 groups\(^*\) of pigs in experiment 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Pigs per group (n)</th>
<th>Group identification</th>
<th>‡Average microscopic lesion scores (ileum)</th>
<th>% IHC positive (ileum)</th>
<th>‡Average microscopic lesion scores (colon)</th>
<th>% IHC positive (colon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>Water Delivery</td>
<td>0.30 (±0.18)(^a)</td>
<td>15(^a)</td>
<td>0.15 (±0.11)(^a)</td>
<td>10(^{a,b})</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>Oral Drench</td>
<td>0.65 (±0.25)(^a)</td>
<td>30(^a)</td>
<td>0.25 (±0.18)(^{a,b})</td>
<td>10(^{a,b})</td>
</tr>
<tr>
<td>3</td>
<td>19**</td>
<td>Challenge Controls</td>
<td>2.32 (±0.29)(^b)</td>
<td>79(^b)</td>
<td>0.70 (±0.29)(^b)</td>
<td>35(^b)</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>Strict Controls</td>
<td>0.0 (±0.0)(^c)</td>
<td>0(^b)</td>
<td>0.0 (±0.0)(^a)</td>
<td>0(^b)</td>
</tr>
</tbody>
</table>

\(^*\) Groups consisted of pigs vaccinated by oral administration of an avirulent live vaccine of *Lawsonia intracellularis* on day 0 via addition to the water supply and challenge exposed on day 21 with virulent heterologous *L. intracellularis* (vaccinates-water delivery), pigs vaccinated by direct oral drench administration of a 2-mL volume to each pig and challenge exposed on day 21 with virulent heterologous *L. intracellularis* (vaccinates-drench), pigs administered culture medium on day 0 and challenge exposed on day 21 with virulent heterologous *L. intracellularis* (challenge-control pigs), and pigs that were not administered vaccine or culture medium and were not challenge exposed (strict-control pigs). \(^{**}\) One ileum sample had inflammation and fibrosis indicative of proliferative enteropathy and as a result, mucosal crypts had been shed and could not be evaluated.

\(^\ddagger\) Microscopic lesions were scored on a scale from 0 (normal) to 3 (severe-diffuse).

\(^a,b,c\) Within a column, values with different superscript letters differ significantly (P<0.05)

\(^\oplus\) Group not included in the statistical analysis of percentage IHC positive samples.
macroscopic and microscopic lesions in the ileum of vaccinated pigs. Significant (P<0.05) higher average daily weight gains (experiment 2) and reduced fecal shedding of *L. intracellularis* (experiments 1 and 2) were also detected in vaccinated pigs after challenge exposure.

Vaccinates did not shed or seroconvert during the period prior to challenge exposure; however, we did not begin to collect samples until 7 days after vaccination. It is possible that collection of samples prior to day 7 or improved PCR assays may reveal shedding in vaccinates prior to day 7 after vaccination. However, the lack of detectable *L. intracellularis* in the feces of all vaccinated pigs during the 3-week period after vaccination suggests that the isolate used in the live avirulent orally administered vaccine is not likely to have a great potential to spread to other pigs via the feces. It is common for avirulent bacterial isolates to have a reduced capacity for replication in the host and, hence, a reduced ability to spread to penmates. This compares to virulent isolates, including those of *L. intracellularis*, which typically are adapted to maximize their animal-to-animal spread and can attain high amounts of organisms in the feces. Analysis of results of these studies on shedding of vaccine isolates of *L. intracellularis* suggests that this organism also fits the pattern of bacterial adaptation and virulence. Therefore, feces from vaccinates should not be considered a reliable source of useful material from which to obtain vaccine isolates. The lack of detectable seroconversion following vaccination in both of the experiments reported here was consistent with our experiences involving this vaccine isolate and was not an unexpected result for oral versus parenteral administration of vaccine. In these experiments, seroconversion was not necessary for protecting vaccinated
pigs from disease, suggesting that nonhumoral factors stimulated by vaccination are perhaps more important for immunity to *L. intracellularis*.

A general advantage of the use of an avirulent live vaccine is that the efficacy and duration of immunity are usually superior to those for inactivated vaccines, because the host’s immune system is exposed to all of the antigenic properties of the organism in its native state. Specifically for intracellular bacterial agents such as *L. intracellularis*, a live avirulent vaccine offers excellent protection for vaccinated animals because of a complete T-cell-based immune response, possibly in conjunction with a complete humoral or mucosal immune response. This response develops even with administration of small doses of avirulent agent and is in contrast with the variable or poor immunity associated with the use of subunit or killed vaccine types for intracellular bacteria. This is also true for obligate intracellular bacteria (*Chlamydia* spp) that cause pathogenic infections within mucosa. Challenge exposure of pigs via oral administration of live *L. intracellularis* consistently leads to intracellular infection in the crypt epithelial cells of the intestinal mucosa. Many studies have also revealed that mucosal macrophages immediately beneath the intestinal epithelium regularly have intracellular infections attributable to *Lawsonia* sp during the active infection phases. This infection of macrophages implies a clear exposure of the host’s immune system to *L. intracellularis* and presentation of its antigens to other components of the immune system in the intestinal mucosa and beyond, probably via a Th1-based immune response typical of that to intracellular organisms. In a clinical study of pigs orally exposed to wild-type *Lawsonia* sp or to another live avirulent vaccine isolate, investigators determined that pigs developed intestinal infections and cell
mediated T-cell responses in a similar manner. The B cell or humoral and mucosal antibody responses of pigs to *L. intracellularis* infection have been characterized in several studies.\(^9\)\(^{11}\)\(^{18}\)\(^{26}\)\(^{27}\) Similar to most bacterial infections, an initial IgM antibody response is followed by a peak of IgG antibody concentrations with a subsequent decrease during the next few weeks. In most cases of acute hemorrhagic proliferative enteropathy, the IgG peak is much higher and its subsequent decrease is much slower than in animals with the chronic form of the disease.\(^{24}\)\(^{27}\) Mucosal infection is associated with a high amount of IgA mucosal antibody.\(^{11}\) Therefore, both T- and B-cell responses are associated with the development of protective immunity in pigs infected with *L. intracellularis*. A study\(^{13}\) that investigated immunity developed against other intracellular bacteria revealed that the T-cell response is the more active response for inducing actual immune protection, although local antibody responses are also likely to be important in the target mucosa.

Possible immunogenic sites on *L. intracellularis* have not been fully explored. A wide range of outer membrane proteins and glycoproteins have been identified in initial studies.\(^{1,4}\) Some of these were found on bacteria located in the intestines in pigs with natural infections.\(^{28}\)\(^{29}\) One of these surface antigens was characterized and cloned in a subsequent study, but its exact role in the immune process in natural infection is not clear.\(^{28}\)\(^{29}\) Detailed studies\(^{14,15,30,31}\) of several other intracellular bacteria have established that single antigen peptides used in vaccines are highly unlikely to produce a meaningful or protective immune response in animals, compared with the excellent protection afforded by use of whole live avirulent bacteria.
The vaccine isolate of *L. intracellularis* used in the experiments reported here (B3903) originated from an acutely infected pig in Denmark, whereas the challenge isolate (N101494) originated from a pig born in the United States that was afflicted with the same form of disease (hemorrhagic proliferative enteropathy). Therefore, the respective *L. intracellularis* isolates used in our experiments were separated by a time frame of 3 years and a distance of thousands of miles. Despite these differences, genomic and proteomic analysis of *L. intracellularis* indicated that it is a single strain or monotypic organism with no variant strains yet detected.\textsuperscript{1,3} This is also consistent with the suggestion that *L. intracellularis* is a recently evolved intracellular organism that has had a large genetic shift from its free-living Desulfovibrio family ancestors.\textsuperscript{1,3} The difficulty of culturing virulent *L. intracellularis* precluded the use of challenge exposures with a wide range of heterologous organisms. However, we consider that heterologous protection was achieved in these initial controlled experiments.

Some of the reasons to investigate vaccination via a water-delivery system are concerns about stress and injury to pigs, amount of human-animal contact time, costs, and labor (including possible difficulties with full compliance and injury to humans administering the vaccine). These factors are frequently seen as a result of numerous vaccinations to each pig in a herd, compared with the ease and consistency of administration of vaccine via the drinking water. Additional advantages of oral administration of vaccine via drenching of each animal or for a herd via the water supply include elimination of the possible transmission of major blood-borne infections (e.g., porcine reproductive and respiratory syndrome virus) via multiple-use needles.\textsuperscript{32} Oral
administration of vaccine would reduce concern among meat retailers about injection-site reactions or broken needles retained in carcasses after attempted injections. Methods for mass vaccination have been widely and effectively used on poultry farms for many years, and we speculate that they will become more widely used on swine farms as the number of pigs per farm increases. An additional possible benefit of an effective vaccine against \textit{L. intracellularis} is that the total antibiotic usage for prevention and treatment of proliferative enteropathy on swine farms may be reduced. It is possible that antibiotic use could be reduced considerably if this endemic disease could be controlled through use of vaccines alone or vaccines used in combination with strategic, periodically administered feed-additive antibiotics rather than constant use of feed-additive antibiotics.

Acknowledgement

The authors thank Mike Daniel and Jeff Knittel for assistance with vaccine development and Philip Utley and Sarah Gannon for technical assistance. We would like to thank the veterinarians and clinical staff at Veterinary Resources, Inc. for their work in the animal trials and Boehringer Ingelheim Vetmedica, Inc. for financing this project.

References


CHAPTER 4. MATERNAL ANTIBODY EXPOSURE AND VACCINATION WITH AN ORAL AVIRULENT LIVE LAWSONIA INTRACELLULARIS VACCINE (ENTERISOL® ILEITIS) IN PIGS

A paper submitted to Research in Veterinary Science

J. J. Kroll1,2 M. B. Roof,2 and S. McOrist3

Running title: Lawsonia intracellularis vaccine

Key words: proliferative enteropathy, Lawsonia intracellularis, vaccine, maternal immunity, sow safety

Abstract

The efficacy of a vaccination strategy applied at or after weaning for subsequent enteric diseases entails assessment of whether maternal (passive) immunity present after weaning could interfere with protective vaccinal (active) immunity. Our primary objective in this study was to assess vaccine efficacy in piglets with maternal antibodies against proliferative enteropathy (PE, ileitis). Secondly, we assessed whether passive immunity alone protects piglets against PE. Results demonstrated vaccine efficacy in piglets born from both Lawsonia-naïve and hyperimmune sows indicating that vaccination is efficacious even in the face of maternal immunity when administered to maternally positive pigs at 3 weeks of age. Furthermore, results suggest passive immunity provides some protection in piglets against virulent challenge exposure for at least six weeks after birth. We concluded

1 Corresponding author (jkroll@bi-vetmedica.com).
2 Boehringer Ingelheim Vetmedica Inc., 2501 North Loop Drive, Ames, IA 50010, USA.
3 QAF Industries, Redlands Road, Corowa, NSW 2646, Australia.
that administering a live, attenuated *Lawsonia intracellularis* vaccine (Enterisol® Ileitis) to piglets with maternal immunity did not reduce the overall efficacy of the vaccine.

**Introduction**

Proliferative enteropathy (PE, ileitis) is a common enteric disease of pigs worldwide, caused by the obligate intracellular bacterium, *Lawsonia (L.) intracellularis* (McOrist et al., 1993 and 1995). The characteristic pathologic feature is a proliferation of immature enterocytes in the intestinal crypts of the ileum and colon. These cells contain the 1.5 μm long, vibroid shaped *L. intracellularis* bacteria within their apical cytoplasm (Rowland and Lawson, 1974). In many cases, these bacteria can also be visualised within macrophages located in the lamina propria between crypts, and in mesenteric lymph nodes (Frisk and Wagner, 1977; Roberts et al., 1980). Genome and protein analysis of *L. intracellularis* indicates a remarkably homogenous “single strain,” suggesting that this agent only recently evolved in a large genetic shift from its *Desulfovibrio* family ancestors (McOrist et al., 1995). The clinical signs in affected post-weaned animals can include diarrhea and uneven weight gain of varying degree. In pigs older than 12 weeks, a more acute clinical course can occur, with the proliferative lesions accompanied by rapid onset of diffuse haemorrhages from the affected mucosa, leading to melanemia and sudden death (McOrist and Gebhart, 1999). The prevalence and economic costs of the disease are rising in Europe possibly due to factors such as a recent reduction in the use of in-feed antibiotics (Stege et al., 2000; McOrist et al., 2003).
The in vitro culture and primary causative role of *L. intracellularis* in all forms of PE was established in 1993 (Lawson et al., 1993; McOrist et al., 1993). In the development of potential *L. intracellularis* vaccines, an oral attenuated vaccine was the formulation most likely to be effective for *L. intracellularis* (Kroll et al., 2004). Ingestion of *L. intracellularis* by intestinal mucosal macrophages, specific humoral, cell-mediated and mucosal responses and antigen-related mitogenesis of pig blood lymphocytes all occur following oral infection (Roberts et al., 1980; McOrist et al., 1992; McOrist and Lawson, 1993; Knittel et al., 1998; Guedes and Gebhart, 2003). Pigs exposed orally to *L. intracellularis*-infected fecal material were considered to have developed protective immunity in previous on-farm studies (Love and Love, 1977). Also, protective immunity to infection by other pathogenic intracellular bacteria, such as *Brucella* sp or *Chlamydia* sp, has only been convincingly demonstrated following appropriate delivery of whole live attenuated bacteria (Su et al., 2000, Morrison and Caldwell, 2002). Killed or subunit vaccine approaches to these agents have been far less successful, despite many attempts.

An attenuated live vaccine formulation of *Lawsonia intracellularis* was therefore developed (Enterisol® Ileitis, Boehringer Ingelheim Vetmedica, Inc.) and tested in controlled challenge studies to evaluate the efficacy of this orally delivered vaccine (Kroll et al., 2004). The efficacy of a vaccination strategy applied at or after weaning for enteric diseases entails assessment of whether maternal immunity present after weaning could interfere with the onset of protective immunity. Therefore, the primary objective of this study was to evaluate efficacy against PE in vaccinated pigs derived from hyperimmunized sows. Secondly, maternal immunity was assessed in pigs derived from hyperimmune sows.
for protective immunity against PE. Lastly, a safety assessment was performed to determine the effects of an attenuated live *L. intracellularis* vaccine on pregnant sows after repeated oral administrations during gestation.

**Materials and Methods**

**General study requirements**

This study was conducted according to the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Products (VICH) guidelines of Good Clinical Practices (GCP). In addition, this study was conducted in a double blind approach in which the group treatment identities were not disclosed to the study monitor or the study investigators until sample processing, laboratory testing, data compilation and statistical analysis was complete. The care and use of test animals in this study complied with the USDA guidelines set forth in the Code of Federal Regulations No. 9. When necessary, animals were euthanised using a humane, professionally acceptable method (electrocution) as per the 2000 Report of the American Veterinary Medical Association Panel on Euthanasia.

**Test animals**

Sixteen healthy pregnant sows in their second trimester of gestation were acquired for this study. The source herd from which the sows were chosen from had not been recently vaccinated or treated with medication for any reason and had no recent clinical history of *L. intracellularis* infection. Regardless, each pregnant sow was determined negative for *L. intracellularis* by *Lawsonia*-specific polymerase chain reaction (PCR) and
serum analysis (immunofluorescence antibody test, IFAT) prior to the first treatment. Upon farrowing, a total of 100 viable and healthy piglets were randomly chosen for participation in the study from hyperimmunized and placebo-administered sows and subjected to a complete serological and fecal shedding analysis as previously mentioned.

Study design

Prior to study initiation, 16 healthy, pregnant and *L. intracellularis* negative sows were randomly allocated into 2 groups; 8 hyperimmunized (Group A) and 8 placebo-administered controls (Group B). On Days -55, -35 and -14 prior to farrowing, each sow in Group A was hyperimmunized with commercially available Enterisol® Ileitis by direct oral drench. The administration of the vaccine dose by this method involved applying the vaccine to the posterior portion of the oral cavity using a sterile, plastic 10 ml syringe. The control group (Group B) received an equivalent dose of placebo consisting of uninfected McCoy cells suspended in growth medium. Hyperimmunization of pregnant sows in Group A was performed in an attempt to induce a high level of maternal immunity prior to farrowing. Each pregnant sow group was housed in separate rooms to avoid cross contamination and under the same conditions (temperature, ventilation, and pen size). Sows in each room were kept in the same pen.

Although efforts were made by the study investigator to have uniform conception and farrowing dates, sows did not all farrow on the same day. Instead, farrowing occurred during a 10-day period. To prevent having multiple vaccination and challenge dates which could lead to excessive variability among pig groups in the study, the mean date of farrow
during the farrowing period was established as Day 0 of the study. Thus, pigs were 3 weeks ± 5 days of age at the time of vaccination (Day 21).

On Day 21, one hundred healthy, weaned piglets were sorted by litter and randomly assigned to six treatment groups. Housing restrictions and conditions were similar to the sow groups mentioned above. Piglets derived from hyperimmunized sows (Group A) were randomly assigned to Groups 1 through 3 and were identified as “hyperimmune-derived” piglets for the remainder of the study. Piglets derived from control sows (Group B) were randomly assigned to Groups 4 through 6 and were identified as “placebo-derived” piglets for the remainder of the study. Groups 1 and 4 (20 pigs/group, respectively) were given a single 2-ml dose of Enterisol® Ileitis by direct oral drench. Groups 2 and 5 (20 pigs/group, respectively) were given an equivalent dose of placebo (uninfected McCoy cells + media). Groups 3 and 6 (10 pigs/group) were designated “strict negative controls” which did not receive a vaccine or placebo treatment and were not challenged during this study. On Day 22, after the weaning period, sows were humanely euthanized and necropsied for evaluations of intestinal lesion development due to PE.

On Day 42 of the study, Groups 1, 2, 4 and 5 received $1 \times 10^{7.3}$ TCID$_{50}$/dose of heterologous virulent pure culture _L. intracellularis_ isolate N101494 via intragastric gavage. All piglets were examined daily 21-days post challenge for clinical symptoms related to PE; diarrhea, behavior and body condition and were given a score of 1 to 4 dependent on severity (1 = clinically normal; 4 = severe illness). Pigs were weighed on Days 21, 42 and 63 of the study to calculate an average daily weight gain per pig group. Average daily weight gains (ADWG) were calculated to analyse the effects of treatment in
relation to normal growth performance in pigs. Pigs were initially weighed to obtain a baseline average group weight prior to receiving a vaccine or placebo treatment. All groups were found to be uniform in size (variation of <0.63 kg/pig). The first key ADWG evaluation period among groups occurred from Day 21 (vaccination) to Day 42 (challenge exposure) to measure the immediate effects of vaccination or placebo inoculation. The second evaluation period was from Day 42 to 63 (necropsy) to measure the effects of challenge exposure with a virulent pure culture *L. intracellularis*.

On Day 63 of the study, all pigs were humanely euthanized and necropsied for evaluations of intestinal lesion development due to PE. A summary of the experimental design is illustrated in Chart 1.

Gross pathology

Gross pathological lesions indicative of PE were evaluated as a primary parameter for determining vaccine efficacy against virulent *L. intracellularis* challenge. Gross examination of each ileum and colon was conducted for the degree of thickening and folding of the mucosa by an investigator blinded to the pig group. Gross lesions were scored according to the severity of mucosal thickening: 1 = normal, 2 = mild thickening, 3 = moderate thickening-inflammation, 4 = severe thickening-inflammation, 5 = severe thickening-inflammation and mucosal haemorrhaging (blood present), 6 = necrosis. Mean scores for gross lesions were calculated for group comparisons.
<table>
<thead>
<tr>
<th>Pig Group</th>
<th># Pigs / Group</th>
<th>Sow Treatment</th>
<th>Pig Treatment</th>
<th>Treatment ID</th>
<th>Route</th>
<th>Day of Vacc.</th>
<th>Challenge Route</th>
<th>Day of Chall.</th>
<th>Day of Study Term.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>Vaccine (Sow Grp. A)</td>
<td>Vaccine</td>
<td>Enterisol® Ileitis</td>
<td>Oral drench</td>
<td>21</td>
<td>Lawsonia intracellularis N101494</td>
<td>IG</td>
<td>42</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>Vaccine (Sow Grp. A)</td>
<td>Placebo</td>
<td>McCoy cells</td>
<td>Oral drench</td>
<td>21</td>
<td>Lawsonia intracellularis N101494</td>
<td>IG</td>
<td>42</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>Vaccine (Sow Grp. A)</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>63</td>
</tr>
<tr>
<td>4</td>
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<td>Placebo (Sow Grp. B)</td>
<td>Vaccine</td>
<td>Enterisol® Ileitis</td>
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<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>63</td>
</tr>
</tbody>
</table>
Histology

Samples 2 to 4 cm in length of ileum and colon were fixed in 10% buffered formalin, embedded in paraffin wax, after which 5 μm thick sections were cut in duplicate. One section was Hematoxylin and Eosin (HE) stained and examined for crypt cell proliferation, inflammatory response, crypt abcessation, and a possible reduction in the number of goblet cells. Another unstained section of each ileum and colon was used in an immunohistochemical (IHC) procedure for *L. intracellularis*, incorporating an indirect immunoperoxidase stain based on a mouse monoclonal antibody (McOrist et al., 1987). For this procedure, each section was dewaxed, rehydrated, rinsed with demineralised water (dH₂O) and PBS and digested with 0.06% trypsin in PBS. Endogenous peroxidase activity was blocked by incubation with 3% H₂O₂ in PBS for 30 min at 20°C. Non-specific antibody binding was prevented by pre-incubation with 20% normal goat serum (Dako, Denmark) for 60 min at 20°C. Sections were then incubated with the IG4 *Lawsonia*-specific monoclonal antibody (McOrist et al., 1987) 1:800 for 30 min, then goat-anti-mouse-biotin (Dako) 1:200 for 1h, then streptavidin-horseradish peroxidase (Dako) 1:200 for 1h, all at 20°C. The slides were washed with PBS between each step. After a rinse with dH₂O, enzyme substrate of 0.03% H₂O₂ and 0.05% aminoethyl carbazol in 0.01 M sodium acetate buffer, pH 5.2, was added, and then sections were counterstained with Mayer's hematoxylin (Dako), washed and mounted. The IHC stained ileum and colon sections were scored individually on the level of severity of *L. intracellularis* infection and microscopic lesion development; 0 = normal, 1 = mid-focal, 2 = moderate-diffuse and 3 = severe-
diffuse). Mean scores for microscopic lesions and the frequency of lesions detected in the affected tissues were calculated for group comparisons.

**DNA-isolation from feces and mucosal samples**

Epithelial cells from the ileum and colon mucosa were obtained by scraping the mucosa of each ileum with a sterile microscope slide. These samples and fecal swabs were place in Nalgene micro-centrifuge tubes containing 200 µl of reagent buffer A (IsoQuick™). From each of these samples genomic DNA was extracted using reagents and procedures outline in the IsoQuick™ DNA extraction kit (Orca Laboratories, Inc).

**PCR**

For all DNA extractions from feces, ileum mucosa or colon contents, the DNA template was incorporated into PCR reactions incorporating specific primers to *L. intracellularis* as described by Jones et al. (1993).

**Immunooassays**

Serum samples from the blood of each sow and pig were tested for the presence of IgG antibodies against *L. intracellularis* by the immunofluorescence antibody test (IFAT) using fixed whole *Lawsonia* antigen on 96-well polystyrene microtiter plates and FITC-labelled antibodies directed against porcine IgG (Knittel et al., 1998). Sows were bled at Day -54, -35, -14 and 0 (farrowing) to determine the rate of seroconversion due to hyperimmunisations with Enterisol® Ileitis. Pig blood samples, 6-10 ml each, were collected weekly from the farrowing period (Day 0) to study termination (Day 63). Percent
IFAT positives were calculated for group comparisons to determine the level of IgG maternal antibodies present from birth through vaccination and the frequency of seroconversion in pigs due to vaccination or challenge exposure.

The IFA test was modified slightly by using FITC-labelled antibodies directed against porcine IgM and IgA. This modified serological procedure was used to detect these antibodies in addition to IgG in each sow’s colostrum for determining the concentration of different *Lawsonia*-specific immunoglobulins present at the time of farrowing. Colostrum was collected from each sow in Groups A and B immediately before piglets were allowed to suckle during the farrowing period. The colostrum was diluted 2-fold in duplicate in PBS and transferred (100 µL/well) to two sets of *Lawsonia* coated 96-well plates as mentioned above. The inoculated plates were allowed to incubate for 30 minutes at 37°C then, washed 3 times with PBS. An anti-swine IgM or IgA FITC-conjugated antibody (Kirkegaard and Perry Laboratories, Inc.) previously diluted 1:200 in PBS was added to the plates and then, incubation and wash steps were repeated. The titer of each specific anti-*Lawsonia* antibody was detected using UV microscopy. Percent IFAT positives and mean titer values for each immunoglobulin were calculated for group comparisons to determine the frequency and the level of IgG, IgM and IgA colostrum antibodies present among sows.

Data analysis

Key study parameters including gross and microscopic lesions, clinical scores, fecal shedding, tissue colonization, weight gain, and seroconversion were statistically analysed. In addition, the following group comparisons were performed to analyse single study
parameter measurements to meet the defined study objectives: Group 1 vs. 2 to determine if maternal antibodies present in *Lawsonia* positive piglets interfere with vaccine efficacy, Group 2 vs. 5 to evaluate the potential maternal immunity derived in *Lawsonia* positive piglets compared to *Lawsonia* negative pigs, Group 4 vs. 5 to determine vaccine efficacy in vaccinated *Lawsonia* negative pigs compared to non-vaccinated *Lawsonia* negative control pigs and Group 1 vs. 4 to compare efficacy of the vaccine in piglets derived from sows with a positive or negative background for *Lawsonia*. A summary of treatment allocations to pig groups is illustrated in Chart 1.

Vaccine efficacy was demonstrated in pigs by significant (p<0.05) reductions in the primary parameters; gross and microscopic lesion development. Group average gross and microscopic (IHC) lesion scores were statistically analysed by mixed model split plot ANOVA and \( \chi^2 \) tests. Average clinical scores and weight gains were also analysed by mixed model split plot ANOVA among pig groups. Non-parametric study parameters such as tissue colonization (PCR), fecal shedding (PCR) and seroconversion (IFAT) were analysed by use of \( \chi^2 \) or Monte Carlo tests.

**Results**

**General observations**

Test animals receiving a vaccination or placebo treatment did not endure any direct adverse reactions caused by to the vaccine, placebo or administration procedures throughout the study. Sows that were hyperimmunized with vaccine did not have any adverse reactions to the vaccine or administration procedures throughout the gestation or farrowing periods.
Clinical observations (diarrhea, behavior, and body condition) among sow or piglet treatment groups (prior to challenge) did not reveal any significant differences. Pigs in Groups 3 and 6 (strict controls) were negative for *L. intracellularis* as determined by IFAT, PCR or IHC analysis throughout the study and confirmed the validity of the pig source and the clinical evaluations.

Maternal antibody detection

*Lawsonia intracellularis*-specific IgG, IgA and IgM antibodies were detected in the sera and colostrum of sows in Group A (hyperimmunized) during the farrowing period. Sixty three percent (5/8 sows) of Group A sows were serum antibody positive for anti-*Lawsonia* IgG antibodies while 0% (0/8) of the sows in Group B were positive at farrowing. In addition, serum IgG antibodies were detected in piglets derived from Group A sows only from farrowing (Day 0) to 5 weeks of age (Day 28), see Figure 1. Anti-*Lawsonia* IgG, IgA and IgM were detected in the colostrum of Group A sows at 50%, 75% and 12.5%, respectively. Average antibody titers in the colostrum samples of Group A sows were 1:14 (IgG, range = 1 to 1:64), 1:10 (IgA, range = 1 to 1:32) and 1:4 (IgM, range 1 to 1:4). Sows in Group B did not have any detectable IgM or IgG anti-*Lawsonia* antibodies in their colostrum during this time period. One pig in sow Group B was positive for IgA at a titer of 1:16.

Seroconversion in piglets to *L. intracellularis*

*Lawsonia*-specific antibody detection by IFAT in pigs due to passive transfer from hyperimmune sows or exposure to vaccine and challenge are summarized in Figure 2. Pigs
Figure 1. Percentages of maternally derived anti-*Lawsonia* IgG antibodies (IFAT) in the offspring of sows hyperimmunized orally with an avirulent *L. intracellularis* vaccine or placebo. Boxed percentage values represent mean IFAT positives from pigs in Groups 1 through 3. Unboxed percentage values represent mean IFAT positives from pigs in Groups 4 through 6.

in the strict control group (Group 6) that were derived from *Lawsonia*-negative sows (Group B) were IFAT negative throughout the study. However, Group 3 strict control pigs derived from hyperimmunized sows (Group A) were IFAT positive for *Lawsonia*-specific IgG antibodies on Days 0, 7, 14, 21 and 28 of the study (10%, 30%, 30%, 20%, and 30%, respectively).

Hyperimmune-derived pigs in Groups 1 and 2 were IFAT positive ranging from 10% to 35% on Days 0 to 14 of the study. On Day 21 (vaccination), these pigs were IFAT positive for anti-*Lawsonia* IgG antibodies at 10% and 15%, respectively. Low levels of positive seroconversion to *L. intracellularis* continued to be detected via IFAT one week
Figure 2. Rates of maternal antibody (IgG) detection and seroconversion to *L. intracellularis* (IFAT) among piglet treatment groups.

**Treatment Key:** Group 1 = Sow A-vaccinate, Group 2 = Sow A-placebo, Group 3 = Sow A-strict controls, Group 4 = Sow B-vaccinate, Group 5 = Sow B-placebo, Group 6 = Sow B-strict controls.

- Overall comparison is not significantly different.
- Group 1 and 2 comparison is significantly (*p*<0.05) different.
- Group 2 and 5 comparison is significantly (*p*<0.05) different.
- Group 1 and 4 comparison is significantly (*p*<0.05) different.
- Group 3 and 6 were not included in the statistical analysis

after vaccination (Day 28) in Groups 1 and 2 (15% and 20% respectively) but then were IFAT negative until 2 weeks after challenge exposure (Day 56) in which 5% of the pigs in Group 2 only were IFAT positive. On the last day of the study, low levels of IFAT positivess were detected in these groups at 11% and 40%, respectively.

Placebo-derived pigs in Groups 4 and 5 was IFAT negative until Day 42 (challenge) when 5% of Group 4 pigs that received the vaccine treatment on Day 21 were IFAT
positive. On Day 49, one week post challenge exposure, both groups were IFAT positive at 10% and 5%, respectively. Rates of seroconversion continued to increase in Group 4 (20% and 25%, respectively) and Group 5 (11% and 50%, respectively) at two and three weeks post challenge exposure (Days 56 and 63, respectively).

Significant (p<0.05) differences in percent IFAT positives among Group 1 and 2 pigs were evident on Day 63 of the study only. Significantly (p<0.05) more IFAT positives were detected in Group 1 compared to Group 4 at farrowing, Day 7 and 14 of the study. Group 1 had significantly (p<0.05) less IFAT positive pigs than Group 4 on Day 56 only. Groups 2 and 5 comparisons revealed significantly (p<0.05) more IFAT positives in Group 2 on Day 7 and 14 of the study.

Vaccine efficacy in *Lawsonia*-naïve pigs

Pigs derived from Group B sows (controls) were first analysed to confirm that the pig source was susceptible to virulent *L. intracellularis* challenge. Secondly, a comparison of Group 4 (vaccinated) and Group 5 (non-vaccinated) piglets which were derived from *Lawsonia*-negative sows was conducted to evaluate the efficacy of the vaccine against virulent *L. intracellularis* challenge.

Average gross and microscopic lesion scores among all groups are summarized in Table 1. Group 5 had 77% of the pigs positive for *Lawsonia*-specific lesions in the ileum and colon compared to only 7.5% of the pigs in Group 4. Group 4 pigs had significantly (p<0.05) lower gross and microscopic (IHC) average lesion scores in the ileum and colon compared to Group 5 at Day 63 of the study.
<table>
<thead>
<tr>
<th>Group</th>
<th>Sow treatment</th>
<th>Pig treatment</th>
<th>Average gross lesion scores (Ileum)</th>
<th>Average gross lesion scores (colon)</th>
<th>Average IHC scores (Ileum)</th>
<th>Average IHC scores (colon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hyperimm.</td>
<td>Vaccinated</td>
<td>0.16&lt;sup&gt;a,b&lt;/sup&gt; (±0.09)</td>
<td>0.26&lt;sup&gt;a&lt;/sup&gt; (±0.18)</td>
<td>0.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.15&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(Sow A)</td>
<td></td>
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<tr>
<td>2</td>
<td>Hyperimm.</td>
<td>Non-vaccinated</td>
<td>0.85&lt;sup&gt;b,c&lt;/sup&gt; (±0.24)</td>
<td>0.45 (±0.22)</td>
<td>0.70&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.55&lt;sup&gt;c&lt;/sup&gt;</td>
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<td></td>
<td>(Sow A)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Hyperimm.</td>
<td>Strict controls</td>
<td>0.00&lt;sup&gt;θ&lt;/sup&gt; (±0.15)</td>
<td>0.00&lt;sup&gt;θ&lt;/sup&gt; (±0.05)</td>
<td>0.00&lt;sup&gt;θ&lt;/sup&gt;</td>
<td>0.00&lt;sup&gt;θ&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>(Sow A)</td>
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<tr>
<td>4</td>
<td>Placebo</td>
<td>Vaccinated</td>
<td>0.15&lt;sup&gt;a,d&lt;/sup&gt; (±0.08)</td>
<td>0.05&lt;sup&gt;a,d&lt;/sup&gt; (±0.05)</td>
<td>0.15&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>0.05&lt;sup&gt;a,d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(Sow B)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Placebo</td>
<td>Non-vaccinated</td>
<td>2.35&lt;sup&gt;c,d&lt;/sup&gt; (±0.39)</td>
<td>0.80&lt;sup&gt;d&lt;/sup&gt; (±0.29)</td>
<td>2.42&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>1.35&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(Sow B)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Placebo</td>
<td>Strict controls</td>
<td>0.00&lt;sup&gt;θ&lt;/sup&gt; (±0.15)</td>
<td>0.00&lt;sup&gt;θ&lt;/sup&gt; (±0.05)</td>
<td>0.20&lt;sup&gt;θ&lt;/sup&gt;</td>
<td>0.00&lt;sup&gt;θ&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>(Sow B)</td>
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</table>

<sup>a</sup> Group 1 and 4 comparison is not significantly different.
<sup>b</sup> Group 1 and 2 comparison is significantly (p<0.05) different.
<sup>c</sup> Group 2 and 5 comparison is significantly (p<0.05) different.
<sup>d</sup> Group 4 and 5 comparison is significantly (p<0.05) different.
<sup>θ</sup> Group not included in the statistical analysis.
<sup>(</sup> Standard Error
Fecal PCR results among all test groups are summarized in Figure 3. No detectable shedding of *L. intracellularis* in the feces by PCR was evident in either group on Days 21 (vaccination) to 35 of the study. Group 4 pigs were initially fecal PCR positive on Day 42 (challenge) and remained positive until Day 63 (study termination) with 5% to 25% of the pigs shedding *L. intracellularis* during this time period. In comparison, Group 5 pigs were

![Graph showing rates of L. intracellularis fecal shedding (PCR) among piglet treatment groups.](image)

**Figure 3.** Rates of *L. intracellularis* fecal shedding (PCR) among piglet treatment groups.

**Treatment Key:** Group 1 = Sow A-vaccinate, Group 2 = Sow A-placebo, Group 4 = Sow B-vaccinate, Group 5 = Sow B-placebo.

- Overall comparison is not significantly different.
- Group 2 and 5 comparison is significantly (p<0.05) different.
- Group 4 and 5 comparison is significantly (p<0.05) different.
- Groups 3 and 6 not shown due to being PCR negative for *Lawsonia* throughout the study and therefore, not included in the statistical analysis.

**Note:** No significant differences among Groups 1 vs. 2 and 1 vs. 4 in fecal shedding during this study.
initially fecal PCR positive on Day 49 with 15% to 72% of the pigs’ shedding *L. intracellularis* until Day 63 of the study. Significantly (p<0.05) less fecal shedding was detected by PCR after receiving a virulent challenge in Group 4 compared to Group 5 on Days 56 and 63 of the study (5%, 10% and 42%, 72%, respectively). However, Group 4 pigs (25%) had significantly (p<0.05) more fecal PCR positives than Group 5 (0%) on the day of challenge exposure (Day 42).

Tissue PCR results of ilea samples revealed significantly (p<0.05) more colonization of *L. intracellularis* in Group 5 pigs (45%) compared to Group 4 (5%) on Day 63 of the study. The mesenteric lymph nodes and colon samples were tissue PCR positive (5% and 10%, respectively) for *L. intracellularis* in Group 5 pigs only. No detection of *Lawsonia* DNA was found in the tonsils of either group.

Average weight gain comparisons among all test groups are summarized in Table 2. Average initial weights were uniform among Groups 4 and 5 on Day 21 (vaccination) with pigs weighing 6.44 and 6.10 kg/pig, respectively. No significant difference in ADWG among Groups 4 (0.44 kg/pig) and 5 (0.41 kg/pig) from Day 21 (vaccination) to Day 42 (challenge). However, significantly (p<0.05) higher ADWG was evident in Group 4 pigs (0.51 kg/pig) than Group 5 pigs (0.40 kg/pig) during the 21-day evaluation period from Day 21 (challenge) to Day 63 (study termination).

Maternal protection

The comparison of unvaccinated, control pigs in Group 2 (pigs derived from sow Group A) to Group 5 (pigs from sow Group B) was conducted to evaluate the potential of
Table 2. Average daily weight gains among treatment groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Sow treatment</th>
<th>Pig treatment</th>
<th>Average initial weight (kg) on Day 21</th>
<th>ADWG Day 21 to 42 (kg)</th>
<th>ADWG Day 21 to 42 (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hyperimm.</td>
<td>Vaccinated</td>
<td>6.53(^a) ((\pm)0.83)</td>
<td>0.41(^a) ((\pm)0.03)</td>
<td>0.45(^c) ((\pm)0.05)</td>
</tr>
<tr>
<td></td>
<td>(Sow A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Hyperimm.</td>
<td>Non-vaccinated</td>
<td>6.35(^a) ((\pm)0.68)</td>
<td>0.4(^a) ((\pm)0.04)</td>
<td>0.46(^d) ((\pm)0.05)</td>
</tr>
<tr>
<td></td>
<td>(Sow A)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>3</td>
<td>Hyperimm.</td>
<td>Strict controls</td>
<td>6.71(^b) ((\pm)0.8)</td>
<td>0.46(^b) ((\pm)0.07)</td>
<td>0.52(^b) ((\pm)0.07)</td>
</tr>
<tr>
<td></td>
<td>(Sow A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Placebo</td>
<td>Vaccinated</td>
<td>6.44(^a) ((\pm)0.62)</td>
<td>0.44(^a) ((\pm)0.03)</td>
<td>0.51(^c,e) ((\pm)0.03)</td>
</tr>
<tr>
<td></td>
<td>(Sow B)</td>
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<td></td>
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</tr>
<tr>
<td>5</td>
<td>Placebo</td>
<td>Non-vaccinated</td>
<td>6.08(^a) ((\pm)0.79)</td>
<td>0.41(^a) ((\pm)0.05)</td>
<td>0.4(^d,e) ((\pm)0.06)</td>
</tr>
<tr>
<td></td>
<td>(Sow B)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Placebo</td>
<td>Strict controls</td>
<td>6.35(^b) ((\pm)0.89)</td>
<td>0.45(^b) ((\pm)0.05)</td>
<td>0.5(^b) ((\pm)0.04)</td>
</tr>
<tr>
<td></td>
<td>(Sow B)</td>
<td></td>
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</tbody>
</table>

\(^{a}\) Overall comparison is not significantly different.
\(^{b}\) Group 1 and 2 comparison is significantly (P<0.05) different.
\(^{c}\) Group 1 and 4 comparison is significantly (P<0.05) different.
\(^{d}\) Group 2 and 5 comparison is significantly (P<0.05) different.
\(^{e}\) Group 4 and 5 comparison is significantly (P<0.05) different.

\(^{\Phi}\) Group not included in the statistical analysis.

\(^{(*)}\) Standard Error
maternal protection against virulent *L. intracellularis* exposure derived from sows hyperimmunized with vaccine (Group A sows).

Average gross and microscopic lesion scores among all groups are summarized in Table 1. Pigs in Group 5 (77%) had a higher percentage of *Lawsonia*-specific lesions in the ileum and colon than Group 2 pigs (27.5%). Group 2 pigs had significantly (p<0.05) lower average gross lesion scores in the ileum and average microscopic (IHC) lesion scores in the ileum and colon compared to Group 5 at Day 63 of the study.

No detectable shedding of *L. intracellularis* in the feces by PCR was evident among Groups 2 and 5 on Days 21 (vaccination) to 42 (challenge) of the study, see Figure 3. Groups 2 and 5 were initially fecal PCR positive beginning on Day 49 and remained positive until Day 63 (study termination) with 5% to 25% and 15% to 72%, respectively, of the pigs shedding *L. intracellularis* during this time period. Significantly (p<0.05) less fecal shedding was detected by PCR in Group 2 (25%) compared to Group 5 (72%) on Day 63 of the study.

A higher percentage of tissue PCR positives were found in the ilea of pigs in Group 5 (45%) compared to Group 2 (25%) at Day 63 of the study. Pigs in Group 2 were PCR negative for *L. intracellularis* in the tonsil, mesenteric lymph node and colon. Various PCR positives were observed in the colon and mesenteric lymph tissue of Group 5 pigs as mentioned above.

Average weight gain comparisons among all test groups are summarized in Table 2. Average initial weights were uniform among Groups 2 and 5 on Day 21 (vaccination) with pigs weighing 6.35 and 6.10 kg/pig, respectively. No significant difference in ADWG
among Groups 2 (0.40 kg/pig) and 5 (0.41 kg/pig) from Day 21 (vaccination) to Day 42 (challenge). However, significantly (p<0.05) higher ADWG was evident in Group 2 pigs (0.46 kg/pig) than Group 5 pigs (0.40 kg/pig) during the 21-day evaluation period from Day 42 (challenge) to Day 63 (study termination) of the study.

Vaccine efficacy in hyperimmune-derived pigs

The comparison of pigs vaccinated with Enterisol® Ileitis in Group 1 to unvaccinated, control pigs in Group 2 was conducted to confirm that vaccination in the face of maternal immunity could be accomplished by evaluating protective immunity against PE after vaccination of pigs at 3 weeks of age. Both groups of pigs were derived from sows hyperimmunized with vaccine (Group A sows). Furthermore, primary and secondary efficacy parameters were analysed between vaccine-treated pigs of Group 1 and Group 4 to determine if vaccine efficacy is similar against virulent *L. intracellularis* challenge.

Average gross and microscopic lesion scores among all groups are summarized in Table 1. Pigs in Group 2 (27.5%) had a higher percentage of *Lawsonia*-specific lesions in the ileum and colon than Group 1 (12.5%). Group 1 pigs had significantly (p<0.05) lower average gross lesion scores (ileum) and numerically lower average microscopic (IHC) lesion scores (ileum and colon) compared to Group 2 at Day 63 of the study. In addition, there were no significant differences among average gross and microscopic lesion scores or lesion severity in pigs receiving a vaccination (Groups 1 and 4).

No detectable shedding of *L. intracellularis* in the faeces by PCR was evident in either group (1 or 2) on Days 21 (vaccination) to 35 of the study, see Figure 3. Group 1 pigs
were initially fecal PCR positive on Day 42 (challenge) and remained positive until Day 63 (study termination) with 11% to 16% of the pigs shedding *L. intracellularis* during this time period. Pigs in Group 2 were initially fecal PCR positive on Day 49 and remained positive until Day 63 (study termination) with 5% to 25% of the pigs shedding *L. intracellularis* during this time period. Pigs in Group 4 were not PCR positive for *Lawsonia* DNA until Day 42 (challenge) and remained positive until Day 63 (study termination) with decreasing rates of shedding from 25% to 5%. No evidence of a significant difference in the rates of fecal shedding of *L. intracellularis* between Groups 1 and 2 and Groups 1 and 4 during the study.

A slightly higher percentage of tissue PCR positives were found in the ilea of pigs in Group 2 (25%) compared to Group 1 (20%) at Day 63 (study termination) of the study. A lower frequency of tissue PCR positives was evident in Group 4 (5%) than Groups 1 and 2 at study termination. No significant differences in PCR positives were noted among Groups 1 and 2 and Groups 1 and 4, respectively. Pigs in all three groups were PCR negative for *L. intracellularis* in the tonsil, mesenteric lymph node and colon.

Average weight gain comparisons among all test groups are summarized in Table 2. Average initial weights were uniform among Groups 1 and 2 on Day 21 (vaccination) with pigs weighing 6.53 and 6.35 kg/pig respectively. Uniform average weights were also observed among Groups 1 and 4 on Day 21 (6.53 kg/pig and 6.44 kg/pig, respectively). No significant difference in ADWG among Groups 1 (0.40 kg/pig) and 2 (0.41 kg/pig) or among Groups 1 and 4 (0.44 kg/pig) from Day 21 (vaccination) to Day 42 (challenge). Significant differences (p<0.05) in ADWG was evident in Group 1 pigs (0.45 kg/pig)
compared to Group 4 pigs (0.51 kg/pig) during the 21-day evaluation period from Day 21 (challenge) to Day 63 (study termination). No significant differences in ADWG among Groups 1 and 2 were noted from Day 21 to Day 63 in this study.

Pregnant sow safety

Although not the primary study objective, safety in pregnant sows due to hyperimmunisation with Enterisol® Ileitis was evaluated among Groups A and B in this study. Sows in Groups A and B was fecal PCR negative for *L. intracellularis* after receiving vaccine or placebo on Days -55, -35 and -14 of the study. Moreover, the ilea and colon samples from these animals were tissue PCR negative for *L. intracellularis* at Day 22 (termination of the sow analysis portion) of the study. Gross and histological analysis reveal no evidence of *L. intracellularis* or lesions suggestive of PE in the intestines of sows in Groups A or B at Day 22 of the study. Despite the fact that vaccine was administered during the 2nd and 3rd trimesters of pregnancy, no abnormal general health observations were recorded for any sow during the study. Farrowing results between Groups A and B was also very similar with an average of 9.4 and 7.6 live born pigs in each group, respectively. Group A sows had an average of 1.8 stillborn pigs per litter and no mummies or farrowing mortalities. Group B sows had an average of 0.9 stillborn pigs per litter, 0.1 mummies, and 0.1 pigs that died for various reasons at farrowing. Diagnostic evaluation of these stillborn pigs indicated they were *L. intracellularis* negative.
Discussion

Comparison of the various groups of pigs in the second phase of this study indicates that the avirulent live *L. intracellularis* vaccine applied at three weeks of age was effective in protecting naïve piglets, born from *Lawsonia*-negative sows, from a virulent challenge at 6 weeks of age (Group 4 vs. 5). Group 4 pigs had significantly (p<0.05) less prevalence and severity of gross and microscopic (IHC) lesion development in the ileum and colon compared to Group 5 pigs. This confirms previous reports (Kroll et al., 2004) that Enterisol® Ileitis provides efficacious protection after a single oral administration in naïve pigs. The non-vaccinated, control pigs (Group 5) were affected with PE of varying severity; 3 pigs had gross pathology indicative of acute PE (mucosal haemorrhaging, blood-filled lumen) while the rest had lesions ranging from mild to severe thickening of the terminal ileum and colon reflective of subclinical or chronic PE. The specific lesions present in Group 5 confirms studies done by several others in validating use of the *L. intracellularis* pure culture challenge model in this study which recognized experimental replication of all syndromes of PE in pigs (McOrist et al., 1993, Guedes et al., 2003, Schwartz et al., 1999).

The results of this study also indicate that maternal immunity passed to offspring by a *Lawsonia*-positive sow (Group A) is not likely to cause significant interference with efficacy of this oral vaccine and subsequent protection from virulent challenge in vaccinated piglets (Group 1 vs. 2). Significantly (p<0.05) less gross lesion development occurred in the ileum of vaccinated pigs (Group 1) compared to non-vaccinated control pigs (Group 2) post mortem. This finding was substantiated by numerically higher average microscopic lesion scores in the ileum and colon in Group 2 pigs. There appears to be no significant
difference in vaccine efficacy against PE for pigs with or without maternal immunity (Group 1 vs. 4). Vaccination of pigs derived from hyperimmune sows did not significantly differ in their level of protection against virulent challenge compared to *L. intracellularis*-negative, vaccinated pigs. Although average gross and microscopic intestinal lesion scores were slightly higher among vaccinates in Group 1 than Group 4, both provided significant (p<0.05) protection against *L. intracellularis* infection compared to their respective control groups (Groups 2 and 5).

Lactogenic antibodies were present in the colostrum from Group A sows. This observation is supported by the presence of systemic IgG antibodies detected in some of these pigs beginning on the day of farrowing, through the weaning period and up to 5 weeks of age. In addition, significant (p<0.05) differences in gross and microscopic lesion development in the comparison of control Groups 2 and 5, demonstrated that residual maternal immunity protected some piglets in Group 2 from virulent challenge at 6 weeks of age. Various concentrations of *Lawsonia*-specific IgG, IgA and to a lesser extent, IgM were found in the colostrum of hyperimmunized sows (Group A) suggesting that these particular immunoglobulins may play a role in maternal protection against *L. intracellularis* infections in weanlings. It is interesting to note that on the day of challenge (Day 42), all pig groups but one, Group 4, were seronegative by IFAT. Seroconversion due to vaccination was slightly evident in Group 4 with only 1 pig (5%) IFAT positive. This observation confirms that IFAT positives in Groups 1 through 3 were maternal antibody derived and likely not due to vaccination. These observations may imply that the serological assay used in this trial does not provide an accurate indicator of vaccination and immunity against virulent
*L. intracellularis* exposure. Pigs in all groups were seronegative on Day 35 (2 weeks after vaccination) suggesting that maternal antibodies may have decayed to undetectable levels between Day 28 and 35 of the study when pigs were 5 to 6 weeks of age.

Pig farms in Europe typically operate on a single farm site, with various ages of pigs within the same perimeter fence and few barriers for the movements of fecal material around the farm. This system is known to be associated with exposures and onset of *L. intracellularis* infections around 1 to 2 months after weaning, a peak of clinical signs and lesions early in the grower-finisher period, and serologic evidence of infection among many breeding-age animals (Møller et al., 1998; McOrist et al., 1999; Stege et al., 2000; Chouet et al., 2003). It is therefore likely that many pigs at least 6 weeks old will be exposed to virulent *L. intracellularis*, as evaluated here through a controlled challenge exposure study. It is also likely that piglets at three weeks of age on many European farms will be exposed to maternal antibodies to *L. intracellularis*, which will persist for some weeks after weaning, depending on the amount originally ingested. Neonatal piglets ingest maternal antibodies, particularly IgG but also other bioactive compounds including IgA and IgM through colostrum intake; their levels of plasma IgG are directly related to their colostral IgG intake (Rooke and Bland, 2002). In some cases, the presence of lactogenic IgG antibodies in piglets is thought to render parenteral vaccines given to those piglets ineffective for pathogenic enteric infections (Bertschinger et al., 2000).

The results of this study indicate that these antibodies will not substantially interfere with use of the oral live attenuated vaccine approach for *L. intracellularis* when administered to pigs derived from hyperimmune sows at 3 weeks of age. This finding is not
entirely unexpected for this type of vaccine in post-weaned pigs. Consistent protection of pigs from virulent post-weaning *Escherichia coli* strains such as oedema disease F18 has only been convincingly demonstrated in piglets which received a specific vaccine delivered orally around the time of weaning (Bertschinger et al., 2000). This is despite the presence of lactogenic immunity in these piglets at the time of their vaccination. The enhanced efficacy of oral vaccines delivered to piglets for post-weaning enteric infections, even in the face of lactogenic immunity, may be due to the an enhanced ability of mucosal surfaces of these piglets to bind and process orally delivered antigens (Weltzin et al., 1989; Bertschinger et al., 2000). Given the etiologic nature of *L. intracellularis* as a mucosal intracellular pathogen and the use of an avirulent live vaccine, cellular immunity may play a greater role in controlling PE (Guedes and Gebhart, 2003; Kroll et al., 2004).

Most previous challenge exposure studies of *L. intracellularis* infections have been performed in recently weaned pigs derived from “clean” farms having *Lawsonia*-negative sows. In these situations, virulent oral challenge by *L. intracellularis* is followed by mucosa infection of the ileum and onset of acute or chronic PE lesions following 2 to 3 weeks after initial exposure (Mapother et al., 1987; McOrist et al., 1993; Schwartz et al., 1999). In contrast, use of this oral live attenuated *L. intracellularis* vaccine itself is not linked to development of any lesions in both sows and piglets. The exact nature of vaccinal stimulation of a pig’s immune system is not yet clear, but clearly involves some antigen processing as IHC analysis of intestinal tissue shortly after immunization revealed *Lawsonia* vaccine antigen internalized by macrophages (Kroll, unpublished data). The recent successful use of an attenuated *L. intracellularis* vaccine across North America and
in controlled challenge exposure studies suggests that colonization of avirulent isolates can provide potent immunity to the agent (Kroll et al., 2004). This immunity was observed here as vaccinated pigs regardless of sow source had significant (p<0.05) reductions in tissue colonization and lesion development compared to non-vaccinates after virulent challenge.

Shedding of *L. intracellularis* in vaccinated pigs (15% to 25% PCR +) prior to challenge indicates that the live oral vaccine isolate used has the potential to shed, but at very low levels. It is usual for attenuated bacterial isolates to have a somewhat reduced capacity for replication in the host, and hence a reduced ability to spread to sentinels (Jones et al., 1993). This compares to virulent isolates, which normally are adapted to maximise their animal-to-animal spread, including *L. intracellularis*, which can attain high levels in faeces (Guedes and Gebhart, 2003; Jones et al., 1993; Smith and McOrist, 1996). This challenge exposure study of *L. intracellularis* suggests that it also fits this pattern of bacterial adaptation and virulence.

Average weight gains and fecal shedding (PCR) were monitored to support the efficacy of the attenuated live *L. intracellularis* vaccine. These secondary efficacy parameters revealed that immunized pigs increased ADWG and reduced levels of shedding *L. intracellularis* in the feces compared to unvaccinated pigs after virulent challenge; a trend consistent with previous studies (Kroll et al., 2004). Significant (p<0.05) reductions in fecal shedding were evident in vaccinated pigs (Group 4) compared to unvaccinated, control pigs (Group 5) at 2 and 3 weeks after challenge exposure. Vaccinates derived from hyperimmunized sows (Group 1) weren’t significantly different than Group 2 pigs in the percentage of fecal PCR positives during the same time period. We speculate that maternal
immunity in these groups reduced the ability of the virulent challenge to sufficiently infect and reach high levels of antigen in the feces post challenge. Pigs gained significantly (p<0.05) more weight daily in Group 4 (0.51 kg/pig) compared to the unvaccinated, controls (Group 5, 0.4 kg/pig) verifying that vaccination of *Lawsonia*-naïve pigs increases ADWG after challenge exposure. No evidence of significant differences in ADWG among maternally positive Groups (1 and 2) was observed throughout this study. Maternal protection among these groups may have been enough to allow uniform increases in weight gains after receiving virulent challenge. Group 4 vaccinates (0.51 kg/pig) had significant (p<0.05) increases in ADWG compared to Group 1 vaccinates (0.45 kg/pig) after challenge however, the mean difference in average weight gain was only 0.06 kg/pig. Even though the ADWG among these groups significantly differed, the comparisons among lesion development did not. This observation may be explained by the slightly higher incidence of *L. intracellularis* colonization and lesion development involved in Group 1 vaccinates compared to Group 4 vaccinates at Day 63 of the study.

The normal reproductive and health performance of sows given three high doses of the live avirulent vaccine in the second and third trimester of gestation indicates an excellent safety profile. This is in contrast to virulent *L. intracellularis* infections in field situations, where acute haemorrhagic proliferative enteropathy in pregnant sows often causes spontaneous abortions (McOrist and Gebhart, 1999).

In conclusion, this study demonstrates that maternal immunity does provide some level of protection to pigs born from hyperimmune sows. Oral vaccination in the face of this passive immunity induces a protective, acquired immune response equivalent to that of
a *Lawsonia*-naïve pig. In addition, this study demonstrated sow safety to repeated exposure of an avirulent live *L. intracellularis* vaccine during gestation.

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References


Guedes, R.M.C., Gebhart, C.J., 2003 Onset and duration of fecal shedding, cell-mediated and humoral immune responses in pigs after challenge with a pathogenic isolate of


McOrist S., Lawson, G.H.K., 1993 Interactions of porcine lymphocytes with Campylobacter-like organism membranes purified from proliferative enteropathy. Veterinary Microbiology 34, 381-388.


Morrison, R.P., Caldwell, H.D., 2002 Immunity to murine chlamydial genital infection. *Infection and Immunity* 70, 2741-2751.


1989 Binding and transepithelial transport of immunoglobulins by intestinal M cells:
demonstration using monoclonal IgA antibodies against enteric viral proteins. *Journal of
CHAPTER 5. A LIPOPOLYSACCHARIDE BASED ENZYME-LINKED IMMUNOSORBENT ASSAY FOR EXPERIMENTAL USE IN DETECTION OF ANTIBODIES TO LAWSONIA INTRACELLULARIS IN PIGS

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J. J. Kroll,¹,² M.A. Eichmeyer,¹ M. L. Schaeffer,¹
S. McOrist³, D.L. Harris,⁴ and M. B. Roof¹

Abstract

An enzyme-linked immunosorbent assay (ELISA) for *Lawsonia intracellularis* was developed and compared with a whole cell antigen based immunofluorescence antibody test (IFAT). The antigen containing lipopolysaccharide (LPS) was derived from Percoll gradient purified cultures of *L. intracellularis* using a modification of the Westphal hot phenol procedure. The antigen was bound directly to polystyrene 96-well microtiter plates and the assay performed in an indirect ELISA format. Specificity and sensitivity values based on 80 known positive and 80 known negative serum samples from controlled experimental trials were 93.7% and 88.7%, respectively. Serological results from a controlled *L. intracellularis* challenge exposure study confirmed the high specificity and sensitivity of this assay (100% and 99.5% respectively). Comparisons between the LPS-ELISA and the IFAT in detecting anti-*Lawsonia* antibodies in this controlled study revealed significantly more LPS-ELISA positive pigs than IFAT positives on Days 21, 28, 35 and 42.

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¹ Department of Research and Development, Boehringer Ingelheim Vetmedica, Inc., 2501 North Loop Drive, Ames, Iowa 50010.
² Corresponding author. Phone: 515 296 6625. E-mail: jkroll@bi-vetmedica.com.
³ QAF Industries, Corowa, NSW 2646, Australia.
⁴ Iowa State University, Ames, Iowa 50011.
(p = 0.003, 0.030, 0.002 and 0.006, respectively). This indirect ELISA (LPS-ELISA) test is an improved method in detecting antibodies soon after exposure to *L. intracellularis* in pigs regardless of isolate type (vaccine or wild-type) in experimental studies. The LPS-ELISA may be used as a tool to support future research trials on vaccine efficacy and to further understand the immune response induced by *L. intracellularis*.

**Introduction**

Proliferative enteropathy (PE) is a common enteric disease of pigs after weaning that is caused by the obligate intracellular bacterium, *Lawsonia intracellularis* (20, 21). The infection and disease are widespread in pig farms across America and Europe and prevalence among groups of pigs on these affected farms can be over 30% (4, 23, 29, 31). This leads to a considerable economic impact of the disease due to diarrhea, weight loss and subclinical illness in growing pigs (22, 31). Since identification of *L. intracellularis* as the cause of PE in 1993, a number of studies aimed at establishing the best diagnostic methods for identifying *Lawsonia* exposure in live animals have been conducted. These have focused on DNA detection via Polymerase Chain Reaction (PCR) of feces and whole-cell immunoassays (8, 12, 13, 15, 17), due to extreme difficulty in isolation of the obligate intracellular *L. intracellularis* from the contaminated environment of feces (13, 17, 18). In situations where samples of ileum are available, immunohistochemistry (IHC) is considered to provide the criterion-referenced or “gold standard” for assessment of the actual infection status of an individual pig (9, 16, 19, 26, 28).
PCR testing of fresh feces involves considerable laboratory effort and cost to extract amplifiable bacterial DNA from each sample (9, 11, 13, 15). False positives due to pre-laboratory sample contamination along with collecting numerous samples from a group of pigs, or due to contamination during the laboratory testing phase may occur. False negatives due to the regular presence of PCR inhibitors in feces may also occur (9, 10, 11). Serologic testing methods have therefore also been widely explored for detecting *L. intracellularis* exposure in pigs. Indirect immunofluorescence or immunoperoxidase assays have been used to examine antibody responses in pigs infected experimentally with *L. intracellularis* in virulent challenge exposure studies and in pigs with PE from farms (3, 4, 7, 11, 14, 29). An indirect enzyme-linked immunosorbent assay (ELISA) was developed previously for testing pig serum antibodies, with crude antigen derived directly from pig intestines affected with PE (12). However, the antigen used in that study was not fully characterized for *L. intracellularis* content. The development of a specific *L. intracellularis*-antigen based ELISA would therefore be of considerable benefit in improving the feasibility of a more universally available and standardized diagnostic assay to study the epidemiology of this economically significant disease.

We describe the development of an ELISA for detecting *L. intracellularis* infection, based on a lipopolysaccharide antigen extract in an indirect ELISA format.
Materials and Methods

Bacterial antigen preparation

The LPS used in this study was derived from *L. intracellularis* isolate #15540. This isolate was acquired from a Danish sow affected with acute hemorrhagic proliferative enteropathy (confirmed by routine histology and immunohistochemistry staining techniques), whose intestines were co-cultured to obtain a pure culture of *L. intracellularis* by methods previously described (18, 21). Multiple 30 L batches of *L. intracellularis* #15540 (ATCC #PTA-4927) were propagated using fresh McCoy cell (ATCC #1696) suspensions in bioreactors (Applicon, Inc., Foster City, CA). Active cultures were allowed to reach 80-100% cell infectivity and then, harvested by centrifugation using an Avanti Beckman J-20I centrifuge, JA-10 rotor at 17,000 g for 15 minutes at 4°C. The supernatants of each batch were discarded and cell pellets containing both harvested extracellular *L. intracellularis* and McCoy cells infected with *L. intracellularis* were resuspended in 30 ml sterile 0.2 M phosphate-buffered saline (PBS) at pH 7.3 and stored at -80°C.

For purifying *L. intracellularis* from McCoy cells, a discontinuous Percoll gradient was prepared following methods previously described with slight modifications (12). Briefly, 225 ml of Percoll (Amersham Biosciences, Uppsala, Sweden) was mixed with 260 ml of distilled water and 15 ml of 5M NaCl to form the stock Percoll gradient. Harvested *L. intracellularis* culture was passed at least 20 times through a 25 Ga. needle and 5 ml of this bacterial/McCoy cell homogenate was mixed with 25 ml of the stock Percoll gradient in polycarbonate tubes. Tubes were then centrifuged at 37,000 g for 1 h at 4°C. The ensuing suspension contained scattered cellular debris in the upper 50% of the
tube while one distinct cellular banding pattern was visualized at a buoyant density of 1.075 g/ml. This band was carefully collected into a 5 ml polypropylene pipette and transferred to new tubes containing 20 ml of sterile PBS. These tubes were centrifuged (Avanti Beckman J-20I, JA-17 rotor) at 37,000 g for 15 minutes at 4°C and this washing process was repeated 3 times to remove Percoll material from each sample. After the final centrifugation step, the bacterial pellets were suspended in sterile PBS and aliquoted into 1.8 ml cryovial tubes and stored at -80°C. Dark field microscopies of sampled material confirmed highly concentrated tiny curved rods and absence of intact McCoy cells.

The LPS component was extracted from this purified *L. intracellularis* #15540 antigen using a hot aqueous phenol method (Westphal method) with slight modification (34). Briefly, 4.5 ml of a pre-heated purified *L. intracellularis* suspension was gently mixed in a tube containing 4.5 ml of 90% v/v hot aqueous phenol suspension for 25 minutes at 65°C, then cooled overnight at 4°C. Each tube was centrifuged at 7,700 g for 25 minutes at 4°C and the LPS-containing supernatant fluid contained in the upper portion of the bi-phasic aqueous layer within each tube was retained and transferred into pre-sterilized dialysis tubing. The tubing was then dialyzed repeatedly against cold reagent grade (reverse osmosis) water for 48 hours to remove the phenol. The *L. intracellularis* LPS extract was collected and centrifuged at 10,000 g for 15 minutes at 4°C to remove any remaining particulates and stored in sterile vials at -80°C.
Antigen characterization

The LPS extract was analyzed by SDS-PAGE using a 4-12 % Bis-Tris gel in MOPS running buffer (Invitrogen) and compared to Percoll-purified whole-cell *L. intracellularis* and uninfected whole McCoy cells. Samples for SDS-PAGE were prepared by diluting each 1:2 into 4X lithium salt dodecyl sulfate denaturing buffer (Invitrogen) and incubated in an 85°C water bath for 10 minutes. Gels were periodate silver stained (30) using a slight modification of the commercially available Bio-Rad Silver Stain. Briefly, gels were oxidized with 0.7% periodic acid immediately after fixing and prior to the second fixation step (10% ethanol and 5% acetic acid), and an additional reduction step with 160 µM dithiothreitol (DTT) was included before the silver reagent step. In other experiments, the LPS extract was subsequently transferred from unstained SDS PAGE gels to polyvinylidene difluoride (PVDF, Invitrogen) membranes for Western blot analysis. The separated molecules were electrophoretically transferred in Towbin buffer using the Novex™ blot module at 30 V constant for 1 hour. After transfer, the blots were cut into strips and blocked for at least 1 hour in 50 ml of a blocking solution consisting of Tris-buffered saline with 0.05% Tween 20 (v/v) (TTBS) and 2% non-fat dry milk (w/v) (NFDM, BioRad). Blots were incubated with numerous primary antibody sources and dilutions; 1) LPS-ELISA positive control hyperimmunized pig serum (1:2500), 2) LPS-ELISA negative control pig serum (1:2500), 3) Swine anti-*Lawsonia* convalescent serum (1:10, 20, 100) and 4) Swine *Lawsonia*-negative convalescent serum (1:100) to illustrate the specificity of anti-*Lawsonia* antibodies to the primary antigen of the extract, *Lawsonia* LPS. The primary antibody dilutions used for this Western analysis were similar to those used in the LPS-ELISA.
primary antibodies were diluted in the blocking reagent described above and incubated with each respective membrane at room temperature for 1 hour. The blots were washed three times for two minutes at room temperature in TTBS, after which the membranes were incubated in 50 ml of the secondary antibody conjugate (goat anti-swine horseradish peroxidase, Kirkegaard and Perry Laboratories, Inc.) at a 1:1000 dilution in blocking reagent for 1 hour at room temperature. The blots were washed twice in TTBS and once in PBS prior to incubation at room temperature for 30 minutes in 10 ml of Opti-4CN substrate (BioRad) solution. The colorimetric reaction was stopped by rinsing the blots with reagent grade (RG) water.

Lastly, the LPS extract was analyzed for a specific concentration by the bacterial endotoxin test (BET) with the Limulus Ameobocyte Lysate assay (Endosafe™). The concentration of LPS within the extract was expressed as endotoxin units per ml (EU/ml) using the kinetic chromogenic method as described by the manufacturer’s instructions.

Optimization of the LPS-ELISA

The ELISA was optimized following standard assay development procedures described elsewhere (6, 27). Concentrations of key reagents used in this LPS-ELISA such as the primary antigen (LPS extract), primary antibodies and HRP-conjugated secondary antibodies were optimized to achieve a desired and consistent working dilution using checkerboard titrations (6, 27). Other influencing factors key to assay success such as blocking and wash buffers, substrates, incubation temperatures and duration of incubation
time periods were also evaluated according to standard procedures to achieve optimum levels (6, 27).

LPS-ELISA for anti-Lawsonia IgG antibodies

The *Lawsonia intracellularis* LPS at a concentration of 34.8 EU/ml was coated at 100 µl/well onto Immulon 2HB plates (Dynex) at a 1:1000 dilution in 0.05M sodium carbonate coating buffer, pH 9.6 (2) and incubated at 20°C for 24 hours. Each plate was then washed in a buffer containing 0.05% Tween 20 (v/v), 0.137 M NaCl, 0.005 M KCl, 0.009 M Na2HPO4, and 0.001 M KH2PO4, pH 7.2 to 7.4, in distilled water. Antigen-coated plates were then blocked at 300 µl/well with a blocking buffer containing 5% (w/v) NFDM (Bio-Rad) in SeaBlock™ (Pierce Biotech) at 4°C for 24 hours to prevent non-specific binding of test serum to the plates. Each plate was then washed for 3 cycles as above. Each test and control serum sample was pre-diluted 1:40 in blocking buffer. Fifty µl per well of each diluted serum sample was incubated with antigen at 37°C for 1 hour. After 3 washing steps, 50 µl/well of a 1:500 dilution of goat anti-swine IgG heavy and light chain specific HRP conjugate (Kirkegaard and Perry Laboratories, Inc.) in blocking buffer was added and incubated at 37°C for 1 hour. After 3 washing steps, 50 µl/well of peroxidase color substrate (3,3’,5,5’ tetramethylbenzidine) was applied to each plate and incubated at 20°C for 5 minutes then stopped with 2 M H2SO4 solution. Absorbance values at 450 nm wavelength (A450) were obtained for each sample well. A range of control serum samples from high to low positive and negative antibodies against the *L. intracellularis* LPS extract were generated and included in each plate. Control sera were initially diluted 1:2560 in
blocking buffer and then, serially diluted 2-fold a total of 5 times (1:2,560, 5,120, 10, 240, 20,480 and 40, 960) immediately prior to sample testing. A 50 µl aliquot of each diluted positive and negative control sample is transferred and typically placed in wells A through E in columns 11 and 12 of the test plate. A test plate was considered valid if the coefficient of determination (r²-value) of these standards’ A450 values were ≥0.9 in a linear regression analysis (Microsoft™ Excel), with empty wells reading blank.

Serum samples derived from clinical pig trials

Serum samples were obtained from pigs in the following studies for use in validating the LPS-ELISA:

Study 1. Two 3-week-old Lawsonia-negative pigs were hyperimmunized for antibodies to the L. intracellularis LPS extract, by deep intramuscular injection with 2 ml of the L. intracellularis LPS extract mixed 1:2 with a commercial Freund’s incomplete adjuvant (Sigma Aldrich). Another control 3-week-old pig received a placebo injection of cell culture medium containing 5% (v/v) bovine serum, mixed 1:2 with this adjuvant. Booster injections of each inoculum were administered 3 and 6 weeks post initial inoculation (p.i.) and final specimen collections were 8 weeks p.i. Serum samples of each pig were collected on Days 0, 21, 42 and 59 of the study and tested for presence and concentration of anti-Lawsonia IgG antibodies in an indirect immunofluorescence assay incorporating L. intracellularis whole cell antigen as described previously (IFAT) (15).

Study 2. Challenge exposure studies with L. intracellularis isolates were conducted in Lawsonia-negative 6- to 9-week-old pigs as described previously (16, 33). Serum
samples from the virulent *L. intracellularis* challenge exposed and strict control pigs (non-vaccinated, non-challenged pigs) in these studies were tested to investigate the cut-off limits for anti-*Lawsonia* LPS antibody positive/negative absorbance values. The *Lawsonia* IFAT was also conducted on these samples.

Study 3. A “proof of concept” vaccine efficacy study was conducted following Good Clinical Practices (GCP) guidelines as previously reported (16). This study utilized conventional pigs that were *Lawsonia*-negative at the time of vaccination. On Day 0 of the study, vaccinates (*n* = 15) were given an oral 2 ml dose of Enterisol® Ileitis (Boehringer Ingelheim Vetmedica, Inc.) per manufacturer’s label instructions while pigs in the challenge control group (*n* = 10) were given an equivalent dose of placebo consisting of uninfected McCoy cells in growth media. Strict control pigs were not given vaccine, placebo or virulent challenge during the study and were kept in separate pens to ensure biosecurity among treatment groups. On Day 21, vaccinated and challenge control pigs were given a 10 ml intragastric dose of virulent *L. intracellularis* isolate N10194 at $10^{7.7}$ Tissue Culture Infective Dose 50 (TCID$_{50}$/dose. On Day 42 of the study, pigs were humanely euthanized, necropsied and evaluated for *Lawsonia* specific gross and microscopic lesions. Average gross and microscopic lesion scores (IHC), average daily weight gains, seroconversion rates (IFAT), *L. intracellularis* fecal shedding rates (PCR) and average daily clinical scores after challenge of this clinical trial were previously reported (16). Serum samples were subsequently tested with the LPS-ELISA for direct comparison to the IFAT for differences in detecting anti-*Lawsonia* antibodies in vaccine and challenge exposed pigs.
Statistical Analysis

Statistical comparisons in seroconversion among assays and treatment groups utilized Fisher’s Exact T-test and Two Sample Test of Proportions (5, 25). Statistical determinations of positive and negative $A_{450}$ cut-offs as well as assay sensitivity, specificity, 95% confidence intervals and the area under the Receiver Operating Characteristic (ROC) curve was conducted with ROC curve analysis using Medcalc version 7.2.1.0. These additional parameters were observed to identify the assay’s ability in detecting *Lawsonia*-specific antibodies in pigs and to gauge the assay’s ability to distinguish between *Lawsonia*-exposed and *Lawsonia*-naïve pigs.

Results

Characterization of the *L. intracellularis* LPS extract as the primary ELISA antigen

The visualization of the *L. intracellularis* LPS component by the SDS-PAGE/periodate silver staining of the LPS extract revealed a glycosylated molecule approximately 18-25 kDa (Figure 1). Glycosylation was confirmed by the reddish staining imparted on the species via the reaction of aldehyde groups formed by periodate oxidation of the sugars with the silver reagent. This LPS fraction was differentially stained reddish-brown to specifically identify glycosylated moieties in comparison to other extracted components. The LPS was observed in the samples containing the LPS extract and whole cell *L. intracellularis* and not in the uninfected McCoy cell sample suggesting that this molecule is of prokaryotic origin. The periodate silver stained gel also revealed the presence of other higher molecular weight proteins that seem to appear at lower concentrations compared to
Figure 1. SDS-PAGE / Periodate Silver Staining of *L. intracellularis* LPS

Lane 1: 10-220 kDa Benchmark™ pre-stained protein marker (Invitrogen)
Lane 2: *L. intracellularis* LPS extract
Lane 3: Whole cell *L. intracellularis* DK15540
Lane 4: Uninfected McCoy cells
Lane 5: Blank

*Arrow indicates the migration range of *L. intracellularis* LPS*
the LPS extract. These extraneous proteins perhaps are due to inadvertent carry-over of particulates from the biphasic layer during the purification step or they could be the results of incomplete degradation of proteins during the hot aqueous phenol phase of the LPS extraction.

Western blots of the LPS extract were performed to determine the specificity of anti-
*Lawsonia* antibodies to the LPS component and to assess the degree of cross-reactivity to the extraneous proteins (Figure 2). Results of the Western blots demonstrated that the LPS-ELISA negative control (1:2500) and *Lawsonia*-negative pig sera (1:100) did not react to any of the extraneous proteins in the LPS extract and reacted very lightly (weak signal strength) to the LPS component itself. Positive LPS-ELISA control sera (1:2500) reacted very strongly (strong signal strength) to the LPS component and to a lesser extent, the extraneous proteins. Slight cross-reaction of the positive control was expected since hyperimmunized pigs would have generated antibodies to both the LPS component and the extraneous proteins. However, the Western signal for this particular interaction was very light making up <1% of the total signal strength compared to the target LPS antigen.

Additionally, Western analysis of the LPS extract to *Lawsonia*-positive convalescent pig serum at dilutions of 1:10, 1:20 and 1:100 revealed strong reactions to the LPS component whereas very light reactions were noted with the higher molecular weight extraneous proteins at 1:10 and 1:20 dilutions only (<1% of total signal strength). An extraneous protein of lower molecular weight than the LPS component (approximately 15 kDa) did react to *Lawsonia*-positive convalescent pig sera but represented only ~9% of the total signal strength of the reaction compared to the LPS component.
Figure 2. Western blots of *Lawsonia intracellularis* LPS using ELISA control or positive and negative convalescent swine sera

Each gel lane was loaded with the same amount of LPS material, and replicate lanes were cut into strips after transfer to PVDF for incubation with different primary antibodies. Secondary antibody in each case was goat anti-swine-HRP (1:1000). M, molecular weight marker. A) Lane 1, Strict Control Negative Serum (1:100); 2, ELISA Negative Control Serum (1:2500); 3, ELISA Positive Control Serum (1:2500); 4, Goat anti-swine-HRP conjugate only (1:1000); 5, Silver stained SDS-PAGE gel. B) Primary antibody was swine anti-*Lawsonia* convalescent serum at different dilutions. Lane 1, 1:10; 2, 1:20; 3, 1:100
Results from the BET for quantifying the amount of endotoxin in the purified sample of *L. intracellularis* LPS extract revealed an endotoxin level of 34,750 EU/ml. Furthermore, the reagent grade water used to store the LPS extract contained <0.05 EU/ml thus indicating the buffer was endotoxin-free and provided no interference in obtaining the true endotoxin concentration of the LPS extract.

Individual LPS-ELISA plate validation via hyperimmunized pig sera

The positive LPS-ELISA control sera was generated from two 3-week-old *Lawsonia*-negative pigs hyperimmunized with *Lawsonia* LPS extract. The negative control sera were generated from one 3-week-old pig receiving adjuvant alone. Pigs that received an LPS extract inoculation were IFAT positive on Days 21, 42 and 59 of the study with mean anti-*Lawsonia* antibody titers 1:200, 1:16,000 and 1:16,000, respectively. Reactivity of the positive and negative control sera against the *Lawsonia* LPS antigen extract (1:1000) in the LPS-ELISA plates was confirmed by performing two-fold titrations in blocking buffer of each control across the plate. The positive control A₄₅₀ value at a 1:40 dilution (optimal detection antibody dilution for this assay) was 1.9 and was determined to have a total anti-*Lawsonia* LPS endpoint titer of 1:10,000 to 1:20,000 by the LPS-ELISA. The negative control A₄₅₀ value at a 1:40 dilution was <0.2. Linear regression analysis of the positive control demonstrated an r² value of 0.96. Based on the linearity of the positive control, an individual LPS-ELISA assay is considered valid in detecting *Lawsonia* specific antibodies when each plate demonstrates an r² value of 0.9 or greater.
Determination of cut-off limits

One hundred and sixty pig sera samples from two *L. intracellularis* challenge exposure studies were evaluated to determine the anti-*Lawsonia* positive and negative antibody cut-off limits for the LPS-ELISA assay. In addition, these serum samples were used to determine assay specificity, sensitivity, 95% confidence intervals and the area under the ROC the curve analysis. Eighty pigs were previously identified to be positive for *L. intracellularis* by both IFAT and tissue IHC analysis (16, 33). Another eighty pigs from strict control (non-vaccinated, non-challenge exposed) and negative control (received a non-*Lawsonia* placebo) groups were previously identified to be negative for *L. intracellularis* by the same detection methods (16, 33).

A ROC curve analysis, a graph that plots the true positive rate in function of the false positive rate at different cut-off points, identified the critical cut-off criterion for these samples to be an $A_{450}$ of $\geq 0.15$ (Figure 3). This value was rounded to the nearest tenth of an $A_{450}$ value to 0.2 to simplify future interpretations of LPS-ELISA results. Therefore, pig serum containing an $A_{450}$ value of 0.2 or lower is considered *Lawsonia* antibody negative whereas serum containing an $A_{450}$ higher than 0.2 is considered *Lawsonia* antibody positive.

In addition, the ROC analysis revealed the specificity of this assay to detect anti-*Lawsonia* antibodies in pigs was 93.7% and the sensitivity was determined to be 88.7% (Figure 3). The 95% confidence interval for this assay was 0.933 to 0.991 and the ROC under the curve analysis revealed a value of 0.972 with a standard error of 0.013.
Figure 3. Scatter Plot of the Mean A450 Values for Anti-*Lawsonia* Antibody Positive and Negative Sera Samples

1. y-axis depicts mean A450 values
2. x-axis depicts sample set identification (0 = negative, 1 = positive for anti-*Lawsonia* antibodies)
3. Dashed black line across A450 0.15 signifies the pos./neg. cut-off value

LPS-ELISA validation in a controlled, *L. intracellularis* challenge exposed study

Pigs in the strict control group remained *L. intracellularis* IFAT and LPS-ELISA negative through the study. From Day 0 (vaccination) to Day 14 (one week before challenge) of the study, vaccinated and unvaccinated, challenge control groups were IFAT and LPS-ELISA negative for anti-*L. intracellularis* antibodies. On Day 21 (challenge inoculation) of the study, all groups were IFAT negative (0%) whereas 53% of the
vaccinated pigs exhibited LPS-ELISA positives. One week after challenge (Day 28), again only the LPS-ELISA was able to detect specific seroconversion to anti-*Lawsonia* antibodies in both the vaccinated and challenge control groups (47% and 30%, respectively). On Days 35 and 42 of the study, the number of pigs in both the vaccinated and challenge control groups gradually increased in seroconversion due to virulent *L. intracellularis* exposure. Serological results from Days 35 and 42 in the vaccinated pigs were 26.7% and 40% IFAT positive and 73% and 93% LPS-ELISA positive, respectively. The challenge control group was 10% and 80% IFAT positive and 60% and 90% LPS-ELISA positive at Days 35 and 42, respectively.

The seroconversion rates based on the LPS-ELISA identified significant (p<0.05) increases in percent anti-*Lawsonia* antibody positives in the vaccinate group compared challenge control and strict control pigs at Day 21, and compared to strict control pigs only on Days 28, 35 and 42 of the study. Non-vaccinated, challenge controls were significantly higher in % LPS-ELISA positives than the strict controls on Days 35 (p=0.001) and 42 (p<0.0001) of the study. No significant differences were noted between vaccinates and challenge controls on Days 35 and 42. Significant (p=0.02) increases in % IFAT positives to *L. intracellularis* challenge exposure were evident in vaccinated pigs compared to strict control pigs on Day 35 of the study. Vaccinates and challenge controls were significantly (p=0.003) more IFAT positive compared to the strict control group on Day 42 of the study. No significant differences in % IFAT positives were evident between vaccinates and challenge controls on Days 35 and 42. Group comparisons in seroconversion rates between assays and treatment groups are summarized in Figure 4.
Figure 4. IFAT vs. Indirect LPS-ELISA; Rates of Seroconversion to *L. intracellularis* Among Treatment Groups

* Significant differences in seroconversion among groups and assay types (Chi square/Fisher’s Exact Test and two sample test of proportions, p<0.05)

1. Bar graph represents mean % IFAT positives for treatment groups throughout the study; vaccinates = grey bar, challenge controls = black bar and strict controls = white bar (not visible = 0%).

2. Line graph represents mean % LPS-ELISA positives for treatment groups throughout the study; vaccinates = solid line with grey triangles, challenge controls = large dashed line with black squares and strict controls = small dashed line with white circles.

3. X-axis represents each day of the study serum samples were tested for anti- *Lawsonia* IgG.

4. Y-axis represents mean % anti- *Lawsonia* antibody positive pigs.
Comparisons between the LPS-ELISA and the IFAT in detecting anti-\textit{Lawsonia} antibodies in this study revealed significantly more LPS-ELISA positive pigs than IFAT positives on Days 21, 28, 35 and 42 (p=0.003, 0.030, 0.002 and 0.006, respectively).

The ROC analysis of the results from this study revealed the specificity of the LPS-ELISA to be 100% and the sensitivity 99.5%. The 95% confidence interval was 0.976 to 0.999 and the ROC under the curve analysis revealed a value of 0.995 with a standard error of 0.007.

\textbf{Discussion}

We have developed a novel EPS-based indirect ELISA that specifically detects anti-\textit{Lawsonia} IgG in the sera from \textit{Lawsonia}-negative pigs exposed to either a modified live vaccine and/or virulent challenge exposure. This immunoassay could improve the early detection of \textit{L. intracelluaris} exposure in pigs by providing a user-friendly and time-efficient research tool for use in controlled clinical evaluations in pigs. Incorporation of this \textit{Lawsonia}-specific and sensitive antibody-antigen reaction into direct or sandwich ELISA approaches may allow other novel immunoassays to be developed for research purposes. Modifications to the conjugate in this LPS-ELISA would also allow for detection of other anti-\textit{Lawsonia} specific IgA or IgM in pigs, or antibodies in other host species. Modifications to the method for preparation of the test sample may also allow testing of tissue, feces or sow milk samples for the test antibody.

The use of this type of LPS-ELISA format has been successfully deployed in serologic assays for other enteric and intracellular bacteria, such as \textit{Salmonella dublin} and
Brucella spp (1, 2, 32). The diagnostic performance of this assay is not expected to be
affected by alterations in the antigen’s lipid A structure or contaminating outer membrane
proteins, that may occur in different antigen preparations (1, 2, 24). The standard carbonate
buffer coating conditions used in our study is considered suitable for the LPS-ELISA format
for smooth LPS antigen (2). There was also a good correlation of the LPS-ELISA results in
a range of field studies for Salmonella dublin infection on farms (32), and these results were
superior to previous Salmonella immunoassay formats (32). The LPS-ELISA described
here is therefore likely to provide a robust, widely applicable diagnostic screening tool, with
broad benefits for disease interpretation among groups of pigs.

Current serological-based assays for L. intracellularis infection and disease are the
indirect immunofluorescence antibody test (IFAT) (15) and the immunoperoxidase
monolayer assay (IPMA) (7, 8), both incorporating whole cell antigen on carrier wells in
plates or slides. They demonstrate good sensitivity (91% and 89%, respectively) and
specificity (100% for both assays) in detecting anti-Lawsonia antibodies ante-mortem in pig
serum. However, both assays are probably not sensitive enough to detect anti-Lawsonia
antibodies in lower concentrations in pig serum during the initial exposure and onset of
infection periods. In addition, these assays rely on highly skilled technicians to accurately
conduct these tests and interpret the results. Results are subjectively obtained by spending
many hours looking into a microscope, analyzing wells illuminated by UV or natural light,
searching for L. intracellularis or L. intracellularis-infected cells stained fluorescent green
or enzymatic red respectively that represents a “positive” sample. A previous attempt to
develop an ELISA for detection of anti-Lawsonia antibodies in pigs failed to produce a
sensitive and specific assay, due to its variable and non-specific quality of antigen (derived directly from pig intestines) and limitations in sampling size related to known disease parameters in test pigs (12).

In the LPS-ELISA described here, we used an antigen source consisting of phenol purified LPS derived from pure cultures of *L. intracellularis*; with a specific cut-off value (A450 0.200) that has been determined for identifying anti-*Lawsonia* antibody positive and negative pig sera. The ability of the LPS-ELISA to accurately distinguish *Lawsonia* exposed pigs has been established by observing the results of the ROC under the curve value and 95% confidence intervals in the controlled challenge exposure study. The strong ROC under the curve value of 0.995 and 95% confidence intervals of 0.976 to 0.999 identifies the LPS-ELISA as a highly accurate serological test for detecting anti- *Lawsonia* antibodies in pigs exposed to *L. intracellularis* in these experimental trials. Serological results from a controlled, *L. intracellularis*-challenge-exposed study reveal the superiority of the LPS-ELISA in detecting early antibody onset following vaccine and virulent challenge exposure in pigs compared to the IFAT. Significantly (p<0.05) more vaccinated pigs (53%) were LPS-ELISA positive prior to receiving a virulent challenge (Day 21) compared to non-vaccinated challenge controls (0%). The LPS-ELISA was the only test sensitive enough to detect seroconversion in pigs specifically due to vaccination at 3 weeks p.i. (Day 21). The LPS-ELISA significantly (p<0.05) detected more anti-*Lawsonia* positives than the IFAT in pigs regardless of treatment groups on day of challenge (Day 21) and one, two and three weeks post virulent challenge. The implications for detecting earlier onset of *L. intracellularis* exposure in pigs using the LPS-ELISA will allow a more precise
and rapid implementation of _L. intracellularis_ control strategies such as vaccination to control and reduce PE.

Numerical trends in percent mean LPS-ELISA positives were higher in vaccinated pigs (47%, 73% and 93%, respectively) compared to the challenge controls (30%, 60% and 90%, respectively) each day post challenge exposure. Whereas, the IFAT results revealed first evidence of seroconversion in vaccinated pigs at 2 weeks post challenge (26.7%) which gradually increased to no more than 40% of the pigs IFAT positive on the last day of the study. The challenge controls demonstrated sharp increases in percent IFAT positives at 10% and 80% on Days 35 and 42 of the study. The LPS-ELISA seems more sensitive in identifying more anti- _Lawsonia_ positive pigs in the vaccinates versus non-vaccinates after virulent challenge; a trend that differs in comparison to previous studies using the IFAT (16).

Specificity and sensitivity values based on 80 known positive and 80 known negative test samples were 93.7% and 88.7%, respectively. Sera tested from the challenge exposure study confirmed this by demonstrating similar specificity (100%) and sensitivity (99.5%) in detecting anti- _Lawsonia_ antibodies in pigs. Western blot analysis of the LPS extract supports this high specificity in detecting anti- _Lawsonia_ antibodies with the LPS-ELISA even though the LPS extract contained extraneous protein carry-over. It appears that at the dilutions used, the reagents of the ELISA are quite specific for what is believed to be a component of _Lawsonia_ LPS. The low level of carry-over protein within the LPS extract contains no confounding effects in the assay's ability to specifically detect _Lawsonia_-specific antibodies in pigs. Furthermore, quantification of total protein in the undiluted LPS
extract by the BCA Protein Assay (Pierce Laboratories, Inc.) revealed concentration levels too low (0.11 mg/ml) for establishing adequate antigen-antibody complexes.

Despite the positive results noted in this investigation, there are some factors which limit the application of the described LPS-ELISA beyond the research laboratory. Other antigen preparations of differing purities were examined and found not to have sufficient sensitivity and specificity for use in *Lawsonia*-specific research trials (data not shown). The requirement of antigen derived from Percoll gradients (low/free from Eukaryotic cellular debris) and phenol extraction methods make *Lawsonia* LPS antigen availability practical for only limited numbers of assays and is probably cost prohibitive beyond research trials.

Secondly, limited evaluation of this assay under field conditions suggests there may be differences in animal backgrounds and LPS reactivity, especially in adult pigs. If this assay were to be applied as a routine diagnostic assay, it is possible that different cut-off values would be required and sensitivity and specificity would be lower.

The technical advantages as outlined above make the LPS-based indirect ELISA test an improved serological method in detecting *L. intracellularis* exposure in pigs over the IFAT because it successfully and accurately identified early onset exposure to this organism regardless of isolate type (vaccine or wild-type) as well as providing a rapid and user-friendly tool for identifying specific seroconversion to *L. intracellularis* in experimental studies.
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References


antibodies to intracellular Campylobacter-like organisms of the porcine proliferative

1993. Reproduction of porcine proliferative enteropathy with pure cultures of ileal

Lawsonia intracellularis gen. nov., sp. nov., the obligately intracellular bacterium of


proliferative enteropathy. Pig J 51: 26-35.

Detection of Lawsonia intracellularis, Serpulina hyodysenteriae, weakly beta-
haemolytic intestinal spirochaetes, Salmonella enterica, and haemolytic Escherichia coli
from swine herds with and without diarrhea among growing pigs. Vet Microbiol 62:
59-72.


CHAPTER 6. SUMMARY AND CONCLUSIONS

In the studies described herewith, we validated the efficacy of a new European-derived, oral, avirulent live *Lawsonia intracellularis* vaccine against virulent challenge exposure in pigs. Various clinical applications were investigated that show efficacy against *L. intracellularis* infections using a virulent pure culture challenge model in PE-susceptible pigs. These applications consisted of delivering the live vaccine orally by direct oral drench or in the drinking water to *Lawsonia*-naïve pigs of susceptible age to PE. Successful delivery of a vaccine through a pig’s drinking water minimizes stress and injury to pigs, amount of human-animal contact time, costs and labor. Needle-less vaccine administration provides a higher quality of pork due to less needle breaks and damage to meat at injection sites in addition to eliminating the transmission of blood-borne diseases among vaccinates.

A new clinical application demonstrated vaccine efficacy in the face of maternally derived, anti-*Lawsonia* antibodies in recently weaned piglets against a subsequent virulent *L. intracellularis* challenge. Implementation of this application method allows veterinarians and pig producers to vaccinate pigs earlier in the nursery without the fear of maternal interference and induce protective immunity before pigs become exposed upon entry into the grower-finisher units.

Investigations in maternal protection showed that pigs derived from hyperimmune sows had some level of protection against a virulent challenge exposure. These data suggested that passive immunity plays an active role in protecting piglets from early infection that may result from transmission of virulent *L. intracellularis* from dam to piglet.
The safety of multiple, high dose inoculations of an avirulent live \textit{L. intracellularis} vaccine in sows during the 2\textsuperscript{nd} and 3\textsuperscript{rd} trimester suggested that pregnant sows are not at risk when repeatedly exposed to this vaccine during the most sensitive periods of gestation.

A new technique was developed for detecting anti-\textit{Lawsonia} IgG antibodies in pig serum. This technique consisted of immobilizing purified LPS from \textit{L. intracellularis} onto microtiter plates as the primary antigen in an indirect enzyme-linked immunosorbent antibody assay. This \textit{ante mortem} serological method has proven to be highly sensitive and specific for detecting antibodies derived from vaccine or virulent \textit{L. intracellularis} challenge exposure in pigs. The LPS-ELISA may be able to accurately identify early onset of \textit{L. intracellularis} exposure in pigs in order to better determine when to incorporate antibiotic or vaccine treatment for disease prevention.

The clinical applications described within this dissertation will give swine producers and veterinarians more options for effective and safe administration of this vaccine to induce protective immunity against \textit{L. intracellularis} infections within affected herds. Most producers use antibiotics and other antimicrobial compounds as in-feed additives for growth promotion during all stages of pig development. Future research is needed to determine the effects of antimicrobials on the development of protective immunity following vaccination and perhaps defining a withdrawal period to ensure optimal delivery of this vaccine to pigs. The use of antibiotics may be reduced significantly through proper use of this vaccine alone or in combination with strategic medication regimens to control PE in endemic herds. New studies are warranted to evaluate efficacy of other novel vaccine strategies and delivery
systems which will provide producers with new options for optimal vaccination and protection against this economically important disease.
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