Prevention and treatment of porcine proliferative enteropathy

Jeffrey Paul Knittel

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Prevention and treatment of porcine proliferative enteropathy

by

Jeffrey Paul Knittel

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

Major: Microbiology
Major Professor: D. L. Harris

Iowa State University
Ames, Iowa
1999

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Graduate College
Iowa State University

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Major Professor

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For the Major Program

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For the Graduate College
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CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

The following dissertation consists of a general introduction, review of the literature, four separate manuscripts, a general summary, and acknowledgments. The doctoral candidate, Jeffrey Paul Knittel, is the senior author and principal investigator for all manuscripts.

Introduction

Porcine Proliferative Enteropathy (PPE) is a common disease of swine. The disease is defined as a thickening of the mucosa of the small, and sometimes the large, intestine (23). Characteristic features of the disease are proliferation of the crypt epithelial cells and thickening of the mucosa in the small intestine and the upper third of the spiral colon.

Histopathological lesions of the disease include epithelial hyperplasia, especially in the mucosal crypts with a distinct absence of goblet cells. The proliferating epithelial cells contain intracytoplasmic, slender, curved, rod-shaped bacteria (26).

The intracellular bacteria associated with PPE were previously and incorrectly referred to as "Campylobacter-like" organisms. The causative agent has now been identified and is considered to be a novel taxonomic genus and species, previously known by the vernacular name Ileal Symbiont (IS) intracellularis (3). Recently the organism has been given the name Lawsonia intracellularis (15).

Growth of L. intracellularis on conventional media has not been successful. The bacteria have been cultivated on tissue culture cells in a reduced oxygen atmosphere (2, 5, 9, 10, 25). Pure cultures of Lawsonia (Ileal Symbiont) intracellularis were used to satisfy Koch's postulates in pigs in 1993 (17).
Due to the difficulty of culturing the organism, isolation of the bacteria in pure culture is not a realistic method for diagnosing PPE. Diagnosis has usually been based on histopathology and silver staining of tissue sections for the demonstration of the morphologic forms of *L. intracellularis*. These methods require post-mortem tissues from the pig. Newer, more sensitive detection techniques such as polymerase chain reaction (PCR) (6, 16) and an indirect fluorescent antibody test (IFAT) (8) have helped researchers and producers obtain better prevalence data and determine impact of the disease.

Porcine proliferative enteropathy occurs in virtually all swine production systems, including modern multiple-site or high “health” herds in which early weaning and segregated rearing are practiced. A recent serologic survey conducted by the National Animal Health Monitoring System (NAHMS) using swine serum (representing 198 farms) found the herd prevalence of PPE in the United States swine herd to be 96%. (1) The within-herd prevalence rate was 28%. Diagnostic investigations of PPE-affected farms in other countries suggest that approximately 35% of growing pigs are affected. (18, 22) Subclinical effects such as reduced rate and efficiency of growth have, however, been difficult to quantify. Direct financial losses due to decreased growth rates (9% to 31%) and feed inefficiency (6% to 25%) have been estimated to cost £2 to £7 per affected pig in the United Kingdom based on the results of five pure culture challenge studies (19). MacKinnon (12) observed weight gain reductions of up to 50% and reduction in feed efficiency of up to 30% in pigs naturally infected with PPE compared to normal pigs. Annual costs to the pig production industries have been estimated at $20 million (United States) (13) and be £2 to £4 million (United Kingdom) (19).
Past studies have shown that exposing pigs to infected premises for 3 weeks before starting antibiotic therapy in the feed appeared to produce an immune population, but antibiotic therapy before pigs have time to respond to the disease produced a susceptible population (11). This suggests the potential for preventing PPE immunologically and by antibiotic therapy. With this information it has been suggested that medication for PPE be approached in one of three strategies: 1) exposure to PPE infected premises for 3 weeks followed by medication for 2 weeks, 2) medication in the feed when clinical disease is apparent, or 3) continuous medication to slaughter (23). A proposed antibiotic treatment regimen includes intermittent (pulse) treatments which may allow a limited infection by *L. intracellularis* to occur, hopefully developing into a protective immune response (4, 28). The more common method of treatment is by continuous application in the feed or water and by parenteral treatment in the face of an outbreak (4, 28).

Studies using *in vitro* cell culture systems determined the minimal inhibitory concentrations (MIC) of several antibiotics. Penicillin, erythromycin, difloxacin, virginiamycin, and chlortetracycline had the best results with MIC’s of < 1 μg per ml. Tiamulin and tilmicosin had MIC’s of < 4 μg per ml (14). Several animal trials have demonstrated the effectiveness of several antibiotics including tiamulin hydrogen fumarate (7, 20, 24), tylosin (21), carbadox, erythromycin, chlortetracycline (28, 29), and neomycin and lincomycin with spectinomycin (27).

The purposes of the following studies were to develop improved methods of cultivating *L. intracellularis*, allow large scale *L. intracellularis* cultivation, and develop a vaccine for the prevention of PPE in susceptible age pigs. Studies were also conducted to
evaluate the prevention and control of PPE using tiamulin hydrogen fumarate using administration in the feed or the drinking water after exposure to *L. intracellularis*.

**References**


7. **Knittel, J. P.** 1999. Iowa State University, Ames, IA.


CHAPTER 2. LITERATURE REVIEW

Introduction

Porcine Proliferative Enteropathy (PPE) is a common disease of swine. The disease is characterized by a thickening of the mucosa of the small, and sometimes the large intestine (68). Characteristic features of the disease are proliferation of the crypt epithelial cells, thickening of the mucosa of the small intestine and the upper third of the spiral colon, and the presence of small, curved, intracellular organisms which are in the apical cytoplasm of proliferating cells (68).

Although proliferative enteropathy is most commonly reported in swine it has been described in hamsters (21), ferrets (20), rabbits (19), foxes (18), dogs (8), rats (79), horses (82), sheep (5), deer (13), emus (43), ostriches (10), and guinea pigs (17). Intracellular bacteria are observed in the proliferating epithelium of all of the mentioned species. Evidence suggests that the infectious agent may be common to all animal species affected with proliferative enteropathy (68).

Clinical disease

The main clinical signs of PPE include loose, watery stools with or without blood, puddled feces with undigested feed, gauntness (railbacks) and lack of uniformity (80). These clinical signs are often seen after a stressful event, such as mixing or sorting, weather changes, shipping, overcrowding, and weaning (84). Pigs between six and 20 weeks of age, and replacement breeding stock are often affected. Pigs under 6-8 weeks are less commonly

affected and it is commonly thought that colostral immunity induces the resistance to infection (70).

Lesions

The disease has a wide variety of clinical symptoms along with a variety of descriptive terminologies. Porcine intestinal adenomatosis (PIA) describes the lesion in which the intestinal mucosa is thickened due to epithelial proliferation, similar to a benign tumor of glandular structure, but is relatively free from inflammation, or has only mild inflammatory lesions on the mucosal surface. Pigs 6 to 20 weeks of age most often develop PIA with mild clinical signs usually consisting of a failure of the pig to gain weight (68). Lesions of PIA usually resolve after several weeks, however in some pigs necrotic enteritis can result. Necrotic enteritis (NE) describes deep coagulative necrosis of an adenomatous mucosa. Necrotic enteritis is the endstage condition of PIA in which the severe thickening of the ileum or “hose-pipe” ileum occurs, usually accompanied by persistent diarrhea (68). The most severe manifestation of PPE is proliferative hemorrhagic enteropathy (PHE). This form of disease is most often seen in late finishing and older pigs most often in high health status herds (3). Proliferative hemorrhagic enteropathy describes massive hemorrhage into the intestinal lumen from proliferated mucosa (68). Proliferative hemorrhagic enteropathy (PHE) is differentiated from hemorrhagic bowel syndrome (HBS) in which there is no abnormal crypt proliferation associated with HBS and hemorrhage occurs throughout all layers of the intestinal wall. This is most likely due to an entirely different etiology (70). HBS occurs in growing animals and is an acute syndrome associated with high mortality often seen in older pigs such as replacement stock (68). The external signs may look similar, however they are not the same disease. Histological examination of HBS affected pigs show that the intestine
is thin walled with a red to deep purple color as compared to a thickening of the wall in PPE
affected pigs (3).

Histopathological lesions common to all forms of PPE include epithelial hyperplasia,
especially in the mucosal crypts. The crypts are elongated, enlarged, and lined with crowded
immature epithelial cells with mitotic figures. There is also a distinct absence of goblet cells.
The proliferating epithelial cells contain intracytoplasmic, slender, curved, rod-shaped
bacteria (81).

Several possible explanations have been made for the development of the hyperplasia
(58). These explanations include 1) bacterial regulation of genes active in cell differentiation
or apoptosis 2) bacterial production of a mitotic agent 3) bacterial damage to cells, with a
"wound healing" proliferation response, or 4) bacterial alteration of a receptor-signaling
mechanism related to normal growth factors.

**Etiology**

Presence of the intracellular bacteria with hyperplasia of crypt cells is the definitive
feature of PPE (67). A variety of *Campylobacter* species are isolated from lesions of PPE.
The most common species are *Campylobacter mucosalis* (67) and *C. hyointestinalis* (25),
both of which are morphologically similar to the intracellular bacterium associated with
proliferative lesions.

Efforts to reproduce PPE with pure cultures of *C. mucosalis* were met with limited
success. The only successful reproduction of lesions typical of PPE with *C. mucosalis* was
reported by Lomax et al. in which 4 of 10 cesarean derived pigs fed cultures of *Salmonella
choleraesuis* and *C. mucosalis* developed lesions (45).
Campylobacter hyointestinalis was isolated from 67% of swine with PPE and only 5% of swine with other enteric diseases (25). Campylobacter coli is also often found in swine populations. However, cultures of C. hyointestinalis as well as C. coli have failed to reproduce the disease despite their ability to colonize the intestine for long periods (4, 6, 12, 62).

Several experiments have been performed with tissue homogenates from pigs with PPE. Roberts et al. reported reproduction of the disease using freshly homogenized adenomatous mucosa in two orally inoculated pigs (65). It was later reported that the disease could be reproduced in both specific pathogen free (SPF) pigs (45) and in cesarean-derived colostrum-deprived (CDCD) pigs (44) as well as in four to five-week-old conventional pigs (14) using intestinal homogenates derived from pigs with PPE.

The difficulty encountered in determining the true causative agent of PPE led to a confusing history of names for the etiologic agent. The bacteria were commonly referred to as 'Campylobacter-like organisms' for many years based on their morphological similarity to Campylobacter species.

The bacteria which were finally isolated and determined to be the "real" cause of PPE were given the vernacular name Ileal Symbiont (IS) intracellularis and were identified as a distinct genus which differed from Campylobacter species. DNA sequences of the 16S ribosomal RNA gene from this organism were found to be most similar (91%) to that of a sulfate-reducing proteobacterium, Desulfovibrio desulfuricans (23). The name Lawsonia intracellularis was formally given to the organism in 1995 in honor of G. H. K. Lawson, the discoverer of the bacterium (50). Lawsonia intracellularis is described as a gram-negative,
microaerophilic, obligate intracellular, non-flagellated, non-spore-forming, curved or S-shaped bacillus (41).

**Isolation and growth of *Lawsonia intracellularis***

Growth of the bacteria in cell-free media has not been successful to date. *Lawsonia intracellularis* has been cultivated on tissue culture cells (rat intestinal cells [IEC-18] (41), human fetal intestine [Int 407] (41), rat colonic adenocarcinoma cells (41), pig kidney [PK-15] (41), piglet intestinal epithelial cells [IPEC-J2] (53), GPC-16 (74), and mouse cells [McCoy] (35) when incubated in reduced oxygen atmospheres (41, 74). Isolation of the organism from infected tissues requires homogenization and trypsinization of the tissue, passage of the homogenate through a series of filters down to a pore size of 0.65 μm, and storage of the filtrate containing the organism in a sucrose potassium glutamate solution with 10% FBS at -70° C (41). The bacteria are cultivated on monolayer tissue cultures that are sparsely planted to allow cell growth.

A new method of cultivation of *L. intracellularis* has been developed by Knittel et. al. (35). This patented method (Patent number: 5,714,375) uses suspended McCoy cells for the propagation of *L. intracellularis* in bioreactors and other large scale vessels. This method allows 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 strain of *L. intracellularis* at a potential vaccine (35).

**Cell pathogenesis**

The development of *in vitro* cultivation methods has provided information on the manner in which the bacteria infect cells in the host. It was shown that the bacteria gain entry into the host cell by close association with the cell membrane (53). The bacteria enter
via membrane-bound vacuoles which soon break down and release the bacteria to multiply free in the cytoplasm. This action would avoid the killing and digestion of the bacteria as a result of the endosome-lysosome fusion. It is likely that the degeneration of the membrane-bound entry vacuoles after the internalization of \textit{L. intracellularis} is associated with the ability of the bacteria to produce a membranolytic agent (53). This method of evading intracellular defense mechanisms is also employed by \textit{Shigella flexneri}, \textit{L. monocytogenes}, and some \textit{Rickettsia} species which produce membrane-damaging agents such as phospholipase or listeriolysin (15, 22, 69, 76).

It was shown that infection of cells by \textit{L. intracellularis} is dependent on cell activity, but not on bacterial viability (40). A component of the entry process appears to be microfilament dependent but there is also evidence for a non-actin dependent pathway. This is similar to the manner in which \textit{Chlamydia} species enter the host cell. However, \textit{Chlamydia} remain within vacuoles throughout their life cycle (75). \textit{Lawsonia intracellularis} are often seen in close association with mitochondria and rough endoplasmic reticulum (30). This mode of entry and growth is similar to some \textit{Rickettsia} species (15, 86) however, \textit{Rickettsia} species belong to the alpha subdivision and \textit{L. intracellularis} belongs to the delta subdivision of \textit{Proteobacteria} making them genetically dissimilar (23, 50). Intracellular growth of the bacteria produces little cell morphologic change or lysis (7, 37, 41). It was also observed that bacteria were released from infected cells within cytoplasmic protrusions (53). These findings are similar to what has been observed \textit{in vivo} in host animal studies (21, 30, 31).
Immune responses to *L. intracellularis* infection

In general, the major protective immune response against intracellular bacteria is cell-mediated immunity (1). Cell-immunity consists of killing of phagocytosed microbes as a result of macrophage activation by T cell-derived cytokines and lysis of infected cells by CD8+ cytolytic T lymphocytes (CTLs). Protein antigens of intracellular bacteria stimulate both CD4+ and CD8+ T cells. CD4+ T cells respond to released antigens that are internalized and presented by class II MHC-expressing antigen presenting cells (APCs). If the bacteria survive within cells and release their antigens into the cytoplasm, they stimulate CD8+ CTLs.

Immunocytological testing of porcine ileal tissues suggested that an immune response consists of an accumulation of IgA within the apical cytoplasm of *L. intracellularis* infected cells with a slight increase in CD8+ T cells in the lamina propria (56). Pigs that showed hemorrhagic gross lesions were often characterized by an infiltration of CD8 and CD25 T lymphocytes, and IgA and IgM B lymphocytes, and the lysis of cells. It was also observed that the proliferating enterocytes do not have the MHC II normally found on non-proliferating enterocytes. This selection of immature crypt cells may represent an adaptation by *L. intracellularis* to influence the proliferation of immature enterocytes to enhance survival by evading the host immune system (56).

Reproduction of disease

Koch's postulates were fulfilled in 1993 when PPE was produced in pigs inoculated with pure cultures of *L. intracellularis* (54). Conventional pigs developed the disease when orally inoculated with *L. intracellularis*, but gnotobiotic (germ-free) pigs did not become colonized or develop the disease. It was later shown that gnotobiotic pigs would develop
lesions if exposed to avirulent strains of *Bacteroides vulgatus* or *Escherichia coli* prior to inoculation with pure cultures of *L. intracellularis* (51). This demonstrated that *L. intracellularis* requires the intestine to be predisposed to other bacteria to produce an adequate environment for infection. Conventional and CDCD pigs contain intestinal flora in contrast to gnotobiotic pigs. The study also showed that severity of disease is dose dependent further demonstrating that *L. intracellularis* is the primary etiologic agent of PPE.

Use of pure culture challenge models has provided information regarding transmission of the organism within herds. Pigs given pure cultures of *L. intracellularis* have been shown to excrete the bacteria up to 10 weeks after inoculation (73). A seeder-pig sentinel model demonstrated direct transmission of the organism to penmates as well as non-direct transmission to non-penmates located within the same room (34). This same study also demonstrated that the infective dose of *L. intracellularis* to be low and that the organism is easily transmitted.

This evidence suggests that weaned pigs can maintain the infection within a herd by fecal excretion of the organisms to penmates, leading to newly infected pigs. The persistence of excretion may lead to some female pigs remaining infected and contagious until their first pregnancy. If this occurs, offspring can be infected in the late suckling period transferring the infection to the next generation (73).

**Diagnosis**

Sensitive and specific diagnostic techniques are key in understanding the true prevalence of the disease as well as in estimating the economic impact. Until recently, the only way to diagnose PPE has been by post-mortem examination of animals for macroscopic and microscopic lesions. Hematoxylin and eosin stains (H & E) of tissue sections allow the
diagnostician to evaluate intestine for proliferative changes (66). Silver (Warthin-Starry) stains are used to verify the presence of intracellular, curved, rod-shaped organisms (68, 81). A modified Ziehl-Neelsen stain on mucosal smears can also provide a simple confirmatory test (68, 81), as *L. intracellularis* will stain red within the cytoplasm of infected cells. These stains are commonly used in most diagnostic laboratories. However, diagnosis using these stains requires post-mortem tissues and is not *L. intracellularis* specific.

A monoclonal antibody against an outer membrane component of *L. intracellularis* is effective in confirming the presence of the organism in affected tissues through incorporation of an indirect fluorescent antibody or immunoperoxidase assay (48). The antibody, specific for *Lawsonia intracellularis*, is key in identifying the causative agent of proliferative enteropathy of many of the host animal species mentioned earlier.

Use of this monoclonal antibody has demonstrated that different isolates of *Lawsonia intracellularis* are antigenically similar (37). The antibody can also be used to confirm shedding of *L. intracellularis* in the feces by staining fecal smears (48). The antibody is a sensitive and specific reagent for detecting *L. intracellularis* in tissue sections (38). Again, this requires post-mortem tissues. In order to use as an ante-mortem test on fecal smears, the animal must be actively shedding the organism at fairly high numbers. The antibody is not commercially available at this time.

A hybridization technique was developed using *L. intracellularis* DNA hybridized *in situ* to test samples from both proliferative hemorrhagic enteropathy (PHE) cases and porcine intestinal adenomatosis (PIA) cases (24). This confirms that the intracellular organisms seen in both the acute and chronic forms of disease share the same sequences. This testing demonstrates that the causative agents of the two conditions are genetically similar.
Polymerase chain reaction (PCR) procedures are highly sensitive and rapid methods for the detection of *L. intracellularis* DNA in feces from infected animals (32). The test is a useful tool for detecting infected pigs from field cases as well as from experimentally infected animals (52). Polymerase chain reaction can also identify infected groups of pigs using pooled fecal samples (29), and demonstrates that pigs shed *L. intracellularis* in the presence and absence of clinical signs of disease (38), and that pigs shed intermittently during an infection (34, 38). Polymerase chain reaction also demonstrates that some pigs shed detectable bacteria in the feces for up to 10 weeks (73). Primers specific for *Serpulina hyodysenteriae*, *Salmonella* sp., and *L. intracellularis* can be used in a multiplex PCR assay that allows the simultaneous detection and identification of each of these agents in swine feces (16). This test may reduce the time and cost of detecting each of these pathogens.

Serologic tests employing enzyme-linked immunosorbent (ELISA) and immunofluorescent assays also have been evaluated. In one study the predominant antibody class stimulated by infection with *L. intracellularis* was IgM, as determined by an indirect fluorescent antibody test (IFAT) (42). IgA was detected, but to a lesser extent in diseased pigs. The presence of IgM antibodies directed against *L. intracellularis* was detected for about eight weeks post inoculation. However, for prevalence surveys, detection of an antibody of longer duration (i.e. IgG) would be necessary for accurate results. In another study, an ELISA test was developed to measure the IgG response against *L. intracellularis* in the sera of pigs (28). The test animals for this study were either hyperimmunized by intramuscular injection of percoll gradient-purified bacteria from the intestinal mucosa of PHE-affected pigs, or orally inoculated with intestinal homogenates from a proliferative hemorrhagic enteropathy-affected 6-week-old pig. Results indicated low and variable IgG
antibody titers in inoculated pigs. These results suggested that IgG plays a minor role in the immune response to *L. intracellularis* infections.

An indirect fluorescent antibody test (IFAT) that detects IgG in pigs exposed to *L. intracellularis* has also been evaluated (36). In contrast to the serology tests previously defined, the test employs antigen grown in pure culture that is then stained using serum from test pigs as the primary antibody and fluorescently labeled anti-swine-IgG as the secondary antibody. The test is more sensitive than PCR for the detection of exposed pigs and is specific for pigs exposed to *L. intracellularis* (36). A possible reason for the greater success of the IFAT over the ELISA test is that background difficulties are avoided with the IFAT. Each sample is directly observed for fluorescing bacteria by the person performing the test allowing a distinction to be made between nonspecific reactions and the positively reacting bacterial antigen. An ELISA does not allow this distinction (36). This test can be used as an ante-mortem test to diagnose pigs exposed to *L. intracellularis*. Also, the reagents used for the test are less expensive than those used for PCR. However, pigs that are serologically positive are not necessarily infected at the time of testing, but may have been previously exposed to *L. intracellularis*. Seroconversion that can be detected by the IFAT occurs 2 to 4 weeks after exposure (36). A summary of the diagnostic techniques is given in table 1.

**Epidemiology and economic impact of PPE**

With the availability of more sensitive techniques for detecting *L. intracellularis* infected pigs, estimates of the impact of the disease have been revised. Early reports stated a low prevalence of PPE of 0.89% to 2.5% in swine herds (68). Other reports have suggested that 5 to 20% of the swine herds had lesions at slaughter with incidence in some
Table 1. Summary of Diagnostic Techniques

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<tr>
<td>Silver Stain</td>
<td>Histopathological evaluation for intracellular bacteria</td>
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<td>Monoclonal antibody</td>
<td>Fluorescent Antibody or Immunoperoxidase stain</td>
<td>Specific</td>
<td>Reagents not commercially available</td>
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<tr>
<td>PCR</td>
<td>Amplification of a DNA fragment of <em>L. intracellularis</em></td>
<td>Specific, shows pigs actively shedding</td>
<td>Possibility of false negative results when testing feces</td>
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<tr>
<td>Serology/IFAT</td>
<td>IgG Response</td>
<td>Sensitive</td>
<td>Demonstrates pigs that have been exposed but not necessarily have an active infection; Seroconversion typically takes 2-4 weeks after exposure.</td>
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Annual costs to the pig production industries have been estimated at $20 million (United States)\(^{(64)}\) and $3-$6.5 million (United Kingdom)\(^{(59)}\).

A survey in the United Kingdom indicated that 30% of the farms had a diagnosis of chronic PPE during a three year period\(^{(59)}\). In Australia, a survey indicated that 56% of the farms had a diagnosis of PPE by the producer, consulting veterinarian, or both between 1988 and 1990\(^{(27)}\). A survey of 73 farms in Spain using PCR of the feces indicated that 22% of the farms had pigs infected with *L. intracellularis*\(^{(39)}\). This estimate is believed to be low, as shedding of *L. intracellularis* in the feces is sporadic as described earlier. A survey of Iowa State University’s Veterinary Diagnostic Laboratory swine cases found that 5% of all pigs submitted were positive for *L. intracellularis* by PCR testing of the intestinal mucosa\(^{(33)}\). This study included all swine cases submitted for a period of 6 months (621 total cases) regardless of age or clinical history. The study found that the majority of pigs
with *L. intracellularis* were from the nursery and grow/finish pigs. The National Animal Health Monitoring System’s national 1995 swine study surveyed a total of 198 swine herds (2). Results demonstrated that 90% of the herds tested were either serologically positive for PPE in the breeding herd, the growing/finishing herd, or both. In growing/finishing herds, over 50% of the herds had greater than or equal to 40% of the samples positive per herd with anti-*L. intracellularis* antibodies. In breeding herds, 21% of the herds had greater than or equal to 40% of the samples per herd with positive results (2).

A serological survey was conducted in which animals were serially bled from the sow population, the nursery, and the grow/finish phase of a swine production system (63). Results demonstrated that pigs in the grow/finish phase had the highest prevalence of seropositive animals consistently at 8 weeks post-entry. Prevalence rates reached as high as 74% within some test groups. The nursery population had no positive animals and the sow population had a low percentage of animals positive throughout the test period (highest percentage was 12.6%). The sero-prevalence within the sows was most often seen midgestation, a time when the animals were recently moved.

Using pure cultures of *Lawsonia intracellularis* to infect animals, McOrist et. al. demonstrated a 6% to 25% reduction in weight gains in infected pigs (59). The costs of extra feed and increased time within a facility in the United Kingdom were estimated to be £2 to £7 per pig. Based on the number of pigs in the UK (14.5 million slaughtered annually) and with an estimate of 35% of the pigs affected with PPE, (55) using the market value of a pig at slaughter at the time of the article, the total direct cost of the disease in the UK was estimated to be £2 to £4 million annually (59). Other investigators have made
similar estimates of the cost of PPE within infected herds. Recent estimates of up to $8.50 per pig have been made in the United States (80).

**Treatment/Prevention**

With the development of pure culture systems, advances have been made in the testing of antimicrobial agents against *L. intracellularis*. Studies using *in vitro* cell culture systems determined the minimal inhibitory concentrations (MIC) of several antibiotics. Penicillin, erythromycin, difloxacin, virginiamycin, and chlortetracycline had the best results with MIC's of < 1 μg per ml. Tiamulin and tilmicosin had MIC's of < 4 μg per ml (49) and are compounds capable of entering the eukaryotic host cell cytoplasm and blocking prokaryotic protein synthesis (78). It is cautioned that *in vitro* results may not always correlate well with *in vivo* efficacy because of the pharmacodynamics of the drug involved. For example, the drug would need to be capable of killing microaerobic gram-negative intracellular bacteria located in the epithelium of the lower bowel. Some drugs may not readily penetrate into cells or may localize in inappropriate parts of the cell such as in cell lysosomes (78) while *L. intracellularis* is free within the cytoplasm (68). PPE is likely a multifactorial disease, and efficacy of some antimicrobials in the host may be the result of the effects of the treatment on other flora in the intestine (9).

Host animal trials have shown that several antibiotics are effective in the control of PPE. Tiamulin hydrogen fumarate (Denagard™) (50 g/ton and 35 g/ton) as a feed grade medication has been tested in several pure culture challenge trials (35, 60, 71). The antibiotic has also demonstrated its effectiveness when administered in the water at 60 ppm after the onset of clinical disease (35). Results suggest that tiamulin is an effective antibiotic
for the treatment and prevention of PPE by reducing gross and microscopic lesions, fecal shedding, clinical signs and by improving growth performance after challenge.

Carbadox (50 g/ton), erythromycin (70 g/ton) and chlortetracycline (100 g/ton) were shown to be effective in preventing PPE after gut homogenate challenges (84, 85). Neomycin-oxytetracycline (150/150 g/ton) and virginiamycin (10 g/ton) (85) were not effective for the control of PPE even though the MIC’s of tetracycline and virginiamycin were low (57). Studies have shown that lincomycin administered in the feed and neomycin and lincomycin with spectinomycin when administered in the water all provide effective treatment of ileitis after challenges using gut homogenates (83). Investigators have also shown that essential oils derived from the plant *Origanum* (72), which has been shown to have *in vitro* antimicrobial activities against various bacteria, may effectively control PPE in growing/finishing pigs under field conditions (77).

Tylosin (100 g/ton) was shown to be an effective drug against PPE in controlled field trials (61). The antibiotic has recently been approved by the Food and Drug Administration (FDA) for the control and treatment of ileitis in the United States.

It was shown that exposing pigs to infected premises for 3 weeks before starting antibiotic therapy in the feed appeared to produce an immune population, but antibiotic therapy prior to bacterial exposure prevents pigs from developing an immune response leading to a susceptible population (46). It has been suggested that medication for PPE be approached in one of three strategies: 1) exposure to PPE for 3 weeks followed by treatment for 2 weeks, 2) medication in the feed when clinical disease is apparent, or 3) continuous medication to slaughter (68). Proposed treatment regimens include intermittent (pulse) treatments that may allow a limited infection by *L. intracellularis* to occur hopefully
developing into a protective immune response (26, 84). The more common method of treatment is by continuous application in the feed or water and by parenteral treatment in the face of an outbreak (26, 84).

**Vaccines**

An avirulent live vaccine has recently been developed demonstrating a reduction of microscopic lesions typical of PPE after virulent challenge (35). The patented vaccine (Patent number: 5,885,823) was orally and intranasally administered to weaned pigs at 3 weeks of age. The attenuated strain was derived from a field strain that was continuously passaged in pure culture using a suspended cell culture system (35). Potential use of the vaccine would help reduce the need for costly antibiotics, maintain consistent weight gains, and lower mortality in gilt replacement herds.

Due to the difficulty in growing *L. intracellularis*, other researchers are investigating non-traditional methods for vaccine development. A recent publication describes the development of a partial DNA library for obtaining clones for production of material for taxonomic, diagnostic, and pathogenesis studies, as well as potential protective antigens against PPE (11).

**Conclusion**

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

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

With the tools available, veterinarians and producers are getting better equipped to diagnose PPE. To confirm that a pig is infected, a post-mortem diagnosis using H & E stain, silver stain, monoclonal antibody stain, or PCR of the intestinal mucosa can be done. To most producers, an ante-mortem test is more desirable. Polymerase chain reaction can be used to demonstrate animals that are actively shedding *L. intracellularis* and can also be used to estimate incidence of the organism by testing a percentage of pigs within a herd. The IFAT test on serum can be used as an ante-mortem test to demonstrate pigs that have been exposed to *L. intracellularis*. The test can also be used for surveys to predict incidence of the organisms by testing a percentage of pigs within a herd and estimating how many have been exposed to *L. intracellularis*. The IFAT test may be used to estimate herd shedding/exposure patterns by testing multiple samples over a period of time.

New developments have been made in controlling and preventing PPE. Several antibiotics are available that have been shown *in vitro* and in host animal studies to be effective at prevention and control of PPE. Vaccines have shown promise in providing additional tools for protection against infection of *L. intracellularis*.

There is still much to learn about *L. intracellularis* and the disease it causes. Future research should be directed toward the mechanisms of the cell proliferation that is caused by
L. intracellularis. Factors such as methods of attachment and invasion of the host cell still require attention to better understand how L. intracellularis infects the host cells and causes disease. Also, a better understanding of factors that make pigs more susceptible to the disease such as reproductive status, gender, breed and seasonal changes would be beneficial. With the development of vaccines, a better understand of the immune response will be needed to enhance delivery and improve methods for protection against L. intracellularis infection. Experiments will need to be performed to understand the effects of antibiotics on these vaccines and how the two options can be used together for effective control and prevention of PPE.

References


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CHAPTER 3. ALTERNATIVE METHOD OF CULTIVATION OF

**LAWSONIA INTRACELLULARIS** IN TISSUE CULTURES

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**Abstract**

Procedures were developed for the large-scale cultivation of *Lawsonia intracellularis* in spinnerflasks and bioreactors. The cultures were grown in McCoy tissue culture cells maintained in suspension and under reduced oxygen atmospheres. Growth was accomplished in 250 ml spinnerflasks, 3 liter bioreactors, and 20 liter bioreactors. Multiple parameters were measured including tissue cell counts and cell viability, pH, glucose, lactate, glutamine, ammonia, viable bacterial counts, and percent cell infection. Oxygen was removed by replacing the headspace with a mixture of 80% N2, 10% H2, and 10% CO2. Results demonstrated that bacterial growth was comparable at all vessel volumes and that bacterial titers correlated to cell viability.

**Introduction**

Porcine Proliferative Enteropathy (PPE) is a common disease of swine. The disease is characterized by a thickening of the mucosa of the small, and sometimes the large, intestine (23). Characteristic features of the disease are proliferation of the crypt epithelial cells with thickening of the mucosa of the small intestine and upper third of the spiral colon. Within these lesions are small, curved, intracellular organisms which are in the apical cytoplasm of proliferating cells (23).

The bacteria determined to be the cause of PPE were given the name *Lawsonia intracellularis* 1995 in honor of G. H. K. Lawson, the discoverer of the bacterium (16).
*Lawsonia intracellularis* is described as a gram-negative, microaerophilic, obligate intracellular, non-flagellated, non-spore-forming curved or S-shaped bacillus (13).

To date, growth of the bacteria in cell-free media has not been successful. *Lawsonia intracellularis* has been cultivated on tissue culture cells (rat intestinal cells [IEC-18] (13), human fetal intestine [Int 407] (13), rat colonic adenocarcinoma cells (13), pig kidney [PK-15] (13), piglet intestinal epithelial cells [IPEC-J2] (17), GPC-16 (25), and mouse cells [McCoy] (8) when incubated in reduced oxygen atmospheres (13, 25). Isolation of the organism from infected tissues requires homogenization and trypsinization of the tissue, passage of the homogenate through a series of filters down to a pore size of 0.65 μm to reduce the amount of contaminating organisms, and storage of the filtrate containing the organism in a sucrose potassium glutamate solution with 10% FBS at -70° C (13).

Pure cultures of *L. intracellularis* have many uses. Pure cultures have been a valuable tool in developing an understanding of the mechanisms of cell invasion, growth, and release (12, 17). They have also provided information on the sensitivity of *L. intracellularis* to a variety of antibiotics *in vitro* and *in vivo*. (15, 19, 21) Pure cultures have been used in a multitude of host animal studies (11, 18, 20, 24) and have provided antigen for diagnostic tests (9).

The need for large-scale cultures is apparent for diagnostic tests, basic research, and for the development of efficacious vaccines. This report describes the growth of *L. intracellularis* using a patented (United States patent 5,714,375, Appendix A) suspended cell system that has capabilities for large-scale cultivation.
Materials and Methods

Origin of bacterial inocula

*Lawsonia intracellularis* strain NP40 was obtained from the ileum of a 1.5 year old sow with proliferative hemorrhagic enteropathy. The intestinal sample was kept at -70° C until processing. Isolation of the bacteria and infection into McCoy cells (ATCC CRL 1696) on monolayers was performed using methodology previously described.(13) Briefly, a 75 cm² tissue culture flask was seeded with McCoy cells at 5000 cells/cm² and incubated eighteen hours at 37°C with 5% CO₂. The bacterial/gut homogenate suspension was added to 15 ml of Dulbecco’s Modified Eagles Medium (DMEM) with 5% fetal bovine serum (FBS), vancomycin (100 µg/ml), neomycin (50 µg/ml), and amphotericin B (2.0 µg/ml). The solution was added to the McCoy cells and the flask was placed in a gas chamber (BBL GasPak, Becton Dickinson and Co., Cockeysville, MD) in which the gas was then replaced with H₂ and CO₂ to give a mixture of 8.0% O₂, 8% CO₂ and 84% H₂. The culture was incubated at 37° C. Six days after infecting the cells, the monolayer was dislodged with a cell scraper and transferred into a 100 ml spinnerflask with 50 ml Dulbecco’s modified Eagle’s medium (DMEM) with 5% fetal bovine serum (FBS) with vancomycin (100 µg/ml), neomycin (50 µg/L), and amphotericin B (2.0 µg/ml). The spinnerflask was placed in a gas chamber in which the gas was then replaced with H₂ and CO₂ to give a mixture of 8.0% O₂, 8% CO₂ and 84% H₂. The culture was kept in suspension at 40 rpm and incubated at 37°C. The use of antibiotics was terminated after 2 passes in the suspension culture.

Passage of the bacteria was accomplished by passing a 10% volume of the infected culture to new uninfected cells every 7 to 14 days by seeding a 250 ml spinnerflask with
fresh McCoy cells seeded at $2 \times 10^5$ cells/ml in 100 ml DMEM with 5% FBS. The flasks were incubated 18 to 24 hours at 37° C with 8.0% O$_2$, 8% CO$_2$ and 84% H$_2$ prior to infecting with the bacteria.

For the experiments described in this paper, we used *L. intracellularis* strain NP40 (ATCC 55783) which was frozen at -70 C in Sucrose-Phosphate-Glutamate solution (SPG) (1) with 10% FBS after continuous growth for 40 weeks.

**Cultivation of suspension cultures**

McCoy cells (ATCC 1696) were seeded into ten 250 ml spinnerflasks at a rate of $2 \times 10^5$ cells/ml in 200 ml of DMEM/F12 media with 5% newborn calf serum (NBS). Seven of the flasks were infected by adding 5 ml of frozen inoculum containing $TCID_{50}$ $10^{5.5}$ bacteria/ml (MOI=0.04) into each spinnerflask. The spinner flasks were placed into gas chambers (BBL GasPak, Becton Dickinson and Co., Cockeysville, MD) which were evacuated at -500 mm Hg. The atmosphere was replaced with a mixture of 80% N$_2$/10% H$_2$/10% CO$_2$. The spinnerflasks were agitated at 40 rpm and incubated at 37° C. At 3-4 days post infection, an additional 75 ml of DMEM/F12/5% NBS was added to all cultures.

Another set of 250 ml flasks were set up the same as stated above and were used to inoculate into 3 L bioreactors (Applikon Inc., Foster City, CA) 6 days after infection. The bioreactors were seeded with McCoy cells at $2 \times 10^5$ cells/ml in 1500 ml DMEM/F12 with 5% NBS and 150 ml of inoculum was added. Another set of 3 L bioreactors were set up as described above and used to infect 20 L bioreactors (Applikon Inc., Foster City, CA) 6 days after infection by passing the infected culture into the 20 L bioreactors at a rate of 10% inoculum per culture volume. The bioreactors were overlayed with a gas mixture of 80% N$_2$, 10% H$_2$, 10% CO$_2$ to replace the oxygen in the headspace volume. The bioreactors were
agitated at 90 rpm to maintain the cells in suspension, and were incubated at 37°C. The bioreactors were programmed to control pH by adding a 10% sodium bicarbonate solution as needed to maintain the pH value at or above 6.9. Three days after infection, fresh DMEM/F12 media with 5% NBS was added to the 3 and 20 L bioreactors at volumes of 500 ml and 4 L respectively. A total of seven 3 L bioreactor replicates were completed and three 20 L bioreactor replicates were completed.

Monitoring cultures

The 250 ml cultures were sampled daily for various measurements including pH, cell counts, cell viability, glucose concentration, lactate concentration, percentage of cells heavily infected with *L. intracellularis*, and live titers using TCID<sub>50</sub> methods. The bioreactors were sampled the same as the 250 ml bioreactors with the additional testing of glutamine and ammonia concentrations.

The percentage of heavily infected cells (greater than 30 bacteria/cell) was monitored by collecting 2 ml of suspended culture, centrifuging at 2000 x g for 5 minutes, removing the supernatant and transferring the cell pellet onto a microscope slide. The slides were air dried and fixed with cold acetone/methanol for 2 min. Staining was carried out by immunofluorescence methods employing a mouse monoclonal antibody (14) as the primary antibody and anti-mouse immunoglobulin G-fluorochrome conjugate (fluorescein isothiocyanate ICN, Durham, NC). The slides were observed on an ultraviolet microscope and the percentage of heavily infected cells was estimated. Glucose and lactate concentrations were determined using a Model 2700 Select Biochemistry Analyzer (Yellow Springs Instruments, Inc., Yellow Springs, Ohio). Glutamine and ammonia concentrations were measured by a commercial kit (Sigma, St. Louis, MO). Quantitation of the Tissue
Culture Infectious Dose 50 percent (TCID$_{50}$) (22) of viable *L. intracellularis* was accomplished by passing 2 ml of the culture 8 to 10 times through a 25 gauge needle to homogenize the cell suspension. The samples were diluted by making serial 1:10 dilutions of the culture in DMEM/F12 with 5% NBS. The dilutions were added to a 96 well microtiter plate with 0.1 ml of diluted sample per well. Six wells were inoculated for each dilution. The microtiter plates were seeded with tissue culture cells at 1000 McCoy cells/well and grown 18-24 hours prior to infection. The infected plates were placed in chambers which were evacuated at -500 mm Hg. The atmosphere was replaced with a gas mixture of 80% N$_2$/10%H/10% CO$_2$. The cells were fixed with cold 50% acetone and 50% methanol for 2 minutes.

To the wells, 0.03 ml/well of anti-*L. intracellularis* monoclonal antibody (14) diluted 1:2000 in phosphate buffered saline (PBS) was added. The plates were incubated for 30 minutes at 37° C and then washed 3 times with PBS. Anti-mouse immunoglobulin G-fluorochrome conjugate (fluorescein isothiocyanate, ICN, Durham, NC) diluted 1:120 in PBS was added at 0.03 ml/well and incubated 30 minutes at 37° C. The plate was washed 3 times with ddH$_2$O and allowed to dry. Samples were observed on a fluorescent microscope and the TCID$_{50}$/ml of bacteria was calculated using the Reed-Muench method (22).

**Electron microscopy**

Culture samples were briefly centrifuged and supernatant removed and re-suspended in 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) fixative. Samples were spun and re-suspended for each subsequent step. Samples were rinsed in buffer and fixed in 1% osmium tetroxide in 0.1M cacodylate buffer for 1hr. at room temp. Samples were rinsed in buffer and dehydrated in graded ethanol series followed by ultrapure acetone washes prior to EPON resin infiltration and embeddment. After a 24hr. polymerization, the blocks were
sectioned using a Reichert Ultracut S ultramicrotome. Ultrathin sections were taken using a Diatome diamond knife, collected onto 200 mesh copper grids, and stained with 4% uranyl acetate followed by Sato's Lead stain. Images were collected using a JEOL 1200EX Scanning and Transmission Electron Microscope at 80 kV. Negatives were scanned using UMAX Powerlook 3000 scanner and imported into PhotoShop.

Contaminating bacteria and adventitious virus testing

Samples of the inoculum and McCoy cells were tested for the presence of contaminating virus and/or bacteria on tissue cultures and selective media by a contract laboratory (American Bioresearch Laboratories, Seymour, TN) using protocols which meet or exceed the USDA 9CFR regulatory requirements (sections 113.51, 113.55, 113.46, and 113.47).

Results

Light microscopy

Infected cells were examined by phase microscopy and showed no morphological change consisting of viability, cytopathic effects, syncytia, vacuoles, or inclusions when compared to non-infected cultures.

Spinnerflask growth

Viable cell numbers did not increase significantly throughout the experiments in any of the cultures. After 4 days, viable cell numbers in the 250 ml flasks started to decrease along with cell viability (Figure 1). Non-infected cells had similar growth with a slight decrease in viable cell numbers after 3 days and a steady decrease in percent viability very similar to the infected cultures (Figure 1). It was observed that the cells often began to aggregate into clusters of 10-20 cells after 3 days, which may account for some of the
variability between cultures. Glucose levels decreased and lactate levels increased in both infected and non-infected cultures (Figure 2), however, the changes appeared to be slightly faster in the infected cultures. The pH levels in the non-infected cultures continuously decreased to approximately 6.8 after 5 days, which coincided with the increase in lactate levels and drop in cell viability (Figure 3). Non-infected cultures also showed a continuous decrease in pH values but the change appeared slightly slower compared to infected cultures.

Infected cells increased from 10% of the cells heavily infected 1 day after infection, to 80-100% heavily infected cells by 6 days. Results revealed that TCID₅₀ titers typically peaked 4 days after infection (Average TCID₅₀ 10⁵.⁵/ml) (Figure 4), when the cells were 60-80% heavily infected (Figures 5-8). It was also observed that there was an increase of non-cell associated bacteria in the cultures after 3 to 4 days.

**Bioreactor growth**

Growth of the bacteria in the 3L and 20L bioreactors was similar to the 250 ml cultures with titers peaking at titers of TCID₅₀ 10⁵.⁸ bacteria/ml 3-5 days after infection in the 3 L bioreactors and at titers of TCID₅₀ 10⁶.⁰¹ bacteria/ml 6 days in the 20L bioreactors. Viable cell numbers remained consistent over time and cell viability remained high for up to 6 days post-inoculation in the 20 L vessels. Cell metabolism was similar in both the 3L and 20L sizes to the 250 ml cultures when comparing glucose and lactate levels over time. Glutamine and ammonia levels changed in the culture at rates comparable to glucose and lactate respectively. The pH reached 6.9 in the bioreactors by 2 days after which it was maintained by sodium bicarbonate addition (Figures 9-14).
Electron microscopy

McCoy cells infected with *L. intracellularis* showed numerous bacterial forms free within the cytoplasm (Figures 15-19). The bacteria were approximately 0.3 μm in diameter and 1 to 1.5 μm in length, curved or rod shaped. Evidence for uptake within a vacuole or endosome was observed (Figures 16-17). Evidence for intracellular bacterial multiplication was observed (Figures 18-19).

**Contaminating bacteria and adventitious virus testing**

Bacterial and McCoy seed vials were tested and found to be negative for the presence of bovine viral diarrhea virus, porcine parvovirus, porcine adenovirus, transmissible gastroenteritis virus, rabies virus, porcine hemagglutinating encephalomyelitis virus, bovine parvovirus, bovine adenovirus groups 3 and 5, bluetongue virus, bovine respiratory syncytial virus, reovirus, and *Mycoplasma sp.* The samples were also tested and found to be negative for cytopathogenic agents and hemadsorbing agents.

**Discussion**

The development of *in vitro* cultivation methods has provided information on the manner in which the bacteria infect cells in the host. It was shown that the bacteria gain entry into the host cell by close association with the cell membrane (17). The bacteria enter via membrane-bound vacuoles, which soon break down and release the bacteria to multiply free in the cytoplasm. It was shown that infection of cells is dependent on cell activity, but not on bacterial viability (12). A component of the entry process appears to be microfilament dependent but there is also evidence for a non-actin dependent pathway (17). This is similar to the manner in which *Chlamydia* species enter the host cell. However, *Chlamydia* remain within vacuoles throughout their life cycle (26). *Lawsonia intracellularis*
are often seen in close association with mitochondria and rough endoplasmic reticulum (6). This mode of entry and growth is similar to some *Rickettsia* species (3); (27) however, *Rickettsia* and *L. intracellularis* are genetically dissimilar (5, 16). Intracellular growth of the bacteria produces little cell morphologic change or lysis (2, 10, 13) and was observed that the bacteria were released from infected cells within cytoplasmic protrusions (17). These findings are similar to what has been observed *in vivo* in host animal studies (4, 6, 7).

This method of cultivation demonstrates that the infection of cells goes beyond those that are originally infected unlike previous reports (13). The bacteria are shed from infected cells that are added to the culture and enter other cells within the flask to eventually infecting the majority of the cells in the culture. In earlier studies using monolayer cells for infection, *L. intracellularis* did not spread beyond those cells originally infected with little evidence for late transfer of infection to previously uninfected cells in the culture (13). The spread of *L. intracellularis* within infected monolayers takes place by division of infected cells. This may be due to the static nature of the cultures which does not allow a distant migration of released bacteria. A suspension culture, with its more dynamic characteristic, increases the likelihood that released bacteria will come into contact with non-infected cells within the culture. Also, it has been shown that the bacteria require actively growing cells for multiplication, thus requiring the cell culture to be lightly seeded before infecting monolayer cultures (12). When the cell monolayer becomes confluent, it is believed the bacterial multiplication slows down. A suspension culture allows cells to continuously grow and reproduce as long as nutrients are available and metabolites do not build to a toxic level. This will allow the continuous growth of the bacteria by passing a portion of the culture to new cells or a continuous culture in which fresh cells and media are added to the existing culture.
The described experiments showed that bacterial titers increased until approximately 3-6 days after inoculation. At this time nutrients were diminishing and metabolites had begun to accumulate to possible toxic levels to both cells and bacteria. As stated earlier, the bacteria require actively growing cells for multiplication. This may be reflected in our findings as bacterial titers peaked at about the same time cell viability and viable cell counts began to decline. The percentage of infected cells continued to increase beyond the point the bacterial titers peaked, possibly the result of reduced bacterial viability but the continuation of bacterial infection and growth in new viable McCoy cells. This would result in the accumulation of both viable and non-viable bacterial cells which are indistinguishable by the FA method used. Since McCoy cell viability is declining but viable cell counts are remaining fairly stable at this point, the cultures are accumulating dead cells that may contain *L. intracellularis* with decrease viability.

When analyzing the 3 L and 20 L cultures, we observed a strong correlation between cell viability and bacterial titers. In the 3 L cultures, bacterial titers increased until day 5 which was also when cell viability started to decrease. In the 20 L cultures, bacterial viability was still increasing at day 6 with cell viability remaining stable.

Differences in glucose metabolism between the non-infected and the infected 250 ml spinnerflasks may be an indication of the metabolic demand that the bacteria have on the host cell. However, caution should be used when analyzing this information, as there was a slight difference in cell numbers between the treatment groups and more replicates of non-infected cultures may help answer these questions.

Although McCoy cell viability was prolonged with the pH control, the main cause of cell death was most likely due to the depletion of oxygen. Other bioreactor cultures operated
under the same parameters but with 5-20% dissolved oxygen resulted in McCoy cell growth reaching as high as $10^6$ cells/ml but with reduced bacteria titers (data not shown). The oxygen needed by the cells and the reduced oxygen requirements of the bacteria demonstrate a difficult balancing act that has potential for improvement in future experiments using tightly control parameters.

This information will be valuable for the continued improvement of *L. intracellularis* growth. Testing the control of pH, atmospheric conditions, and exchange of media and nutrients with depleted media will continue in future experiments. Optimizing these parameters may lead to improved McCoy cell growth and viability indirectly leading to improved bacterial titers.

Other work should focus on the bacteria that are released from the cells determining the role of these bacteria within the culture. It is believed that these bacteria enable the infection of new cells in the suspension culture, however studies need to be done to isolate and test the viability of the free bacteria to determine their potential role within this system.

The suspension culture has other advantages over a monolayer culture in that labor is reduced when harvesting and when passing the bacteria to new vessels; a portion of an infected culture is added to a new non-infected suspension culture. Monitoring the suspension culture has advantages as a direct sample can be taken from the culture as opposed to a monolayer culture, which requires a parallel culture for processing. Monolayer cultures still have some advantages over suspension cultures, especially for primary isolation of new bacteria. The exchange of media is more complete allowing contaminating bacteria to be removed and new antibiotic containing media to be added.
The suspension culture method has the potential to yield high volume cultures, as demonstrated in pilot scale 20 L cultures, needed for future vaccine production and for antigen production for basic research and diagnostic tests. As we scaled-up from the 250 ml volume to 20 L culture volumes we increased from $4 \times 10^8$ to $2 \times 10^{10}$ total viable bacteria.

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**References**


Figure 1. Average McCoy cell count and percent cell viability of infected vs. non-infected (control) 250 ml spinner flasks.

Figure 2. Average glucose and lactate levels of infected vs. non-infected (control) 250 ml spinner flasks.
Figure 3. Average measurements of pH of infected vs. non-infected (control) 250 ml spinner flasks.

Figure 4. Average TCID<sub>50</sub> titer and percent of heavily infected cell in infected 250 ml spinner flasks.
Figure 5. Infected McCoy cells 1 day after inoculation. Bacteria are stained by IFA using anti-\textit{L. intracellularis} monoclonal antibodies. Arrow shows heavily infected cell. Bar=10\textmu m.

Figure 6. Infected McCoy cells 2-3 days after inoculation with approximately 30\% of the cells heavily infected. Bacteria are stained by IFA using anti-\textit{L. intracellularis} monoclonal antibodies. Arrows show heavily infected cell. Bar=10\textmu m.
Figure 7. Infected McCoy cells 4-5 days after inoculation with approximately 60% of the cells heavily infected. Bacteria are stained by IFA using anti-L. intracellularis monoclonal antibodies. Bar=10μm.

Figure 8. Infected McCoy cells 6-7 days after inoculation with 100% of the cells heavily infected. Bacteria are stained by IFA using anti-L. intracellularis monoclonal antibodies. Bar=10μm.
Figure 9. Average cell count, percent cell viability, and pH of infected 3 L bioreactors.

Figure 10. Average glucose, lactate, glutamine, and ammonia concentrations of infected 3 L bioreactors.
Figure 11. Average viable bacteria counts (TCID\(_{50}\)) and % infected cells of infected 3 L bioreactors.

Figure 12. Average cell count, percent cell viability, and pH of infected 20 L bioreactors.
Figure 13. Average glucose, lactate, glutamine, and ammonia concentrations of infected 20 L bioreactors.

Figure 14. Average viable bacterial counts (TCID₅₀) and % infected cells of infected 20 L bioreactors.
Figure 15. TEM of thin sections of McCoy cells 6 days after infection with *L. intracellularis* showing a breakdown of the cell membrane and release of bacteria. Arrow shows possible uptake of bacteria into a neighboring cell. Other bacteria within the cells are free in the cytoplasm with no surrounding membrane. Bar = 1 μm.
Figure 16. Higher magnification of Figure 15 showing area where a bacterium is in an endosomal/vacuole-like structure partially surrounded by a filopodia extension. Bar = 500 nm.
Figure 17. TEM of thin sections of McCoy cells 6 days after infection with *L. intracellularis* showing a bacterium within a vacuole-like structure (arrow). Bar = 500 nm.
Figure 18. TEM of thin sections of McCoy cells 6 days after infection with *L. intracellularis* showing numerous bacteria free in the cytoplasm with a bacterium forming a septum suggestive of binary cell division. Bar = 200 nm.
Figure 19. Higher magnification of Figure 18 showing septum development (arrow). Bar = 100 nm.
CHAPTER 4. SAFETY OF A LIVE ATTENUATED LAWSONIA INTRACELLULARIS VACCINE IN SWINE

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Abstract

*Lawsonia intracellularis* is an obligate intracellular bacterium and the causative agent of porcine proliferative enteropathy (PPE). An attenuated strain of *L. intracellularis* strain NP40 was developed by continuous cultivation in suspended tissue culture cells for over 40 weeks. Three experiments were conducted to test the safety of the attenuated *L. intracellularis* strain in susceptible pigs. In experiment 1 the bacteria were intranasally administered at a dose of TCID$_{50}$ $10^7$ bacteria/dose in pigs 3 weeks of age given injections of dexamethasone. The attenuated strain was demonstrated to colonize tissues consisting of the ileum and mesenteric lymph node but failed to produce lesions typical of PPE. In experiment 2, 3 week old pigs were given multiple injections of dexamethasone, and were intragastrically administered a higher dose of TCID$_{50}$ $10^8$ bacteria per dose. Control pigs given a dose of $10^7$ bacteria per dose of the low passage parent strain showed significantly higher rates of lesion development, colonization, and shedding compared to pigs given the attenuated strain. In the third experiment, the attenuated bacterial strain was tested for reversion to virulence by 5 repeated passages through 1 day old Cesarean derived-colostrum deprived pigs. Results demonstrated that the bacteria were undetectable after the 2$^{nd}$ serial passage in the pig hosts.
Introduction

Porcine Proliferative Enteropathy (PPE) is a common disease of swine. The disease is characterized by a grossly visible thickening of the mucosa of the small, and sometimes the large intestine. Characteristic features of the disease are proliferation of the crypt epithelial cells, thickening of the mucosa of the small intestine, thickening of mucosa of the upper third of the spiral colon, and the presence of small, curved, intracellular organisms which are in the apical cytoplasm of proliferating cells. The causative agent of PPE has been shown to be a gram-negative, microaerophilic, obligate intracellular, non-flagellated, non-spore-forming curved or S-shaped bacillus called *Lawsonia intracellularis*.

Differing forms of the disease exist. These include porcine intestinal adenomatosis (PIA) which is a chronic form of PPE most commonly associated with pigs 6 to 20 weeks of age, and proliferative hemorrhagic enteropathy (PHE), which is a more acute condition frequently occurring in adult pigs.

The main clinical signs of PPE consist of loose, watery stools with or without blood, puddled feces with undigested feed, gauntness (railback), and lack of uniformity. These clinical signs are often seen after a stressful event, such as mixing or sorting, weather changes, shipping, overcrowding, and weaning.

Histopathological lesions common to all forms of PPE include epithelial hyperplasia, especially in the mucosal crypts. The crypts are elongated, enlarged, and lined with crowded immature epithelial cells with mitotic figures. There is also a distinct absence of goblet cells. The proliferating epithelial cells contain intracytoplasmic, slender, curved, rod-shaped bacteria named *Lawsonia intracellularis*.
Growth of the bacteria in cell-free media has not been successful. *Lawsonia intracellularis* has been cultivated on tissue culture cells (rat intestinal cells [IEC-18], human fetal intestine [Int 407], rat colonic adenocarcinoma cells, pig kidney [PK-15], piglet intestinal epithelial cells [IPEC-J2], Guinea pig cells [GCP-16] and mouse cells [McCoy]) when incubated in reduced oxygen atmospheres.

To date, the only methods to control PPE are the use of antibiotics. Host animal trials have shown that several antibiotics are effective in the control of PPE. Tiamulin (50 g/ton and 35 g/ton) as a feed additive has been tested in several pure culture trials. The antibiotic has also demonstrated its effectiveness when administered in the water at 60 ppm. Results suggest that tiamulin is an effective antibiotic for the treatment and prevention of PPE by reducing gross and microscopic lesions using a pure culture challenge model.

Carbadox (50 g/ton), erythromycin (70 g/ton) and chlortetracycline (100 g/ton) were shown to be effective in preventing PPE in pigs challenged with homogenized gut harvested from pigs clinically effected by PPE. Neomycin-oxytetracycline (150/150 g/ton) and virginiamycin (10 g/ton) were not effective for the control of PPE even though the MIC's of tetracycline and virginiamycin were low. Studies have shown that lincomycin administered in the feed and neomycin and lincomycin plus spectinomycin when administered in the water all provide effective treatment of PPE.

Tylosin (100 g/ton) was shown to be an effective feed additive against PPE in controlled field trials. The antibiotic has recently been approved by the Food and Drug
Administration (FDA) Center for Veterinary Medicine for the control and treatment of PPE in the United States.

Porcine proliferative enteropathy occurs in virtually all swine production systems. A recent serologic survey conducted by the National Animal Health Monitoring System (NAHMS) using its swine serum bank (representing 198 farms) found the prevalence of PPE in the United States swine herd to be 96%. The prevalence rate was 28% in all samples tested. Diagnostic investigations of PPE-affected farms in other countries suggest that approximately 35% of growing pigs are affected. Subclinical effects such as reduced rate and efficiency of growth have, however, been difficult to quantify. Direct financial losses due to decreased growth rates and feed efficiency have been estimated to cost $3.28-$11.48 per affected pig in the UK based on the results of five pure culture challenge studies. Annual costs to the pig production industries of several countries have been estimated at $20 million (United States) $3-$6.5 million (United Kingdom), and $25AUS per sow (Australia).

The following experiments describe the development and testing of the safety of a live attenuated *Lawsonia intracellularis* vaccine strain. These experiments are required by the United States Department of Agriculture (USDA) Center for Veterinary Biologics for the licensure of live attenuated vaccines.

**Materials and Methods**

**Bacterial isolate**

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

The intestinal sample was kept at -70° C until processing. Isolation of the bacteria was performed as previously described. The bacteria were infected into McCoy cells (ATCC CRL 1696) using methodology previously described. Briefly, a 75 cm² tissue culture flask was seeded with McCoy cells at 5000 cells/cm² and incubated eighteen hours at 37°C with 5% CO₂. The bacterial/gut homogenate suspension was added to 15 ml of Dulbecco’s Modified Eagles Medium (DMEM) with 5% fetal bovine serum (FBS), vancomycin (100 μg/ml), neomycin (50 μg/ml), and amphotericin B (2.0 μg/ml). The solution was added to the McCoy cells and the flask was placed in a gas chamber (BBL GasPak, Becton Dickinson and Co., Cockeysville, MD) in which the gas was then replaced with H₂ and CO₂ to give a mixture of 8.0% O₂, 8% CO₂ and 84% H₂. The culture was incubated at 37° C. Six days after infecting the cells, the monolayer was dislodged with a cell scraper into a 100 ml spinnerflask containing 50 ml DMEM with 5% FBS, vancomycin (100 μg/ml), neomycin (50 μg/L), and amphotericin B (2.0 μg/ml). The spinner flask was placed in a gas chamber (BBL GasPak, Becton Dickinson and Co., Cockeysville, MD) in which the gas was then replaced with H₂ and CO₂ to give a mixture of 8.0% O₂, 8% CO₂ and 84% H₂. The culture was incubated at 37° C. The use of antibiotics was terminated after 2 passes in the suspension culture.

Passage of the bacteria was accomplished by passing a 10% volume of the infected culture to new uninfected cells every 7 to 14 days. The new uninfected cells were seeded into spinner flasks by seeding a 250 ml spinnerflask with fresh McCoy cells at 200,000
cells/ml in 100 ml DMEM with 5% FBS. The cells were incubated 18 to 24 hours at 37°C with 8.0% O₂, 8% CO₂ and 84% H₂. The bacterial culture was passed by adding 10 ml of the infected cell culture to the new cells and incubating at the conditions described above. Passage of the bacteria was performed for 40 continuous weeks after which a single clone was harvested and stored in sucrose potassium glutamate buffer (SPG)²² with 10% FBS at -70°C. The high passage bacteria was designated strain NP40 (ATCC 55783).

For experiments 1 and 2, strain NP40 (ATCC 55783) was grown continuously in pure culture for 6 weeks. For experiment 3, strain NP40 was grown in pure culture for 2 weeks. The cultures were harvested by centrifugation at 3000 x g for 20 minutes. The pellets were resuspended in Sucrose-Phosphate-Glutamate solution (SPG) with 10% FBS and passed 4 times through a 25 gauge needle. The lysates were centrifuged at 500 X g for 5 minutes to pellet the debris. The supernatant was saved and stored at -70°C until approximately one hour before vaccination at which time it was thawed and stored on ice until administration. The titers of the vaccines were determined by the use of a TCID₅₀ method previously described.²³-²⁵

Contaminating bacteria and adventitious virus testing

Samples of the *L. intracellularis* inoculum and McCoy cells were tested for the presence of contaminating viruses and/or bacteria on tissue cultures and selective media by a contract laboratory (American Bioreserch Laboratories, Seymour, TN) using protocols which meet or exceed the USDA 9CFR regulatory requirements (sections 113.51, 113.55, 113.46, and 113.47).
Experiment 1: High dose safety

To initially assess vaccine safety, forty-six mixed sex, conventional pigs, were obtained from an Iowa farm with no recorded history of proliferative enteritis. The pigs were transported to the research facility where they were tagged to individually identify each pig. Fecal samples from each pig were tested by polymerase chain reaction (PCR) for the presence of *L. intracellularis* prior to initiation of the study with no positive pigs detected. The pigs were held at this facility for two days prior to initiation of the study to allow acclimation to the facility and were fed antibiotic-free feed throughout the study. The average age of the pigs at the time of inoculation was 3 weeks of age. On day 0 of the study, all pigs were weighed, bled to collect serum, clinically scored, and fecal swabs were collected. The pigs were then randomly divided into 6 groups that were penned separately. Groups 1 and 2 (vaccinates) had 12 pigs each and were divided into 2 pens and given an intranasal dose of $10^{7.8}$ bacteria/pig of *L. intracellularis* strain NP40. Two extra pigs were added to both groups 1 and 2 and were not dosed, serving as contact sentinels throughout the study. Groups 3 and 4 (controls) had 8 pigs each, which were divided into 2 pens and were intranasally dosed with non-infected McCoy cells. Groups 5 and 6 (challenged controls) had 4 pigs each and were divided into 2 pens and intranasally dosed with $10^{7.7}$ bacteria/pig of low passage virulent strain *Lawsonia intracellularis* strain N343. On days 0, 3, 6, and 9, dexamethasone at a dose of 4 mg/pig was given intramuscularly to the pigs in groups 1, 3, and 6.

Fecal swabs, body weights, and serum samples were collected at 7-day intervals throughout the study. The fecal swabs were processed for PCR testing using *L. intracellularis* specific primers defined by Jones, et. al. The serum samples were tested for
L. intracellularis specific IgG response using an immunofluorescence assay. Daily clinical observations of the pigs were made throughout the entire study, which included stool consistency, behavior, appetite, body condition, and hair coat. Each observation was on a scale from 1 to 4 with 1 being normal and 3 the most severe, as well as a score of 4 for each parameter in deceased animals. A healthy animal received a daily accumulative score of 5 and a deceased animal as the result of PPE received an accumulative score of 20. Rectal temperatures were recorded for 14 consecutive days after vaccination.

At weekly intervals, starting at 21 days post-vaccination, 3 pigs from groups 1 and 2 (vaccinates) and two pigs from groups 3 and 4 (controls) were necropsied. Two pigs from groups 5 and 6 (challenge controls) were necropsied on day 21 and day 42. At necropsy, samples of tonsil, lung, mesenteric lymph node, ileum, and colon were collected for PCR testing. Duplicate samples of the organs were placed in buffered formalin for histological examinations using hematoxylin and eosin stains, Warthin-Starry silver stains and immunofluorescent stains using a monoclonal antibody specific for L. intracellularis.

Experiment 2: High dose safety

The safety of the attenuated vaccine was further evaluated using twenty-six mixed sex, conventional pigs, 3 weeks of age purchased from a herd with no previous history of PPE and transported to the research facility where they were tagged to individually identify each pig. The pigs were held at this facility for 7 days prior to initiation of the study to allow acclimation to the facility and were fed antibiotic-free feed throughout the study. On day 0 of the study, all pigs were weighed, bled to collect serum, clinically scored, and swabbed rectally. The animals were divided into groups of 5 pigs each with an even distribution of pig weights in each group. Each pen was designated a separate treatment
group and each group was placed in separate rooms. Group 1 was orally dosed with the vaccine strain, NP40, at a dose of TCID$_{50}$ $10^{8.6}$/pig. Group 2 was orally challenged with low passage virulent *L. intracellularis* strain N343 at a dose of TCID$_{50}$ $10^{7.7}$/pig. The third and fourth groups were orally dosed with non-infected McCoy cells. At day 7 of the study, 3 sentinel pigs were comingled with group 1 (vaccinates) and 3 sentinel pigs were comingled with group 2 (challenge controls). The animals in groups 1, 2, and 3 were given 2 ml intramuscular injections of dexamethasone (4mg/ml) on days 0, 3, 6, and 9 of the study. Group 4 and all sentinels were not given dexamethasone at any time during the study.

The experimental pigs were observed daily for the duration of the study. These observations included stool consistency, behavior, appetite, body condition, and hair coat. Each observation was assessed a quantitative score as described for experiment 1. Rectal temperatures were recorded for seven days after vaccination/or challenge. Fecal swabs, weights, and serums were collected at 7-day intervals throughout the study. The fecal swabs were processed for PCR testing using *L. intracellularis* specific primers defined by Jones, et. al.$^{26}$ The serums were tested for IgG response using an immunofluorescence assay.$^{24}$

At 21 days post-vaccination or virulent challenge, all of the pigs, except sentinel pigs, were necropsied and samples of tonsil, lung, mesenteric lymph node, ileum, and colon were collected for PCR testing. Duplicate samples of the organs were placed in buffered formalin for histological examinations using hematoxylin and eosin stains, Warthin-Starry silver stains and immunofluorescent stains using a monoclonal antibody specific for *L. intracellularis*.$^{27}$ The sentinel pigs were necropsied and the same samples were collected 28 days post-vaccination.
Experiment 3: Reversion to Virulence

The experiment was designed to assess the likelihood of the reversion to virulence of the live attenuated *L. intracellularis* strain NP40 after 6 successive passes in the host species. The pigs were housed individually in hepa-filtered cages with independent air supplies. The animals in passes 1-5 were necropsied at 13-14 days post-inoculation to make recovery of the vaccine microorganism more likely and to limit the possibility of host elimination of the avirulent isolate which may occur with longer incubation periods. Animals in passage 6 were necropsied 21 days post inoculation to provide adequate time for manifestation of clinical disease and gross and histological lesions to develop if the vaccine microorganism reverted to virulence. For passes 1-5, ilea (approximately 20-30 cm) were collected at necropsy. The collected ilea were cut open and the fecal material was washed out with phosphate buffered saline (PBS). The mucosa was scraped in an attempt to recover *L. intracellularis* for passage to the next set of pigs. The homogenate was collected in a sucrose-potassium-glutamate (SPG) buffer with 10% fetal bovine serum. The mucosa was homogenized in a Tenbroeck tissue grinder, then stored on ice until administration to the pigs.

In passage 1, thirteen cesarean derived, colostrum deprived (CDCD) pigs at 1 day of age were randomly divided into 2 groups with 9 pigs intragastrically receiving TCID$_{50}$ titers of $10^{7.6}$/dose. Four pigs received no inoculum and were used as controls. All pigs were necropsied on day 13.

In passage 2, five (CDCD) pigs at 1 day of age were randomly divided into groups in which 4 pigs received pooled homogenate from vaccinated pigs from pass 1, and 1 pig
received pooled homogenate from the control group from group 1. All pigs were necropsied on day 14.

Passage 3 was performed the same as passage 2 in which the pigs received homogenate from the pass 2 pigs. All pigs were necropsied on day 14.

In passage 4, six (CDCD) pigs at 1 day of age were randomly divided into 2 groups in which 4 pigs received pooled homogenate from vaccine pigs from pass 3, and 2 pigs received pooled homogenate from the control pig from pass 3. All pigs were necropsied on day 14.

Passage 5 was performed the same as passage 2 in which the pigs received homogenate from pass 4. All pigs were necropsied on day 14.

In passage 6, nine pigs at 1 day of age were divided into 3 groups in which 5 pigs received pooled homogenate from vaccine pigs from pass 5, 2 pigs received homogenate from the control pig from pass 5, and 2 pigs received no treatment. All pigs were necropsied on day 14.

For passages 1-5, fecal swabs were collected on days 0, 5, 6, 7 and day of termination. The fecal swabs were processed for PCR testing using primers defined by Jones, et. al. At necropsy during each pass, samples of ileum and colon were collected for PCR testing. Duplicate samples of the organs were placed in buffered formalin for histological examinations using hematoxylin and eosin (H & E) stains, Warthin-Starry silver stains and immunofluorescent stains using a monoclonal antibody specific for *L. intracellularis*.

During passage 6, daily observations were made throughout the entire study, which included stool consistency, behavior, appetite, body condition, and hair coat. Each observation for each parameter was on a scale from 1 to 4 as described for experiment 1.
Rectal temperatures were recorded daily. Weights were recorded on day 0 and the day of termination.

Fecal swabs were collected on days 0, 7, 14 and day of termination (21 days). The fecal swabs were processed for PCR testing using *L. intracellularis* specific primers defined by Jones, et. al. At necropsy, samples of ileum, colon, tonsil, lung, mesenteric lymph node and liver were collected for PCR testing. Duplicate samples of the organs were placed in buffered formalin for histological examinations using Hematoxylin and Eosin stains, Warthin-Starry silver stains and immunofluorescent stains using a monoclonal antibody specific for *L. intracellularis*. Weights were recorded on day 0 and day of termination. Serum samples were collected on days 0 and 21 and were tested for *L. intracellularis* specific IgG antibody production by the pigs after exposure to *L. intracellularis*.

Samples were tested for the presence of pathogens in the intestines of the pigs from pass 6 after necropsy. The ilea and colons of all of the pigs were cultured by swabbing the internal contents and tested for the presence of *E. coli*, *Salmonella sp.* and *Serpulina sp.*

**Statistical analysis**

For the histopathology and serology results a Pearson chi-square statistic was calculated for daily test results to test for independence between treatment group and test result. The adjusted residual (observed-expected)/normalized Std. Err for residual) for each table cell was used to identify cells that differed significantly from the expected model of independence. Adjusted residuals are roughly the same as *z* scores in their distribution. For the clinical scores, temperature and average daily gains, the results were analyzed using an analysis of variance procedure and the SAS statistical system. The Duncan's multiple range
test for variables was performed on the data for each test. A student's t-test was performed on the data sets from experiment 3 passage #6.

Results

Experiment 1: High dose immunosuppression/sentinel

Detection of *L. intracellularis* using PCR demonstrated that no pigs were shedding the bacteria throughout the duration of the study. Polymerase chain reaction of the lungs, tonsils, and mesenteric lymph nodes did not detect *L. intracellularis*.

Histological sections of the collected tissues were fluorescent antibody (FA) stained using a monoclonal antibody against *L. intracellularis* as the primary antibody and anti-mouse immunoglobulin G-fluorochrome conjugate as the secondary antibody. One of three pigs from the vaccine with dexamethasone group was FA positive for the presence of *L. intracellularis* in the ileum, colon, and mesenteric lymph node on day 21. One of three pigs on day 21 from the challenge group was FA positive in the ileum and mesenteric lymph node. One of three pigs from the challenged group with dexamethasone on day 21 was positive in the mesenteric lymph node. One of three pigs from the vaccinated group/no dexamethasone was positive on day 28 in the ileum and mesenteric lymph node. All tissues that were positive by FA were mildly infected with 1-5 foci of bacteria in the section.

Hematoxylin and eosin stains of sections from the necropsied pigs revealed non-specific mild to moderate diffuse infiltration of predominantly mononuclear inflammatory cells throughout the mucosa in all of the pigs. Villi were of normal length. The inflammation could not be attributed to one specific cause and may have been a non-specific response of the intestine to a variety of insults including feed antigens, molds, or non-pathogenic bacteria. At 21 days of the study, 1 of 3 pigs from vaccinated/dexamethasone
group exhibited increased but insignificant proliferation and a small cluster of inflammatory cells in the crypt lumen with bacteria in apical portions of the proliferating epithelial cells by silver stain. This pig was also positive by the FA stain. The changes in these single crypts would not have resulted in any grossly identifiable lesions and did not have had any clinical significance for the pig. The other pigs necropsied at day 21 did not demonstrate lesions typical of PPE. At 28, 35, and 42 days, no lesions suggestive of PPE and no intracellular bacteria were detected in small or large intestines from any of the pigs.

Average weight gains were calculated during the periods between vaccination/challenge and termination. Differences in weight gains between groups were small and no reduction in weight was observed compared to non-vaccinated controls (Figure 1). No significant temperature differences were observed between treatment groups throughout the study as the range of temperatures were from 103.3° F in the vaccinates that received dexamethasone to 102.7° F in control pigs that did not receive dexamethasone. No significant differences in clinical scores were observed between the treatment groups throughout the study as mean scores ranged from 5 to 5.05 between treatment groups.

Non-specific lesions were observed at necropsy, however no differences were observed when vaccinated pigs were compared to control pigs.

Two animals were positive for the presence of IgG antibodies against *L. intracellularis* at the beginning of the study when the animals were 3 weeks old, but were no longer positive at day 7. Seroconversion began at 14 days post-inoculation. Dosed animals that seroconverted remained serologically positive throughout the study. One animal in the control group was positive at day 21 but was no longer positive at later bleedings. The number of sero-positive animals peaked between 21 and 35 days in animals that were dosed
with high passage or parent \( L. \text{intracellularis} \) strains. Sentinel animals also seroconverted showing that the vaccine was shed to penmates. Seroconversion in sentinels started 28 days after the start of the study (Table 1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
<th>Day 35</th>
<th>Day 42</th>
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<tbody>
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<td>8 (1/12)</td>
<td>0</td>
<td>33 (4/12)</td>
<td>75 (9/12)*</td>
<td>89 (8/9)*</td>
<td>83 (5/6)*</td>
<td>100 (3/3)*</td>
</tr>
<tr>
<td>NP40</td>
<td>0</td>
<td>0</td>
<td>33 (4/12)</td>
<td>83 (10/12)*</td>
<td>89 (8/9)*</td>
<td>100 (6/6)*</td>
<td>100 (3/3)*</td>
</tr>
<tr>
<td>Control/Dex</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>13 (1/8)*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Challenge/Dex</td>
<td>25 (1/4)</td>
<td>0</td>
<td>0</td>
<td>100 (4/4)*</td>
<td>100 (2/2)*</td>
<td>100 (2/2)*</td>
<td>100 (2/2)*</td>
</tr>
<tr>
<td>Challenge</td>
<td>0</td>
<td>0</td>
<td>50 (2/4)</td>
<td>75 (3/4)*</td>
<td>100 (2/2)*</td>
<td>100 (2/2)*</td>
<td>100 (2/2)*</td>
</tr>
<tr>
<td>NP40/Dex: Sentinel</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>50 (1/2)*</td>
<td>50 (1/2)*</td>
<td>50 (1/2)*</td>
</tr>
<tr>
<td>NP40: Sentinel</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>50 (1/2)*</td>
<td>100 (2/2)*</td>
</tr>
</tbody>
</table>

*indicates that this cell differs significantly from non-exposed control pigs (p < 0.05)

**Experiment 2: High dose immunosuppression/sentinel**

Vaccinates received approximately 400 X the efficacious dose.\(^9\) No \( L. \text{intracellularis} \) DNA was detected in the feces by PCR in the vaccinates throughout the study. Three of five pigs in group 1 had \( L. \text{intracellularis} \) detected in the ileum by PCR. No other organs were positive by PCR in the vaccinated group. None of the sentinel animals housed with the vaccinates were positive by PCR in the feces or in the organs. By contrast 4 of 5 of the challenged control animals were PCR positive in the feces and colon.

All of the challenged animals (5 of 5) were colonized by \( L. \text{intracellularis} \) in the ileum as determined by PCR, fluorescent antibody stains and silver stains. In addition, one sentinel animal housed with challenged pigs was also PCR positive for \( L. \text{intracellularis} \) in
the colon (Table 4). Samples of the mesenteric lymph node (MLN), lung, and tonsil were pooled within treatment groups for PCR testing. All pooled samples were negative.

One vaccinate was FA positive in the ileum and colon. None of the sentinels housed with the vaccinates were positive by FA stains. All other organs were negative for *L. intracellularis*. Fluorescent antibody stains demonstrated that all of the challenge pigs were positive in the ileum and colon for the presence of *L. intracellularis*. Two of three sentinels housed with the challenged pigs were FA positive in the ileum.

Histological examination found that one pig in the vaccine group had mild hyperplasia in the ileum. This was the same pig that was positive by the FA stain. However, all animals that received the challenge had mild to severe crypt proliferation. Silver stains demonstrated intracellular bacteria within crypt cells of the ileum of one vaccinated pig (1 of 5) and intracellular bacteria in the ilea of all (5 of 5) challenged pigs. No intracellular bacteria were observed in any of the other sections that were collected (Table 2).

Histological sections of the sentinel animals did not reveal intracellular organisms in any of the organ samples submitted. One challenge sentinel had mild crypt hyperplasia in the ileum but no organisms were identified (Table 2).

Average weight gains were calculated during the periods between inoculation and termination. No significant differences were observed between treatment groups. (Figure 2)

Rectal temperatures were recorded throughout the experiment. Variable results were observed throughout the study. The vaccinates had a mean rectal temperature of 104.0°F which was slightly higher than the other groups (range 103.4°F to 103.6°F), but this small difference was not of biological or statistical significance.
An increase in mean clinical scores was observed in the vaccinate group compared to the other groups. This increase was attributed primarily to a single pig, which had diarrhea 10 days after inoculation and continued to termination. This same pig was found to be colonized by *L. intracellularis* (Figure 3).

Seroconversion was observed at 21 days after dosage of the pigs in 4 of 5 pigs that received vaccine and in 4 of 5 pigs that were challenged with strain N343. Seroconversion was observed in one of the sentinel pigs that was placed in the challenge pen showing that the virulent bacteria were shed and transmitted to the sentinel pig (Table 3).

### Table 2: Experiment 2 Histopathology and PCR results, % Positive

<table>
<thead>
<tr>
<th>Group</th>
<th>FA ileum</th>
<th>FA Colon</th>
<th>H &amp; E ileum</th>
<th>Silver Stain ileum</th>
<th>PCR ileum</th>
<th>PCR colon</th>
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<td>sentinel</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N343</td>
<td>100 (5/5)*</td>
<td>100 (5/5)*</td>
<td>100 (5/5)*</td>
<td>100 (5/5)*</td>
<td>100 (5/5)*</td>
<td>80 (4/5)*</td>
</tr>
<tr>
<td>sentinel</td>
<td>67 (2/3)</td>
<td>33 (1/3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>33 (1/3)</td>
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<td>Control/Dex</td>
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<td>Control</td>
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</tr>
</tbody>
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* indicates cell differs significantly (p < 0.05)

### Table 3: Experiment 2 Serology Results, % Positive

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
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*indicates that this cell differs significantly (p < 0.05).
Fecal swabs were collected at 14 days post-vaccination and delivered to the Iowa State University Diagnostic Laboratory and checked for other pathological agents. Hemolytic *E. coli* strains were isolated from 2 pigs (1 pig from the vaccinated group and 1 pig from the challenged group). The swabs were negative for *Salmonella* sp., *Serpulina hodysenteriae* and *S. pilosicoli*.

**Experiment 3: Reversion to virulence**

In pass 1, fecal shedding was detected in 1 pig from the vaccine group on day 7. Shedding was not detected on other sampling days. Polymerase chain reaction of the ilea demonstrated *L. intracellularis* in 4 of 9 pigs in the vaccine group. No control pigs were shown to be colonized by *L. intracellularis* by PCR. Fluorescent antibody stains detected *L. intracellularis* in the ilea in 5 of 9 pigs in the vaccine group and in no pigs in the control group. Hematoxylin and eosin stains demonstrated the presence of mild enteritis in 6 of 9 pigs in the vaccinated group, and 1 of 4 pigs in the control group. Silver stains demonstrated the presence of intracellular bacteria in 4 of 9 pigs in the vaccine group, and no pigs in the control group. None of the pigs had significant microscopic lesions associated with the presence of the organisms. The crypt hyperplasia observed in virtually all pigs was mild, likely the result of nonspecific irritation, rather than the typical lesions of porcine proliferative enteropathy.

In pass 2, fecal shedding of *L. intracellularis* was detected by PCR in 2 pigs from the group of pigs that received the serial passage of gut homogenate originating from vaccinated pigs by PCR on day 7. Testing of the ilea by PCR did not demonstrate *L. intracellularis* in the vaccine group. *L. intracellularis* was not detected by PCR in the control pig. No abnormalities were noted in tissue sections in any pigs with H and E stains. Silver stains
demonstrated suspicious intracellular particles in 1 of 4 pigs in the vaccine group. One pig in the control group had suspicious particles and one pig appeared to have intracellular bacteria. However, PCR and FA stains were unable to detect *L. intracellularis* in any of the sections. This may be attributed to the non-specific nature of the silver stain.

In passes 3-6, *L. intracellularis* was not found by PCR in the feces or intestines in any of the pigs. No abnormalities were noted in any pigs with H and E stains, silver stains, and FA stains. Polymerase chain reactions, H and E stains, silver stains, and FA stains of sections from the lung, liver, tonsil, colon, and mesenteric lymph node from pass 6 did not demonstrate detectable *L. intracellularis* or lesions typical of PPE (Table 4).

### Table 4: Results of PCR and Histopathology testing Experiment 3

<table>
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<tr>
<th>Pass 1</th>
<th>Fecal PCR Day 5</th>
<th>Fecal PCR Day 6</th>
<th>Fecal PCR Day 7</th>
<th>Ileal PCR</th>
<th>H&amp;E</th>
<th>Silver</th>
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</table>
Average daily weight gains (ADG) in passage 6 were calculated during the periods between inoculation and termination. A significant difference was observed between groups with vaccinates having an ADG of 13 grams and controls having an ADG of 59 grams.

Temperatures were recorded daily during pass 6. Significant differences were not observed between groups with an average daily temperature of 101.3° F in the vaccinates and 102.4° F in the control group.

Clinical scores were recorded daily and averaged by treatment group during pass 6 with differences observed between treatment groups after day 4 of the study with the vaccine group having an average daily score of 6.9 and controls having an average daily score of 5.7.

Due to the morbidity of animals from pass 6, samples were tested for the presence of pathogens in the intestines of the pigs after necropsy. The ilea and colons of all pigs from pass 6 were cultured by swabbing the internal contents. Three of the five pigs from the vaccine group and 2 of 4 control pigs were colonized with _Salmonella oranienburg_ which may have been the cause for the elevated clinical scores and reduced weight gains in the vaccine group.

No pigs from pass 6 had detectable levels of antibodies against _L. intracellularis_ at day 0 and day 21. Serum from passes 1-5 were not tested because of the short time period of each pass which would not allow time for adequate seroconversion.

**Contaminating bacteria and adventitious virus testing**

Seed vials of strain NP40 were tested and found to be negative for the presence of bovine viral diarrhea virus, porcine parvovirus, porcine adenovirus, transmissible gastroenteritis virus, rabies virus, porcine hemagglutinating encephalomyelitis virus, bovine parvovirus, bovine adenovirus groups 3 and 5, blue tongue virus, bovine respiratory syncytial
virus, reovirus and *Mycoplasma sp.* The samples were also tested and found to be negative for cytopathogenic agents and hemadsorbing agents.

**Discussion**

This report describes the safety testing of an attenuated strain of *L. intracellularis* developed as a vaccine for the prevention of PPE in swine. The attenuation of the bacteria was accomplished by cell culture adaptation of the virulent organism following repeated *in-vitro* passage of the organism in tissue culture cells. This is a common method of attenuation for both bacteria and viruses. McOrist et al. observed that pigs which received higher passage *L. intracellularis* developed lesions that were less severe compared to animals that received a lower passage isolate, possibly as the result of repeated *in vitro* passage of the isolate. The bacterial isolate used in the studies described in this paper was shown to be safe at high doses (60 X the efficacious dose in study 1, 400 X the efficacious dose in study 2 and 40 X the efficacious dose in study 3) in dexamethasone treated pigs and after repeated oral passage in the host animal.

These experiments used high doses of the vaccine along with dexamethasone and showed that animals became colonized, however, they did not develop lesions typical of PPE. The pigs dosed with virulent *L. intracellularis* in the first experiment failed to develop gross or microscopic lesions typical of PPE. This may have been due to a dose too low for proper challenge, or the intranasal route of administration did not allow proper exposure, or the method of *in vitro* propagation prior to administration reduced the virulence of the bacteria, or pig resistance and lack of susceptibility to *L intracellularis*. The lack of lesion development in the challenge control pigs made it not possible to conclude the attenuation of strain NP40. This result motivated us to repeat the study and perform experiment 2.
In experiment 2, microscopic lesions, fecal shedding, and ileal PCR resulting from attenuated strain were of significantly lesser magnitude when compared to those occurring in pigs given the low passage virulent strain. The difference was also seen with almost a 10 fold higher dose of the attenuated bacteria compared to the challenge inoculum. The dose of NP40 was also 400 X the efficacious dose needed for protection against virulent challenge. An intragastric route was used for this experiment to ensure the placement of the bacteria in the gastrointestinal tract to increase the likelihood of the bacteria infecting the host tissue naturally infected by virulent *L. intracellularis*.

A difference in average daily weight gains was observed between pigs from experiment 1 compared to pigs from experiment 2. This difference is most likely the result of the experimental time differences between experiments with 42 days as the length of experiment 1 and 21 days as the length of experiment 2.

In experiment 3, repeated passage of *L. intracellularis* strain NP40 in pigs did not revert to virulence in 1 day old CDCD pigs free of active or passive immunity to *L. intracellularis*. The use of conventional pigs poses a possible risk of confounding prior or existing infection in PPE experiments. One day old CDCD pigs were used for this experiment to ensure that if pigs did became colonized the source of infection originated from the vaccine and not from the source herd. It has been shown that very young pigs are susceptible to *L. intracellularis* challenge. McOrist et. al. demonstrated the disease in 7 day old gnotobiotic pigs after crude homogenate challenge. Pigs in the first passage had a high degree of colonization, however, lesions were not typical of PPE. Subsequent passage of the tissues in pigs did not demonstrate increased virulence, but actually a failure to recover the bacteria.
In experiments 1 and 2, antibody responses against *L. intracellularis* showed a high rate of seroconversion by 21 days after the exposure of the pigs to both strain NP40 and strain N343. Two animals in experiment 1 were positive for IgG antibodies against *L. intracellularis* at the start of the study. This was most likely due to maternal antibodies, as all of the animals were negative 7 and 14 days later.

Before a vaccine can be used it must demonstrate both safety and efficacious protection against virulent challenge. The attenuated *L. intracellularis* strain NP40 has shown protection against the development of microscopic lesions in pigs orally administered $10^9$ bacteria per dose. The attenuated *L. intracellularis* strain was dosed in 3 week old pigs and demonstrated a significant reduction of microscopic lesions typical of PPE consisting of crypt hyperplasia and presence of *L. intracellularis* in tissues.

These experiments have shown that a safe vaccine microorganism for swine derived from *L. intracellularis* can be successfully cultivated, maintained, and attenuated in McCoy tissue culture cells kept in suspension. *Lawsonia intracellularis* is a fastidious bacteria requiring specialized techniques and atmospheres in order to grow. The ability to grow large quantities of the organism would provide a means to obtain sufficient antigen for experimental studies, diagnostic tests and possible commercial-scale vaccine production.

**Acknowledgements**

We thank the assistance of Mr. Christian Baum and Ms. Kelly Burkhart for the maintenance of the bacterial cultures and sample testing. Also, we thank Dr. Kent Schwartz and Dr. Bruce Janke for the histopathological analysis on the tissue samples and Douglas Cleveringa, Dr. Jeffrey Husa, Dr. Reid Philips, Mr. Lance Oostenink, Mr. Jeremy Wiersema, Mr. Todd Duistermars, Dr. David Carter, and Dr. Rexanne Struve for their help with the
animal studies. For the statistical analysis, we thank Dr. Kevin Flaming. These experiments were paid for by Boehringer Ingelheim Vetmedica, Inc.

References


Figure 1. Average daily gains of pigs 42 days after inoculation from experiment 1. No significant differences were observed between vaccinate groups and control groups.

Figure 2. Average daily gains of pigs 21 days after inoculation from experiment 2. No significant differences were observed between vaccinate groups and control groups.
Figure 3. Average daily clinical scores of pigs from experiment 2. (*) represents groups significant at (p > 0.05).
CHAPTER 5. EFFICACY OF A LIVE-ATTENUATED STRAIN OF

*LAWSONIA INTRACELLULARIS* FOR THE PREVENTION OF

PORCINE PROLIFERATIVE ENTEROPATHY.

A paper to be submitted for publication in the American Journal of Veterinary Research

Jeffrey P. Knittel, Jeremy Kroll, Phil Hayes, Michael B. Roof

**Abstract**

*Lawsonia intracellularis* is the causative agent of porcine proliferative enteropathy. An attenuated strain of *L. intracellularis* was developed using continuous *in vitro* suspension cultures of *L. intracellularis*. Three host animal experiments were performed in pigs vaccinated at 3 weeks of age to determine the efficacy of the attenuated *L. intracellularis* strain NP40 against a pure culture challenge of the virulent parent *L. intracellularis* strain N343. Experiment 1 was a feasibility study to determine if vaccination with a high passage isolate derived from strain N343 would provide protection against a virulent challenge of *L. intracellularis*. A twenty-five pig study was performed with 10 vaccinates, 10 challenge controls, and 5 strict controls. The vaccinates were given a nasopharyngeal gavage of TCID\textsubscript{50} 7.2 x 10\textsuperscript{6} bacteria/dose of a high passage culture of *L. intracellularis* N343. Twenty-six days after vaccination, the vaccinates and challenge controls were challenged with pure cultures of the low passage parent *L. intracellularis* strain N343. A significant reduction of microscopic lesions was observed with 70% of challenge controls positive with no vaccinates positive as determined by fluorescent antibody stains of histological sections of the intestines 21 days after challenge. In experiment 2, seventy pigs, 3 weeks of age were divided into 5 groups. Group 1 had 15 pigs that received an oral dose of *L. intracellularis* strain NP40 with
TCID$_{50}$ $10^6$ bacteria/pig, group 2 had 20 pigs that received an oral dose of TCID$_{50}$ $10^5$ bacteria/pig, and group 3 had 15 pigs that received an oral dose of TCID$_{50}$ $10^4$ bacteria/pig. Group 4 had 15 pigs that were not vaccinated serving as challenge controls and group 5 had 5 pigs that served as strict controls. All pigs except the strict controls were intragastrically challenged 28 days after vaccination with a pure culture of low passage $L$. intracellularis strain N343. Polymerase chain reaction demonstrated that 9 of 15 challenge controls were colonized while 1 of 15 pigs in group 1 were positive. Histopathological testing showed a significant reduction in colonization and lesions with 60% of challenge controls compared to 7% of the pigs in group 1. In experiment 3, thirty-two pigs 3 week of age were divided into 2 groups. Group 1 had 19 pigs that were vaccinated with TCID$_{50}$ $10^6$ bacteria/pig of $L$. intracellularis strain NP40. Group 2 had 9 pigs that served as challenge controls and group 3 had 4 pigs that served as strict controls. A significant reduction was observed in fecal shedding, detected by PCR (56% challenge controls/5% vaccinates), and colonization by histological and PCR testing of the ileum (67% challenge controls/5% vaccinates) in pigs challenged 8 weeks after vaccination with the low passage $L$. intracellularis strain N343. In conclusion, a live attenuated strain of $L$. intracellularis reduced the colonization and shedding of $L$. intracellularis in vaccinated pigs following challenge with pure cultures of virulent $L$. intracellularis.

**Introduction**

Porcine Proliferative Enteropathy (PPE) is a common disease of swine. The disease is defined as a thickening of the mucosa of the small, and sometimes the large, intestine.$^1$ The disease has characteristic features consisting of proliferation of the crypt epithelial cells and thickening of the mucosa of the small intestine and the upper third of the spiral colon.$^1$
Pigs between 6 and 20 weeks of age, and replacement breeding stock are often affected. Pigs under 6-8 weeks are less commonly affected and it is commonly thought that colostral immunity provides resistance to infection.\(^2\)

The disease has a wide variety of clinical signs along with a variety of descriptive terminologies. Porcine intestinal adenomatosis (PIA) describes thickening of the intestinal mucosa due to epithelial proliferation and is a chronic form of the disease occurring most often in pigs 6 to 20 weeks of age. Porcine intestinal adenomatosis presents with mild clinical signs usually consisting of slower weight gains.\(^1\) Necrotic enteritis (NE) describes deep coagulative necrosis of an adenomatous mucosa. NE is the secondary condition of PIA in which the severe thickening of the ileum, or “hose-pipe” ileum, occurs, usually accompanied by persistent diarrhea.\(^1\) Proliferative hemorrhagic enteropathy (PHE) describes massive hemorrhage into the intestinal lumen from proliferated mucosa.\(^1\)

Histopathological lesions of the disease include epithelial hyperplasia, especially in the mucosal crypt with a distinct absence of goblet cells. Intracytoplasmic, slender, curved, rod-shaped bacteria are observed within the proliferating epithelial cells.\(^3\) The causative agent, considered to be a novel taxonomic genus and species, has been given the name *Lawsonia intracellularis*.\(^4\) The bacteria was previously known by the vernacular name Ileal Symbiont (IS) intracellularis.\(^5\)

Growth of *L. intracellularis* on conventional media has not been successful. The bacteria have been cultivated on tissue culture cells in a reduced oxygen atmosphere of 5.0-8.0% \(\text{O}_2\) and 7.0% \(\text{CO}_2\).\(^6,7\) Pure cultures of *Lawsonia* (Ileal Symbiont) *intracellularis* were used to satisfy Koch’s postulates in pigs in 1993.\(^8\)
Several surveys have been completed to estimate the prevalence of the disease. A serological test identifying swine IgG antibodies against *L. intracellularis* was used to survey 198 swine herds from the National Animal Health Monitoring System's national 1995 swine study. Results demonstrated that 90% of the herds tested were either serologically positive for PPE in the breeding herd, the growing/finishing herd, or both, with an average of 28% of the pigs from each herd positive. A survey of British farms indicated that the prevalence of PPE was 31 per cent based on responses to questionnaires. Surveys in Spain, Denmark, and Taiwan had farm prevalence rates of 22%, 40%, and 30%, respectively.

Using pure cultures of *Lawsonia intracellularis* to infect animals, McOrist et al. demonstrated a 6% to 25% reduction in weight gains in infected pigs. The costs of extra feed and increased time within a facility in the United Kingdom were estimated to be £2 to £7 per pig. Recent estimates of the economic impact of *L. intracellularis* of up to $8.50 per pig have been made in the United States.

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

Management practices may reduce the incidence of PPE in herds. The clinical signs are often seen after a stressful event, such as mixing or sorting, weather changes, shipping, overcrowding, and weaning. It was shown that exposing pigs to infected premises for 3 weeks before starting antibiotic therapy in the feed appeared to produce an immune
population, but antibiotic therapy before pigs have time to respond to the disease produced a susceptible population.\textsuperscript{21} It has been suggested that medication for PPE be approached in one of three strategies: 1) exposure to PPE for 3 weeks followed by treatment for 2 weeks, 2) medication in the feed when clinical disease is apparent, or 3) continuous medication to slaughter.\textsuperscript{1}

Host animal trials have shown that several antibiotics are effective in the control of PPE. Tiamulin (50 g/ton and 35 g/ton) as a feed grade medication has been tested in several pure culture trials.\textsuperscript{22, 23} Results indicate that tiamulin is an effective antibiotic for the treatment and prevention of PPE by reducing gross and microscopic lesions in a pure culture challenge model.

Carbadox (50g/ton), erythromycin (70 g/ton) and chlortetracycline (100 g/ton) were shown to be effective in preventing PPE in pigs challenged with tissue homogenate harvested from the ilea of clinically affected pigs. Treated pigs displayed improved performance, clinical scores, and gross and microscopic lesions using these antimicrobial therapies.\textsuperscript{20, 24} Neomycin-oxytetracycline (150/150 g/ton) and virginiamycin (10 g/ton)\textsuperscript{24} were not effective for the control of PPE even though the \textit{in vitro} MIC's of tetracycline and virginiamycin were low for \textit{L. intracellularis}.\textsuperscript{25}

Tylosin (100 g/ton) was shown to be an effective drug against PPE in controlled field trials.\textsuperscript{26} Pigs given tylosin had reduced lesions and increased weight gains. The antibiotic has recently been approved by the Food and Drug Administration (FDA) Center for Veterinary Medicine for the control and treatment of PPE in the United States.
This report describes the use of a live-attenuated strain of *Lawsonia intracellularis* to vaccinate pigs and to protect against colonization and disease manifestations after virulent challenge with a low passage virulent strain of *L. intracellularis*.

**Materials and Methods**

*Lawsonia intracellularis* strains

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

The intestinal sample was kept at -70° C until processing. Isolation of the bacteria was performed as previously described. The bacteria were infected into McCoy cells (ATCC CRL 1696) using methodology previously described. Briefly, a 75 cm² tissue culture flask was seeded with McCoy cells at 5000 cells/cm² and incubated eighteen hours at 37°C with 5% CO₂. The bacterial/gut homogenate suspension was added to 15 ml of Dulbecco's Modified Eagles Medium (DMEM) with 5% fetal bovine serum (FBS), vancomycin (100 μg/ml), neomycin (50 μg/ml), and amphotericin B (2.0 μg/ml). The solution was added to the McCoy cells and the flask was placed in a gas chamber (BBL GasPak, Becton Dickinson and Co., Cockeysville, MD) in which the gas was then replaced with H₂ and CO₂ to give a mixture of 8.0% O₂, 8% CO₂ and 84% H₂. The culture was incubated at 37° C. Six days after infecting the cells, the monolayer was dislodged with a cell scraper into a 100 ml spinnerflask containing 50 ml DMEM with 5% FBS, vancomycin (100 μg/ml), neomycin (50 μg/L), and amphotericin B (2.0 μg/ml). The spinner flask was placed in a gas chamber in
which the gas was then replaced with H₂ and CO₂ to give a mixture of 8.0% O₂, 8% CO₂ and 84% H₂. The culture was incubated at 37° C. The use of antibiotics was terminated after 2 passes in the suspension culture.

Passage of the bacteria was accomplished by passing a 10% volume of the infected culture to new uninfected cells every 7 to 14 days. The new uninfected cells were seeded into spinner flasks by seeding a 250 ml spinner flask with fresh McCoy cells at 200,000 cells/ml in 100 ml DMEM with 5% FBS. The cells were incubated 18 to 24 hours at 37° C with 8.0% O₂, 8% CO₂ and 84% H₂. The bacterial culture was passed by adding 10 ml of the culture to the new cells and incubating at the conditions described above. Four to seven days after infecting the cells, an additional 150 ml of media was added.

**Vaccine isolate-Experiment 1**

Experiment 1 was designed to test the feasibility of a high passage strain of *L. intracellularis* to effectively protect pigs against a virulent challenge of *L. intracellularis*. Strain N343 was grown continuously in pure culture for 29 weeks. The isolate was grown in McCoy cells in a spinner flask at reduced oxygen until approximately 100% infection was observed. The culture was harvested by centrifugation at 3000 x g for 20 minutes. The pellet was resuspended in Sucrose-Phosphate-Glutamate solution (SPG) with 10% FBS and passed 4 times through a 25 gauge needle. The lysates were centrifuged at 500 X g for 5 minutes to pellet the debris. The supernatant was saved and stored at -70° C until approximately one hour before vaccination in which it was thawed and then stored on ice until administration.
Vaccine isolate-Experiments 2 and 3

For studies 2 and 3 the *L. intracellularis* strain N343 was grown for 40 continuous weeks as described above after which a single clone was harvested and stored in SPG with 10% FBS at −70°C. The high passage bacteria was designated strain NP40 (ATCC 55783). The isolate was scale-up in an 8 L spinnerflask as previously described and harvested by adding glycerol to a final concentration of 10% and then stored at −70 until vaccination.

Quantitation of live *L. intracellularis*

Quantitation of viable *L. intracellularis* was accomplished by determination of the Tissue Culture Infectious Dose 50 percent (TCID$_{50}$). Ninety-six well microtiter plates were seeded with McCoys cells at 1000 cells/well and grown 18-24 hours prior to infection. The culture samples were passed 8 times through a 25 gauge needle, then serially diluted 1:10 in DMEM/5% FBS. The dilutions were added to the 96 well microtiter plates with 0.1 ml/well. Each dilution was replicated in 12 wells. The plate was incubated for 6 days at 37°C at gas concentrations of 8.0% O$_2$ and 8.8% CO$_2$. The cells were fixed with cold 50% acetone and 50% methanol for 2 minutes. To the wells, 0.03 ml/well of anti-*L. intracellularis* monoclonal antibody diluted 1:2000 in PBS was added. The plate was incubated for 30 minutes at 37°C and then washed 3 times with PBS. Anti-mouse immunoglobulin G-fluorochrome conjugate (FITC) diluted 1:30 was added at 0.03 ml/well and incubated 30 minutes at 37°C. The plate was washed 3 times with deionized H$_2$O and allowed to dry. Samples were observed on a fluorescent microscope and the TCID$_{50}$/ml was determined using the Reed-Meunisch method of calculation.
Clinical observations

Daily observations for all 3 experiments included stool consistency, behavior, appetite, body condition, and hair coat. Each observation evaluated was based on a scale from 1 to 4 with 1 being normal and 3 the most severe, and a score of 4 for deceased animals. The scores were totaled each day with a normal animal receiving a score of 5 and a deceased animal receiving a score of 20. Scores of animals that died from causes other than *L. intracellularis* were excluded from consideration. The scores were averaged within treatment groups for comparison. The scores were made by animal caretakers that were blinded to the treatment groups.

Polymerase chain reaction

Fecal samples collected weekly as well as organs obtained at necropsy were analyzed by PCR using primers as previously described. DNA was extracted using a guanidine thiocyanate nucleic acid extraction kit. (Orca Research, Inc., Bothell, WA) The primer set, 5'-TATGGCTGTCAAAACACTCCG-3' and 5'-TGAAGGTATTGGTATTCTCC-3' was selected for the DNA amplifications. Cycle parameters were 93°C for 5 minutes, 55°C for 45 seconds, and 72°C for 45 seconds for the first cycle. The subsequent thirty-three cycles were performed for 45 seconds at each of previously mentioned temperatures. The final cycle was performed at 93°C for 45 seconds, 55° for 45 seconds, and 72°C for 2 minutes. PCR positive samples produced a 319 bp product detected by gel electrophoresis.

Histopathology

Organ samples were fixed by immersion in 10% buffered formalin. Sections were processed routinely, stained by hematoxylin and eosin (H & E) and Warthin-Starry silver stains, and examined by light microscopy. Sections were also examined using an indirect
immunofluorescence assay, incorporating a monoclonal antibody against *L. intracellularis*, as described previously.  

**Serology**

Testing to demonstrate the production of antibodies against *L. intracellularis* was performed as previously described. Plates for serology testing were prepared by seeding 96 well tissue culture plates with McCoy cells at 1000 cells/well and grown 18-24 hours prior to infection. Pure cultures of *L. intracellularis* strain N343 were added to Dulbecco’s Modified Eagle’s Medium (DMEM)/5% FBS at a dilution of approximately 3000-5000 bacteria/ml and 100 μl was added to each well. The plates were incubated for 6 days at gas concentrations of 8.0% O₂ and 8.8% CO₂. The cells were fixed with cold 50% acetone and 50% methanol for 2 minutes. Serum samples were diluted 1:30 in PBS and 100 μl was added to the test well. The plates were incubated for 30 minutes at 37° C and then washed 3 times with PBS. Anti-swine immunoglobulin G-fluorochrome conjugate (fluorescein isothiocyanate, Organon Teknika Corporation, Durham, NC), diluted 1:10 in PBS, was added at 100 μl/well and incubated 30 minutes at 37° C. The plate was washed 5 times with ddH₂O and allowed to dry. Samples were observed on a fluorescent microscope and wells with fluorescing bacteria were labeled as serologically positive.  

**Experiment 1: Efficacy feasibility**

Twenty-five mixed sex, conventional pigs, 3 weeks of age were obtained from an Iowa farm with no recorded history of proliferative enteritis. The pigs were transported to the research facility where they were tagged to individually identify each pig. The pigs were screened to exclude animals with existing *L. intracellularis* infections by collecting individual fecal samples by PCR for the presence of *L. intracellularis* prior to initiation of the
study. The pigs were held at this facility for two days prior to initiation of the study to allow acclimation to the facility and were fed antibiotic-free feed throughout the study. On day 0 of the study, all pigs were weighed, bled to collect serum, clinically scored, and fecal swabs were collected. The pigs were then randomly divided into three groups. The high passage bacteria were given by nasopharangeal gavage to group 1 which had 10 pigs. TCID$_{50}$ results indicated that pigs given high passage strain NP29wk received TCID$_{50}$ $3.6 \times 10^6$ bacteria/dose. Group 2 had 15 control animals, which were given non-infected McCoy cells in the same manner as the live vaccine. Group 3 had five pigs which were treated as strict negative controls through the experiment. All animals, except the 5 controls from group 3, were given a challenge culture 26 and 27 days post-vaccination consisting of low passage virulent cultures of strain N343. The animals were challenged with $6.0 \times 10^6$ bacteria per pig and $4.7 \times 10^6$ bacteria per pig on days 26 and day 27, respectively. All animals were euthanized and necropsied 21 days after challenge and examined for gross lesions. Samples of ileum, colon, tonsil, and mesenteric lymph node were collected for PCR testing and histological examination. Serum samples were collected at the time of vaccination, at challenge, and at necropsy.

**Experiment 2: minimum immunizing dose**

Seventy, mixed sex, conventional pigs, 3 weeks of age were obtained from a herd with no clinical history of proliferative enteritis. The pigs were transported to the research facility where they were tagged to individually identify each pig. The pigs were screened to exclude animals with existing *L. intracellularis* infections. Individual fecal samples were tested by PCR. The pigs were held at this facility for two days prior to initiation of the study to allow acclimation to the facility and were fed antibiotic-free feed throughout the study.
On day 0 of the study, all pigs were weighed, bled to collect serum, clinically scored, and rectally swabbed.

The pigs were randomly divided into 5 groups and placed into pens. Group 1 had 15 pigs that received a TCID$_{50}$ of $1.0 \times 10^6$ of strain NP40 per dose. Group 2 had 20 pigs that received a TCID$_{50}$ of $1.0 \times 10^5$ bacteria per dose. Group 3 had 15 pigs that received a TCID$_{50}$ of $1.0 \times 10^4$ bacteria per dose. Group 4, challenge control group, received no treatment and had 15 pigs. Group 4 was placed in a separate room from the vaccinated pigs. Group 5, strict control group, had 5 pigs and was also kept in a separate room from the other pigs throughout the study. Fecal samples were collected weekly for PCR testing from all pigs. All animals, except the strict controls, were given a low passage virulent challenge culture of $L. \ intracellularis$ strain N343 twenty-eight days post-vaccination consisting of $1.6 \times 10^7$ bacteria per pig. The challenge inoculum was grown in the same manner as experiment 1. All animals were euthanized and necropsied 21 days after challenge and examined for gross lesions. Samples of ileum and colon were collected for PCR testing and histological examination. Serum samples were collected at the time of vaccination, at challenge, and at necropsy.

**Experiment 3: Eight week duration of immunity**

Thirty-two mixed sex, conventional pigs, 3 weeks of age were obtained from a herd with no clinical history of proliferative enteritis. The pigs were transported to the research facility where they were tagged to individually identify each pig. Fecal samples were tested by PCR for the presence of $L. \ intracellularis$ prior to initiation of the study. The pigs were held at this facility for two days prior to initiation of the study to allow acclimation to the facility and were fed antibiotic-free feed throughout the study. On day 0 of the study, all pigs
were weighed, bled to collect serum, clinically scored, and rectally swabbed. Serum and fecal swabs were collected every seven days during the study.

The pigs were randomly divided into 3 groups and placed into pens. Group 1 had 19 pigs that received a TCID$_{50}$ of 1.0 x 10$^6$ bacteria/dose of strain NP40. Group 2 had 9 that received no vaccine and were challenge control pigs. Group 3 had 4 pigs housed in a separate room as strict controls and were not treated throughout the study. Groups 1 and 2 were challenged 56 days post-vaccination with 1.2 x 10$^7$ low passage *L. intracellularis* strain N343 per pig. The challenge was grown in the same manner as it was for experiment 1. All animals were euthanized and necropsied 21 days after challenge and examined for gross lesions. Samples of ileum and colon were collected for PCR testing and histological examination.

**Statistical analysis**

For the histopathology and serology results a Pearson chi-square statistic was calculated for daily test results to test for independence between treatment group and test result. The adjusted residual (observed-expected)/normalized Std. Err for residual) for each table cell was used to identify cells that differed significantly from the expected model of independence. Adjusted residuals are roughly the same as z scores in their distribution. For the clinical scores, temperature and average daily gains, the results were analyzed using an analysis of variance procedure and the Duncan's multiple range test for variables on each data set.
Results

Experiment 1: Efficacy

Individual fecal samples were tested by PCR for the presence of *L. intracellularis*. Fecal shedding of *L. intracellularis* was detected in 2 of 10 vaccinates 14 and 21 days post vaccination (Figure 1). All pigs were PCR negative in the feces 26 days post vaccination, at the time of challenge. Seven days post challenge (day 33) 1 of 10 pigs in the vaccinated group and 1 of 10 pigs in the control group were fecal PCR positive. Fourteen days post challenge (day 40) 0 of 10 vaccinates were PCR positive and 3 of 10 pigs in the challenge control group were positive. Twenty-one days post challenge (day 47) at the time of necropsy, no pigs were PCR positive (Figure 1). At necropsy, the small intestines in 0 of 10 vaccinates, group 1, and in 4 of 10 challenge controls, group 2, were colonized by *L. intracellularis* as determined by PCR. Polymerase chain reaction did not detect *L. intracellularis* in the colons of any of the pigs (Figure 2). Pooled samples of the mesenteric lymph nodes, tonsils, and lungs consisting of 5 samples per pool were negative in all groups.

Hematoxylin and eosin stains revealed moderate inflammation and presence of lymphoplasmacytic atrophic enteritis. The lesion was consistent among all pigs including strict controls. In addition to moderate crypt hyperplasia, silver stains also demonstrated crypt epithelium to frequently contain granular material in the apical portions of enterocytes. Within this dark staining material, curved rod-shaped bacteria typical of *L. intracellularis* could not be discerned. Intracellular bacteria were observed in 4 of 10 pigs of group 2. The remainder of the study pigs did not have lesions typical of PPE nor were intracellular bacteria observed (Figure 3). Fluorescent antibody stains using the *L. intracellularis* specific monoclonal antibody of the intestinal sections demonstrated that the vaccine significantly
reduced the number of *L. intracellularis* colonized intestines. Fluorescent antibody stains of the small intestine were positive for *L. intracellularis* in 7 of 10 animals in group 2 and 0 of 10 in group 1 (Figure 3).

No gross lesions attributed to PPE were observed in the ileum, colon, tonsil, or lymph node from any of the study pigs. No significant differences in average daily weight gains were observed between the groups.

Pigs in group 1 were shown to have IgG antibodies against *L. intracellularis* in 6 of 10 pigs at the time of challenge. No other pigs were positive at the time of challenge. Twenty-one days after challenge, 8 of 10 vaccinated pigs and 9 of 10 challenge control pigs were positive for antibodies to *L. intracellularis*. Strict control pigs remained seronegative throughout the study (Figure 4).

**Experiment 2: Minimum immunizing dose**

Results of PCR testing of fecal samples were not significant, as a maximum of one pig per group was positive at any sampling time throughout the study (Figure 5). Polymerase chain reaction testing of the small intestine and colon, however, confirmed the efficacy of the avirulent vaccine at the high dose. The small intestines in only 1 of 14 pigs in group 1. Group 2 (medium dose) had 8 of 20 pigs positive in the small intestine and group 3 (low dose) had 6 of 14 pigs positive in the low dose group. Nine of 15 pigs were colonized by *L. intracellularis* in group 4 (challenge controls) and 0 of 4 pigs were positive in group 5 (strict controls). The results from the PCR of the colon were similar; 0 of 14 pigs in group 1, 4 of 20 on group 2, 2 of 14 pigs in group 3, 6 of 15 in group 4, and 0 of 4 pigs in group 5 were positive for *L. intracellularis* (Figure 6).
Fluorescent antibody stains of the organs were consistent with the PCR findings in that the high dose of vaccine significantly reduced the number of *L. intracellularis* colonized intestines as assessed by fluorescent antibody staining. Fluorescent antibody stains of the small intestine and colon specific for *L. intracellularis* were positive in 0 of 14 animals in group 1, 3 of 20 animals in group 2, 3 of 14 animals in group 3, 6 of 15 animals in group 4, and 0 of 4 animals in group 5 (Figure 7). Hematoxylin and eosin and silver staining of the intestines confirmed the efficacy of the high dose of the vaccine. Both lesions and the presence of intracellular bacteria were significantly reduced in the high dose vaccine group (Figure 8). Lower doses of vaccine did not show a significant reduction of typical PPE lesions. All lesions typical of PPE were found in the small intestine, as were all intracellular organisms. No lesions attributed to PPE were observed in the colon, tonsil, or lymph node from any of the study pigs. There were no significant differences in gross lesions between groups.

Serology results indicated that animals vaccinated with the high dose had a much higher number of animals seroconverting to the challenge inoculum compared to the other groups (Figure 9).

Three pigs died before completion of the study for reasons unrelated to *L. intracellularis* infection, a rectal prolapse in a pig in the high dose group, a *Haemophilus parasuis* infection in a pig from the low dose group, and death of a pig in the strict control group due to complications of blood collection.

**Experiment 3: Eight week duration of immunity**

Fecal PCR did not demonstrate shedding of *L. intracellularis* until day 70 (14 days after challenge). On day 70, 2 challenge control pigs, and 1 vaccinate shed *L. intracellularis*
in the feces. On day 77 of the study, 5 pigs in the challenge control group and 1 vaccinate were positive by PCR of fecal swabs. PCR testing of the intestinal mucosa demonstrated that 1 of 19 vaccinated pigs and 5 of 9 challenge control pigs were colonized by *L. intracellularis* (Figure 10).

Fluorescent antibody stains of the organs demonstrated 6 of 9 animals positive in the challenge control group and 1 of 19 positive in the vaccinated group. All other animals were FA negative for the presence of *L. intracellularis* (Figure 11). Hematoxylin and eosin stains demonstrated mild crypt hyperplasia with a higher incidence in the challenge control group. Seven of nine pigs had crypt hyperplasia in challenge control group and three of nineteen pigs had crypt hyperplasia in the vaccinated group. All lesions typical of PPE were found in the small intestine, as were all intracellular organisms (Figure 11). No significant differences in gross lesions were observed between groups.

Seroconversion results indicated that animals vaccinated with the high passage attenuated strain of *L. intracellularis* had significantly higher seroconversion rates compared to nonvaccinated animals. Non-vaccinated challenged control pigs began to seroconvert 21 days after virulent challenge with *L. intracellularis* (Figure 12).

**Discussion**

This is the first report of the use of an attenuated strain of *L. intracellularis* for the prevention of PPE in pigs. The attenuation process included the *in vitro* passage of a pure culture of *L. intracellularis* in tissue culture cells grown in suspension at reduced oxygen concentrations. Pigs orally vaccinated with a dose of *L. intracellularis* at dose of TCID$_{50}$ of $10^6$ bacteria/pig had significantly reduced histological lesions in the small intestine compared to non-vaccinated control pigs given a low passage virulent challenge strain grown in pure
culture. This reduction of microscopic lesions would be expected to elicit a commensurate reduction in the disease typically associated with such lesions caused by PPE. This dose of attenuated bacteria also protected pigs for at least 8 weeks after vaccination against virulent challenge. Serological responses were more prevalent in vaccinated pigs, however, it is not possible to conclude that a serological response correlates to protection against virulent challenge provided by the vaccine.

The challenges for all of the described studies were prepared from pure cultures which avoid the confounding effects of other potentially pathogenic microflora and tissue factors inherent in crude inoculum preparations. Pure cultures also provide a uniform infective dose, thereby avoiding potentially significant variation in the actual challenge that individual pigs receive when administered crude gut inoculum from one or more donor animals. These studies used a homologous challenge in which the parent organism was used as the challenge strain and the vaccine organism was a high passage bacteria serially passaged in tissue culture over several weeks. Future studies will require a heterologous challenge strain to demonstrate efficacy against other isolates. Because all virulent isolates examined to date are not distinguishable genetically or phenotypically, one would expect the vaccine to be protective against isolates characterized to date. Also, the current diagnostic tests including PCR, histopathology, and serology are not able to distinguish between the vaccine organism from virulent field isolates. It will be of high importance to attempt to find methods to differentiate the vaccine from other isolates.

The studies demonstrated efficacy based on the reduction of microscopic lesions. Serology results were also an indication of an immune response due to vaccination as vaccinates had detectable antibodies against *L. intracellularis* at the time of or shortly after
challenge. Clinical signs and gross lesions, which are also important parameters for measuring efficacy, were not apparent in any of the groups in the studies. Future experiments will be conducted to study these parameters in a larger data set to evaluate efficacy against naturally occurring *L. intracellularis* infections with the typical low morbidity seen with PPE.

The high passage attenuated strain has been tested in several studies to demonstrate its safety in the host animal. The attenuated strain has been administered at high doses (greater than $10^7$) in dexamethasone treated pigs 3 weeks of age with no clinical effects observed. Reversion to virulence studies were performed in which the bacteria was administered to 1 day old cesarean-derived, colostrum-deprived (CDCD) pigs. The pigs were necropsied 14 days after administration and the ilea were homogenized and administered to a new group of 1 day CDCD pigs. The passage process was repeated a total of 5 times with no development of PPE typical lesions throughout the study. Polymerase chain reaction, Warthin-Starry silver stain, or fluorescent antibody stain of the intestine did not detect the bacteria after the second passage in the pigs (data not shown). No adverse effects were observed during the use of the vaccine in the studies described within this paper either.

A successful vaccine must elicit an immune response that prevents the bacterium from successfully initiating an infection in the intestine that results in manifestations of disease and reduced animal performance. Two major types of active immune responses may develop either concomitantly or separately: antibody formation (mainly secretory immunoglobulin A, or SIgA) and T cell-mediated immunity. In mucosal secretions, SIgA serves to neutralize microbes before invasion occurs. This serves to prevent microbes from
having an opportunity to attach, invade, and colonize the host. In general, live vaccines stimulate a more effective cell-mediated immune response when compared with killed preparations. As *L. intracellularis* is an obligately intracellular pathogen, a cell-mediated response is likely to be an important factor for host resistance to disease. The oral route allows the attenuated vaccine to utilize natural routes of invasion, thereby presenting antigen to lymphocytes in the gut-associated lymphoid tissue.

Future work will need to be done to determine the effects of antibiotics that are routinely used as feed additives and water medications on the vaccine. As most producers use some type of feed additive, we will need to know their effects on the development of an effective immune response following vaccination. Also, it will be advantageous to learn how the vaccine and antibiotics can be used together to prevent and treat *L. intracellularis* infections along with other performance reducing pathogens.

The use of a modified live vaccine for prevention of PPE caused by *Lawsonia intracellularis* would aid the swine industry by facilitating efficient production of pigs including more uniform rates of gain and time to market. The vaccine by itself or when used in combination with additional PPE strategies must provide efficacy that is economically advantageous when compared to the efficacy achieved with other methods for controlling the financial losses resulting from PPE. As with other vaccines, optimal disease control, production performance, and economic return from the vaccine will be achieved when used as part of a comprehensive PPE program. Each program must consider the epidemiology of the disease, the individual farm and production management system, and the associated constraints as well as the efficacy and safety of the vaccine.
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References


Figure 1. PCR testing of fecal samples from experiment 1. No significant differences were observed between treatment groups (P>0.05).

Figure 2. PCR testing of organ samples from experiment 1. No significant differences were observed between treatment groups (P>0.05).
Figure 3. Fluorescent Antibody and silver stain results of histological sections from experiment 1. (*) represents groups significantly different from the challenge control group (P>0.05).

Figure 4. Seroconversion rates experiment 1. (*) represents groups significantly different from non-exposed strict control group (P>0.05).
Figure 5. PCR of fecal samples from experiment 2. No differences were observed between treatment groups (P>0.05).

Figure 6. PCR of organ samples from experiment 2. (*) represents groups significantly different from Challenge control group (P>0.05).
Figure 7. Fluorescent antibody stains of histological section from study 2. (*) represents groups significantly different from Challenge control group (P>0.05).

Figure 8. Silver and Hematoxylin and Eosin stains of histological sections from experiment 2. (*) represents groups significantly different from Challenge control group (P>0.05).
Figure 9. Seroconversion rate of pigs in study 2. (*) represents groups significantly different from non-infected strict control group (P>0.05).

Figure 10. PCR testing of fecal samples and organ samples from study 3. (*) represents groups significantly different from the challenge control group (P>0.05).
Figure 11. Histopathological testing of organ sections from experiment 3. (*) represents groups significantly different from challenge control group (P>0.05).

Figure 12. Seroconversion rates of pigs from experiment 3. (*) represents groups significantly different from non-exposed strict control group (P>0.05).
CHAPTER 6. CONTROL AND TREATMENT OF PORCINE PROLIFERATIVE ENTEROPATHY USING DIFFERENT DELIVERY METHODS OF TIAMULIN HYDROGEN FUMARATE

A paper to be submitted to Swine Health and Production

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Summary

Objective: To determine the effect of tiamulin hydrogen fumarate (thf)-medicated feed or water in pigs infected with pure cultures of \textit{Lawsonia intracellularis} by monitoring fecal shedding of \textit{L. intracellularis} by polymerase chain reaction (PCR), antibody response, clinical effects, lesion development and growth performance.

Methods: Two studies were conducted. Study 1 had 47 pigs that were divided into 2 groups. All pigs were inoculated with a virulent pure culture of \textit{L. intracellularis}. Tiamulin hydrogen fumarate was administered in the feed at 38.5 g per ton starting 9 days after inoculation when clinical symptoms were apparent. Medication continued for 28 days until termination of the study for PPE -specific lesion assessment. All pigs were monitored daily for clinical signs until necropsied 28 days post infection (PI). Fecal shedding of \textit{L. intracellularis} by PCR, antibody response, body weights and feed intake were determined on day 0 (challenge), day
9 (treatment initiation) and weekly for 4 weeks post-treatment initiation.

Study 2 had 48 pigs that were divided into 2 groups. All pigs were inoculated with a pure culture of *L. intracellularis*. Tiamulin hydrogen fumarate was administered in the water at 60 ppm starting 8 days after inoculation when clinical symptoms were apparent. Medication continued for 5 days followed by a 10 day post-medication observation period after which the study was terminated for PPE-specific lesion assessment. All pigs were monitored daily for clinical signs. Fecal shedding of *L. intracellularis* by PCR, antibody response, body weights and feed intake were determined on days 0 (challenge), 8 (treatment initiation), 13 (treatment cessation) and 23 (end of 10 day post-medication observation period/termination of study).

**Results:** In study 1, medication was initiated 9 days after infection. Fecal shedding of *L. intracellularis* was first observed 7 days after the initiation of treatment. Medication in the feed for control/treatment of PPE after the development of clinical signs significantly reduced the prevalence and severity of gross lesions and microscopic lesions, severity of clinical signs, and prevalence of fecal shedding of *L. intracellularis*. Prevalence of seroconversion was high in both groups showing that all pigs responded immunologically to the challenge prior to treatment.

In study 2, medication was initiated 8 days after infection. Fecal shedding of *L. intracellularis* was first observed 8 days after infection (at the time of initiation of treatment). Medication in the water for treatment/control of PPE after the development of clinical signs significantly reduced the prevalence and severity of gross lesions and microscopic lesions, severity of clinical signs and prevalence of fecal shedding of *L. intracellularis*. Seroconversion was significantly reduced in the pigs receiving water medication.
**Implications:** Tiamulin hydrogen fumarate (thf) in the feed or water was effective in treating/controlling the development of PPE. Medication status may affect the results of antemortem diagnostic tests (fecal shedding, seroconversion) for *L. intracellularis* infection. Treatment of pigs with thf in the water post-infection reduced the prevalence of seroconversion against *L. intracellularis*, while treatment post-infection in the feed has little effect on seroconversion rates.

**Introduction**

Porcine proliferative enteropathy (PPE, ileitis) is a common enteric disease of grow-finish swine. The disease may result in poor growth rate, diarrhea, and stunting or it can present as acute death or bloody diarrhea in late finishing pigs and replacement gilts.¹

Histopathological lesions of the disease include epithelial hyperplasia, especially in the mucosal crypts. There is also a distinct absence of goblet cells. The proliferating epithelial cells contain intracytoplasmic, slender, curved, rod-shaped bacteria.² The causative agent has been identified and is considered to be a novel taxonomic genus and species, recently given the name *Lawsonia intracellularis*.³

Porcine proliferative enteropathy occurs in virtually all swine production systems, including modern multiple-site or "high-health" herds in which early weaning and segregated rearing are practiced. A recent serologic survey conducted by the National Animal Health Monitoring System (NAHMS) using its swine serum bank (representing 198 farms) found the prevalence of PPE in the United States to be 96% on a herd basis.⁴ The total pig prevalence rate was 28%. Diagnostic investigations of PPE-affected farms in other countries suggest that approximately 35% of growing pigs are affected.⁵, ⁶ Subclinical effects such as
reduced rate and efficiency of growth have, however, been difficult to quantify. Direct financial losses due to decreased growth rates (9%-31%) and feed inefficiency (6%-25%) have been estimated to cost $3.28 to $11.48 per affected pig in the UK. It was observed by MacKinnon that weight gain reductions of up to 50% and reduction in feed efficiency of up to 30% could occur in pigs naturally infected with PPE compared to normal pigs. Annual costs to the pig production industries of several countries have been estimated at $20 million (United States), $3 to $6.5 million (United Kingdom), and $25AUS per sow (Australia).

Porcine proliferative enteropathy can be diagnosed by a variety of techniques. Historically, postmortem examination and histopathology have diagnosed PPE. Polymerase chain reaction (PCR) tests are now available to detect the organism’s DNA in feces and tissues. In addition, an indirect fluorescent antibody (IFA) serological test has been developed to detect IgG antibodies specific for *Lawsonia intracellularis*.

Growth of *L. intracellularis* on conventional media has not been successful. The bacteria have been cultivated on tissue culture cells in a reduced oxygen atmosphere. Pure cultures of *Lawsonia intracellularis* were used to fulfill Koch’s postulates in pigs in 1993. Pure cultures eliminate confounding effects of other potentially pathogenic microflora and tissue factors inherent in crude inoculum preparations. In addition, pure culture inoculation permits a uniform infective dose, thereby avoiding potentially significant variation in the actual challenge that individual pigs receive when administered crude gut inoculum from one or more donor animals.

Tiamulin hydrogen fumarate (thf) (Denagard™) is a member of the diterpene class of antibiotics. Diterpene class antibiotics are selectively reserved for use in food producing animals and are not used in human medicine. Tiamulin hydrogen fumarate is lipophilic and
therefore concentrates within cells and tissues and acts by inhibiting microbial protein synthesis. When given to pigs orally via feed or water, or parenterally by injection, thf achieves high tissue concentrations in the enteric and respiratory tracts as well as in the tonsil. Tiamulin hydrogen fumarate has good in vitro activity against Gram-positive bacteria, mycoplasmas, anaerobes, spirochetes (e.g., *Serpulina* spp.), and selective activity against Gram-negative pathogens, including *L. intracellularis*,^20^, ^21^ *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, and *Pasteurella multocida*.

Tiamulin hydrogen fumarate was previously shown to be effective in preventing PPE in pigs challenged with a virulent pure culture *L. intracellularis* challenge if the medication was administered in the feed prior to, during, and at subsequent exposure.^22^ The purpose of this paper is to summarize 2 animal studies performed to evaluate the control and treatment of PPE using thf in the feed or water after exposure to *L. intracellularis* and the development of clinical signs typical of PPE. The PPE-specific effects of thf were determined using various diagnostic techniques including PCR, histopathology, gross pathology and serology. All studies were performed using a virulent pure culture challenge of *L. intracellularis*.

**Materials and methods**

**Animals**

Forty-seven pigs were tested in study 1 and forty-eight pigs were tested in study 2. The animals were healthy 6-week-old pigs weaned at 3-4 weeks of age and of mixed breed and sex. The pigs were purchased from a closed swine herd with no clinical or diagnostic evidence of PPE, *Salmonella*, or *Brachyspira* (formerly *Serpulina*) *spp.* infections. Offspring from this herd have been shown to be susceptible to *L. intracellularis* infection and have been used previously to successfully reproduce PPE. The pigs in each study were from five
litters and ranged in weight from 8-20 lbs. (3.6 - 9 kg) with a mean weight of 13 lbs. (5.9 kg) when purchased. No antibiotics had been administered to these pigs before purchase.

Treatments

Study 1--All pigs were identified by eartag, weighed, and randomly allocated to pens of 3 pigs and blocked to replication by weight. The pigs were divided into 2 treatment groups. All pigs were inoculated with a pure culture of \textit{L. intracellularis} strain N343 as described below. Pigs in treatment group 1 received no medication throughout the study. Pigs in treatment group 2 began receiving feed containing 35 g/ton (38.5 ppm) thf when >60% of pens had at least 1 pig showing clinical signs typical of PPE. Feed medication was administered for 28 days.

Study 2--All pigs were identified by eartag, weighed, and randomly allocated to pens of 3 pigs and blocked to replication by weight. The pigs were divided into 2 treatment groups. All pigs were inoculated with a pure culture of \textit{L. intracellularis} strain N343 as described below. Pigs in treatment group 1 received no medication throughout the study. Pigs in treatment group 2 received 60 ppm thf in the drinking water when >60% of pens had at least 1 pig showing clinical signs typical of PPE. Treatment lasted for 5 days followed by a 10 day post-medication observation period.

Water/Feed

Study 1--Water and feed were available \textit{ad libitum} for the duration of the study. A corn-soymeal-based diet was formulated to meet or exceed NRC requirements for growing pigs. The non-medicated diet was fed to all pigs until initiation of the medicated feed in group 2 in which feed containing 38.5 ppm thf was presented.
Study 2—Water and feed were available ad libitum for the duration of the study. A corn-soybean-based diet was formulated to meet or exceed NRC requirements for growing pigs. All animals received antibiotic-free feed for the entire study. Group 2 received water containing 60 ppm thf at the designated time. The medicated water solution was prepared fresh daily for 5 days.

Infection

*Lawsonia intracellularis* strain N343 is a recent field isolate from the ileum of a sow from a farm in Minnesota. The *in vitro* minimum inhibitory concentration (MIC)/susceptibility of this isolate for thf was determined to be low (0.25 μg/ml). Low-passage cultures of *L. intracellularis* strain N343 were grown using continuous cell culture techniques for 8-12 weeks, harvested, processed as previously described, and quantified by the Tissue Culture Infectious Dose 50 (TCID50) percent using the Reed-Meunsch method. On day 0, all pigs received $10^{7.2}$ and $10^{8.2}$ bacteria/dose via gastric intubation in study 1 and study 2, respectively.

Facilities

For both of the experiments the test facility was totally confined, environmentally controlled single room with twenty 5 ft x 7 ft (1.5 m x 2.1 m) pens separated by solid partitions on woven wire flooring over a shallow pit. Ambient temperatures were held at approximately 85°F (29°C) for the first week and lowered incrementally each week to 75°F (24°C) during the final week. Heat lamps provided supplemental zonal heating. A single-sided adjustable stainless steel self-feeder and one automatic nipple waterer serviced each pen. Pens were randomly assigned a treatment group and animal caretakers were blinded to the identity. The study designs were blinded randomized complete block designs with
treatment allocation blinded to both the investigator and monitor. The pigs were stratified by weight. The treatments were randomly assigned within each pair of pens within each block by draw from a hat prior to the _L. intracellularis_ challenges.

**Measurements and observations**

_Study 1_—Each pig was weighed on day -1 when allocated to pens, at the beginning of the treatment period, and at weekly intervals to test and at 4 weeks post-treatment initiation. Feed disappearance was measured at the same intervals. Fecal swabs were taken on the day of challenge, the day of treatment initiation, and at weekly intervals to the end of the study. Polymerase chain reaction using primers specific for _L. intracellularis_ on the fecal swabs was used to detect _L. intracellularis_, and reported as positive or negative. Blood samples were collected at study termination. Each pig was observed daily for diarrhea (scale: 0 = normal, 1 = mild, 2 = moderate, or 3 = severe), clinical appearance (scale: 0 = normal, 1 = gaunt, and 2 = moribund) and presence or absence of fecal blood (0 = absent, 1 = present). A composite pig clinical symptom score was derived for statistical analysis by adding diarrhea severity score, fecal blood score, and clinical appearance score.

On day 28, all remaining live pigs (45 of 47) were euthanized, necropsied, and sampled. Microscopic examination of the jejunum, ileum, and colon was used to determine the presence of lesions (hematoxylin and eosin) and intracellular curved bacteria (Warthin-Starry silver stains) to score the degree of crypt cell hyperplasia (scale: 0 = normal, 1 = mild, 2 = moderate, 3 = severe) and relative numbers of intracellular curved bacteria.

Mesenteric lymph node, ileum, and colon from all pigs were submitted to the Iowa State University Veterinary Diagnostic Laboratory for isolation of _Salmonella_, pathogenic _Escherichia coli_, and _Brachyspira_ spp. Indirect-fluorescent antibody tests were performed on
histological sections of ileum and colon to detect and confirm the presence of *L. intracellularis* at necropsy.

*Study 2*—The same measurements and samples were taken as in study 1 except the animals were necropsied 15 days after treatment initiation instead of 28 days after treatment initiation. Each pig was weighed on day -1 when allocated to pens, at the beginning of the treatment period, at the end of the treatment period and at termination of the study. Feed disappearance was measured at the same intervals. Fecal swabs were taken on the day of challenge, the day of treatment initiation, at the day of treatment cessation and at study termination. Blood samples were collected at termination.

**Statistical analysis**

In both studies, the individual pig was considered to be the experimental unit for statistical analysis of the categorical data (gross and microscopic lesions, PCR, serology etc.) The appearance scores, diarrhea scores, weight gain, feed intake and feed conversion data were statistically evaluated using the pen as the experimental unit. For the comparisons of proportions and prevalence rates exact stratified Mantel-Haenszel tests were performed. Exact stratified permutation tests and exact stratified Wilcoxon-Mann-Whitney tests were used to compare severity levels of clinical symptoms and severity levels of lesions. Exact stratified Wilcoxon signed rank tests were used to compare the continuous outcomes associated with growth performance.

**Results**

**Initiation of medication**

*Study 1*—Medicated feed was initiated 9 days after inoculation as clinical signs consistent with PPE (diarrhea or clinical appearance scores > 0) were apparent in >60% of
the pens (10 of 16 pens; 63%). Tiamulin hydrogen fumarate was added to the feed of Group 2 pigs at 35 g/ton for the subsequent 4 weeks.

**Study 2**—Medication in the water was initiated 8 days after inoculation as >60% of the pens (14 of 16 pens; 88%) showed clinical signs consistent with PPE. Tiamulin hydrogen fumarate was added to the drinking water at 60 ppm and continued for 5 days.

**Bacteriology**

In both studies bacterial culture attempts were negative for pathogenic *E. coli*, *Salmonella* spp., and *Serpulina* spp. verifying the presence of an uncomplicated *L. intracellularis* infection in both studies.

**Lesions**

**Study 1**—Two non-medicated pigs were euthanized and necropsied due to severe clinical disease 34 days post-challenge. Both pigs had gross and microscopic lesions typical of PPE. No other pathologic lesions were observed in the pigs.

The remaining pigs were necropsied 37 days after challenge (28 days after treatment initiation). Gross lesions typical of PPE were significantly more prevalent in non-medicated pigs (seven of twenty-four had lesion in the small intestines and four of twenty-four in the large intestines) (Figure 1) compared to medicated pigs (one of twenty-three with lesions in the small intestines and zero of twenty-four with lesions in the large intestines).

Microscopic lesions consistent with PPE (figure 2) were significantly more prevalent in non-medicated pigs (eight of twenty-four had lesions in their small intestines and seven of twenty-four in their large intestines) when compared to medicated pigs (two of twenty-three in the small intestines and one of twenty-three in the large intestines) (Figure 3).
Study 2—Gross lesions typical of PPE were significantly more prevalent in non-medicated pigs (twelve of twenty-four had lesions in the small intestines and eight of twenty-four in the large intestines) when compared to medicated pigs (two of twenty-four with lesions in the small intestines and one of twenty-four with lesions in the large intestines).

Microscopic lesions typical of PPE were significantly more prevalent in non-medicated pigs (twenty of twenty-four had lesions in their small intestines and fourteen of twenty-four in their large intestines) when compared to medicated pigs (two of twenty-four in their small intestines and two of twenty-four in the large intestines) (Figure 4).

Serology

Serum samples were collected at termination of both studies. In study 1, anti-*L. intracellularis* antibodies at necropsy were observed in almost all pigs regardless of treatment group (Figure 5). In study 2, antibodies against *L. intracellularis* were significantly more prevalent (*P*<0.05) in the non-medicated group compared to pigs that received water medication (Figure 6).

Fecal shedding

Study 1—Fecal swabs positive by PCR first appeared 7 days after the initiation of treatment (16 days post-challenge) in either group (eight of twenty-four in the non-medicated pigs and seven of twenty-three in the medicated pigs). At later samplings more pigs had positive PCR results in the non-medicated group (five of twenty-four non-medicated pigs at day 14, three of twenty-four pigs non-medicated pigs at day 21, and four of twenty-four non-medicated pigs at day 28. No medicated pigs were positive at days 14, 21, and 28 after the initiation of treatment.). Shedding differences were significant 14 and 28 days after treatment initiation (*P*<.05) (Figure 7).
Study 2—Fecal swabs positive by PCR first appeared at 8 days post challenge (sixteen of twenty-four in the non-medicated pigs and eleven of twenty-two in the medicated pigs). Treatment started at day 8 after which more pigs had positive PCR results in the non-medicated pigs (twenty-four of twenty-four non-medicated pigs at day 13, seventeen of twenty-four pigs at day 23) compared to medicated pigs (15 of twenty-four positive at day 13, three of twenty-four at day 23). Shedding differences were significant at days 13 and 23 (P<0.05) (Figure 8).

Clinical scores

Study 1—Eleven pigs had clinical symptoms 9 days after inoculation when medication began. Seven of the pigs were in the to-be-medicated group and 4 pigs were in the to-be-non-medicated group. The number of symptomatic pig-days for the medicated pigs decreased steadily and dramatically within days after the initiation of feed medication with thf. However, the non-medicated pigs have persistent problems with clinical symptoms throughout the study period with the observed maximum levels of severity increasing (Figure 9).

Study 2—Twenty pigs had clinical symptoms 8 days after inoculation when medication began. Eight of the pigs were in the to-be-medicated group and 12 pigs were in the to-be-non-medicated group. The number of symptomatic pig-days for the medicated pigs decreased steadily and dramatically within days after the initiation of medication with thf in the drinking water. However, the non-medicated pigs have persistent problems with clinical symptoms throughout the study period (Figure 10).
Growth performance

*Study 1*—For the overall treatment period, the average daily feed intake (ADFI) was significantly greater for the thf-medicated group, with the estimated median difference in average daily feed intake being 0.208 lbs/pig-day (p=0.029). Average daily weight gain (ADG) was also significantly greater for the thf-medicated group, with the estimated median difference being 0.27 lbs/pig-day (P = .007). Feed efficiency (ADG/ADFI) was also greater for the thf-medicated group (P = .01) (Table 1).

### Table 1. Overall Treatment Group Productivity Comparisons, Study 1

<table>
<thead>
<tr>
<th></th>
<th>Non-medicated</th>
<th>Medicated</th>
<th>Confidence interval</th>
<th>P-value</th>
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<tr>
<td>ADG, # (g)</td>
<td>0.9 (408)</td>
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<td>ADFI, # (kg)</td>
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<td>0/23</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

ADG=average daily weight gain  
ADFI=average daily feed intake

*Study 2*—For the overall treatment period, the average daily feed intake (ADFI) was not significantly different between treatment groups (p=0.363). Average daily weight gain (ADG) was significantly greater for the thf-medicated group, with the estimated median difference being 0.29 lbs/pig-day (P = .007). Feed efficiency (ADG/ADFI) was also greater for the thf-medicated group (P = .01) (Table 2).

### Table 2. Overall Treatment Group Productivity Comparisons, Study 2

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<th></th>
<th>Non-Medicated</th>
<th>Medicated</th>
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<th>P-Value</th>
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<td>ADG # (kg)</td>
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<td>1.41 (.640)</td>
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<td>ADFI # (kg)</td>
<td>2.15 (.977)</td>
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<td>ADG/ADFI</td>
<td>0.528</td>
<td>0.63275</td>
<td>0.041, 0.163</td>
<td>0.01</td>
</tr>
</tbody>
</table>

ADG=average daily weight gain  
ADFI=average daily feed intake
Discussion

These studies demonstrated the effectiveness of thf to effectively reduce lesions, clinical disease, and productivity losses associated with PPE. The negative cultures for other recognized potentially confounding enteric bacterial pathogens that we observed in these studies verify that the experimental inoculation resulted in an uncomplicated *L. intracellularis* infection. All studies including the two described in this paper and a previous study\textsuperscript{22} used pure culture challenges to ensure that other possible confounding infections did not confuse the interpretation of the results which are possible with gut homogenate challenges.

In a previous study, dietary inclusion of thf beginning prior to infection completely prevented the development of PPE gross lesions in the small and large intestine.\textsuperscript{22} That study also showed that thf completely prevented development of large intestinal microscopic lesions and significantly reduced the prevalence and severity of PPE microscopic lesions in the small intestine compared to pigs in the infected non-medicated group.

Tiamulin hydrogen fumarate in the feed or in the water after the onset of clinical signs due to *L. intracellularis* infection significantly reduced the development of gross and microscopic lesions compared to non-medicated pigs. Clinical signs were significantly reduced in medicated pigs 10 days after initiation of treatment in the feed. Feed medication also prevented the development of new cases after 4 days of medication while non-medicated pigs continued to develop clinical signs.

Water delivery of thf significantly reduced the development of gross and microscopic lesions compared to non-medicated pigs. Clinical signs were significantly reduced in medicated pigs after initiation of treatment in the water. Medication also prevented the
development of new cases after 3 days of medication while non-medicated pigs continued to
develop clinical signs. Both experiments also demonstrated significant benefits of thf
medication in growth rates and feed efficiency.

Pigs in both of these studies were medicated after exposure to *L. intracellularis* and
clinical signs typical of PPE had developed. The studies demonstrated that medication with
thf following the development of clinical signs was effective in treating and controlling PPE
based on development of PPE specific lesions, shedding of *L. intracellularis* in the feces, and
rate and efficiency of growth.

These experiments also demonstrate the impact of thf on seroconversion against *L.
intracellularis*. Development of antibodies specific for *L. intracellularis* was significantly
reduced in pigs medicated in the feed prior to infection\(^2\) while pigs that were exposed to *L.
intracellularis* and clinically affected by PPE prior to the initiation of medication in the feed
had high rates of seroconversion. Seroconversion results of pigs given thf in the drinking
water subsequent to challenge and development of clinical signs demonstrated reduced
seroconversion rates against *L. intracellularis* compared to non-medicated pigs. This may
reflect a dose-related effect since water delivery of 60 ppm (mg/L) thf delivers approximately
3.5 mg/lb body weight compared to feed medication at 35 g/ton (38.5 ppm) delivering
approximately 0.7 mg/lb body weight. The seroconversion data generated from these studies
suggest a potential confounding effect of antibiotics on serological surveys such as the
NAHMS survey\(^4\) causing possible underestimates of the prevalence of the disease within
medicated herds.

Tiamulin hydrogen fumarate as been reported to possess good *in vitro* activity against
*L. intracellularis*.\(^2\) These experiments confirm the ability of thf to control the clinical and
pathological effects of PPE and demonstrate the potential impact of effective medication on the results of new antemortem diagnostic tests. Medication may interfere with expected fecal shedding and seroconversion to *L. intracellularis*, which may confound interpretation of these tests in medicated populations. Accurate identification of the timing of *L. intracellularis* infection may facilitate the design of more effective treatment, control, and possibly eradication strategies.

**Acknowledgements**

We thank Dr. Connie Gebhart and Ms. Rebecca Mackie of the University of Minnesota for the MIC testing of the challenge isolate and Dr. Jon Lemke of the University of Iowa for statistical analysis.

**References**


Figure 1. Small intestine with gross lesions typical of PPE. Arrow shows areas of adenomatous mucosal thickening and serosal reticulation.

Figure 2. Fluorescent antibody stain using a monoclonal antibody against *L. intracellularis* confirming the presence of the bacteria within crypt epithelial cells of the small intestine in a formalin fixed histological section.
Figure 3. Prevalence of gross and microscopic PPE lesions at necropsy from study 1.

Figure 4. Prevalence of gross and microscopic PPE lesions at necropsy from study 2.
Figure 5. Seroconversion to *L. intracellularis* at necropsy from study 1. No significant differences were observed between treatment groups.

Figure 6. Seroconversion to *L. intracellularis* at necropsy from study 2.
Figure 7. PCR testing of the feces over study period of study 1.

Figure 8. PCR testing of the feces over study period of study 2.
Figure 9. Clinical Symptoms observed over study period from study 1. The bars indicate significance levels of difference between treatment groups showing the medicated group had significant reduction of clinical signs after day 18, ten days after the initiation of treatment.

Figure 10. Clinical Symptoms observed over study period from study 2. The bars indicate significance levels of difference between treatment groups showing the medicated group had significant reduction of clinical signs after day 11, 3 days after the initiation of treatment.
CHAPTER 7. SUMMARY AND CONCLUSIONS

In the studies described herein, we explored two methods for the prevention and control of porcine proliferative enteropathy. These methods included biological and pharmaceutical agents, both of which showed efficacy against virulent challenges of pure cultures of *Lawsonia intracellularis*.

A new technique was developed for the cultivation of *L. intracellularis*. This technique consists of a suspended eukaryotic host cell system for co-cultivation of *L. intracellularis*. This method allows cultures to be grown in large-scale fermentors and bioreactors for production of antigens for various uses including diagnostic reagents and vaccines.

A United States isolate of *Lawsonia intracellularis* was used to develop a vaccine for the prevention of lesions associated with porcine proliferative enteropathy. The field strain was attenuated by continuous passage in pure cultures using the suspended cell system described within this dissertation. The attenuated strain was then used to perform several studies to determine safety and efficacy against virulent challenges of *L. intracellularis* in the pig host.

Three studies were performed to demonstrate the safe use of the vaccine strain. Two studies were performed using high doses of the vaccine along with dexamethasone in pigs three weeks of age showing that the vaccine strain was attenuated. A third study was completed in CDCD pigs demonstrating the failure of the bacteria to revert back to virulence after serial backpassages within the host.

Three studies were also performed to demonstrate the efficacy of the vaccine to protect against virulent challenges of *L. intracellularis*. These studies demonstrated that at
doses of $10^6$ bacteria/dose, pigs had reduced amounts of colonization of virulent *L. intracellularis* and microscopic lesion development based on PCR of the feces and ileum, and by microscopic examination using fluorescent antibody stains, silver stains and hematoxylin and eosin stains. These studies demonstrated protection at 4 and 8 weeks after vaccination.

A common method of PPE control is the use of antibiotics in the feed or drinking water. In this dissertation we looked at two methods for the administration of tiamulin hydrogen fumarate. It was shown that tiamulin hydrogen fumarate was effective at treating and reducing PPE in pigs when administered in the feed after the onset of disease. It was also shown that water delivery of tiamulin hydrogen fumarate reduced PPE in pigs when administered after challenge and after the development of clinical signs typical of PPE.

The methods of prevention and control of PPE described within this dissertation will give swine producers and practitioners more options for controlling *L. intracellularis* infections within affected herds. Future work will need to be done to determine the effects of antibiotics that are routinely used as feed additives and water medications on the vaccine. As most producers use some type of feed additive, we will need to know the effects on the development of an effective immune response following vaccination. Also, it will be advantageous to learn how the vaccine and antibiotics can be used together to prevent and treat *L. intracellularis* infections along with other performance reducing pathogens. The vaccine by itself or when used in combination with additional PPE strategies must provide efficacy that is economically advantageous when compared to the efficacy achieved with other methods for controlling the financial losses resulting from PPE. Optimal disease control, production performance, and economic return from the *L. intracellularis* vaccine
and/or tiamulin hydrogen fumarate will be achieved when used as part of a comprehensive PPE program. Each program must consider the epidemiology of the disease and the individual farm and production management system.
APPENDIX A. PATENT FOR CULTIVATION IN SUSPENDED CELLS

Ileal symbiont intracellularis propagation in suspended host cells

Abstract

A method for large scale cultivation and attenuation of IS intracellularis bacteria by inoculating cells with IS intracellularis bacteria to infect the cells, incubating the infected cells in a reduced oxygen concentration and maintaining the infected cells in suspension. Anti-IS intracellularis vaccines are prepared from attenuated strains. Diagnostic agents are also disclosed.

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Assignee: NOBL Laboratories, Inc. (Ames, IA)
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Filed: June 5, 1995

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Intern'l Class: C12N 001/12; A61K 039/02
Field of Search: 435/252.1, 245, 383, 394, 395, 403 424/53.3, 93.4, 234.1

References Cited [Referenced By]

U.S. Patent Documents

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4904597 Feb., 1990 Inoue et al. 435/252.
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5230912 Jul., 1993 Yajima et al. 426/43.

Other References

S. McOrist et al., "Synergism of Ileal Symbiont Intracellularis and Gut Bacteria in The Reproduction of

Primary Examiner: Saucier; Sandra E.
Attorney, Agent or Firm: Dickstein Shapiro Morin & Oshinsky LLP

Claims

1. A method for cultivating Ileal symbiont intracellularis comprising:

infected cultured cells with an inoculum comprising Ileal symbiont intracellularis, incubating said infected cells at an oxygen concentration of less than about 18 percent while maintaining said infected cells in suspension by agitation of said cells for a sufficient period of time to increase the production of said Ileal symbiont intracellularis, and harvesting at least a portion of said Ileal symbiont intracellularis.

2. The method of claim 1 wherein said infected cells are incubated at an oxygen concentration above about 2 percent.

3. The method of claim 1 comprising the step of passaging a portion of said cultivated Ileal symbiont intracellularis to fresh cultured cells to further increase the production of said Ileal symbiont intracellularis.

4. The method of claim 1 wherein said Ileal symbiont intracellularis is obtained from an animal infected with Ileal symbiont intracellularis.

5. The method of claim 4 where said animal is a pig.

6. The method of claim 1 wherein said Ileal symbiont intracellularis comprises the Ileal symbiont intracellularis strain deposited in the American Type Culture Collection under ATCC Accession No. 55672.

7. The method of claim 1 wherein said incubation occurs at an oxygen concentration in the range from about 4
percent to about 10 percent and a carbon dioxide concentration in the range from about 4 percent to about 10 percent.

8. The method of claim 7 wherein said incubation occurs at an oxygen concentration in the range from about 5 percent to about 8 percent and a carbon dioxide concentration in the range from about 6 percent to about 9 percent.

9. The method of claim 1 wherein said cultured cells are selected from the group consisting of HEp-2, McCoy, and IEC-18 cells.

10. The method of claim 9 wherein said McCoy and IEC-18 cells are cultured on microcarriers.

11. A method for cultivating Ileal Symbiont intracellularis comprising:

(1) inoculating cultured cells with an inoculum comprising Ileal symbiont intracellularis,

(2) incubating said infected cells at a temperature of about 36 degree C. to about 38 degree C. in an oxygen concentration of less than about 18%,

(3) agitating said cells for a sufficient period of time to increase the production of said Ileal symbiont intracellularis, and

(4) harvesting at least a portion of said Ileal Symbiont intracellularis.

12. The method of claim 11 wherein said infected cells are incubated in a spinner flask.

13. The method of claim 12 wherein following step (3) the volume in the spinner flask is gradually increased using additional growth media.

14. The method of claim 12 wherein said inoculum is prepared by:

(a) inoculating a cultured cell monolayer which is at about 30 percent confluency with an inoculum comprising Ileal symbiont intracellularis so as to infect said cells, and

(b) incubating said infected cells at a temperature of about 36 degree C. to about 38 degree C. at an oxygen concentration of less than about 18% until said cells reach confluency.

15. The method of claim 11 wherein said inoculum is prepared by:

(a) growing Ileal symbiont intracellularis infected cells in a suspension culture, and

(b) freezing said infected cells.

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**Description**

**FIELD OF THE INVENTION**

The present invention is directed to anti-IS intracellularis vaccines and methods for protecting against and diagnosing IS intracellularis infection in susceptible animals. The products and processes of the invention are attainable, in part, as the result of an improved method for cultivating large scale supplies of IS intracellularis.

**DESCRIPTION OF THE RELATED ART**
Porcine proliferative enteropathy (PPE) is a common diarrheal disease of swine worldwide. IS intracellularis, the causative agent of PPE, also affects hamsters, fox, rabbits and other animals, but is a particularly great cause of losses in swine herds. These losses are associated with decreased growth rates, substantial antibiotic costs during the growth period and, in some cases, deaths. Estimates of the prevalence and incidence of PPE in the U.S. have been as high as 20 percent of the swine herd with estimated losses of $20 million annually.

A consistent feature of PPE is the occurrence of intracytoplasmic, non-membrane bound curved bacilli within enterocytes in affected portions of intestine. The bacteria associated with PPE have been referred to as "Campylobacter-like organisms." S. McOrist et al., Vet. Pathol., Vol. 26, 260-64 (1989). More recently, the causative bacteria have been identified as a novel taxonomic genus and species, currently known as Ileal symbiont (IS) intracellularis. C. Gebhart et al., Intl. J. of Systemic Bacteriology, Vol. 43, No. 3, 533-38 (1993).

IS intracellularis is an obligate, intracellular bacterium which cannot yet be cultured by normal bacteriological methods on conventional cell-free media and has been thought to require attached epithelial cells for growth.


The current understanding of PPE and the treatment and effective control of the disease have been seriously hampered by the fastidious growth requirements of IS intracellularis in in vitro cultures. There is currently a need for a method for large-scale cultivation of IS intracellularis. There is also a need for anti-IS intracellularis vaccines and tools for diagnosing IS intracellularis infection.

SUMMARY OF THE INVENTION

One object of the present invention is to provide a method for large-scale cultivation of IS intracellularis.

Another object of the invention is to provide an anti-IS intracellularis vaccine.

Another object of the invention is to provide a method for detecting the presence of IS intracellularis in a biological sample.

A further object is to provide a ready supply of IS intracellularis for production of vaccines and diagnostic agents.

To achieve these and other objects, and in accordance with the purpose of the invention as embodied and broadly described herein, the present invention provides a method for cultivating IS intracellularis and large scale supplies of bacteria produced thereby. According to the method, culture cells are inoculated with an inoculum comprising IS intracellularis bacteria to infect the cells with the bacteria. The infected cells are then incubated in an oxygen concentration of from about 2 percent to about 18 percent, while agitating the infected cells so as to cultivate the IS intracellularis while maintaining the infected cells in suspension.

According to a preferred embodiment, a method is provided for cultivating IS intracellularis bacteria by inoculating an HEp-2, McCoy, or IEC-18 cell monolayer, which is at about 30 percent confluency, with an inoculum comprising IS intracellularis bacteria so as to infect the cells with the bacteria. The infected cells are then incubated at a temperature of about 37 degrees C. at an oxygen concentration of about 8.0 percent until the cells reach confluence. The infected cells are then placed in a spinner flask containing growth media. The spinner flask is incubated at a temperature of about 37 degrees C. at an oxygen concentration of about 5.0% to
about 8.0%, a carbon dioxide concentration of about 8.0% to about 9.0%, while spinning the flask so as to cultivate the IS intracellularis bacteria while maintaining the infected cells in suspension. A portion of the cultivated IS intracellularis is then passaged to fresh culture cells to increase the production of IS intracellularis bacteria.

The invention also provides anti-IS intracellularis vaccines and methods for producing vaccines against IS intracellularis. An avirulent IS intracellularis bacteria is produced by passaging the cultivated IS intracellularis bacteria a sufficient number of times and selecting for an attenuated strain, or by subjecting the cultivated bacteria to chemical means of attenuation. According to a particularly preferred embodiment the bacteria are continuously cultured for at least about 6 to 8 months while being passaged at least about 7 to 12 times to produce an attenuated strain for use as a vaccine.

The invention also provides a method for determining the presence of IS intracellularis bacteria in a biological sample by harvesting at least a portion of the cultivated IS intracellularis bacteria, contacting a biological sample from an animal with harvested IS intracellularis bacteria under conditions whereby antibody present in the biological sample reacts with the IS intracellularis, and determining if an antibody-antigen reaction has occurred.

Additional features and advantages of the invention will be set forth in the description which follows and will be apparent from the description or may be learned by practice of the invention.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Reference will now be made in detail to the presently preferred embodiments of the invention.

As used herein, the term "IS intracellularis" means the intracellular, curved, gram-negative bacteria described in prior art by C. Gebhart et al., Intl. J. of Systemic Bacteriology, Vol. 43, No. 3, 533-38 (1993) and includes, but is not limited to, the bacteria deposited as ATCC 55672 in the American Type Culture Collection, Rockville, Md.; the bacteria deposited as NCTC 12656 and 12657 in the National Collection of Type Cultures, Colindale, London; the causative bacteria which can be obtained from PPE infected swine or other animals throughout the world given the knowledge in the art and the teachings herein; and any variants or mutants of any of the above bacteria, whether spontaneously or artificially obtained.

As used herein, the term "large-scale cultivation" means a level of cultivation of IS intracellularis greater than approximately 2.0 to 3.0 liters and includes production on a scale of 100 liters or more. "Cultivation" as used herein, means the process of promoting the growth, reproduction and/or proliferation of IS intracellularis.

In practicing the cultivation method of the invention, culture cells are first inoculated with an inoculum comprising IS intracellularis bacteria so as to infect the cells with the bacteria. Numerous cell lines can be used in practicing the invention, including, but not limited to, IEC-18 (ATCC 1589)~rat intestinal epithelial cells, HEp-2 (ATCC 23)~human epidermoid carcinoma cells, McCoy (ATCC 1696)~mouse (nonspecified) cells, MDCK (ATCC 34)~Madin-Darby canine kidney cells, BGMK (Biowhittaker #71-176)~buffalo green monkey kidney cells, and swine intestinal epithelium cells. The preferred culture cells are HEp-2, McCoy or IEC-18 cells.

Prior to being inoculated, the cells are preferably in the form of a monolayer. To form a monolayer, the cells may be seeded into conventional flasks. Each flask is generally seeded with between about 1.times.10.sup.5 cells to about 10.times.10.sup.5 cells per 25 cm.sup.2 flask mixed with growth media. The growth media may be any media for cell cultivation which includes a nitrogen source, necessary growing factors for the chosen culture cells, and a carbon source, such as glucose or lactose. The preferred media is DMEM with 2-5% fetal bovine serum, although various other commercially available media may be used with good results.

One of the keys to successful cultivation of IS intracellularis is to maintain the culture cells in a constant state of growth. Therefore, the culture cell monolayer should be at about 20 percent to about 50 percent confluency at
the time of inoculation. Preferably, the cells should be at about 30 percent to about 40 percent confluency at the
time of inoculation, with about 30 percent confluency being most preferred.

The inoculum may be a pure culture of IS intracellularis obtained, for example, from ATCC deposit 55672,
NCTC deposits 12656 or 12657, or from infected swine or other animals using the isolation and purification
teachings discussed herein.

According to one embodiment, the inoculum for practicing the invention is an intestinal homogenate prepared
by scraping the mucosa off of the ileum of a swine or other animal infected with PPE. When preparing an
intestinal homogenate, ileal sections selected for culture should show severe lesions with gross thickening of the
gut. Due to the fragile nature of the bacteria, samples should preferably be stored at -70°C. as quickly as
possible after necropsy. An antibiotic to which IS intracellularis is resistant such as Vancomycin, Amphotericin
B or members of the aminoglycoside group of antibiotics, including Gentamicin and Neomycin, to name a few,
is preferably added to the inoculum to suppress contaminating bacteria while permitting IS intracellularis
growth. Whether the inoculum is a pure culture or an intestinal homogenate, inoculation of the culture cells can
be performed by various techniques known in the art given the teachings herein.

The inoculated culture cells are then incubated under a reduced O\textsubscript{2} concentration. At oxygen
concentrations greater than 18% or less than 2%, IS intracellularis growth is less than optimal with cessation of
growth eventually occurring at oxygen concentrations outside this range. Preferably, the inoculated culture cells
are incubated in an oxygen concentration in the range of from about 4% to about 10%. More preferably, the
cells are incubated in an oxygen concentration in the range of from about 5% to about 8%, with an oxygen
concentration of about 8.0% being most preferred.

The proper concentration of carbon dioxide is also important to the proper growth of IS intracellularis. At carbon
dioxide concentrations greater than 10% and less than 4%, non-optimum growth occurs with cessation of
growth eventually occurring at carbon dioxide concentrations outside this range. Preferably, the inoculated
cells are incubated in a carbon dioxide concentration in the range from about 6% to about 9%, with a carbon
dioxide concentration of about 8.8% being most preferred.

In addition, the inoculated cells are preferably incubated at a hydrogen concentration in the range from about
73% to about 94%. Nitrogen may be used in place of some or all of the hydrogen present, but hydrogen is
preferred. According to a particularly preferred embodiment, the cells are incubated in an atmosphere of about
8.0% O\textsubscript{2}, about 8.8% CO\textsubscript{2}, and about 83.2% H\textsubscript{2}.

The inoculated cells may be incubated in a dual gas incubator or other gas chamber which contains the proper
oxygen and carbon dioxide concentrations and which allows the cells to be suspended during incubation. The
chamber should comprise a means for maintaining the inoculated cells in suspension, and a gas monitor and
supply source to supply and maintain the proper gas concentrations. In addition, the chamber should include a
means for regulating the temperature to which the cells are subjected during incubation. The incubation
temperature should be in the range of from 30°C. to 45°C. and is more preferably in the range of from about 36°C. to about 38°C. Most preferably, the temperature is about 37°C. The
necessary equipment for the cultivation method of the invention is readily available to those of ordinary skill in
the art given the teachings herein. The presently preferred equipment comprises a dual gas incubator, e.g.,
model 480 available from Lab-Line, Melrose Park, Ill., in conjunction with spinner flasks to maintain the cells
in suspension.

By maintaining the cells in a suspended state during incubation, maximum growth of the cells, and hence IS
intracellularis, is achieved by increasing each individual cell's exposure to growth media and the proper mixture
of oxygen and carbon dioxide. Also, as will be further described below, cultivating the cells in suspension
results in much more efficient passage, harvest and scale-up for cultivating large-scale supplies of IS
intracellularis. The culture cells can be agitated and maintained in suspension by a variety of methods known in
the art, including, for example, culture flasks, roller bottles, membrane cultures and spinner flasks. Most
preferably, the cells are kept in suspension during incubation by incubating the cells in a spinner flask inside a
dual gas incubator or similar apparatus. The term "spinner flask", as used herein, means a flask or other container which employs a paddle, propeller or other means to agitate the culture and keep the cells contained therein in suspension.

In a particularly preferred embodiment of the invention, the inoculated cells are incubated until the cells reach confluency and then the cells are placed in a spinner flask containing growth media and incubated in a dual gas incubator while spinning the flask. Preferably, the inoculated cells are scraped into the spinner flask. This can be achieved by a variety of methods known in the art such as using a cell scraper to detach the cells. Once the cells are introduced into the spinner flask, the paddle of the spinner flask is typically rotated in the range of from about 30 to about 60 rpm in order to maintain the infected cells in suspension.

A portion of the cultivated IS intracellularis is then passaged to fresh culture cells to increase the production of IS intracellularis bacteria. The term "passaging" or variations thereof herein means the process of transferring a portion of the cultivated IS intracellularis to fresh culture cells in order to infect the fresh cells with the bacterium. The term "fresh", as used herein, means cells which have not yet been infected by IS intracellularis. Preferably such cells are, on the average, no more than approximately one day old.

After sufficient growth of the culture cells and subsequent infection by IS intracellularis at greater than about 70% cell infectivity, as determined by IFA, TCID.sub.50 or other comparable method, at least a portion of the cultivated IS intracellularis bacteria is then harvested. The harvesting step may be performed by separating the bacteria from the suspension by various techniques known to those of ordinary skill in the art, given the teachings herein. Preferably, the IS intracellularis bacteria is harvested by centrifuging the contents of all or a portion of the suspension to pellet the culture cells, resuspending the resulting cell pellets, and lysing the infected cells. Typically, at least a portion of the contents is centrifuged at about 3000.times.g for about 20 minutes in order to pellet the cells and bacteria. The pellet may then be resuspended in, for example, a sucrose-phosphate-glutamate (SPG) solution and passed approximately four times through a 25 gauge needle in order to lyse the cells. If further purification is desired, the samples can be centrifuged at about 145.times.g for about five minutes to remove cellular nuclei and debris. The supernatant may then be centrifuged at about 3000.times.g for about twenty minutes and the resulting pellet resuspended in an appropriate diluent, such as SPG with fetal bovine serum (to prepare harvested bacteria suitable for freezing or use as an inoculant) or such as growth media (to prepare harvested bacteria more suitable for passaging to fresh cells).

The passage of IS intracellularis in suspension cultures may be accomplished by removing a portion of the original culture and adding it to a new flask containing fresh culture cells. If the original culture has a high number of bacteria/ml, for example, greater than about 10.sup.4 bacterial/ml, it is preferable to add between about 1 to 10% (volume to volume) of culture from the infected flask to a new flask containing fresh cells. This is preferably done when 50-100% of the cells are infected. If fewer than 50% of the cells are infected, passaging is preferably accomplished by splitting the culture 1:2 into a new flask and scaling-up the volume by adding fresh media. In either case, cell lysis and other steps are not required, in direct contrast to the passage of monolayer cultures, as in the prior art.

As previously mentioned, one key to effectively growing IS intracellularis for large-scale production is to keep the tissue cells actively growing. With monolayers, when cultures become confluent the rate of cell division decreases substantially. Attempts to grow IS intracellularis on monolayer tissue cultures have had limited success and scale-up has not been possible. However, using suspension cultures greatly facilitates keeping the cells actively growing and permits continuous culture expansion and scale-up. We have been able to grow up to 10.sup.6 bacteria/ml. We have also been able to keep the cultured bacteria actively growing for many months and expect to be able to do so indefinitely.

Prior to the instant invention, it was generally believed that cells must be attached to a surface in order to be infected by IS intracellularis. The cell suspensions of the instant invention are unique and contradict this theory. When using McCoy or IEC-18 cells, it is preferable to add gelatin, agarose, collagen, acrylamide or silica beads, such as Cultisphere-G porous microcarriers manufactured by HyClone Laboratories, Logan, Utah, along with the growth media. However, HEp-2 cells do not require microcarriers according to the cultivation method of the
invention. This provides an especially advantageous and economical route for large-scale cultivation.

For culture maintenance purposes, with HEp-2 cultures, preferably 25-50% of the culture is removed and replaced with fresh media at weekly intervals. For cell cultures with microcarriers or beads, preferably 25-50% of the culture is removed and replaced with fresh microcarriers or beads and fresh media 1-2 times weekly. For scale-up purposes, an additional 25-50% of media, or media with microcarriers, may be added to the culture.

Depending upon the rate at which the culture cells become infected, passage to fresh cells generally occurs between about every 2 to about 5 weeks. Assuming that the culture cells become at least 70% infected within 2-3 weeks, preferably passage occurs between about every 3 to 4 weeks.

The present invention also provides vaccines and methods for producing vaccines against IS intracellularis. According to a particularly preferred embodiment, after maintaining the infected cells in suspension for an extended time (for example, 6-8 months), at least a portion of the cultivated IS intracellularis bacteria are harvested and monitored for potential attenuation. Such monitoring is preferably accomplished by host animal or animal model challenges to select for an attenuated strain.

The present invention allows rapid culture expansion, an increase in yields of 100-1000 fold, and reduced cost. As a result, the abundant supply of IS intracellularis bacteria produced according to the cultivation method of invention is readily attenuated for vaccine production purposes. Attenuation is difficult in monolayer cultures due to the low yield of bacteria produced using conventional monolayer growing techniques. In contrast, the method of growing IS intracellularis of the present invention greatly increases the ease, speed, and number of bacterium available for this purpose. The more cells and cell divisions which occur, the greater the level of mutations occurring which are advantageous in vaccine development. Accordingly, growth in suspensions according to the invention increases the expression of important immunogens controlled by environmentally regulated genes and their expression products.

The attenuated strain can be cultivated in tissue culture monolayers as described in Example 1 below, but according to the preferred method of the invention. Other means of attenuation include chemical attenuation by the use of, for example, N-methyl nitrosoguanidine and others known in the art. In either case, and whether by multiple passage or chemical means, an attenuated IS intracellular is produced and selected for vaccine preparation.

According to one vaccine embodiment of the invention, the antigen is harvested by centrifugation or microfiltration as described above. The antigen is then standardized at a defined level based on the optimum host animal immune response, determined by a dose titration in the host animal species. The bacteria may be inactivated using 0.3% formalin or other inactivating agent. The antigen is then incorporated into a suitable adjuvant, such as aluminum hydroxide or mineral oil to enhance the immune response.

The antigen is then used to vaccinate the host via intramuscular or subcutaneous injection at about 3-4 weeks of age, with a booster dose if necessary. The animal is exposed to virulent challenge about fourteen days after vaccination. For example, pigs may be orally challenged with about 1-times.10.sup.7 or more organisms. The infected animals should be necropsied about 21 days after challenge and the small intestines observed for gross lesions as well as microscoic lesions. Polymerase chain reaction (PCR) testing should be performed on the mucosa of the ileum and colon as well as fecal contents. Fluorescent antibody (FA) testing using a monoclonal antibody against IS intracellularis should also be performed on tissue sections of the ileum, jejunum, and colon for the presence of the bacteria.

Alternatively, according to a particularly preferred vaccine embodiment, using the cultivation methods previously described for suspension growth of IS intracellularis, the bacteria are serially passaged to induce and select for an attenuated, avirulent live culture. The culture is tested in the host animal (after preferably at least 6 to 8 months or more of growth in the suspension culture) for signs of attenuation. The culture is harvested as described earlier and diluted. Pigs are orally vaccinated with 1-times.10.sup.5 to 1-times.10.sup.6 bacteria. About twenty-eight days after vaccination, the pigs are orally inoculated with about 1-times.10.sup.7 organisms.
from a less passaged (about 30 to 45 days old) virulent cultures of IS intracellularis. The infected animals are necropsied 21 days after challenge and the small intestines observed for gross lesions as well as microscopic lesions. PCR and fluorescent antibody should also be performed.

About eighty percent of the control animals will show gross or microscopic lesions and test positive for the presence of IS intracellularis in the mucosal cells of the intestines using either PCR or FA testing methods. Vaccinated animals will have normal mucosal surfaces as determined by histological observations and will be negative by PCR testing.

Generally, an attenuated immunogenic IS intracellularis strain is produced after continuous culture for between at least about 150 and 250 days, during which time the culture is passaged at least about 7 to about 12 times. Other attenuated cultures may be produced by varying these figures so long as the monitoring and selection methods taught herein are employed.

A vaccine is then prepared comprising an immunologically effective amount of the attenuated IS intracellularis in a pharmaceutically acceptable carrier. The combined immunogen and carrier may be an aqueous solution, emulsion or suspension. An immunologically effective amount is determinable by means known in the art without undue experimentation given the teachings contained herein. In general, the quantity of immunogen will be between 50 and 500 micrograms, and preferably between 10.sup.7 and 10.sup.9 TCID.sub.50, when purified bacteria are used.

The vaccines according to the invention are generally administered to susceptible animals, preferably swine, in one or more doses. Preferably, the vaccine is administered 1 or 2 times at 2 week intervals. The preferred routes of administration are oral or intranasal, but intramuscular or subcutaneous injection may also be used.

Effective diagnosis of PPE has also been hindered by the time required to culture the causative bacteria. As a result of the present invention, development of diagnostic tools promoting rapid and accurate assays for the presence of IS intracellularis in biological samples taken from swine and other animals susceptible to PPE is now possible.

The invention provides a method for determining the presence of IS intracellularis bacteria in a biological sample comprising the steps of harvesting at least a portion of the cultivated IS intracellularis bacteria, obtaining a biological sample from an animal, contacting the sample with harvested IS intracellularis bacteria under conditions whereby antibody present in the biological sample reacts with the IS intracellularis, and determining if an antibody-antigen reaction has occurred, thereby determining the presence of IS intracellularis in the sample.

The IS intracellularis bacteria grown according to the method of the instant invention can be used as an antigen in an ELISA or other immunoassay to detect antibodies to IS intracellularis in the serum and other body fluids of animals suspected of being infected with the bacteria. Alternatively, the bacteria grown according to the invention can be used in a Western Blot assay.

The preferred ELISA protocol according to this embodiment of the invention is as follows:

1. Add 0.1 ml/well antigen diluted in coating buffer. Incubate for 18 hours at 4.degree. C.
2. Wash 3 times with PBS.
3. Add 0.25 ml of blocking buffer to each well of plate. Incubate 1 to 2 hours at 37.degree. C.
4. Wash 3 times with wash buffer.
5. Dilute serum in blocking buffer and add 0.1 ml to the first wells of plate. Make serial 1:2 dilutions across the plate. Incubate for 1 hour at 37.degree. C.
6. Wash 3-5 times with wash buffer.

7. Dilute conjugate in blocking buffer and add 0.1 ml to wells of plate and incubate for 1 hr at 37.\degree\ C.

8. Wash 3-5 times with wash buffer.


12. Measure absorbance of light with a spectrophotometer.

13. Wells in which antigen was not added are used as blanks.

14. Positive and negative control pig serum should also be used with each test.

The present invention is further described in the following examples which are provided for illustrative purposes only and are not to be construed as limiting.

EXAMPLE 1
Isolation of IS intracellularis from the intestines of American pigs with porcine proliferative enteropathy

Materials and Methods

Selection of inoculum samples

Sample N24912 was obtained from a herd on a farm in Iowa in which fifteen of 300 five month old finisher pigs were observed to have persistent bloody stools despite penicillin treatment. Upon necropsy of the pigs, the intestine (ileum) had a thickened mucosa. Histopathology examinations with silver stains demonstrated the presence of curved intracellular bacteria and crypt enterocyte hyperplasia confirming the diagnosis of PPE. Sample N72994 was obtained from a 1.5 year old second litter SPF sow on a farm in Minnesota. The herd size was between 70-80 sows and antibiotic treatment is unknown. Upon necropsy, the mucosa of the ileum was thickened with some hemorrhage. Giminez staining of the mucosa demonstrated many curved bacteria. Sample N101494 was obtained from a 12 week old pig from an Indiana farm with 600 furrow to finish sows. The pig was treated with Tylan injectable upon the onset of bloody diarrhea, but the animal died soon after treatment.

Preparation of pig derived bacterial inocula

Intestinal samples were kept at -70\degree\ C. The intestines were opened and washed with phosphate buffered saline (PBS). One gram samples of mucosa were scraped into sodium potassium glutamate (SPG) and homogenized for 30 seconds with 4.0 ml 1% Trypsin (JRH Biosciences, Lenexa, Kans.) in SPG. The suspensions were incubated for 35 minutes at 37\degree\ C. Ten ml SPG/10% fetal calf serum (FCS) (JRH Biosciences, Lenexa, Kans.) was added and the samples were ground in a tissue grinder for 1 minute. Ten ml SPG/10% (FCS) was added and filtered once through filterpaper (Whatman 113V; Whatman Labsales, Hillsboro, Oreg.) and sequentially through 5.0, 1.0, and 0.65 micron membrane filters. Filtrates were aliquoted and frozen at -70\degree\ C. in 1.0 ml aliquots. The mucosa was smeared onto a slide for Giminez stain. Separate smears of filtrates were stained by IFA using a specific monoclonal antibody for IS intracellularis. S. McOrist et al., Vet. Rec. 121:421-422 (1987) (incorporated by reference herein in its entirety).

Cell Culture

IEC-18 cells (Rat intestinal epithelial cells, ATCC CRL 1589) were grown in DMEM (JRH Biosciences, Lenexa, Kans.) with L-glutamine and 10% FCS and routinely passaged by trypsin weekly. Cell monolayers were grown at 37\degree\ C. in air with 5% CO.sub.2.
Infection of cell culture

IEC-18 cells were seeded at 1.25 x 10^5 cells in 25 cm^2 flasks and at comparable rates in chamberslides (Nunc, Inc., Naperville, Ill.), incubated 24 hours, then media removed. Frozen pig-derived bacterial isolates were quickly thawed and diluted in DMEM/7% FCS with Vancomycin (100 μg/ml) and Amphotericin B (2.0 μg/ml) at ratios of 1.0 ml homogenate to 15 ml media and added to the monolayers. Monolayers and bacterial suspensions were centrifuged for 30 minutes at 2000 g and transferred to anaerobic jars. The jars were evacuated and the gas was replaced with hydrogen and carbon dioxide to give a mixture of 8.0% O_2, 10 % CO_2, and 82% H_2. The cultures were incubated for 3 hours at 37°C. Then refed with DMEM/7% FCS with L-glutamine, Vancomycin (100 μg/ml), Neomycin (50 μg/L), and Amphotericin B (2.0 μg/ml). Cultures were replaced in the anaerobic jars and incubated for 6 days with media changes every 2 days.

Passage of IS intracellularis

IS intracellularis bacteria were passed by cell lysis using potassium chloride as described previously in G. Lawson et al., J. Clin. Microbiol., 31:1136-1142 (1993) (incorporated by reference herein in its entirety) then added to fresh IEC-18 monolayers. Media was poured off the monolayers and 0.1% KCl was added and the cells incubated for 10 minutes at 37°C. The KCl was removed and SPG/10% was added and the monolayers detached mechanically with a cell scraper. The cells were lysed by passing 3 times through a syringe with a 21 gauge needle. Cell nuclei were removed by centrifugation at 100 x g for 5 minutes and the bacterial suspension in the supernatant fluid added to fresh 1 d monolayers of IEC-18 cells.

Monitoring infection of cell cultures

Infection was monitored by fixing the cells on chamberslides with cold acetone/methanol for 5 minutes. Staining was carried out by immunofluorescence and immunoperoxidase methods. Both methods employed a mouse monoclonal antibody (as described in S. McOrist et al., Vet. Rec. 121:421-422 (1987)) as the primary antibody and either anti-mouse immunoglobulin G-fluorochrome conjugate (fluorescein isothiocyanate; Organon Teknika Corporation, Durham, N.C.) or peroxidase conjugate (goat anti-mouse immunoglobulin G; Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.). Quantitation of bacteria was accomplished by counting the number of specifically stained bacteria within cells on each slide.

Polymerase chain reaction

Sample inocula and passaged bacteria were incorporated as template DNA into PCR using the sample preparation method, primers, and cycle parameters as described by Jones et al., J. Clin. Microbiol., 31:2611-2615 (1993) and McOrist et al., Vet. Microbiol. 1-8 (1994) (each of which are incorporated by reference herein in their entirety). Cycle parameters were 93 degree. C. for 5 minutes, 55 degree. C. for 45 seconds, and 72 degree. C. for 45 seconds for the first cycle. Thirty-three cycles were performed at the previously mentioned temperatures for 45 seconds per temperature, as well as one cycle at 93 degree. C. for 45 seconds, 55 degree. C. for 45 seconds, and 72 degree. C. for 2 minutes. Positive inocula only were used to inoculate IEC-18 cells. PCR was also performed for the monitoring of passage material to confirm infections. DNA produced by PCR was submitted to the Iowa State University Nucleic Acid Facility for sequencing. Results of the sequencing were compared to sequences produced by Gary F. Jones as reported in his Ph.D. thesis, University of Minnesota, Minneapolis, Minn. (June, 1993).

Results

Selection of inoculum samples

Pig number N24912 and N72994 had severe PPE with bloody intestinal contents and thickened mucosa. N101494 had severe PPE and severe hemorrhage resulting in a large blood clot in the intestinal lumen. Giminez
staining of the mucosal smears demonstrated large numbers of curved or S-shaped bacteria. IFA stains revealed large numbers of brightly fluorescing bacteria in pig-derived bacterial inocula.

Monitoring infection of cell cultures

Inoculated monolayers were monitored by light microscopy throughout the growth cycle and little morphological change of the cells was observed. Uninfected monolayers grown under reduced oxygen tension (8% O2) had similar morphology.

Immunofluorescence and immunoperoxidase stained infected cultures demonstrated large numbers of curved or S-shaped specifically stained bacteria apparently within cells. The monolayers did not have confluent infection. Infected cells were often closely associated with infected foci of 1-10 cells. Heavily infected cells (i.e., cells with 30 or more bacteria) were also seen in association with cells with fewer than 30 bacteria. Bacterial numbers peaked at or about 6 days. Infection was dependent on specific growth conditions. The bacteria were successfully passaged by the cell lysis procedure described herein. Centrifugation of newly inoculated cells was not necessary but enhanced the numbers of infected cells. Centrifugation also decreased contamination by allowing cells exposed to infection with antibiotic-free media to be refed at 3 hours with antibiotic containing media. Reducing FCS from 10% to 7% in the media was necessary to slow the growth of the IEC-18 cells allowing the bacteria to proliferate to higher numbers before monolayers became confluent.

Polymerase chain reaction

PCR of chromosomal DNA generated a 319 bp fragment (including primers) from all isolates. A fragment of appropriate size was visually compared to a known positive sample generated by McOrist et al. (1994) using PCR. Sequence analysis of the PCR products of N24912, N72994, and N101494 confirmed a close homology (97-99%) to the p78 sequence determined by Jones (1993).

EXAMPLE 2

Growth of IS intracellularis in suspension cultures of HEp-2 cells

Preparation of intestinal homogenates for inoculum:

Intestinal homogenate was prepared by scraping the mucosa off of 6.0 to 8.0 cm of ileum from the intestinal samples of Example 1. Trypsin (1%) was added to the scraped mucosa and the samples were homogenized briefly, then incubated for 35 minutes at 37 degree C. Ten ml SPG/10% FBS was then added and the samples were ground in a tissue grinder. Another 10 ml SPG/10% FBS was added. The homogenates were passed through a Whatman V113 filter and then sequentially through 5.0, 1.0, and 0.65 mu.m filters. The samples were dispensed into 1 ml aliquots and frozen at -70 degree C.

Infection of cell culture:

Method A:

Tissue cells were seeded at 1 times 10 sup.7 cells in 50 ml DMEM/10% FBS in a 100 ml spinner flask. The cultures were incubated 24 hr., then Vancomycin and fungizone were added. One vial of frozen intestinal homogenate was quickly thawed and diluted in 3.0 ml DMEM/5% FBS with Vancomycin (100 mu.g/ml) and Amphotericin B (2.0 mu.g/ml). The sample was passed through a 0.65 mu.m filter and added to the flask. The culture was placed in a gas chamber, evacuated, and regassed with hydrogen and carbon dioxide to give a mixture of 8.0% O2, 8.8% CO2, and 83.2% H2. The cultures were incubated for 3 hours at 37 degree C. and then Neomycin and Gentamycin were added. The culture was refed at 24 hours with DMEM/5% FBS with L-glutamine, Vancomycin (100 mu.g/ml), Neomycin (50 mu.g/L), Gentamycin (50 mu.g/L) and Amphotericin B (2.0 mu.g/ml).
Method B

Two 25 cm.² conventional flasks were seeded with 1.25 x 10⁷ HEp-2 cells in DMEM/10% FBS and allowed to grow 18-24 hours. The cells were at 30% confluency at time of inoculation. The inoculum was diluted in DMEM/5% FBS. When the inoculum is from an intestinal homogenate, the media also contained Vancomycin (100 μg/ml) and Amphotericin B (2.0 μg/ml). The cultures were placed in a gas chamber, evacuated, and regassed with hydrogen and carbon dioxide to give a mixture of 8.0% O₂, 8.8% CO₂, and 83.2% H₂. The cultures were incubated for 3 hours at 37 °C. Then Neomycin and Gentamycin were added. The culture was refed at 24 hours with DMEM/5% FBS with L-glutamine, Vancomycin (100 μg/ml), Neomycin (50 μg/L), Gentamycin (50 μg/L), and Amphotericin B (2.0 μg/ml). No antibiotics were required when the inoculum was a pure culture. The cultures were incubated for 6 days or until confluency. The cells were scraped from the flasks and added to a 100 ml spinner flask containing 50 ml DMEM/5% FBS.

The culture was diluted 1:2 at weekly intervals by either harvesting one half of the culture and adding fresh media or by passing into a larger spinner flask and adding more media.

Passage of the culture

The culture was passed to fresh HEp-2 cells by seeding new HEp-2 cells at 1 x 10⁷ into DMEM/5% FBS. The new culture was allowed to incubate overnight at 8.0% O₂, 8.8% CO₂, and 83.2% H₂. The new culture was then inoculated with infected culture and incubated at reduced O₂ concentrations as previously stated. Inoculum amounts were dependent on the degree of infection of the original culture.

Harvesting and storage of cultures:

The cultures were harvested by collecting the desired amount of culture while centrifuging at 3000 x g for 20 minutes. The pellet was resuspended in Sucrose-Phosphate-Glutamate (SPG) solution and passed 4 times through a 25 gauge needle. The cultures were aliquoted and frozen at -70 °C. For further purification, the sample was centrifuged at 145 x g for 5 minutes to remove the cellular nuclei and debris. The supernatant was then centrifuged at 3000 x g for 20 minutes. The pellet was then resuspended in diluent.

Estimation of viable IS intracellularis in tissue culture:

Quantitation of viable IS intracellularis was accomplished by determination of the Tissue Culture Infectious Dose 50 percent (TCID₅₀). This was done by removing 2.0 ml of culture to be tested and lysing the cells by passing through a 25 gauge needle 4 times. The sample was serially diluted 1:10 in DMEM/5% FBS containing Vancomycin (100 μg/ml) and Amphotericin B (2.0 μg/ml). The dilutions were added to a 96-well microtiter plate with 0.1 ml/well. The microtiter plates were seeded with HEp-2 cells at 1250 cells/well and grown 18-24 hours prior to infection. Between 3 wells/dilution and 6 wells/dilution were used. The plate was incubated for 6 days at gas concentrations of 8.0% O₂, 8.8% CO₂, and 83.2% H₂. The cells were fixed with cold 50% acetone and 50% methanol for 2 minutes. To the wells, 0.03 ml/well of anti-IS intracellularis monoclonal antibody (McOrist, 1994) diluted 1:2000 in PBS was added. The plate was incubated for 30 minutes at 37 °C. The plate was then washed 3 times with PBS. Anti-mouse FITC diluted 1:30 was added in the amount of 0.03 ml/well and incubated 30 minutes at 37 °C. The plate was then washed 3 times with ddH₂O and allowed to dry. Samples were observed on a fluorescent microscope and the TCID₅₀/ml was determined.

Results

The TCID₅₀ results indicated that the cultures contained up to 1.0 x 10⁶ bacteria/ml. This was accomplished in 45 days. The culture volume was scaled-up to 3.0 litres in the same amount of time.

EXAMPLE 3
Growth of IS intracellularis in suspension cultures of McCoy cells

Preparation of intestinal homogenates for inoculum

Intestinal homogenate was prepared as described in Example 2. A sample of IS intracellularis cultivated according to the method of the following example was deposited under the Budapest Treaty on May 19, 1995 in the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md. U.S.A. 20852 and assigned accession number 55672.

Infection of cell culture

Two 25 cm.sup.2 conventional flasks were seeded with 1.25.times.10.sup.5 McCoy cells in DMEM/10% FBS and allowed to grow 18-24 hours. The cells were at 30% confluency at time of inoculation. The inoculum was diluted in DMEM/5% FBS. When the inoculum is from an intestinal homogenate, then the media also contained Vancomycin (100 .mu.g/ml) and Amphotericin B (2.0 .mu.g/ml). The cultures were placed in a gas chamber, evacuated, and regassed with hydrogen and carbon dioxide to give a mixture of 8.0% O.sub.2, 10% CO.sub.2, and 82% H.sub.2. The cultures were incubated for 3 hours at 37.degree. C., then Neomycin and Gentamycin were added. The culture was refed at 24 hours with DMEM/5% FBS with L-glutamine, Vancomycin (100 .mu.g/ml), Neomycin (50 .mu.g/L), Gentamycin (50 .mu.g/L), and Amphotericin B (2.0 .mu.g/ml). No antibiotics were required when the inoculum was a pure culture. The cultures were incubated for 6 days until confluency. The cells were scraped from the flasks and added to a 100 ml spinner flask containing 50 ml DMEM/2% FBS and 0.05g Cultisphere-G Microcarriers. The flasks were stirred at 40-50 rpms.

The culture was diluted 1:2 every 2-3 days by either harvesting one half of the culture and adding fresh media and Cultisphere-G beads or by passing the culture into a larger spinner flask and adding more media and Cultisphere-G beads. The final concentration of beads in the culture was about 0.001 g beads/ml.

Passage of the culture:

The culture was passed to fresh McCoy cells by seeding 1.times.10.sup.7 new McCoy cells into DMEM/2% FBS and 0.05 g Cultisphere-G beads. The new culture was allowed to incubate overnight at 8.0% O.sub.2, 8.8% CO.sub.2 and 83.2% H.sub.2. The new culture was then inoculated with 25 ml of infected culture and incubated at reduced O.sub.2 concentrations as previously stated.

Harvesting and storage of cultures:

The cultures were harvested by collecting the desired amount of culture and centrifuging at 3000.times.g for 20 minutes. The pellet was resuspended in SPG and passed 4 times through a 22 gauge needle. The cultures were aliquotted and frozen at -70.degree. C. For further purification, the sample was centrifuged at 145.times.g for 5 minutes to remove the beads, cellular nuclei and debris. The supernatant was then centrifuged at 3000.times.g for 20 minutes. The pellet was then resuspended in diluent.

Estimation of viable IS intracellularis in tissue culture:

Quantitation of viable IS intracellularis was determined as described in Example 2 using a 22 gauge needle to lyse the cells and using McCoy cells at 1250 cells/well to seed the microtiter plates.

Results:

The TCID50 results indicated that the cultures contained up to 1.times.10.sup.6 bacteria/ml. This was accomplished in less than 1 month. The culture volume was scaled-up to 3.0 liters in the same amount of time.

EXAMPLE 4
Determining infectious dose of IS intracellularis pure cultures in host animals

Summary:

A thirty-one pig study was completed by infecting 6 week-old conventional pigs with pure cultures of IS intracellularis from sample N72994. The pigs were randomly divided into 4 groups and the groups were penned separately. Group 1 contained 7 pigs and was considered the negative control group dosed with uninfected tissue culture or nothing. The group 2 contained 8 pigs dosed with 10^7 bacteria/pig. Group 3 had 8 pigs and was dosed with 10^6 bacteria/pig. And, Group 4 contained 8 pigs receiving 10^5 bacteria/pig.

Fecal swabs were collected on days 0, 7, 14, and 21, and 24 for PCR testing. On day 24, the pigs were necropsied and the ileum, jejunum, and the colon were collected for PCR testing, histopathology, and FA stains, all as described above.

PCR testing of the ileal mucosa revealed the presence of IS intracellularis in 100% of the high dose, 75% of the medium dose, and 50% of the low dose. Histopathology results indicated an increase of mononuclear cells in the lamina propria and submucosa of 88% of the high dose, 75% of the medium dose, and 88% of the low dose. Crypt hyperplasia was observed in 50% of the high dose, 63% of the medium dose, and 50% of the low dose. FA staining revealed IS intracellularis in tissue sections of the ileum, jejunum, and colon in 88% of the high dose, 63% of the medium dose, and 63% of the low dose. Control animal were negative for the presence of IS intracellularis via PCR, FA, and silver stains.

In conclusion, a pure culture was successfully used to infect and cause lesions of PPE. Koch's postulates were fulfilled by the identification and isolation of IS intracellularis from the infected animals.

In challenged animals 100% of the high dosed animals had confirmed recovery and identification via silver stains, FA, and PCR.

Materials and Methods:

Growth of Inoculum:

One 75 cm^2 conventional flask was seeded with 3.75 × 10^5 HEP-2 cells in DMEM/10% FBS and allowed to grow 18-24 hrs at 37°C. (The cells were at 30% confluency at time of inoculation.) One vial of N72994 was diluted in 15 ml DMEM/5% FBS. The culture was placed in a gas chamber, evacuated, and regassed with hydrogen and carbon dioxide to give a mixture of 8.0% O_2, 8.8% CO_2 and 83.2% H_2. The culture was refed at 24 hr. with DMEM/5% FBS.

The cultures were incubated for 6 days, then the cells were scraped from the flasks and added to a 100 ml spinner flask containing 50 ml DMEM/5% FBS. The flask volume was scaled-up by doubling the media volume at weekly intervals. The culture was grown for 3 weeks in the spinner flask.

Harvesting Cultures:

The culture was harvested by centrifuging at 3000 × g for 20 minutes. The pellet was resuspended in Sucrose-Phosphate-Glutamate solution (SPG) with 10% FBS and passed 4 times through a 25 gauge needle. Inoculum was diluted to the final volume in SPG/10% FBS and 1:10 dilutions were made.

The inoculum for the controls consisted of non-infected HEP-2 cells diluted to the same concentration of viable cells as the infected culture. The cells were harvested the same as the infected culture. The control pigs received a similar dose of cells as the high dose group.

Quantitation of IS intracellularis:
Quantitation of viable IS intracellularis was accomplished by determination of the Tissue Culture Infectious Dose 50 percent (TCID\textsubscript{50}). This was done by removing 2 ml of culture to be tested and lysing the cells by passing through a 22 gauge needle 4 times. The sample was serially diluted 1:10 in DMEM/5% FBS containing Vancomycin (100 \mu g/ml) and Amphotericin B (2.0 \mu g/ml). The dilutions were added to a 96 well microtiter plate with 0.1 ml/well. The microtiter plates were seeded with HEp-2 cells at 2500 cells/well and grown 18-24 hours prior to infection. Twelve wells/dilution were used. The plate was incubated for 6 days at gas concentrations of 8.0% O\textsubscript{2}, 8.8% CO\textsubscript{2} and 83.2% N\textsubscript{2}. The cells were fixed with cold 50% acetone and 50% methanol for 2 minutes. To the wells, 0.03 ml/well of anti-IS intracellularis monoclonal antibody (McOrist, 1987) diluted 1:2000 in PBS was added. The plate was incubated for 30 minutes at 37\degree C. and then washed 3 times with PBS. Anti-mouse FITC diluted 1:30 was added at 0.03 ml/well and incubated 30 minutes at 37\degree C. The plate was washed 3 times with ddH\textsubscript{2}O and allowed to dry. Samples were observed on a fluorescent microscope and the TCID\textsubscript{50}/ml was determined.

Animals:
Thirty-one mixed sex pigs six weeks of age from PIC x Lieske females and large white boars were provided by Dr. Kent Schwartz. The pigs were randomly distributed to 4 pens by weight on day 0.

Facility:
Four pens in a small nursery facility, each separated by at least 3 feet, were used to house the pigs. The pens had wire flooring and solid pen dividers. Heat was provided by a furnace with zonal supplemental heat by heatlamps. The temperature was maintained between 78 and 85\degree F. for the duration of the study.

Feed and Water:
A 19% protein, ground corn-soy diet, free of antibiotics, was provided ad libitum via stainless steel feeders. Water was provided ad libitum via nipple waterers.

Infection of Pigs:
On day 0, the pigs were weighed and blood samples collected via capillary tube placed in the retroorbital sinus. Serum was harvested and stored frozen at -20\degree C. Fecal swabs were also collected for PCR. The pigs were dosed with 10 ml inoculum given intragastrically via stomach tube.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control - uninfected cells</td>
<td>5</td>
</tr>
<tr>
<td>Control - no treatment</td>
<td>2</td>
</tr>
<tr>
<td>High dose</td>
<td>8</td>
</tr>
<tr>
<td>Medium dose</td>
<td>8</td>
</tr>
<tr>
<td>Low dose</td>
<td>8</td>
</tr>
</tbody>
</table>

The pigs were weighed and bled on days 0, 10, 17 and 24.

Polymerase Chain Reaction:
Infection of the pigs was monitored by PCR using primers and cycle parameters as described by Jones (1993).
Fecal samples collected on days 0, 7, 14, 21, and 24 as well as mucosa of the intestines were checked by PCR.

Histopathology:

Sections of ileum, jejunum, and colon were formalin fixed, processed routinely, stained with hematoxylin and eosin as well as silver impregnation and evaluated. Sections were also stained using monoclonal antibody specific for IS intracellularis.

Results:

Clinical Signs:

Clinical signs consisting of loose stools were first observed in the high dose group at 3 days. The signs peaked at 14 days and began to resolve thereafter.

Weight Gain

Average daily weight gains were calculated showing that the high and medium dose groups had reduced weight gains compared to the control group. There was a dose titration effect in the weight gains when comparing the groups.

PCR:

Fecal shedding was not observed until 14 days. At 21 days, 37.5% of the high dose pigs were PCR positive in the feces. After necropsy, the mucosas of the ileums were checked by PCR with 100% positive in the high dose, 75% in the medium dose, 50% in the low dose and 0% in the controls.

Gross Lesions:

Gross lesions were found in 2 pigs of the high dose group (#50 and #202). The pigs had approximately 3 ft of thickening in the ileum with necrosis in #202.

Histopathology:

FA:

FA staining of sections of the ileum, jejunum, and colon revealed the presence of IS intracellularis in 87.5% of the high dose, 62.5% of both the medium and low doses and 0% in the controls.

Microscopic lesions

Lesions were observed in 100% of the high dose, 75% of the medium dose, 87.5% of the low dose and 14% in the controls. This was determined by the observation of increased mononuclear cells in the lamina propria and submucosa, often associated with hyperplasia of Peyer's Patchers. Crypt hyperplasia was also observed.

Silver Stain:

Silver staining of sections for the presence of intracellular, curved bacteria was also done. This demonstrated the presence of bacteria in 87.5% of the high dose, 62.5% in the medium dose, 87.5% in the low dose and 0% in the controls.

Discussion:

The pigs were successfully infected with pure cultures of IS intracellularis. At doses of 10^7 bacteria. 100%
of the pigs demonstrated infection by PCR and microscopic lesions. The severity of the lesions and the amounts of bacteria in the tissue sections were relatively low. This study is a satisfactory challenge model for IS intracellularis due to the presence of IS intracellularis and microscopic lesions in the pigs. Lesions may be improved with a second dose 7 days after the first dose.

EXAMPLE 5

Use of IS intracellularis antigen in diagnostic test for the detection of PPE

Preparation of intestinal Homogenates for inoculum

Intestinal homogenate is prepared as described in Example 2.

Infection of cell culture:

Two 25 cm.sup.2 conventional flasks are seeded with 1.25.times.10.sup.5 McCoy or HEp-2 cells in DMEM/10% FBS and allowed to grow 18-24 hrs. The cells should be at 30% confluency at time of inoculation. The inoculum is diluted in DMEM/5% FBS. If the inoculum is from an intestinal homogenate, then the media should also contain Vancomycin (100 .mu.g/ml) and Amphotericin B (2.0 .mu.g/ml). The cultures are placed in an atmosphere of reduced oxygen and 10% CO.sub.2. The cultures are incubated for 3.0 hr. at 37.degree. C. then Neomycin and Gentamycin are added. The culture is refed at 24 hr. with DMEM/5% FBS with L-glutamine, Vancomycin (100 .mu.g/ml), Neomycin (50 .mu.g/L), Gentamycin (50 .mu.g/L), and Amphotericin B (2.0 .mu.g/ml). No antibiotics are required if the inoculum is a pure culture. The cultures are incubated for 6 days or until confluency. The cells are scraped from the flasks and added to a 100 ml spinner flask containing 50 ml DMEM/2% FBS and (0.05 g Cultisphere-G Microcarriers for McCoy cultures). The flasks are stirred at about 40-50 rmps.

The McCoy culture is scaled up by diluting 1:2 every 2-3 days by passing the culture into larger spinner flasks and adding more media and Cultisphere-G beads. The final concentration of beads in the McCoy culture should be 0.001 g beads/ml. The HEp-2 culture is scaled up by diluting 1:2 weekly by passing the culture into larger spinner flasks and adding more media.

Harvesting and storage of cultures:

The cultures are harvested by collecting the desired amount of culture while centrifuging at 3000.times.g for 20 minutes. The pellet is resuspended in PBS and passed 4 times through a 22 gauge needle. The antigen is centrifuged at 145.times.g for 5 minutes to remove the beads, cellular nuclei, and debris. The supernatant is then centrifuged at 3000.times.g for 20 minutes and the pellet is resuspended in the desired diluent.

IS intracellularis ELISA protocol:

1. Add 0.1 ml/well antigen diluted in coating buffer. Incubate for 18 hours at 4.degree. C.
2. Wash 3 times with PBS.
3. Add 0.25 ml of blocking buffer to each well of plate. Incubate 1 to 2 hours at 37.degree. C.
4. Wash 3 times with wash buffer.
5. Dilute serum in blocking buffer and add 0.1 ml to the first wells of plate. Make serial 1:2 dilutions across the plate. Incubate for 1 hour at 37.degree. C.
6. Wash 3-5 times with wash buffer.
7. Dilute conjugate in blocking buffer and add 0.1 ml to wells of plate and incubate for 1 hr at 37° C.

8. Wash 3-5 times with wash buffer.


12. Measure absorbance of light with a spectrophotometer.

13. Wells in which antigen was not added are used as blanks.

14. Positive and negative control pig serum should also be used with each test.

Coating Buffer:

\[
\begin{align*}
\text{Na}_2\text{CO}_3 & \quad 1.6 \ g \quad \text{pH} = 9.6 \\
\text{NaHCO}_3 & \quad 2.9 \ g \\
n\text{ddH}_2\text{O} & \quad 1.0 \ L
\end{align*}
\]

Block Buffer:

\[
\begin{align*}
\text{Coating buffer} & \quad 1 \ L \\
\text{Tween-20} & \quad 0.5 \ ml \\
\text{Bovine serum albumin} & \quad 20 \ g \ (2\%)
\end{align*}
\]

Wash Buffer (20 times):

\[
\begin{align*}
\text{1OX PBS} & \quad 1 \ L \\
\text{Tween 20} & \quad 5 \ ml \\
\text{Thimerisal (optional)} & \quad 0.125 \ g
\end{align*}
\]

Dilute 1:20 before use.

PBS:

\[
\begin{align*}
\text{NaCl} & \quad 80 \ g \\
\text{KCl} & \quad 2.0 \ g \\
\text{Na}_2\text{HPO}_4 & \quad 6.1 \ g \\
\text{KH}_2\text{PO}_4 & \quad 2.0 \ g \\
n\text{ddH}_2\text{O} & \quad \text{Q.S.} \quad \text{to} \ 1 \ L
\end{align*}
\]

Conjugate:
Anti-swine IgG or IgM peroxidase labeled conjugate

Substrate:

TMB peroxidase substrate.

IS intracellularis Western Blot Protocol

1. Run antigen on 12% SDS-PAGE and transfer to nitrocellulose membrane.

2. Place membrane in blocking buffer for 2 hr.

3. Remove blocking buffer and rinse with PBS for 1 minute.

4. Dilute serum in blocking buffer and add to membrane. Incubate for 2 hours at room temperature.

5. Wash 3 times with wash buffer (5 minutes for each wash).

6. Dilute conjugate in blocking buffer and add to membrane. Incubate for 1 hr. at room temperature.

7. Wash 3 times with wash buffer.

8. Add substrate for 10 minutes or until strong banding occurs.

9. Rinse with PBS.

10. Air dry and store in the dark.

REAGENTS

Blocking buffer (10 times.)

1 L ddH2O

80 g NaCl

2 g KCl

11.5 g Na2HPO4

2 g KH2PO4

pH to 7.5

Add 3% Blocker immediately before use. (SIGMA Cat #170-6404)

Wash buffer (10 times.)

1 L ddH2O

80 g NaCl

2 g KCl
11.5 g Na$_{2}$HPO$_4$

2 g KH$_2$PO$_4$

5 ml Tween 20

pH to 7.5

Substrate

Kirkegaard and Perry

TMB Peroxidase Substrate (Cat. #50-77-03)

The above description and examples are only illustrative of preferred embodiments which achieve the objects, features, and advantages of the present invention, and it is not intended that the present invention be limited thereto. Any modifications of the present invention which come within the spirit and scope of the following claims is considered part of the present invention.
APPENDIX B. PATENT FOR L. INTRACELLULARIS VACCINES

Lawsonia intracellularis cultivation, anti-Lawsonia intracellularis vaccines and diagnostic agents

Abstract

A method for large scale cultivation and attenuation of L. intracellularis bacteria by inoculating cells with L. intracellularis bacteria to infect the cells, incubating the infected cells in a reduced oxygen concentration and maintaining the infected cells in suspension. Anti-L. intracellularis vaccines are prepared from cultures grown in suspension. Diagnostic agents are also disclosed.

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Assignee: NOBL Laboratories, Inc. (Sioux Center, IA)
Appl. No.: 658194
Filed: June 4, 1996

U.S. Class:
435/243; 435/245; 435/252.1; 435/366; 435/383; 435/395; 435/403; 424/93.4; 424/234.1; 424/825

Intern'l Class:
C12N 001/20; C12N 001/00; A61K 039/02

Field of Search:
424/234.1,93.4,825 435/245,252.1,366,383,395,403,423

References Cited

Other References

S. McOrist et al., "Antimicrobial Susceptibility of Ileal Symbiont Intracellularis Isolated From Pigs With
An avirulent strain of Lawsonia intracellularis, wherein said avirulent strain is Lawsonia intracellularis deposit strain ATCC No. 55783 or a Lawsonia intracellularis strain having all of the identifying characteristics of deposit strain ATCC No. 55783.

2. A vaccine for the immunization of an animal, comprising a pharmaceutically effective amount of an avirulent strain of Lawsonia intracellularis, wherein said avirulent strain is Lawsonia intracellularis deposit strain ATCC No. 55783 or a Lawsonia intracellularis strain having all of the identifying characteristics of deposit strain ATCC No. 55783, and a pharmaceutically acceptable carrier.

3. A method for stimulating the immune system of an animal to respond to an immunogenic antigen of pathogenic Lawsonia, comprising administering to said animal an immunogenic composition containing an avirulent strain of Lawsonia intracellularis, wherein said avirulent strain is Lawsonia intracellularis deposit strain ATCC No. 55783 or a Lawsonia intracellularis strain having all of the identifying characteristics of
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deposit strain ATCC No. 55783.

4. A method for making an attenuated Lawsonia intracellularis strain, comprising the steps of incubating
Lawsonia in culture cells which are in suspension at an oxygen concentration of less than about 18 percent.

5. The method of claim 4 comprising the further step of passaging a portion of said cells to fresh cells to
increase the production of L. intracellularis bacteria.

6. The method of claim 4 wherein said L. intracellularis bacteria is obtained from an animal infected with L.
intracellularis.

7. The method of claim 4 wherein said incubation occurs at an oxygen concentration in the range of from about
0 percent to about 8 percent.

8. The method of claim 4 wherein said incubation occurs at an oxygen concentration in the range of from about
0 percent to about 3 percent.

9. The method of claim 4 wherein said culture cells are selected from the group consisting of HEP-2, McCoy,
and IEC-18 cells.

10. The method of claim 9 wherein said McCoy and IEC-18 cells are incubated on microcarriers.

11. A method for making a vaccine for inducing an immune response to L. intracellularis bacteria in an animal,
comprising the steps of

incubating Lawsonia bacteria in culture cells which are in suspension at an oxygen concentration of less than
about 18 percent to cultivate said bacteria, and

mixing said cultivated bacteria with a pharmaceutically acceptable carrier.

12. The method of claim 11 wherein said Lawsonia bacteria is prepared from an intestinal homogenate of an
animal infected with L. intracellularis.

13. The method of claim 12 wherein said Lawsonia bacteria is selected from the group consisting of Lawsonia
intracellularis deposit strain ATCC No. 55783, a Lawsonia intracellularis strain having all of the identifying
characteristics of deposit strain ATCC No. 55783, Lawsonia intracellularis deposit strain ATCC No. 55672, and
a Lawsonia intracellularis strain having all of the identifying characteristics of deposit strain ATCC No. 55672.

14. The method of claim 11 further comprising the step of killing said cultivated bacteria to prepare a vaccine
containing killed L. intracellularis bacteria.

15. The method of claim 11 further comprising the step of cultivating said bacteria for a sufficient time to
produce an attenuated strain.

16. A biologically pure culture of a mammalian host cell infected by an intracellular bacteria, wherein said
bacteria is Lawsonia intracellularis deposit strain ATCC No. 55672 or a Lawsonia intracellularis strain having
all of the identifying characteristics of deposit strain ATCC No. 55672.

17. A vaccine for inducing an immune response to L. intracellularis bacteria in an animal, comprising

killed L. intracellularis bacteria selected from the group consisting of L. intracellularis deposit strain ATCC No.
55672, an L. intracellularis strain having all of the identifying characteristics of L. intracellularis deposit strain
ATCC No. 55672, L. intracellularis deposit strain ATCC No. 55783, and an L. intracellularis strain having all
of the identifying characteristics of L. intracellularis deposit strain ATCC No. 55783, and
a pharmaceutically acceptable carrier.

FIELD OF THE INVENTION

The present invention is directed to anti-Lawsonia intracellularis vaccines and methods for protecting against and diagnosing Lawsonia intracellularis infection. The products and processes of the invention are attainable, in part, as the result of the improved method which we have discovered for cultivating large scale supplies of L. intracellularis.

DESCRIPTION OF THE RELATED ART

L. intracellularis, the causative agent of porcine proliferative enteropathy ("PPE"), affects virtually all animals, including humans, rabbits, ferrets, hamsters, fox, horses, and other animals as diverse as ostriches and emus.

L. intracellularis is a particularly great cause of losses in swine herds. Estimates of the prevalence and incidence of PPE in the U.S. have been as high as 20 percent of the swine herd with estimated losses of $20 million annually.

A consistent feature of PPE is the occurrence of intracytoplasmic, non-membrane bound curved bacilli within enterocytes in affected portions of intestine. The bacteria associated with PPE have been referred to as "Campylobacter-like organisms." S. McOrist et al., Vet. Pathol., Vol. 26, 260-64 (1989). Subsequently, the causative bacteria have been identified as a novel taxonomic genus and species, vernacularly referred to as Ileal symbiont (IS) intracellularis. C. Gebhart et al., Int'l. J. of Systemic Bacteriology, Vol. 43, No. 3, 533-38 (1993). More recently, these novel bacteria have been given the taxonomic name Lawsonia (L.) intracellularis. S. McOrist et al., Int'l. J. of Systemic Bacteriology, Vol. 45, No. 4, 820-25 (1995). These three names have been used interchangeably to refer to the same organism as further identified and described herein. We have endeavored to use the taxonomic name, L. intracellularis, throughout the discussion of the present invention.

L. intracellularis is an obligate, intracellular bacterium which cannot be cultured by normal bacteriological methods on conventional cell-free media and has been thought to require attached epithelial cells for growth. S. McOrist et al., Infection and Immunity, Vol. 61, No. 10, 4286-92 (1993) and G. Lawson et al., J. of Clinical Microbiology, Vol. 31, No. 5, 1136-42 (1993) discuss cultivation of L. intracellularis using IEC-18 rat intestinal epithelial cell monolayers in conventional tissue culture flasks. In addition, H. Stills, Infection and Immunity, Vol. 59, No. 9, 3227-36 (1991) discusses using Intestine 407 human embryonic intestinal cell monolayers and GPC-16 guinea pig colonic adenocarcinoma cell monolayers in conventional tissue culture flasks. These prior cultivation methods are labor intensive and are not suitable for scale-up.

The current understanding of L. intracellularis infection and the treatment and effective control of the disease have been seriously hampered by the fastidious growth requirements of L. intracellularis in vitro cultures. There is currently a need for an improved method for cultivation of L. intracellularis. There is also a need for anti-L. intracellularis vaccines and effective tools for diagnosing L. intracellularis infection.

SUMMARY OF THE INVENTION

One object of the invention is to provide anti-L. intracellularis vaccines.

Another object of the invention is to provide methods for detecting the presence of antibodies to L. intracellularis in biological samples.

A further object is to provide an improved cultivation method allowing large scale cultivation of L. intracellularis for production of vaccines and diagnostic agents.

To achieve these and other objects, and in accordance with the purpose of the invention as embodied and
broadly described herein, the present invention provides a method for cultivating *L. intracellularis* and large scale supplies of bacteria produced thereby. According to the method, *L. intracellularis* bacteria are incubated in an oxygen concentration of from about 0 percent to about 18 percent, while agitating the bacteria to cultivate the *L. intracellularis* while maintaining the bacteria in suspension.

According to another embodiment, a method is provided for cultivating *L. intracellularis* bacteria by inoculating an HEp-2, McCoy's, or IEC-18 cell monolayer, which is at about 30 percent confluency, with an inoculum comprising *L. intracellularis* bacteria so as to infect the cells with the bacteria. The infected cells are then incubated at a temperature of about 36 to about 38°C, at an oxygen concentration of about 0 percent to about 8.0 percent until the cells reach confluence. The infected cells and growth media are then placed in a fermentor, bioreactor,spinner flask or other container suitable for maintaining the cells in suspension. The infected cells are incubated while agitating the cells so as to cultivate the *L. intracellularis* bacteria while maintaining the infected cells in suspension. A portion of the cultivated *L. intracellularis* is then passaged to fresh culture cells to increase the production of *L. intracellularis* bacteria.

The invention provides anti-*L. intracellularis* vaccines and methods for producing vaccines against *L. intracellularis*. An avirulent *L. intracellularis* bacteria is produced by passaging the cultivated *L. intracellularis* bacteria a sufficient number of times and selecting for an attenuated strain, or by subjecting the cultivated bacteria to chemical means of attenuation. Killed *L. intracellularis* vaccines are also prepared using the cultivation methods of the invention. According to a particularly preferred embodiment, the bacteria are continuously cultured for at least about 6 to 8 months while being passaged at least about 7 to 12 times to produce an attenuated strain for use as a vaccine. The attenuated bacteria is then admixed with a pharmaceutically acceptable carrier and administered to an animal in an effective amount to produce an immune response. We have deposited the currently preferred attenuated strain (N343NP40wk) in the American Type Culture Collection.

The invention also provides a method for determining the presence of antibodies that specifically react with *L. intracellularis* bacteria in a biological sample by harvesting at least a portion of the cultivated *L. intracellularis* bacteria, contacting a biological sample from an animal with harvested *L. intracellularis* bacteria or a component thereof under conditions whereby antibody present in the biological sample reacts with the *L. intracellularis* or component, and determining if an antibody-antigen reaction has occurred.

Additional features and advantages of the invention will be set forth in the description which follows and will be apparent from the description or may be learned by practice of the invention.

DESCRIPTION OF THE PREFERRED EMBODIMENTS
As used herein, the term "*L. intracellularis*" means the intracellular, curved, gram-negative bacteria described in detail by C. Gebhart et al., *Int'l J. of Systemic Bacteriology*, Vol. 43, No. 3, 533-38 (1993) and S. McOrist et al. *Int'l J. of Systemic Bacteriology*, Vol. 45, No. 4, 820-25 (1995) (each of which is incorporated herein by reference in its entirety) and includes, but is not limited to, the bacteria deposited as ATCC 55672 in the American Type Culture Collection, Rockville, MD; the bacteria deposited as NCTC 12656 and 12657 in the National Collection of Type Cultures, Colindale, London; the causative bacteria which can be obtained from PPE infected swine or other animals throughout the world given the knowledge in the art and the teachings herein; and variants or mutants of any of the above bacteria, whether spontaneously or artificially obtained.

As used herein, the term "attenuated strain" means any *L. intracellularis* strain that is prepared according to the cultivation and passaging techniques taught herein to achieve avirulence while maintaining immunogenic properties when administered to a host animal. As demonstrated below, various different *L. intracellularis* strains have been cultivated and attenuated according to the present teachings to obtain attenuated immunogenic strains having efficacy as vaccines in swine and other animals susceptible to *L. intracellularis* infection.

The attenuated strains of the invention are expected to have utility as immunogens in antimicrobial vaccines for animals, including birds, fish, cattle, swine, horses, mammals and primates in general, and humans. Such vaccines can be prepared by techniques known to those skilled in the art, given the teachings contained herein.
Such a vaccine would comprise an immunologically effective amount of the attenuated strain in a pharmaceutically acceptable carrier. The vaccine could be administered in one or more doses. An immunologically effective amount is determinable by means known in the art without undue experimentation, given the teachings contained herein. The amount of avirulent bacteria should be sufficient to stimulate an immune response in disease-susceptible animals while still being avirulent. This will depend upon the particular animal, bacteria, and disease involved. The recommended dose to be administered to the susceptible animal is preferably about $10^{3}$ to $10^{9}$ bacteria/Kg of body weight and most preferably about $10^{5}$ to $10^{7}$ bacteria/Kg of body weight. The carriers are known to those skilled in the art and include stabilizers and diluents. Such a vaccine may also contain an appropriate adjuvant. The vaccines of the invention may be used in combination with other vaccines, for example, as a diluent of another lyophilized vaccine, or combined before lyophilization with another vaccine. The vaccine preparations may also be desiccated, for example, by freeze drying for storage purposes or for subsequent formulation into liquid vaccines.

Accordingly, the invention also comprises a method for inducing an immune response to virulent, wild-type L. intracellularis bacteria in an animal host for the purpose of protecting the host from such bacteria. The method comprises administering an immunologically effective amount of the attenuated bacteria or killed bacteria of the invention to the host and, preferably, administering the vaccine of the invention to the host.

As used herein, the term "large-scale cultivation" means a level of cultivation of L. intracellularis greater than approximately 2.0 to 3.0 liters and includes production on a scale of 100 liters or more. "Cultivation" as used herein, means the process of promoting the growth, reproduction and/or proliferation of L. intracellularis.

In practicing the cultivation method of the invention, culture cells may first be inoculated with an inoculum comprising L. intracellularis bacteria so as to infect the cells with the bacteria. Numerous cell lines can be used in practicing the invention, including, but not limited to, IEC-18 (ATCC 1589)—rat intestinal epithelial cells, HEp-2 (ATCC 23)—human epidermoid carcinoma cells, McCoy's (ATCC 1696) —mouse (nonspecified) cells, MDCK (ATCC 34)—Madin-Darby canine kidney cells, BGMK (Biowhittaker #71-176)—buffalo green monkey kidney cells, and swine intestinal epithelium cells. The preferred culture cells are HEp-2, McCoy's or IEC-18 cells. Alternatively, the bacteria may be cultivated in a cell free system so long as the bacteria are maintained at the appropriate dissolved O$_{2}$ concentration as taught herein.

If culture cells are used, prior to being inoculated, the cells are preferably but need not be in the form of a monolayer. To form a monolayer, the cells may be seeded into conventional flasks. Each flask is generally seeded with between about 1 times $10^{5}$ cells to about 10 times $10^{5}$ cells per 25 cm$^{2}$ flask mixed with growth media. The growth media may be any media for cell cultivation which includes a nitrogen source, necessary growing factors for the chosen culture cells, and a carbon source, such as glucose or lactose. The preferred media is DMEM with 2-5% fetal bovine serum, although various other commercially available media may be used with good results.

We have found that successful cultivation of L. intracellularis is enhanced by maintaining the culture cells in a constant state of growth. Therefore, the culture cell monolayer should be at about 20 percent to about 50 percent confluence at the time of inoculation. Preferably, the cells should be at about 30 percent to about 40 percent confluence at the time of inoculation, with about 30 percent confluence being most preferred.

The inoculum may be a pure culture of L. intracellularis obtained, for example, from ATCC deposit 55672, NCTC deposits 12656 or 12657, or from infected swine or other animals using the isolation and purification teachings discussed herein.

According to one embodiment, the inoculum for practicing the invention is an intestinal homogenate prepared by scraping the mucosa off of the ileum of a swine or other animal infected with PPE. When preparing an intestinal homogenate, ileal sections selected for culture should show severe lesions with gross thickening of the gut. Due to the fragile nature of the bacteria, samples should preferably be stored at -70 degree C. as quickly as possible after necropsy. An antibiotic to which L. intracellularis is resistant such as Vancomycin, Amphotericin B or members of the aminoglycoside group of antibiotics, including Gentamicin and Neomycin, to name a few,
is preferably added to the inoculum to suppress contaminating bacteria while permitting L. intracellularis growth. Whether the inoculum is a pure culture or an intestinal homogenate, inoculation of the culture cells can be performed by various techniques known in the art given the teachings herein.

The bacteria and/or inoculated culture cells are then incubated under a reduced dissolved O\(_2\) concentration. At dissolved oxygen concentrations greater than 18% L. intracellularis growth is less than optimal with cessation of growth eventually occurring at oxygen concentrations outside this range. Preferably, the inoculated culture cells are incubated in a dissolved oxygen concentration in the range of from about 0% to about 10%. More preferably, the cells are incubated in an oxygen concentration in the range of from about 0% to about 8%, with an oxygen concentration of about 0% to about 3.0% being most preferred.

The proper concentration of carbon dioxide is also important to the proper growth of L. intracellularis. At carbon dioxide concentrations greater than 10% and less than 4%, non-optimum growth occurs with cessation of growth eventually occurring at carbon dioxide concentrations outside this range. Preferably, the carbon dioxide concentration is in the range from about 6% to about 9%, with a carbon dioxide concentration of about 8.8% being most preferred.

In addition, the cells are preferably incubated at a hydrogen concentration in the range from about 73% to about 94%. Nitrogen may be used in place of some or all of the hydrogen present. According to a particularly preferred embodiment, the cells are incubated in about 0-8.0% O\(_2\), about 8.8% CO\(_2\), and about 83.2% H\(_2\).

Inoculated cells may be incubated in a dual gas incubator or other gas chamber which contains the proper oxygen and carbon dioxide concentrations and which allows the cells to be suspended during incubation. The chamber should comprise a means for maintaining the inoculated cells in suspension, and a gas monitor and supply source to supply and maintain the proper gas concentrations. The incubation temperature should be in the range of from 30\(\)°C. to 45\(\)°C. and is more preferably in the range of from about 36\(\)°C. to about 38\(\)°C. Most preferably, the temperature is about 37\(\)°C. The necessary equipment for the cultivation and attenuation methods of the invention is readily available to those of ordinary skill in the art given the teachings herein. One example of equipment suitable for carrying out the present invention is a dual gas incubator, e.g., model 480 available from Lab-Line, Melrose Park, Ill., in conjunction with spinner flasks to maintain the cells in suspension. The presently preferred equipment comprises a fermentor, bioreactor or rotary shaker containing at least about 2 litres media and capable of maintaining the culture cells in suspension via sparging gas of the appropriate concentration, or other means of mechanical agitation, and continuously monitoring dissolved O\(_2\) levels in the media. New Brunswick, Braun and other companies make suitable fermentors and bioreactors for this purpose.

By maintaining the inoculated cells in a suspended state during incubation, maximum growth of the cells, and hence L. intracellularis, is achieved by increasing each individual cell's exposure to growth media and the proper mixture of oxygen and carbon dioxide. The culture cells can be agitated and maintained in suspension by a variety of methods known in the art, including, for example, culture flasks, roller bottles, membrane cultures and spinner flasks. The cells may be kept in suspension during incubation by incubating the cells in a spinner flask inside a dual gas incubator or similar apparatus. The term "spinner flask", as used herein, means a flask or other container which employs a paddle, propeller or other means to agitate the culture and keep the cells contained therein in suspension.

In a particularly preferred embodiment of the invention, the inoculated cells are incubated until the cells reach confluency and then the cells are placed in a spinner flask containing growth media and incubated in a dual gas incubator while spinning the flask. Preferably, the inoculated cells are scraped into the spinner flask. This can be achieved by a variety of methods known in the art such as using a cell scraper to detach the cells. Once the cells are introduced into the spinner flask, the paddle of the spinner flask is typically rotated in the range of from about 30 to about 60 rpm in order to maintain the infected cells in suspension.

A portion of the cultivated L. intracellularis is then passaged to fresh culture cells to increase the production of
L. intracellularis bacteria. The term "passaging" or variations thereof herein means the process of transferring a portion of the cultivated L. intracellularis to fresh culture cells in order to infect the fresh cells with the bacterium. The term "fresh", as used herein, means cells which have not yet been infected by L. intracellularis. Preferably such cells are, on the average, no more than approximately one day old.

The passage of L. intracellularis in suspension cultures may be accomplished by removing a portion of the original culture and adding it to a new flask containing fresh culture cells. If the original culture has a high number of bacteria/ml, for example, greater than about 10.sup.4 bacterial/ml, it is preferable to add between about 1 to 10% (volume to volume) of culture from the infected flask to a new flask containing fresh cells. This is preferably done when 50-100% of the cells are infected. If fewer than 50% of the cells are infected, passaging is preferably accomplished by splitting the culture 1:2 into a new flask and scaling-up the volume by adding fresh media. In either case, cell lysis and other steps are not required, in direct contrast to the passage of monolayer cultures, as in the prior art.

After sufficient growth of the culture cells and subsequent infection by L. intracellularis at greater than about 70% cell infectivity, as determined by IFA, TCID.sub.50 or other comparable method, at least a portion of the cultivated L. intracellularis bacteria is then harvested. The harvesting step may be performed by separating the bacteria from the suspension by various techniques known to those of ordinary skill in the art, given the teachings herein. Preferably, the L. intracellularis bacteria is harvested by centrifuging the contents of all or a portion of the suspension to pellet the culture cells, resuspending the resulting cell pellets, and lysing the infected cells. Typically, at least a portion of the contents is centrifuged at about 3000.times.g for about 20 minutes in order to pellet the cells and bacteria. The pellet may then be resuspended in, for example, a sucrose-phosphate-glutamate (SPG) solution and passed approximately four times through a 25 gauge needle in order to lyse the cells. If further purification is desired, the samples can be centrifuged at about 145.times.g for about five minutes to remove cellular nuclei and debris. The supernatant may then be centrifuged at about 3000.times.g for about twenty minutes and the resulting pellet resuspended in an appropriate diluent, such as SPG with fetal bovine serum (to prepare harvested bacteria suitable for freezing or use as an inoculant) or growth media (to prepare harvested bacteria more suitable for passaging to fresh cells).

As previously mentioned, effective growth of L. intracellularis for large-scale production is enhanced by keeping the tissue cells actively growing. With monolayers, when cultures become confluent the rate of cell division decreases substantially. Attempts to grow L. intracellularis on monolayer tissue cultures have had limited success and scale-up has not been possible. However, using suspension cultures greatly facilitates keeping the cells actively growing and permits continuous culture expansion and scale-up. Using a fermentor and between about 0-3% dissolved O.sub.2 as explained above, we have been able to grow up to 10.sup.8 bacteria/ml. We have also been able to keep the cultured bacteria actively growing for many months and expect to be able to do so indefinitely.

Prior to the instant invention, it was generally believed that cells must be attached to a surface in order to be infected by L. intracellularis. The cell suspensions of the instant invention are unique and contradict this theory. When using McCoy's or IEC-18 cells, it is preferable to add gelatin, agarose, collagen, acrylamide or silica beads, such as Cultisphere-G porous microcarriers manufactured by HyClone Laboratories, Logan, Utah, along with the growth media. However, HEP-2 cells and others do not require microcarriers according to the cultivation method of the invention. This provides an especially advantageous and economical route for large-scale cultivation.

For culture maintenance purposes, with HEP-2 cultures, preferably 25-50% of the culture is removed and replaced with fresh media at weekly intervals. For cell cultures with microcarriers or beads, preferably 25-50% of the culture is removed and replaced with fresh microcarriers or beads and fresh media 1-2 times weekly. For scale-up purposes, an additional 25-50% of media, or media with microcarriers, may be added to the culture.

Depending upon the rate at which the culture cells become infected, passage to fresh cells generally occurs between about every 2 to about 5 weeks. Assuming that the culture cells become at least 70% infected within 2-3 weeks, preferably passage occurs between about every 3 to 4 weeks.
The present invention also provides vaccines and methods for producing vaccines against *L. intracellularis*. According to a particularly preferred embodiment, after maintaining the infected cells in suspension for an extended time (for example, 6-8 months), at least a portion of the cultivated *L. intracellularis* bacteria are harvested and monitored for potential attenuation. Such monitoring is preferably accomplished by host animal or animal model challenges to select for an attenuated strain. Such attenuated strains are used in vaccines according to the methods taught herein. The attenuated *L. intracellularis* vaccines according to the present invention have shown efficacy against *L. intracellularis* infection in a variety of animals and are expected to be effective in humans as well.

The present invention allows rapid culture expansion, an increase in yields of 100-1000 fold, and reduced cost. As a result, the abundant supply of *L. intracellularis* bacteria produced according to the cultivation method of invention is readily attenuated for vaccine production purposes. Attenuation is difficult in monolayer cultures due to the low yield of bacteria produced using conventional monolayer growing techniques. In contrast, the method of growing *L. intracellularis* of the present invention greatly increases the ease, speed, and number of bacteria available for this purpose. The more cells and cell divisions which occur, the greater the level of mutations occurring which are advantageous in vaccine development. Growth in suspensions according to the invention increases the expression of important immunogens controlled by environmentally regulated genes and their expression products.

The resulting attenuated strains can be cultivated in tissue culture monolayers as described in Example 1 below, but are preferably cultivated in suspension cultures according to the method of the invention. Other means of attenuation can include chemical attenuation by the use of, for example, N-methyl nitrosoguanidine and others known in the art. Whether by multiple passage or chemical means, an attenuated *L. intracellularis* is produced and selected for vaccine preparation.

According to one vaccine embodiment of the invention, the antigen is harvested by centrifugation or microfiltration as described above. The antigen is then standardized at a defined level based on the optimum host animal immune response, determined by a dose titration in the host animal species. The bacteria may be inactivated by prolonged exposure, e.g., one week, to ambient O2 levels, or by using 0.3% formalin or other inactivating agent to prepare a killed vaccine. The antigen is then incorporated into a suitable adjuvant, such as aluminum hydroxide or mineral oil to enhance the immune response. The antigen is then used to vaccinate the host via intramuscular or subcutaneous injection, in the case of pigs at about 3-4 weeks of age, with a booster dose if necessary.

Alternatively, according to a particularly preferred vaccine embodiment using the cultivation methods previously described, the bacteria are serially passaged to induce and select for an attenuated, avirulent live culture. The culture is tested in the host animal (after preferably at least 6 to 8 months or more of growth in the suspension culture) for signs of attenuation. The culture is harvested as described earlier and diluted. Pigs, for example, are orally vaccinated with 1×10^5 to 1×10^6 bacteria. About twenty-eight days after vaccination, the pigs are orally inoculated with about 1×10^7 organisms from a less passaged (about 30 to 45 days old) virulent culture of *L. intracellularis*. The infected animals are necropsied 21 days after challenge and the small intestines observed for gross lesions as well as microscopic lesions. PCR and fluorescent antibody should also be performed. About eighty percent of the control animals will show gross or microscopic lesions and test positive for the presence of *L. intracellularis* in the mucosal cells of the intestines using either PCR or FA testing methods. Vaccinated animals will have normal mucosal surfaces as determined by histological observations and will be negative by PCR testing.

Generally, an attenuated immunogenic *L. intracellularis* strain is produced after continuous culture for between at least about 150 and 250 days, during which time the culture is passaged at least about 7 to about 12 times. Other attenuated cultures may be produced by varying these figures so long as the monitoring and selection methods taught herein are employed.

A vaccine is then prepared comprising an immunologically effective amount of the attenuated *L. intracellularis*
in a pharmaceutically acceptable carrier. The combined immunogen and carrier may be an aqueous solution, emulsion or suspension. An immunologically effective amount is determinable by means known in the art without undue experimentation given the teachings contained herein. In general, the quantity of immunogen will be between 50 and 500 micrograms, and preferably between $10^{7}$ and $10^{9}$ TCID$_{50}$, when purified bacteria are used.

The vaccines according to the invention are generally administered to susceptible animals, preferably swine, in one or more doses. The live or killed vaccine may be administered 1 or 2 times at 2 week intervals. For the attenuated, live vaccines, one dose is preferred. The preferred routes of administration of live attenuated strains are oral or intranasal, but intramuscular or subcutaneous injection may also be used. The intramuscular and subcutaneous injection routes are most preferred for the killed vaccine.

Effective diagnosis of PPE has also been hindered by the time required to culture the causative bacteria. As a result of the present invention, development of diagnostic tools promoting rapid and accurate assays for the presence of L. intracellularis in biological samples taken from swine and other animals susceptible to PPE is now possible.

The L. intracellularis bacteria grown according to the method of the instant invention, or components derived from such bacteria, can be used as an antigen in an ELISA or other immunoassay, such as an immunofluorescent antibody test ("IFA"), to detect antibodies to L. intracellularis in the serum and other body fluids of animals suspected of being infected with the bacteria. The presently preferred immunoassay is an IFA as described in the example below. Alternatively, the bacteria grown according to the invention can be used in a Western Blot assay.

The preferred ELISA protocol according to this embodiment of the invention is as follows:

1. Add 0.1 ml/well antigen diluted in coating buffer. Incubate for 18 hours at 4 degree. C.
2. Wash 3 times with PBS.
3. Add 0.25 ml of blocking buffer to each well of plate. Incubate 1 to 2 hours at 37 degree. C.
4. Wash 3 times with wash buffer.
5. Dilute serum in blocking buffer and add 0.1 ml to the first wells of plate. Make serial 1:2 dilutions across the plate. Incubate for 1 hour at 37 degree. C.
6. Wash 3-5 times with wash buffer.
7. Dilute conjugate in blocking buffer and add 0.1 ml to wells of plate and incubate for 1 hr at 37 degree. C.
8. Wash 3-5 times with wash buffer.
10. Measure absorbance of light with a spectrophotometer.
11. Wells in which antigen was not added are used as blanks.
12. Positive and negative control pig serum should also be used with each test.

The preferred Western blot protocol is as follows:

1. Run antigen on 12% SDS-PAGE and transfer to nitrocellulose membrane.
2. Place membrane in blocking buffer for 2 hr.
3. Remove blocking buffer and rinse with PBS for 1 minute.
4. Dilute serum in blocking buffer and add to membrane. Incubate for 2 hours at room temperature.
5. Wash 3 times with wash buffer (5 minutes for each wash).
6. Dilute conjugate in blocking buffer and add to membrane. Incubate for 1 hr. at room temperature.
7. Wash 3 times with wash buffer.
8. Add substrate for 10 minutes or until strong banding occurs.
9. Rinse with PBS.
10. Air dry and store in the dark.

The present invention is further described in the following examples which are provided for illustrative purposes only and are not to be construed as limiting.

EXAMPLE 1

Isolation of L. intracellularis from the Intestines of American Pigs with Porcine Proliferative Enteropathy

Materials and Methods:

Selection of Inoculum Samples:

Sample N24912 was obtained from a herd on a farm in Iowa in which fifteen of 300 five month old finisher pigs were observed to have persistent bloody stools despite penicillin treatment. Upon necropsy of the pigs, the intestine (ileum) had a thickened mucosa. Histopathology examinations with silver stains demonstrated the presence of curved intracellular bacteria and crypt enterocyte hyperplasia confirming the diagnosis of PPE. Sample N72994 was obtained from a 1.5 year old second litter SPF sow on a farm in Minnesota. The herd size was between 70-80 sows and antibiotic treatment is unknown. Upon necropsy, the mucosa of the ileum was thickened with some hemorrhage. Giminez staining of the mucosa demonstrated many curved bacteria. Sample N101494 was obtained from a 12 week old pig from an Indiana farm with 600 farrow to finish sows. The pig was treated with Tylan injectable upon the onset of bloody diarrhea, but the animal died soon after treatment.

Preparation of Pig Derived Bacterial Inocula:

Intestinal samples were kept at -70 degree. C. The intestines were opened and washed with phosphate buffered saline (PBS). One gram samples of mucosa were scraped into sodium potassium glutamate (SPG) and homogenized for 30 seconds with 4.0 ml 1% Trypsin (JRH Biosciences, Lenexa, Kans.) in SPG. The suspensions were incubated for 35 minutes at 37 degree. C. Ten ml SPG/10% fetal calf serum (FCS) (JRH Biosciences, Lenexa, Kans.) was added and the samples were ground in a tissue grinder for 1 minute. Ten ml SPG/10% (FCS) was added and filtered once through filterpaper (Whatman 113V; Whatman Labsales, Hillsboro, Oreg.) and sequentially through 5.0, 1.0, and 0.65 micron membrane filters. Filtrates were aliquoted and frozen at -70 degree. C. in 1.0 ml aliquots. The mucosa was smeared onto a slide for Giminez stain.

Cell Culture:
IEC-18 cells (Rat intestinal epithelial cells, ATCC CRL 1589) were grown in DMEM (JRH Biosciences, Lenexa, Kans.) with L-glutamine and 10% FCS and routinely passaged by trypsin weekly. Cell monolayers were grown at 37\degree C. in air with 5% CO\sub{2}.

Infection of Cell Culture:

IEC-18 cells were seeded at 1.25\times10^5 cells in 25 cm\sup{2} flasks and at comparable rates in chamberslides (Nunc, Inc., Naperville, Ill.), incubated 24 hours, then media removed. Frozen pig-derived bacterial isolates were quickly thawed and diluted in DMEM/7% FCS with Vancomycin (100 \mu g/ml) and Amphotericin B (2.0 \mu g/ml) at ratios of 1.0 ml homogenate to 15 ml media and added to the monolayers. Monolayers and bacterial suspensions were centrifuged for 30 minutes at 2000 g and transferred to anaerobic jars. The jars were evacuated and the gas was replaced with hydrogen and carbon dioxide to give a mixture of 8.0% O\sub{2}, 10% CO\sub{2}, and 82% H\sub{2}. The cultures were incubated for 3 hours at 37\degree C. C. then refed with DMEM/7% FCS with L-glutamine, Vancomycin (100 \mu g/ml), Neomycin (50 \mu g/L), and Amphotericin B (2.0 \mu g/ml). Cultures were replaced in the anaerobic jars and incubated for 6 days with media changes every 2 days.

Passage of L. intracellularis:

L. intracellularis bacteria were passed by cell lysis using potassium chloride as described previously in G. Lawson et al., J. Clin. Microbiol., 31:1136-1142 (1993) (incorporated by reference herein in its entirety) then added to fresh IEC-18 monolayers. Media was poured off the monolayers and 0.1% KCl was added and the cells incubated for 10 minutes at 37\degree C. The KCl was removed and SPG/10% was added and the monolayers detached mechanically with a cell scraper. The cells were lysed by passing 3 times through a syringe with a 21 gauge needle. Cell nuclei were removed by centrifugation at 100\times10^3 g for 5 minutes and the bacterial suspension in the supernatant fluid added to fresh 1 d monolayers of IEC-18 cells.

Monitoring Infection of Cell Cultures:

Infection was monitored by fixing the cells on chamberslides with cold acetone/methanol for 5 minutes. Staining was carried out by immunofluorescence and immunoperoxidase methods. Both methods employed a mouse monoclonal antibody (as described in S. McOrist et al., Vet. Rec. 121:421-422 (1987)) as the primary antibody and either anti-mouse immunoglobulin G-fluorochrome conjugate (fluorescein isothiocyanate; Organon Teknika Corporation, Durham, N.C.) or peroxidase conjugate (goat anti-mouse immunoglobulin G; Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.). Quantitation of bacteria was accomplished by counting the number of specifically stained bacteria within cells on each slide.

Polymerase Chain Reaction:

Sample inocula and passaged bacteria were incorporated as template DNA into PCR using the sample preparation method, primers, and cycle parameters as described by Jones et al., J. Clin. Microbiol., 31:2611-2615 (1993) and McOrist et al., Vet. Microbiol. 1-8 (1994) (each of which are incorporated by reference herein in their entirety). Cycle parameters were 93\degree C. for 5 minutes, 55\degree C. for 45 seconds, and 72\degree C. for 45 seconds for the first cycle. Thirty-three cycles were performed at the previously mentioned temperatures for 45 seconds per temperature, as well as one cycle at 93\degree C. for 45 seconds, 55\degree C. for 45 seconds, and 72\degree C. for 2 minutes. Positive inocula only were used to inoculate IEC-18 cells. PCR was also performed for the monitoring of passage material to confirm infections. DNA produced by PCR was submitted to the Iowa State University Nucleic Acid Facility for sequencing. Results of the sequencing were compared to sequences produced by Gary F. Jones as reported in his Ph.D. thesis, University of Minnesota, Minneapolis, Minn. (June, 1993).

Results:

Selection of Inoculum Samples:
Pig number N24912 and N72994 had severe PPE with bloody intestinal contents and thickened mucosa. N101494 had severe PPE and severe hemorrhage resulting in a large blood clot in the intestinal lumen. Gimenez staining of the mucosal smears demonstrated large numbers of curved or S-shaped bacteria. IFA stains revealed large numbers of brightly fluorescing bacteria in pig-derived bacterial inocula.

Monitoring Infection of Cell Cultures:

Inoculated monolayers were monitored by light microscopy throughout the growth cycle and little morphological change of the cells was observed. Uninfected monolayers grown under reduced oxygen tension (8% O$_2$) had similar morphology.

Immunofluorescence and immunoperoxidase stained infected cultures demonstrated large numbers of curved or S-shaped specifically stained bacteria apparently within cells. The monolayers did not have confluent infection. Infected cells were often closely associated with infected foci of 1-10 cells. Heavily infected cells (i.e., cells with 30 or more bacteria) were also seen in association with cells with fewer than 30 bacteria. Bacterial numbers peaked at or about 6 days. Infection was dependent on specific growth conditions. The bacteria were successfully passaged by the cell lysis procedure described herein. Centrifugation of newly inoculated cells was not necessary but enhanced the numbers of infected cells. Centrifugation also decreased contamination by allowing cells exposed to infection with antibiotic-free media to be refed at 3 hours with antibiotic containing media. Reducing FCS from 10% to 7% in the media was necessary to slow the growth of the IEC-18 cells allowing the bacteria to proliferate to higher numbers before monolayers became confluent.

Polymerase Chain Reaction:

PCR of chromosomal DNA generated a 319 bp fragment (including primers) from all isolates. A fragment of appropriate size was visually compared to a known positive sample generated by McOrist et al. (1994) using PCR. Sequence analysis of the PCR products of N24912, N72994, and N101494 confirmed a close homology (97-99%) to the p78 sequence determined by Jones (1993).

EXAMPLE 2

Growth of L. intracellularis in Suspension Cultures of HEp-2 Cells

Preparation of Intestinal Homogenates for Inoculum:

Intestinal homogenate was prepared by scraping the mucosa off of 6.0 to 8.0 cm of ileum from the intestinal samples of Example 1. Trypsin (1%) was added to the scraped mucosa and the samples were homogenized briefly, then incubated for 35 minutes at 37$^\circ$C. Ten ml SPG/10% FBS was then added and the samples were ground in a tissue grinder. Another 10 ml SPG/10% FBS was added. The homogenates were passed through a Whatman V113 filter and then sequentially through 5.0, 1.0, and 0.65 .mu.m filters. The samples were dispensed into 1 ml aliquots and frozen at -70$^\circ$C.

Infection of Cell Culture:

Method A:

Tissue cells were seeded at 1.times.10$^7$ cells in 50 ml DMEM/10% FBS in a 100 ml spinner flask. The cultures were incubated 24 hr., then Vancomycin and fungizone were added. One vial of frozen intestinal homogenate was quickly thawed and diluted in 3.0 ml DMEM/5% FBS with Vancomycin (100 .mu.g/ml) and Amphotericin B (2.0 .mu.g/ml). The sample was passed through a 0.65 .mu.m filter and added to the flask. The culture was placed in a gas chamber, evacuated, and regassed with hydrogen and carbon dioxide to give a mixture of 8.0% O$_2$, 8.8% CO$_2$, and 83.2% H$_2$. The cultures were incubated for 3 hours at 37$^\circ$C. and then Neomycin and Gentamycin were added. The culture was refed at 24 hours with
DMEM/5% FBS with L-glutamine, Vancomycin (100 μg/ml), Neomycin (50 μg/L), Gentamycin (50 μg/L) and Amphotericin B (2.0 μg/ml).

Method B:

Two 25 cm.sup.2 conventional flasks were seeded with 1.25.times.10.sup.5 HEp-2 cells in DMEM/10% FBS and allowed to grow 18-24 hours. The cells were at 30% confluency at time of inoculation. The inoculum was diluted in DMEM/5% FBS. When the inoculum is from an intestinal homogenate, the media also contained Vancomycin (100 μg/ml) and Amphotericin B (2.0 μg/ml). The cultures were placed in a gas chamber, evacuated, and regassed with hydrogen and carbon dioxide to give a mixture of 8.0% O.sub.2, 8.8% CO.sub.2, and 83.2% H.sub.2. The cultures were incubated for 3 hours at 37.degree. C. then Neomycin and Gentamycin were added. The culture was refed at 24 hours with DMEM/5% FBS with L-glutamine, Vancomycin (100 μg/ml), Neomycin (50 μg/L), Gentamycin (50 μg/L), and Amphotericin B (2.0 μg/ml). No antibiotics were required when the inoculum was a pure culture. The cultures were incubated for 6 days or until confluency. The cells were scraped from the flasks and added to a 100 ml spinner flask containing 50 ml DMEM/5% FBS.

The culture was diluted 1:2 at weekly intervals by either harvesting one half of the culture and adding fresh media or by passing into a larger spinner flask and adding more media.

Passage of the Culture:

The culture was passed to fresh HEp-2 cells by seeding new HEp-2 cells at 1.times.10.sup.7 into DMEM/5% FBS. The new culture was allowed to incubate overnight at 8.0% O.sub.2, 8.8% CO.sub.2, and 83.2% H.sub.2. The new culture was then inoculated with infected culture and incubated at reduced O.sub.2 concentrations as previously stated. Inoculum amounts were dependent on the degree of infection of the original culture.

Harvesting and Storage of Cultures:

The cultures were harvested by collecting the desired amount of culture while centrifuging at 3000.times.g for 20 minutes. The pellet was resuspended in Sucrose-Phosphate-Glutamate (SPG) solution and passed 4 times through a 25 gauge needle. The cultures were aliquoted and frozen at -70.degree. C. For further purification, the sample was centrifuged at 145.times.g for 5 minutes to remove the cellular nuclei and debris. The supernatant was then centrifuged at 3000.times.g for 20 minutes. The pellet was then resuspended in diluent.

Estimation of Viable L. intracellularis in Tissue Culture:

Quantitation of viable L. intracellularis was accomplished by determination of the Tissue Culture Infectious Dose 50 percent (TCID.sub.50). This was done by removing 2.0 ml of culture to be tested and lysing the cells by passing through a 25 gauge needle 4 times. The sample was serially diluted 1:10 in DMEM/5% FBS containing Vancomycin (100 μg/ml) and Amphotericin B (2.0 μg/ml). The dilutions were added to a 96 well microtiter plate with 0.1 ml/well. The microtiter plates were seeded with HEp-2 cells at 1250 cells/well and grown 18-24 hours prior to infection. Between 3 wells/dilution and 6 wells/dilution were used. The plate was incubated for 6 days at gas concentrations of 8.0% O.sub.2, 8.8% CO.sub.2, and 83.2% H.sub.2. The cells were fixed with cold 50% acetone and 50% methanol for 2 minutes. To the wells, 0.03 ml/well of anti-IS intracellularis monoclonal antibody (McOrist, 1994) diluted 1:2000 in PBS was added. The plate was incubated for 30 minutes at 37.degree. C. and then washed 3 times with PBS. Anti-mouse FITC diluted 1:30 was added in the amount of 0.03 ml/well and incubated 30 minutes at 37.degree. C. The plate was then washed 3 times with ddH.sub.2 O and allowed to dry. Samples were observed on a fluorescent microscope and the TCID.sub.50 /ml was determined.

Results:

The TCID.sub.50 results indicated that the cultures contained up to 1.times.10.sup.6 bacteria/ml. This was
accomplished in 45 days. The culture volume was scaled-up to 3.0 litres in the same amount of time.

EXAMPLE 3

Growth of L. intracellularis in Suspension Cultures of McCoys Cells

Preparation of Intestinal Homogenates for Inoculum:

Intestinal homogenate was prepared as described in Example 2. A sample of L. intracellularis cultivated according to the method of the following example was deposited under the Budapest Treaty on May 19, 1995 in the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md. U.S.A. 20852 and assigned accession number 55672.

Infection of Cell Culture:

Two 25 cm.sup.2 conventional flasks were seeded with 1.25.times.10.sup.5 McCoys cells in DMEM/10% FBS and allowed to grow 18-24 hours. The cells were at 30% confluency at time of inoculation. The inoculum was diluted in DMEM/5% FBS. When the inoculum is from an intestinal homogenate, then the media also contained Vancomycin (100 .mu.g/ml) and Amphotericin B (2.0 .mu.g/ml). The cultures were placed in a gas chamber, evacuated, and regassed with hydrogen and carbon dioxide to give a mixture of 8.0% O.sub.2, 10% CO.sub.2, and 82% H.sub.2. The cultures were incubated for 3 hours at 37.degree. C., then Neomycin and Gentamycin were added. The culture was refed at 24 hours with DMEM/5% FBS with L-glutamine, Vancomycin (100 .mu.g/ml), Neomycin (50 .mu.g/L), Gentamycin (50 .mu.g/L), and Amphotericin B (2.0 .mu.g/ml). No antibiotics were required when the inoculum was a pure culture. The cultures were incubated for 6 days until confluency. The cells were scraped from the flasks and added to a 100 ml spinner flask containing 50 ml DMEM/2% FBS and 0.05 g Cultisphere-G Microcarriers. The flasks were stirred at 40-50 rpms.

The culture was diluted 1:2 every 2-3 days by either harvesting one half of the culture and adding fresh media and Cultisphere-G beads or by passing the culture into a larger spinner flask and adding more media and Cultisphere-G beads. The final concentration of beads in the culture was about 0.001 g beads/ml.

Passage of the Culture:

The culture was passed to fresh McCoys cells by seeding 1.times.10.sup.7 new McCoys cells into DMEM/2% FBS and 0.05 g Cultisphere-G beads. The new culture was allowed to incubate overnight at 8.0% O.sub.2, 8.8% CO.sub.2 and 83.2% H.sub.2. The new culture was then inoculated with 25 ml of infected culture and incubated at reduced O.sub.2 concentrations as previously stated.

Harvesting and Storage of Cultures:

The cultures were harvested by collecting the desired amount of culture and centrifuging at 3000.times.g for 20 minutes. The pellet was resuspended in SPG and passed 4 times through a 22 gauge needle. The cultures were aliquoted and frozen at -70.degree. C. For further purification, the sample was centrifuged at 145.times.g for 5 minutes to remove the beads, cellular nuclei and debris. The supernatant was then centrifuged at 3000.times.g for 20 minutes. The pellet was then resuspended in diluent.

Estimation of Viable L. intracellularis in Tissue Culture:

Quantitation of viable L. intracellularis was determined as described in Example 2 using a 22 gauge needle to lyse the cells and using McCoys cells at 1250 cells/well to seed the microtiter plates.

Results:
The TCID\textsubscript{50} results indicated that the cultures contained up to \(1 \times 10^{6}\) bacteria/ml. This was accomplished in less than 1 month. The culture volume was scaled-up to 3.0 liters in the same amount of time.

**EX\textsubscript{A}M\textsubscript{P}LE 4**

**Determining Infectious Dose of \textit{L. intracellularis} Pure Cultures in Host Animals**

**Summary:**

A thirty-one pig study was completed by infecting 6 week-old conventional pigs with pure cultures of \textit{L. intracellularis} from sample N72994. The pigs were randomly divided into 4 groups and the groups were penned separately. Group 1 contained 7 pigs and was considered the negative control group dosed with uninfected tissue culture or nothing. The group 2 contained 8 pigs dosed with \(10^7\) bacteria/pig. Group 3 had 8 pigs and was dosed with \(10^6\) bacteria/pig. And, Group 4 contained 8 pigs receiving \(10^5\) bacteria/pig.

Fecal swabs were collected on days 0, 7, 14, and 21, and 24 for PCR testing. On day 24, the pigs were necropsied and the ileum, jejunum, and the colon were collected for PCR testing, histopathology, and FA stains, all as described above.

PCR testing of the ileal mucosa revealed the presence of \textit{L. intracellularis} in 100% of the high dose, 75% of the medium dose, and 50% of the low dose. Histopathology results indicated an increase of mononuclear cells in the lamina propria and submucosa of 88% of the high dose, 75% of the medium dose, and 88% of the low dose. Crypt hyperplasia was observed in 50% of the high dose, 63% of the medium dose, and 50% of the low dose. FA staining revealed \textit{L. intracellularis} in tissue sections of the ileum, jejunum, and colon in 88% of the high dose, 63% of the medium dose, and 63% of the low dose. Control animal were negative for the presence of \textit{L. intracellularis} via PCR, FA, and silver stains.

In conclusion, a pure culture was successfully used to infect and cause lesions of PPE. Koch’s postulates were fulfilled by the identification and isolation of \textit{L. intracellularis} from the infected animals.

In challenged animals 100% of the high dosed animals had confirmed recovery and identification via silver stains, FA, and PCR.

**Materials and Methods:**

**Growth of Inoculum:**

One 75 cm\textsuperscript{2} conventional flask was seeded with 3.75 \(\times\) \(10^5\) HEp-2 cells in DMEM/10% FBS and allowed to grow 18-24 hrs at 37 degree C. at 5% CO\textsubscript{2}. (The cells were at 30% confluency at time of inoculation.) One vial of N72994 was diluted in 15 ml DMEM/5% FBS. The culture was placed in a gas chamber, evacuated, and regassed with hydrogen and carbon dioxide to give a mixture of 8.0% O\textsubscript{2}, 8.8% CO\textsubscript{2} and 83.2% H\textsubscript{2}. The culture was refed at 24 hr. with DMEM/5% FBS.

The cultures were incubated for 6 days, then the cells were scraped from the flasks and added to a 100 ml spinner flask containing 50 ml DMEM/5% FBS. The flask volume was scaled-up by doubling the media volume at weekly intervals. The culture was grown for 3 weeks in the spinner flask.

**Harvesting Cultures:**

The culture was harvested by centrifuging at 3000 \(\times\) g for 20 minutes. The pellet was resuspended in Sucrose-Phosphate-Glutamate solution (SPG) with 10% FBS and passed 4 times through a 25 gauge needle. Inoculum was diluted to the final volume in SPG/10% FBS and 1:10 dilutions were made.

The inoculum for the controls consisted of non-infected HEp-2 cells diluted to the same concentration of viable
cells as the infected culture. The cells were harvested the same as the infected culture. The control pigs received a similar dose of cells as the high dose group.

Quantitation of L. intracellularis:

Quantitation of viable L. intracellularis was accomplished by determination of the Tissue Culture Infectious Dose 50 percent (TCID.sub.50). This was done by removing 2 ml of culture to be tested and lysing the cells by passing through a 22 gauge needle 4 times. The sample was serially diluted 1:10 in DMEM/5% FBS containing Vancomycin (100 .mu.g/ml) and Amphotericin B (2.0 .mu.g/ml). The dilutions were added to a 96 well microtiter plate with 0.1 ml/well. The microtiter plates were seeded with HEp-2 cells at 2500 cells/well and grown 18-24 hours prior to infection. Twelve wells/dilution were used. The plate was incubated for 6 days at gas concentrations of 8.0% O.sub.2, 8.8% CO.sub.2 and 83.2% N.sub.2. The cells were fixed with cold 50% acetone and 50% methanol for 2 minutes. To the wells, 0.03 ml/well of anti-L. intracellularis monoclonal antibody (McOrist, 1987) diluted 1:2000 in PBS was added. The plate was incubated for 30 minutes at 37.degree. C. and then washed 3 times with PBS. Anti-mouse FITC diluted 1:30 was added at 0.03 ml/well and incubated 30 minutes at 37.degree. C. The plate was washed 3 times with ddH.sub.2 O and allowed to dry. Samples were observed on a fluorescent microscope and the TCID.sub.50 /ml was determined.

Animals:

Thirty-one mixed sex pigs six weeks of age from PIC.times.Lieske females and large white boars were provided by Dr. Kent Schwartz. The pigs were randomly distributed to 4 pens by weight on day 0.

Facility:

Four pens in a small nursery facility, each separated by at least 3 feet, were used to house the pigs. The pens had wire flooring and solid pen dividers. Heat was provided by a furnace with zonal supplemental heat by heatlamps. The temperature was maintained between 78.degree. and 85.degree. F. for the duration of the study.

Feed and Water:

A 19% protein, ground corn-soy diet, free of antibiotics, was provided ad libitum via stainless steel feeders. Water was provided ad libitum via nipple waterers.

Infection of Pigs:

On day 0, the pigs were weighed and blood samples collected via capillary tube placed in the retroorbital sinus. Serum was harvested and stored frozen at -20.degree. C. Fecal swabs were also collected for PCR. The pigs were dosed with 10 ml inoculum given intragastrically via stomach tube.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. pigs</th>
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<tbody>
<tr>
<td>Control - uninfected cells</td>
<td>5</td>
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<tr>
<td>Control - no treatment</td>
<td>2</td>
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<tr>
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<td>Medium dose</td>
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<tr>
<td>Low dose</td>
<td>8</td>
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</table>

The pigs were weighed and bled on days 0, 10, 17 and 24.
Polymerase Chain Reaction:

Infection of the pigs was monitored by PCR using primers and cycle parameters as described by Jones (1993). Fecal samples collected on days 0, 7, 14, 21, and 24 as well as mucosa of the intestines were checked by PCR.

Histopathology:

Sections of ileum, jejunum, and colon were formalin fixed, processed routinely, stained with Hematoxylin and Eosin as well as silver impregnation and evaluated. Sections were also stained using monoclonal antibody specific for L. intracellularis.

Results:

Clinical Signs:

Clinical signs consisting of loose stools were first observed in the high dose group at 3 days. The signs peaked at 14 days and began to resolve thereafter.

Weight Gain:

Average daily weight gains were calculated showing that the high and medium dose groups had reduced weight gains compared to the control group. There was a dose titration effect in the weight gains when comparing the groups.

PCR:

Fecal shedding was not observed until 14 days. At 21 days, 37.5% of the high dose pigs were PCR positive in the feces. After necropsy, the mucosas of the ileums were checked by PCR with 100% positive in the high dose, 75% in the medium dose, 50% in the low dose and 0% in the controls.

Gross Lesions:

Gross lesions were found in 2 pigs of the high dose group (#50 and #202). The pigs had approximately 3 ft of thickening in the ileum with necrosis in #202.

Histopathology:

FA:

FA staining of sections of the ileum, jejunum, and colon revealed the presence of L. intracellularis in 87.5% of the high dose, 62.5% of both the medium and low doses and 0% in the controls.

Microscopic Lesions:

Lesions were observed in 100% of the high dose, 75% of the medium dose, 87.5% of the low dose and 14% in the controls. This was determined by the observation of increased mononuclear cells in the lamina propria and submucosa, often associated with hyperplasia of Peyer's Patchers. Crypt hyperplasia was also observed.

Silver Stain:

Silver staining of sections for the presence of intracellular, curved bacteria was also done. This demonstrated the presence of bacteria in 87.5% of the high dose, 62.5% in the medium dose, 87.5% in the low dose and 0% in the controls.
Discussion:

The pigs were successfully infected with pure cultures of L. intracellularis. At doses of $10^7$ bacteria, 100% of the pigs demonstrated infection by PCR and microscopic lesions. The severity of the lesions and the amounts of bacteria in the tissue sections were relatively low. This study is a satisfactory challenge model for L. intracellularis due to the presence of L. intracellularis and microscopic lesions in the pigs. Lesions may be improved with a second dose 7 days after the first dose.

Example 5

Hamster Vaccine Efficacy Experiment

Goal:

Evaluate a lab animal model for determining the safety and efficacy of an avirulent-live vaccine of L. intracellularis in hamsters.

Summary:

A 40 hamster study was completed by vaccinating 3 week-old hamsters with pure cultures of a high passage strain of L. intracellularis and challenging 22 days after vaccination with pure cultures of low passage virulent material. The hamsters were divided into 3 groups. Group A was vaccinated with 1 dose of L. intracellularis strain N72994 at day 0. Group B was designated the control group and was not dosed with a vaccine culture. Both groups were challenged with 2 doses of a pure culture of L. intracellularis strain N343 on days 22 and 25 post-vaccination. Group C was given challenge strain, N101494, to compare relative virulence to strain N343. Groups A and B contained 15 hamsters each and Group C contained 10 hamsters. Tissue Culture Infectious Dose 50% (TCID$_{50}$) results indicated that the hamsters were vaccinated with $10^{5}$ TCID$_{50}$/dose. The N343 challenge contained $10^{5.5}$ TCID$_{50}$/dose. The challenge dose for Group C was $10^{2.75}$ TCID$_{50}$/dose. Fecal swabs were collected on days 0, 7, 14, 21, 29, 36, and 43 for polymerase chain reaction (PCR) testing. On day 21, 5 animals were necropsied from Groups A and B each for PCR testing of the mucosas as well as FA, Hematoxalin and Eosin stains, and Silver stains of ileal sections to determine the persistence of colonization of the bacteria in the vaccinated hamsters. The remaining animals were necropsied 21 days post-challenge with similar testing.

PCR results indicated the presence of L. intracellularis in the intestinal mucosas of 100% of the Group A hamsters 21 days post-vaccination. Group B hamsters were all negative at 21 days post-vaccination. Twenty-one days post-challenge 50% of the hamsters were PCR positive in Group A 100% were positive in Group B. Histopathology of the sections indicated mild to severe lesions in 50% of animals in Group A and mild lesions in 50% in Group B 21 days post-challenge. No animals demonstrated lesions 21 days post-vaccination. Group C animals did not have lesions at 21 days post-challenge. FA and silver stains were not able to demonstrate the presence of L. intracellularis in any of the sections.

In conclusion, a 50% reduction of infection was observed in hamsters vaccinated with a high passage strain of L. intracellularis as demonstrated by PCR. The intestines were colonized by low numbers of intracellular organisms as demonstrated by the lack of observed organisms in FA and silver stained sections. Hamsters in Group C were unable to show infection throughout the study most likely due to the low dosage of bacteria.

Materials and Methods:

Hamster Description:

Forty 3 week old female hamsters from Harlan Sprague Dawley were used.
Growth of Inoculum:

Vaccine Culture:

A continuous culture of L. intracellularis grown in HEp-2 cells for 29 weeks was used. The culture was grown in a similar manner as stated in the challenge culture section except the culture is passed to new HEp-2 cells every 2-3 weeks.

Challenge cultures:

One 75 cm$^2$ conventional tissue culture flask was seeded with $3.75 \times 10^5$ McCoys cells in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and allowed to grow 18-24 hrs at 37$^\circ$ C with 5% CO$_2$. The media was removed from the cells and one vial of N343 MSC X diluted in 14 ml DMEM/2% FBS was added to the flask. The culture was placed in a gas chamber, evacuated, and re-gassed with hydrogen and carbon dioxide to give a mixture of 8.0% O$_2$, 8.8% CO$_2$ and 83.2% H$_2$. The culture was grown for 6 days, then the cells were scraped into a 100 ml spinner flask with 90 ml DMEM/2% FBS and 0.01 g of cultisphere-G beads. The culture was grown at the gas concentrations stated above. The flask volume was scaled-up by doubling the media volume at weekly intervals. The culture was grown for 25 days in the spinner flask to a final volume of 250 ml.

Strain N101494 was grown in the same manner as strain N343.

Harvesting Cultures:

Vaccine Culture:

The culture was harvested by centrifuging at 3000 times g for 20 minutes. The pellet was resuspended in Sucrose-Phosphate-Glutamate solution (SPG) with 10% FBS and passed 4 times through a 25 gauge needle. Inoculum was diluted to the final volume (15 ml) in SPG/10% FBS.

Challenge Cultures:

The cultures were harvested by centrifuging at 3000 times g for 20 minutes. The pellets were resuspended in Sucrose-Phosphate-Glutamate solution (SPG) with 10% FBS and passed 4 times through a 25 gauge needle. The inoculum were diluted to the final volume in SPG/10% FBS (20 ml for strain N343 and 10 ml for strain N101494).

Dosage of Hamsters:

Vaccine:

At day 0 all hamsters in Group A were orally vaccinated with 1 ml of the prepared vaccine.

Challenge:

Twenty-two days post-vaccination, 10 hamsters in Group A and 10 hamsters in Group B were orally dosed with 0.5 ml of challenge culture strain N343. Group C was challenged with 0.5 ml of challenge culture strain N101494.

Quantitation of IS Intracellularis:

Quantitation of viable IS intracellularis was accomplished by determination of the Tissue Culture Infectious Dose 50 percent (TCID$_{50}$). This was done by removing 2 ml of culture to be tested and lysing the cells by passing through a 22 gauge needle 4 times. The sample was serially diluted 1:10 in DMEM/5% FBS containing
Vancomycin (100 ug/ml) and Amphotericin B (2.0 ug/ml). The dilutions were dispensed at 0.1 ml/well to a 96 well microtititer plate which was seeded with McCoys cells at 1250 cells/well and incubated 18-24 hours at 37 degree C. at 5% CO2 prior to infection. Twelve wells/dilution were used. The plate was incubated for 6 days at gas concentrations of 8.0% O2, 8.8% CO2 and 83.2% N2. On day 6, the cells were fixed with cold 50% acetone and 50% methanol for 2 minutes. To the wells, 0.03 ml/well of anti-IS intracellularis monoclonal antibody diluted 1:2000 in PBS was added. The plate was incubated for 30 minutes at 37 degree C. and then washed 3 times with PBS. Anti-mouse FITC diluted 1:30 was added at 0.03 ml/well and incubated 30 minutes at 37 degree C. The plate was washed 3 times with ddH2O and allowed to dry. Samples were observed on a fluorescent microscope and the TCIDsub.50 /ml was determined.

Monitoring Infection of Hamsters:

Infection of the hamsters was monitored by PCR using primers and cycle parameters as described by Gary Jones. Fecal samples were collected at 0, 7, 14, 21, 29, 36, and 43 days post-vaccination. After termination of the hamsters the mucosa of the intestines were also checked by PCR.

Histopathology:

Sections of ileum and colon were formalin fixed, processed routinely, stained with Hemtoxylin and Eosin and silver impregnation, and evaluated. The sections were also stained with a monoclonal antibody specific for L. intracellularis.

Average Daily Weight Gains:

Weights of the hamsters were collected 21, 28, 35, and 42 days post-vaccination to determine the average daily weight gains.

Results: Refer to Table Below.

TCIDsub.50:

TCIDsub.50 results indicated that the vaccine group (Group A) received 10sup.4.86 TCIDsub.50 /hamster. Hamsters in Groups A and B were challenged with strain N343 and received 10sup.5.5 TCIDsub.50. Group C hamsters challenged with strain N101494 received 10sup.2.75 TCIDsub.50 /hamster.

PCR:

PCR testing demonstrated the presence of L. intracellularis in 100% of the vaccinated hamsters that were necropsied 21 days post-vaccination. Testing 43 days post-vaccination demonstrated that 100% of the control hamsters and 50% of the vaccinated hamsters were infected with L. intracellularis. None of the hamsters challenged with N101494 were positive. Fecal shedding was not detected throughout the study in any of the hamsters.

Histopathology:

H & E stains revealed no histological lesions in all sections of hamsters necropsied 21 days post-vaccination. In sections harvested 43 days post-vaccination 50% of the Vaccine group had mild to severe lymphocytic enteritis and 50% of the control group had mild lymphocytic enteritis. No lesions were seen in the N101494 challenge group.

FA stains failed to demonstrate L. intracellularis in any of the hamsters 43 days post-vaccination.

Discussion:
A 50% reduction of infection was observed in hamsters vaccinated with a high passage strain of L. intracellularis as demonstrated by PCR.

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EXAMPLE 6

Swine Vaccine Efficacy Experiment

Purpose:

The object of this study was to evaluate the safety, persistent colonization and efficacy of an avirulent-live isolate and a killed isolate of L. intracellularis in pigs 2-3 weeks of age. A host animal study was conducted in which pigs 3 weeks of age were vaccinated then exposed to a virulent challenge with L. intracellularis strain N343 to compare differences in protection between the vaccines.

Methods:

On Dec. 11, 1995, a total of 45 pigs, 3 weeks of age were purchased from H & K Farms. They were transported to Veterinary Resources, Inc., a research facility located near Cambridge, Iowa, where they were tagged to individually identify each pig. The pigs were held at this facility for two days prior to initiation of the study to allow acclimation to the facility and were fed antibiotic-free feed throughout the study.

On December 13, all pigs were weighed, bled to collect serum, clinically scored, and rectal swabs collected. The pigs were then randomly divided into groups of five and placed into tubs. Twenty pigs were placed into a separate room and were designated control and strict control groups. Fifteen pigs were placed in a second room for the ISi-1 vaccine. A third room had 10 pigs for ISi-2.

The live vaccine was prepared at the NOBL Laboratories Research and Development facility and identified as experimental serial ISi-1. ISi-1 (strain N343) was isolated from a pig and grown continuously in pure culture for 29 weeks. The vaccine was grown in McCoy's cells in spinner flasks at reduced oxygen until approximately 100% infection was observed. A sample of the high passage N343 strain used for ISi-1 was passed an additional 11 weeks ("N343NP40wk") and deposited under the Budapest Treaty on May 22, 1996 in the ATCC, 12301 Parklawn Drive, Rockville, Md. U.S.A. 20852 and assigned Accession Number 55783. The cultures were harvested by centrifuging at 3000 x g for 20 minutes. The pellets were resuspended in Sucrose-Phosphate-Glutamate solution (SPG) with 10% FES and passed 4 times through a 25 gauge needle. The lysates were centrifuged at 500 x g for 5 minutes to pellet the debris and microcarrier beads. The supernatant was saved and stored at -70 degree. C. until approximately one hour before vaccination where it was stored on ice until administration.

The killed vaccine (ISi-2) was grown, passed for 121/2 weeks and harvested in a similar manner as above and was purified by a percol gradient. The purified bacteria were then stored at -70 degree. C. until approximately 1 week before vaccination in which it was stored at 4 degree. C. at normal atmospheric oxygen levels which becomes toxic to L. intracellularis. ALOH was added to the bacteria to a final mixture of 10% ALOH. Protein concentration was determined using the Biurett method.

Quantitation of Live IS Intracellularis:

Quantitation of viable L. intracellularis was accomplished by determination of the Tissue Culture Infectious Dose 50 percent (TCID sub 50). Ninety-six well microtiter plates were seeded with McCoy's cells at 1250 cells/well and grown 18-24 hours prior to infection. The samples were serially diluted 1:10 DMEM/5% FBS containing Vancomycin (100 mu.g/ml) and Amphotericin B (2.0 mu.g/ml). The dilutions were added to the 96 well microtiter plates with 0.1 ml/well. Twelve wells/dilution were used. The plate was incubated for 6 days at 37 degree. C. and gas concentrations of 8.0% O.sub.2, 8.8% CO.sub.2 and 83.2% N.sub.2. The cells were fixed with cold 50% acetone and 50% methanol for 2 minutes. To the wells, 0.03 ml/well of anti-L. intracellularis monoclonal antibody (developed by Dr. Steven McOrist) diluted 1:2000 in PBS was added. The plate was incubated for 30 minutes at 37 degree. C. and then washed 3 times with PBS. Anti-mouse immunoglobulin G-fluorochrome conjugate (FITC) diluted 1:30 was added at 0.03 ml/well and incubated 30 minutes at 37 degree.
C. The plate was washed 3 times with ddH$_2$O and allowed to dry. Samples were observed on a fluorescent microscope and the TCID$_{50}$/ml was determined using the Reed-Muench method of calculation.

TCID$_{50}$ results indicated that ISi-1 had 1.8 times 10$^{5}$ bacteria/ml. A fourth inoculum was a placebo and was derived from tissue culture cells processed in the same manner as the vaccines.

The killed vaccine was tested for total protein content using the Biuret method and contained 0.311 mg/ml.

The pigs were vaccinated on Dec. 13, 1995. The live vaccine was all given at a dose of 2 ml IN with 1 ml/nostril. The ISi-2 (killed) vaccine was given IM with 1.5 ml/pig and again 14 days later. All control animals were given non-infected cells in the same manner as the live vaccines.

Observation and Samples:

Fecal swabs and serums were collected at 7 day intervals throughout the study. The fecal swabs were processed for PCR testing using the primer set, 5'-TATGGCTGTCAACACACTCCG-3' and 5'-TGAAGGTATTGGTATTCC-3' for the DNA amplifications. Cycle parameters were 93. degree C. for 5 minutes, 55. degree C. for 45 seconds, and 72. degree C. for 45 seconds for the first cycle. Thirty-three cycles were performed at the previously mentioned temperatures for 45 seconds per temperature. The final cycle was 93. degree C. for 45 seconds, 55. degree C. for 45 seconds, and 72. degree C. for 2 minutes, primers defined by Jones et al.

Challenge:

All animals, except strict controls, were given a challenge culture 26 and 27 days post-vaccination consisting of low passage cultures of L. intracellularis strains N343 and N72994 that were grown between 8 and 12 weeks continuously. The cultures were harvested by centrifuging at 3000 times g for 20 minutes. The pellets were resuspended in Sucrose-Phosphate-Glutamate solution (SPG) with 10% fetal bovine serum and passed 4 times through a 25 gauge needle. Some harvested cultures were stored at -70 degree C. until time of challenge while others were grown until the day of challenge and harvested. Challenge inoculums were combined and TCID$_{50}$ of the cultures were determined. The samples were stored on ice until administered.

Challenge culture given on Jan. 8, 1996 consisted of 4 times 10$^{4}$ bacteria/ml and challenge culture given on Jan. 9, 1996 had 3 times 10$^{4}$ bacteria/ml. The pigs were given 15 ml of challenge on both days via gastric lavage. The animals thus received 6 times 10$^{5}$ bacteria/pig and 4.7 times 10$^{5}$ bacteria/pig on Jan. 8, 1996 and Jan. 9, 1996 respectively.

Results:

Safety:

Fecal PCR results: Detection of L. intracellularis using PCR demonstrated that no pigs were shedding the bacteria at the beginning of the study. At seven days post-vaccination all pigs were negative. Fourteen days post-vaccination 3 pigs in the ISi-1 group were positive. Two animals were positive in the ISi-1 group 21 days post-vaccination and all other pigs negative. At day 26 post-vaccination no animals were shedding the bacteria as detected by PCR. Twenty-six day post-vaccination 5 pigs from groups ISi-1 and controls and 4 pigs from group ISi-2 were necropsied. Samples collected were ileum, colon, mesenteric lymph node, and tonsil as well as lung samples from pigs with lesions suspicious for pneumonia.

PCR testing was performed on the individual ileum and lung samples. Tonsil, colon, and lymph nodes were pooled by treatment group and PCR performed. Results of the PCR testing are below.

<p>| PCR of Ileum | Mesenteric |</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination</th>
<th>Colon</th>
<th>Tonsil</th>
<th>Lymph Node</th>
<th>Lung</th>
</tr>
</thead>
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<tr>
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<td></td>
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</tr>
<tr>
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<td>-</td>
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<tr>
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<td>-</td>
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<td>test</td>
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</table>

Histological sections of the ileums were stained using a monoclonal antibody specific for L. intracellularis as the primary antibody and anti-mouse immunoglobulin G-fluorochrome conjugate as the secondary antibody. L. intracellularis were observed in 3 of the five pigs from ISi-1. All other pigs were negative by fluorescent antibody staining.

The remaining pigs were necropsied 21 days after challenge and the same samples were collected for evaluation. PCR results are listed below.

<table>
<thead>
<tr>
<th>Group</th>
<th>Challenge</th>
<th>Colon</th>
<th>Tonsil</th>
<th>Lymph Node</th>
<th>Lung</th>
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</table>

FA stains of the ileums were performed as stated above with 7 of 10 animals positive in the control group. All other animals were negative for the presence of L. intracellularis.

The serum was tested for IgG antibody production by the pigs after exposure to L. intracellularis. The test was set up by seeding tissue culture treated Terasaki plates with McCoys cells at 125 cells/well and grown 18-24 hours prior to infection. A pure culture of L. intracellularis diluted to 1000-3000 bacteria/ml in DMEM with 5% fetal bovine serum was then added to the wells with 0.01 ml/well. The plate was incubated for 6 days at gas concentrations of 8.0% O2, 8.8% CO2 and 83.2% N2. The cells were fixed with cold 50% acetone and 50% methanol for 2 minutes. The sera from the pigs were diluted 1:75 in sterile PBS. The diluted serum was added to the wells at 0.01 ml/well. The plates were then incubated for 30-60 minutes at 37 degree C. The plates were washed 5 times with sterile PBS. To the wells, 0.01 ml/well of anti-swine IgG immunoglobulin G-fluorochrome conjugate was added. The plate was incubated for 30 minutes at 37 degree C. The plates were washed 5 times with ddH2O2 O and allowed to dry. Samples washed 5 times with ddH2O2 O and allowed to dry. Samples were observed on a fluorescent microscope and the wells in which bacteria were observed were labeled positive, and wells in which no bacteria were observed were labeled negative.
Results:

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0</th>
<th>26 days Post-Vaccination</th>
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<tr>
<td>Strict Controls</td>
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</tbody>
</table>

Animals that were positive at day 0 were again tested at weekly intervals. Results demonstrated that all became serologically negative by 14 days post-vaccination. This is not unexpected since the age of the pigs at day 0 was three weeks and positive results at that age could be due to maternal antibodies.

The sera were tested along with a positive control serum obtained by hyperimmunizing a pig with L. intracellularis grown in pure culture. Negative control serum used was collected from a gnotobiotic pig at South Dakota State University.

The above description and examples are only illustrative of preferred embodiments which achieve the objects, features, and advantages of the present invention, and it is not intended that the present invention be limited thereto. Any modifications of the present invention which come within the spirit and scope of the following claims is considered part of the present invention.
ACKNOWLEDGEMENTS

The completion of experiments and writing of this thesis was made possible by the help and support of many people. I would like to thank my major professor, Dr. D.L. Harris, for his guidance and support throughout the course of my graduate program. I would like to thank Dr. Michael Roof for all of the advice and mentoring that you have provided over the years. I thank Dr. Jan Schuiteman and Dr. Mike Daniel for their support and enthusiasm to get this project started and for the opportunity to continue my education. A special thanks to my wife, Machelle, whose love and patience helped me continue through all of the ups and downs of graduate school and to my daughter Sydney who has given me new inspiration. I would also like to thank my parents, Albert and Genevieve Knittel, as their love and encouragement was always very helpful. The technical assistance of Dr. Steven McOrist and Ms. Rebecca Mackie, for providing the expertise of cultivation of *Lawsonia intracellularis*, was key to the initiation of this project. The assistance of Dr. Kent Schwartz, Dr. Phil Hayes, Dr. Don Walter, Dr. Dianna Jordan, Mr. Jeremy Kroll, Mr. Christian Baum, and Ms. Kelly Burkhart, was appreciated in their support of the animal trials and sample processing. Mr. Jeremy Kroll, Dr. Anja Preissmann, Mr. Randy Downs, Ms. Julia Nolan, Mr. David Gorcyca, Ms. Kathy Schlesinger, and Ms. Janelle Hoogendoorn were tremendously helpful in the culture scale-up experiments. I thank Boehringer Ingelheim Vetmedica for the financial support for the project and for the opportunity to work on a project with so many scientific rewards. And finally, to all of my co-workers, past and present, at Boehringer Ingelheim for putting up with me during the whole process of pursuing my degree, I thank you all.