DETERMINATION OF FARM-SPECIFIC LAWSONIA INTRACELLULARIS SEROPREVALENCE IN CENTRAL KENTUCKY THOROUGHBREDS AND THE IDENTIFICATION OF FACTORS CONTRIBUTING TO EQUINE PROLIFERATIVE ENTEROPATHY

Allen E. Page
University of Kentucky, allen.e.page@gmail.com

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Allen E. Page, Student

Dr. David W. Horohov, Major Professor

Dr. Daniel Howe, Director of Graduate Studies
DETERMINATION OF FARM-SPECIFIC *LAWSONIA INTRACELLULARIS*
SEROPREVALENCE IN CENTRAL KENTUCKY THOROUGHBREDS AND THE
IDENTIFICATION OF FACTORS CONTRIBUTING TO EQUINE PROLIFERATIVE
ENTEROPATHY

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture
at the University of Kentucky

By

Allen Edward Page

Lexington, KY

Director: Dr. David W. Horohov, Department of Veterinary Science

Lexington, KY

2013

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ABSTRACT OF DISSERTATION

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ABSTRACT OF DISSERTATION

Determination of farm-specific *Lawsonia intracellularis* seroprevalence in central Kentucky Thoroughbreds and the identification of factors contributing to equine proliferative enteropathy

*Lawsonia intracellularis* and the disease it causes in horses, equine proliferative enteropathy (EPE), is an emerging pathogen of increasing importance to the horse industry from both an economic and welfare standpoint. Long recognized as an economically important disease of swine, the hallmark of EPE is a protein-losing enteropathy, where affected horses suffer weight loss and some ultimately succumb to the disease despite aggressive treatment. There are currently no known EPE preventative measures and the epidemiology of the disease remains poorly defined. While EPE is a sporadic disease affecting less than 25% of exposed horses, some farms experience clinical cases year after year. Further, weanlings are uniquely susceptible to this disease, although no conclusive reason for this predisposition has been identified. The overall hypothesis is that the host immune response plays a significant role in the susceptibility of weanlings to *L. intracellularis* infection and the occurrence of clinical equine proliferative enteropathy. To test this hypothesis, four individual hypotheses were proposed: (H1) previous farm history of EPE does not have an effect on weanling seroprevalence, (H2) passively-acquired antibodies do not have an effect on susceptibility to *L. intracellularis* and the occurrence of EPE, (H3) the serological status of mares can be used to determine the role they play in the epidemiology of EPE on endemic farms, and (H4) *L. intracellularis*-specific IFN-γ expression is not associated with increased resistance to EPE.

KEYWORDS: Horse, Seroprevalence, Weanling, *Lawsonia intracellularis*, Interferon-gamma

Allen Edward Page
July 25, 2013
DETERMINATION OF FARM-SPECIFIC LAWSONIA INTRACELLULARIS SEROPREVALENCE IN CENTRAL KENTUCKY THOROUGHBREDS AND THE IDENTIFICATION OF FACTORS CONTRIBUTING TO EQUINE PROLIFERATIVE ENTEROPATHY

By

Allen Edward Page

Dr. David Horohov
Director of Dissertation

Dr. Daniel Howe
Director of Graduate Studies

July 29th, 2013
Date
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CHAPTER ONE:
Introduction & Literature Review

Section I: *Lawsonia intracellularis* and Equine Proliferative Enteropathy

*Lawsonia intracellularis*

*Lawsonia intracellularis* is the causative bacterial organism of proliferative enteropathies in a variety of species, but most notably in pigs, horses, and hamsters.\(^1\) First described in pigs by Biester and Schwarte,\(^2\) it wasn't until 1973 that proliferative enteropathy was attributed to a bacterium.\(^3\) Difficulty with isolation, cultivation, and characterization delayed naming of the bacterium until 1995 when it was formally named *Lawsonia intracellularis*.\(^4\) *L. intracellularis* has been classified as a member of the delta subdivision of the *Proteobacteria* class, with closest genetic homology (91%) to *Desulfovibrio desulfuricans*.\(^4\) *L. intracellularis* is an obligately intracellular, flagellated,\(^1\) curved, gram-negative rod which measures approximately 1.25-1.75 µm in length and 0.26-1.5 µm in width,\(^4,5\) requiring a reduced oxygen atmosphere for *in vitro* cultivation.\(^5,6\)

*L. intracellularis* is best recognized for causing porcine proliferative enteropathy (PPE), an economically devastating disease in the porcine industry due to its detrimental effect on average daily gain and market weight.\(^7,8\) As a result, the majority of what is known with respect to *L. intracellularis* has been discovered through work with pigs and largely extrapolated to the horse. Porcine proliferative enteropathy encompasses several clinical entities, including the subclinical form in which affected pigs exhibit no clinical signs but shed the bacterium, the chronic form where affected pigs fail to grow as well as littermates (porcine intestinal adenomatosis), and the acute form (normally referred to as
porcine hemorrhagic enteritis or PHE), which is often characterized by hemorrhagic diarrhea and acute death.\textsuperscript{1} While acute morbidity and mortality has long been recognized as a common clinical presentation of \textit{Lawsonia intracellularis} infection in swine,\textsuperscript{1} only recently has work demonstrated a similar presentation in horses, called the necrotizing form of EPE (N-EPE)(See Appendix), which, like PHE, leads to rapid clinical deterioration and death.\textsuperscript{9} Likewise, most EPE-affected horses exhibit signs similar to those of chronic PPE pigs with a more prolonged and less-severe outcome while a small portion of horses will experience subclinical EPE.\textsuperscript{10}

\textbf{Equine Proliferative Enteropathy Introduction}

Equine proliferative enteropathy (EPE) is an emerging disease of young horses with important economic and welfare concerns.\textsuperscript{9,11} Whether EPE is truly emerging or is more common as a result of better recognition by veterinarians and horse owners is not known, although unpublished data suggests that exposure to \textit{L. intracellularis} in Kentucky has remained fairly static over that past 7+ years.\textsuperscript{12} Proliferative enteropathy was first reported in a horse from California by Duhamel and Wheeldon in 1982, when the disease was still referred to as “intestinal adenomatosis”,\textsuperscript{13} while Williams et al.\textsuperscript{14} in 1996 published the first report of EPE in a horse from Kentucky using the correct name of \textit{L. intracellularis}. Since then, an expansive distribution of EPE has now been published across the world, although no cases have been reported in the literature, to date, in Asia or Africa.\textsuperscript{15-18}
**EPE Clinical Signs**

The clinical signs associated with EPE are relatively non-specific and include anorexia, fever, dependent edema, depression, lethargy, rapid weight loss, and an unthrifty appearance/hair coat, while colic and diarrhea have been occasionally reported.\(^{11,18}\) EPE is typically seen in weanlings, a common characteristic of all *L. intracellularis*-induced PE's, with most cases in central Kentucky occurring between August and February.\(^{11,19,20}\) Most affected horses will respond well to treatment (see below) although some may develop secondary complications, such as salmonellosis or N-EPE, which may prolong the recovery period or result in death. For those horses that do recover, the recovery period can take weeks to months before they regain the appearance of unaffected cohorts (author's personal experience).

Unique amongst the species that *L. intracellularis* infects, hypoproteinemia and hypoalbuminemia are considered one of the antemortem hallmarks of EPE\(^ {11}\) and the reason that dependent edema is seen with EPE cases. Loss of protein and albumin through an unknown mechanism, likely a combination of decreased food intake, malabsorption,\(^ {21}\) and possibly protein loss through compromised small intestine,\(^ {9}\) results in lower plasma oncotic pressure and a net movement of free fluid from the circulation into tissues.

**Treatment of EPE**

Treatment of EPE requires the use of antimicrobials that reach therapeutic concentrations within the cytoplasm of infected enterocytes because of the intracellular location of the pathogen. A variety of various antimicrobials have been demonstrated to
exhibit *in vitro* inhibition of *L. intracellularis*; primarily the use of chloramphenicol, macrolides with or without rifampin, and tetracycline-related drugs have been described. If it is needed, supportive care of affected horses is typically aimed at restoring circulating oncotic pressure through the use of equine plasma or synthetic colloids. Horses with severe anorexia and weight loss may require partial or total parenteral nutrition in a hospital setting. Further, horses with antemortem signs of N-EPE require immediate and aggressive care to blunt the sequelae that result in these cases; treatment with broad-spectrum antimicrobials to ensure coverage against both Gram-positive and Gram-negative bacteria, anti-thrombotics, and anti-endotoxic measures such as low-dose flunixin meglumine and/or intravenous polymyxin-B. Due to their potential nephrotoxic effects, care should be taken when administering non-steroidal anti-inflammatory drugs and/or polymyxin-B; likewise, tetracycline-class drugs (particularly intravenous oxytetracycline) can be nephrotoxic and should be used carefully in hypovolemic patients.

**Antemortem Diagnostic Tests for EPE**

Given that the hallmark of proliferative enteropathies caused by *L. intracellularis* is mucosal hyperplasia, abdominal ultrasonography can be a useful diagnostic test in horses. Small intestinal wall thickness in excess of 3mm anywhere in the abdominal cavity of young horses is suggestive of EPE, while increased thickness is highly suspicious for EPE when accompanied by compatible clinical and/or clinicopathologic signs. Care should be exercised when no small intestinal thickening is observed as there
have been reports of clinical EPE cases where wall thickness was reported to be within normal limits.  

In horses, it is well reported that hypoalbuminemia and hypoproteinemia are non-specific, but highly suggestive findings seen with EPE.  These changes likely reflect both protein loss and malabsorption in the affected small intestine. As a result, and as demonstrated in previous studies, total protein and albumin levels can drop rapidly in the span of 4-7 days.  While reference ranges will vary based on the population and testing method, total protein concentrations below 5.3 mg/dL and albumin concentrations below 3.2 mg/dL should be considered hypoproteinemic and hypoalbuminemic, respectively (see Chapter 3 below). It is important to note though that there are a number of conditions, including renal disease, colitis, salmonellosis, and intestinal parasites, that can also result in decreased in albumin levels. With respect to other clinicopathologic testing, complete blood counts, and fibrinogen levels are not typically considered useful for EPE screening because routine clinical cases of EPE are devoid of a detectable intestinal inflammatory response and *L. intracellularis* infection is primarily localized to the intestinal enterocytes. Additionally, metabolic derangements are rare unless severe diarrhea is present, the case has been ongoing for a prolonged period of time, or there is concurrent disease.

Fecal polymerase chain reaction (PCR) for *L. intracellularis* is probably the most specific test available in that it detects sequences of DNA unique to the bacterium. Issues arise, however, with *L. intracellularis* PCR in that the bacterium may be intermittently shed by infected horses and there are a variety of PCR inhibitors in feces.  Further, fecal shedding of the bacterium in horses has been reported to stop within 4-6
18 days of antimicrobial administration. It is likely for these reasons that the sensitivity of *L. intracellularis*-specific fecal PCR testing is variable.\textsuperscript{32,34-36}

Currently, there are several *L. intracellularis*-specific serologic assays available for use in horses. The original assay, the immunoperoxidase monolayer assay (IPMA), was initially created for use in pigs\textsuperscript{37} and has been adapted for commercial use in horses; a newer version of the IPMA is also commercially available as it reportedly has less background staining artifact, although no manuscripts detailing its use have been published. Outside of the United States, a blocking ELISA is commercially available for use in horses, while a newly equine-validated ELISA\textsuperscript{20} is currently available in the U.S. on a research and limited commercial basis. With the exception of the blocking ELISA available outside of the U.S., serologic tests appear to be highly specific for *L. intracellularis* exposure.\textsuperscript{38} It is important to note that these tests detect whole immunoglobulin (IgG) to the bacterium, which is a result of exposure and not indicative of clinical EPE or ongoing infection. Further, individual horses respond differently to *L. intracellularis* exposure and the severity of exposure and infection cannot, as of yet, be inferred from the titer value obtained. Based on experimental studies, horses challenged with *L. intracellularis* have been shown to seroconvert within 14 days of challenge while most begin showing clinical signs of EPE by day 19-21.\textsuperscript{10,39} These horses were administered doses likely far in excess of that which is normally encountered, which may have decreased the incubation period of the disease.
**Pathological/Post-mortem Features of EPE**

The pathological features of EPE and PPE typically identified on post-mortem examination of affected animals are fairly similar, namely small intestinal mucosal hyperplasia. *L. intracellularis* in horses is most commonly identified in enterocytes of the terminal ileum, immediately prior to the ileo-cecal junction. Intestinal mucosal hyperplasia is typically found in this region, and results from rapid and unchecked division of the crypt cells. Histologically, these regions exhibit crypt epithelial hyperplasia with subsequent elongation and branching of the crypts and villi, a decreased goblet cell density, and typically a lack of evidence of a cellular inflammatory process (except in PHE and N-EPE cases where the inflammation is likely secondary to mucosal necrosis).¹

Though mucosal proliferation is a hallmark of this disease, the underlying mechanism remains unknown. Several possible mechanisms for *L. intracellularis* virulence have been proposed including perturbations in cell cycle processes of crypt epithelia, alterations of factors involved in the differentiation of normally immature and proliferative crypt cells, disturbances of relevant growth factor receptors, and failure of apoptotic cell death in the crypt.⁴⁰,⁴¹ A recent study examined the effect on differential expression of cell cycle and cell differentiation genes within mouse fibroblast cells infected with *L. intracellularis*.⁴¹ While this led to the identification of a large number of candidate genes potentially responsible for the proliferation and hyperplasia seen with *L. intracellularis* infection, mouse fibroblasts are not typically affected by *L. intracellularis*. Genomic sequencing studies have recently identified the genes for and expression of type III secretion system components by *L. intracellularis*.⁴²,⁴³ This finding is important as
type III secretion systems in other enteric pathogens have been shown to contribute to apoptosis perturbations, cellular invasion, and immune suppression.42

**Epidemiology of EPE**

To date, the epidemiology of EPE remains poorly characterized, although a fecal-oral route of transmission is widely suspected.30 With EPE, the most commonly affected age groups are weanling and short yearling horses (those yearlings that have not yet reached 12 months of age),11,20 although there are unpublished reports of rare cases in nursing foals and older horses. Recent work has suggested a possible role for rabbits in the transmission of the bacterium as several weanling horses were nasogastrically intubated with feces from experimentally challenged rabbits, ultimately leading to seroconversion but no disease in the study horses.44 Further, a non-challenged sentinel horse that was co-housed with an experimentally challenged horse seroconverted during another study, but again, no signs of EPE were noted.39 Several studies have revealed the presence of *L. intracellularis* in a variety of species, including blacktailed jackrabbits, cottontailed rabbits, cats, striped skunks, Virginia opossums, coyotes, guinea pigs, mice, rats, hamsters, hedgehogs, ferrets, rabbits, wild pigs, dogs, foxes, calves, wolves, deer, ostriches, emus, monkeys, and giraffes;1,45-47 however, none have been successfully implicated in the induction of EPE. Although there is recent evidence of species-specificity with respect to *L. intracellularis* strains,48 a role for rodents, rats in particular, warrants further investigation in the future as work has shown a proportion of rats on PPE-endemic farms can shed up to $10^{10}$ *L. intracellularis* organisms per gram of feces.49 This is of particular importance as experimental challenge studies in pigs have induced
PPE at bacterial doses as low as $10^5$ per pig\textsuperscript{50} while EPE has been induced in using doses of $10^{10}$ per horse.\textsuperscript{10,39} Further, one study has shown that the bacterium may be capable of infectivity up to two weeks in the environment at temperatures of 5-15\degree C,\textsuperscript{51} raising the possibility of a prolonged period during which there is a risk of exposure.

Due to the significant impact of *L. intracellularis* on the swine industry, progress in understanding PPE epidemiology is much more evolved than it is with EPE. It has been hypothesized that PPE persists and is transmitted within swine operations via poor/inadequate disinfection techniques as well as subclinical shedders of the bacterium. Indeed, work has shown that subclinically affected pigs can efficiently spread the bacterium amongst cohorts.\textsuperscript{52} Although a study with bi-monthly fecal PCR testing did identify several weanling horses that shed the bacterium in the absence of clinical signs,\textsuperscript{19} the role, if any, subclinically affected horses play in transmission remains to be determined, especially given the management differences between swine and equine operations. Sows are not believed to play a role in the transmission of the bacterium to their offspring, although they have been reported to occasionally shed the bacterium.\textsuperscript{53} The possibility that mares could be exposing their foals to *L. intracellularis* is explored in Chapter 4.

**Prevention/Mitigation of EPE**

A licensed and effective vaccine for the prevention of PPE exists in the United States (Enterisol Ileitis, Boehringer Ingelheim Vetmedica).\textsuperscript{54-56} Off-label research into the use of the porcine vaccine in horses has garnered mixed results with a small-scale experimental challenge study suggesting that the vaccine protects against clinical EPE\textsuperscript{57}
while a field trial identified at least one vaccinated horse with clinical EPE. Anecdotal reports from equine veterinarians in central Kentucky with experience using the vaccine off-label suggest a decrease in the incidence of EPE on those farms that elect to utilize it.

As with any enteric disease, and especially given the suspected feco-oral transmission route and suggestion of horse-to-horse exposure, it seems prudent that those horses with suspected or confirmed EPE be isolated for a week following initiation of antimicrobial treatment to allow time for fecal shedding to cease. Work with pigs suggests that quarantine is an effective mechanism of preventing PPE, however, given the open nature of equine farms, as well as the potential for wildlife hosts/intermediaries, it seems unlikely that additional quarantine of new horses above what is routinely done would have a beneficial effect on the disease incidence.

The use of a regular screening program to detect *L. intracellularis* exposure and early EPE has been reported to be useful in the mitigation of EPE and variations on these programs are used on some EPE-endemic farms in central Kentucky. These screening programs typically include weekly screening of total protein levels for abnormally low levels; those horses with low levels are then monitored closely for signs of EPE and may have additional diagnostic tests run, such as albumin determination, abdominal ultrasonography, fecal PCR, and/or serological testing. Given the non-specific nature of total protein and albumin level fluctuations, it is never recommended to treat horses with antimicrobials for suspected EPE based solely on these parameters.
Section II: *Lawsonia intracellularis* and Immunology

**Immune Response to *Lawsonia intracellularis***

Given the intracellular nature of *L. intracellularis*, it is likely that innate and cell-mediated immunity is more important than humoral immunity with respect to clearance of the bacterium from infected animals. While antibodies provide an excellent, antemortem indicator of exposure to *L. intracellularis*, their role in the prevention or clearance of *L. intracellularis* remains unclear. This review will therefore focus on the innate and, primarily, cell-mediated branches of the immune system.

The uptake of *L. intracellularis* into enterocytes appears to be via endocytosis. Typically, this bacterium-containing endosome would fuse with a lysosome in the host-cell cytosol for degradation and antigen processing; however, *L. intracellularis*, by an unknown mechanism, evades this process and is reportedly found free in the enterocyte cytoplasm within three hours of invasion. Throughout this process, and as evidenced by the typical lack of an inflammatory response to *L. intracellularis*, the bacterium seems to evade the innate immune system in the gastrointestinal tract.

Innate immunity exists to quickly detect and respond to non-self antigens expressed by a large variety of pathogens, including bacteria, viruses, and fungi. By doing so, the innate immune system bridges the gap in time between pathogen presentation/invasion and the acquired cell-mediated and humoral immune responses. The innate immune system relies on highly conserved molecular signatures on pathogens, generally referred to as pathogen-associated molecular patterns (PAMP's), including but not limited to lipopolysaccharide (LPS), peptidoglycan, flagellin, DNA, and RNA.
Most PAMP recognition by host cells is carried out via detection by either toll-like receptors (TLRs) or nucleotide-binding oligomerization domain receptors (NOD-like receptors or NLRs). Of the various TLRs and NLRs which have been identified and characterized, the most important with respect to *L. intracellularis* based on their location and function likely would be TLR4, TLR5, Nlrc4, and Nlrp3.62,63

Immediately prior to and during invasion, *L. intracellularis*, a gram-negative, flagellated bacterium, must evade detection by TLR's 4 and 5. Both are located on the outer surface of enterocytes where TLR4 is specific for LPS and TLR5 is specific for flagellin.62 Likewise, once free within the enterocyte cytoplasm, *L. intracellularis* evades intracellular Nlrc4 (detects flagellin) and Nlrp3 (detects bacterial RNA and LPS).63 Normally, once bound to their respective ligands, both TLRs and NLRs induce a pro-inflammatory response as TLRs will induce NF-κB via the MyD88 pathway and NLRs activate caspase-1, leading to the conversion of pro-IL-1β to IL-1β. Interestingly, TLR4 has been suggested to play a role in the occurrence and severity of necrotizing enterocolitis in mice and humans.64,65 Given that PHE and N-EPE are characterized by mucosal necrosis while other forms of *L. intracellularis* infection are relatively devoid of an inflammatory response,1 it stands to reason that "accidental" detection of the bacterium by either the TLRs or NLRs could be the cause of these severe outcomes.

Similarly, it would be expected that the cell-mediated immune system should play a significant role in immunity to *L. intracellularis*. The host's response to a pathogen via cell-mediated immunity (CMI) depends on whether that response is weighted in favor of T-helper 1 cells (Th1) or T-helper 2 cells (Th2).66 T-helper cells are CD4+ with an affinity for major histocompatibility complex II (MHC-II), primarily located on the
surface of antigen presenting cells and B lymphocytes. While initially present as undifferentiated T-helper 0 cells (Th0), whether these cells differentiate into Th1 and Th2 cells is dependent upon the cytokines present in the local environment. In turn, the cytokines produced depend on the type and location (intra- vs. extracellular) of the pathogen of interest. Another type of T-helper cell, the regulatory T cell (Treg), is important for immune-modulation and suppression. Tregs do this by migrating to sites of inflammation and suppressing lymphocytes, with T-helper cells being the main target. Further, Tregs can be induced in tissues by cytokines, such as TGF-β. Given recent work suggesting an increase in TGF-β production by enterocytes when infected with *L. intracellularis*, as well as the lack of inflammation during typical *L. intracellularis* infections, it seems feasible that *L. intracellularis* may utilize Tregs to prevent overwhelming inflammatory reactions in response to enterocyte infection.

With *L. intracellularis*, we are primarily interested in the Th1 response targeted towards CMI and killing of intracellular organisms. Th2 responses are targeted towards a humoral response and antibody production for the killing of extracellular organisms. Th1 cells are produced in response to the presence of interleukin (IL) 2, IL-12, IL-18, interferon-gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α); Th2 cells are produced due to the presence of IL-4, IL-5, IL-10, IL-13, and IL-β. Pathways taken by Th0 cells depend on cytokines from antigen presenting cells, either as a result of infection or antigen processing. Once a Th0 differentiates into a Th1 or Th2 cell, this process typically continues with the recruitment of other nearby, antigen-specific Th0 cells as the newly differentiated T-helper cells release more cytokines that potentiate their
differentiation while inhibiting that of the other T-helper subtype. Thus, once an immune response begins on Th1 or Th2 paths, it typically continues in that fashion.

Multiple studies have detailed the induction of a CMI response to *L. intracellularis* infection. This has primarily been done in experimentally infected pigs through *L. intracellularis*-stimulation of peripheral blood mononuclear cells (PBMCs) *in vitro* where an increase in IFN-γ production was noted as soon as 14 days post-inoculation with either a pathogenic or vaccine strain of the bacterium. This increased production of IFN-γ *in vitro* can be augmented by co-culture of PBMCs in the presence of IL-18. Additionally, a recent study utilizing a commercially available vaccine showed that pigs vaccinated orally had significantly more TNF-α in small intestinal mucosal scrapings when compared to non-vaccinated and intramuscularly vaccinated controls, further suggesting a Th1 response to bacterial colonization. More germane to this review with regards to equids is the recent study that found PBMCs from *L. intracellularis* vaccinated and naturally occurring EPE horses produced increased amounts of IFN-γ mRNA *in vitro* when stimulated with the bacterium when compared to non-exposed controls. Further, vaccinated horses demonstrated an increased level of mRNA expression through the study's termination at 180 days post-vaccination, suggesting a long-lived memory recall with a Th1 bias. Interestingly, the amount of IFN-γ mRNA produced in response to the bacterium did not differ significantly between the vaccinated and EPE horses. Given that primary infection with *L. intracellularis* protects against repeat colonization in pigs, vaccination of equids might provide protection against disease. Except for what is presented in Chapter 5, the role of IFN-γ on the occurrence of PE in pigs and horses has not been reported.
The best evidence for a significant role of IFN-γ in infection and immunity to *L. intracellularis* comes from studies using knockout mice. Smith et al. 2000, showed that IFN-γ knockout mice developed more severe disease than wild-type mice of the same genetic background. This increased severity, compared to controls, manifested as a higher enterocyte infection rate, increased mucosal hyperplasia (small intestine and colonic), and a failure to clear the infection in 35 days. While the control group in this study did develop disease, the infection was cleared by day 28 post-challenge. The decreased amount of hyperplasia compared to the knockout group confirmed a previously reported anti-proliferative role for IFN-γ in enterocytes. An additional Short Communication provided some evidence of high levels of systemic IFN-γ in receptor knockout mice (severely afflicted with disease) compared to wild-type (and disease-unaffected) controls, a finding the authors interpreted as indicating IFN-γ binding is vitally important to prevention of *L. intracellularis*-induced disease.

The Th1 response to intracellular infection, mediated in large part by IFN-γ, generally functions to increase inflammation at the site of infection, as well as activate macrophages and increase cytotoxicity mediated by CD8+ cytotoxic lymphocytes (CTLs). Given the lack of an inflammatory response during normal *L. intracellularis* infections, much like with the innate immune system, the bacterium seems to largely avoid the cell-mediated immune response in affected animals. One possible explanation for this would be the presence of an immature or incompetent Th1 response in those animals that are affected with *L. intracellularis*. While recent work with foals has detailed many interesting findings supporting this assertion, little work has been conducted in the age group we are interested in, weanlings and young yearlings.
It has now been shown that, like human neonates, neonatal foals are lacking in their ability to produce IFN-γ early in life. Even though the deficiency does abate quickly and a rapid maturation in the ability to produce IFN-γ is seen, it can take 10 months or longer for the percentage of IFN-γ positive lymphocytes and the production of *in vitro* IFN-γ to approach that of adult horses. Interestingly, recent work in our lab showed that foal exposure to a barn atmosphere, which contained significantly more aerosolized fungi and bacteria compared to pasture, was associated with a significantly increased number of IFN-γ positive lymphocytes compared to those foals housed solely on pasture. While the finding that the environment can have a profound impact on CMI may be important with respect to a variety of young horse diseases, the effect only lasted through 3 months of age, so the potential significance with respect to *L. intracellularis* is unclear given that these horses are typically weaned at 4-6 months of age. The process of weaning itself has been hypothesized as one of the reasons weanlings are predisposed to *L. intracellularis* infection. Recently it was shown that weaning can have a detrimental, albeit short, effect on IFN-γ production where abruptly weaned horses exhibited a rapid (within 7 days post-weaning) decrease in IFN-γ positive lymphocytes and IFN-γ mRNA expression which lasted until at least 14 days post-weaning. It was hypothesized that this decrease was caused by stress-induced hormones, so while very few horses exhibit clinical EPE in the weeks following weaning (see Chapter 3 below), it is possible that stressful events in the months following weaning may exhibit the same pattern and lead to *L. intracellularis* susceptibility. Along these lines, the closest "relatives" of *L. intracellularis*, the *Desulfovibrio* genus, has some species which may exhibit opportunistic infection patterns.
While the mechanism(s) by which *L. intracellularis* escapes the immune system remains unknown, some work has indicated that the bacterium exerts an immunosuppressant effect on the small intestine of pigs.\textsuperscript{82} The suggested immunosuppressant effect of *L. intracellularis*, in addition to the identification of a Type III secretory system which is known to be immunosuppressive in other enteric organisms,\textsuperscript{42} involves a substantial decrease in small intestinal T (primarily CD8\textsuperscript{+}) and B cells, as well as MHC II expression, within 14 days of challenge with the bacterium.\textsuperscript{82} Interestingly, *in vitro* work using PBMCs from PPE cases has showed the opposite occurs, with an increase in lymphocyte proliferation due to stimulation with the bacterium.\textsuperscript{83} The finding that CD8\textsuperscript{+} cell numbers are decreased *in vivo* is important because, although the study had no negative controls for comparison, CD8\textsuperscript{+} CTLs from infected pigs have been shown to produce large amounts of IFN-\(\gamma\) following *in vitro* stimulation with *L. intracellularis*.\textsuperscript{84} Work with humans has also shown that MHC II-restricted CTLs play an important role in enterocyte immunity,\textsuperscript{85} and CD8\textsuperscript{+} MHC II-restricted T cells have been identified in pigs and horses.\textsuperscript{86,87} Though another *L. intracellularis* study showed CD8\textsuperscript{+} CTLs produced lower quantities of IFN-\(\gamma\) than Th1 cells,\textsuperscript{71} a decrease in CD8\textsuperscript{+} cells within the small intestine following infection with *L. intracellularis* would be accompanied by a reduced IFN-\(\gamma\) response, possibly contributing to the immune evasion of the bacterium. Additionally, a concurrent decrease of MHC II expression both in the lamina propria\textsuperscript{82} and on the enterocyte surface\textsuperscript{86} following *L. intracellularis* infection would further assist the bacterium with evasion.

As mentioned previously, both vaccination and prior clinical disease have been shown to prevent secondary disease caused by *L. intracellularis*.\textsuperscript{50,54,68,73} Disease in pigs
has been reportedly induced using doses of $10^5$-$10^{10}$ organisms/animal, and while there appears to be a dose-dependent severity of disease, all of the doses protected pigs from re-infection (not just clinical disease) when later re-challenged at $10^{10}$ organisms/animal. Due to the solely enteric nature of *L. intracellularis* infections, all protective measures have been enterically-targeted, through either the use of oral or intra-rectal vaccination. A recent paper, however, has suggested that intramuscular (IM) vaccination with the commercially available vaccine was as protective as the traditional oral route for vaccination in pigs. Given that IM vaccination would be unlikely to induce mucosal immunity and that elevated IgG serum titers resulted, IgG may confer possible protection against infection. However, another recent report suggested that maternal IgG in piglets (explored with respect to EPE in Chapter 3) had no protective effect against disease, although it was a secondary parameter that was not examined in depth. While the role, if any, IgG plays in infection and immunity to *L. intracellularis* remains unresolved, the role of the humoral system has been examined in several papers. It has been widely reported that systemic IgG production occurs in response to natural infection and re-infection/challenge with *L. intracellularis* in both pigs and horses, although IgG detection in vaccinated animals is variable. It appears as though the detectable humoral response occurs faster than a detectable CMI response and the magnitude and duration of detectable IgG titers is dose-dependent in pigs.

Unlike IgG, due to the enteric location of *L. intracellularis*, it seems likely that IgA would play a role in infection and immunity to the bacterium. Indeed, work has shown that IgA accumulates in infected enterocytes. Although whether this was a
bacterium-specific accumulation or due to an effect of the bacterium on IgA passage through the infected enterocyte was not elucidated, it has been suggested that IgA may play a role in neutralizing intracellular organisms as it passes through infected enterocytes. $^{90}$ *L. intracellularis*-specific IgA has been reported in the small intestinal lumen within 22 days post-infection$^{89}$ although other studies have been unable to detect significant amounts of specific IgA in either the small intestine or serum following vaccination,$^{68}$ whether this would be due to a dose or strain difference is unknown.

Our overall hypothesis is that the host immune response plays a significant role in the susceptibility of weanlings to *L. intracellularis* infection and the occurrence of equine proliferative enteropathy. The large number of horses, particularly Thoroughbreds, around the central Kentucky region have provided an opportunity to study this relatively rare disease at a rate that is likely higher than elsewhere in North America. The specific aims of the following studies are to:

1. Develop and validate an ELISA for the detection of *L. intracellularis*-specific antibodies and then use this ELISA to determine whether previous farm history of EPE has an effect on seroprevalence (Chapter 2).
2. Determine whether passively-acquired antibodies have an effect on the susceptibility to *L. intracellularis* and the occurrence of EPE (Chapter 3).
3. Evaluate whether mares play a role in the epidemiology of EPE on endemic farms using mare serological status (Chapter 4).
4. Determine whether *L. intracellularis*-specific IFN-$\gamma$ expression is associated with resistance to EPE (Chapter 5).

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CHAPTER TWO:
Adaptation and validation of a bacteria-specific enzyme-linked immunosorbent assay for determination of farm-specific *Lawsonia intracellularis* seroprevalence in central Kentucky Thoroughbreds

Summary

*Lawsonia intracellularis* is the causative agent of equine proliferative enteropathy (EPE), a bacterium for which no large-scale seroprevalence studies have been conducted. The objectives of this study were to validate and use an equine-specific ELISA for *L. intracellularis* to determine the seroprevalence of *L. intracellularis* on numerous farms and the effect of past EPE history on seroprevalence. An ELISA was adapted from previous work in swine to use purified antigen. A total of 337 Thoroughbreds from 25 central Kentucky farms were enrolled and monthly serum samples collected from August 2010 to January/February 2011. Samples were screened for *L. intracellularis*-specific antibodies using a modified ELISA. Farms were classified into one of three groups based on their recent (in the three years prior to the study) history with EPE. The overall ELISA intra-assay coefficient of variation (CV) was 6.73 and inter-assay CV was 9.60. An overall seroprevalence of 68% was obtained, with farm specific seroprevalences ranging from 14-100%. A significant difference was found in the average seroprevalence (p<0.05) on farms with recently presumptive cases of EPE. Additionally, both lower average ELISA unit (EU) values (p=0.079) and maximum EU values (p=0.056) on farms with no recent EPE history were detected when compared with farms with recently presumptive or suspected cases of EPE. Further, a bimodal exposure distribution to *L.*
*intracellularis* was detected in the fall and winter months. With this work, we showed that recent history of EPE was associated with higher average seroprevalence indicating increased exposure on farms with prior cases of EPE; seasonally bimodal exposure was also observed. The adapted ELISA appears to be useful for determination of *L. intracellularis*-specific antibody levels. The high farm-specific seroprevalences and bimodal distribution of exposure to *L. intracellularis* detected here were unexpected and suggest that farms with a previous history of EPE remain at risk due to heightened exposure levels beyond early winter.

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Introduction

Lawsonia intracellularis, an obligate intracellular, gram-negative rod, is the causative agent of proliferative enteropathy.\textsuperscript{1} L. intracellularis is viewed as an emerging cause of proliferative enteropathy in a variety of mammalian species,\textsuperscript{91,92} including horses,\textsuperscript{14,93-95} where the bacteria causes equine proliferative enteropathy (EPE). Clinical signs of EPE, usually seen in weanlings or young yearlings,\textsuperscript{11,18,94-96} include anorexia, fever, lethargy, depression, dependent edema caused by hypoproteinemia/hypoalbuminemia, weight loss, colic, and diarrhea. In addition, thickened small intestine detected by abdominal ultrasound is considered highly suggestive of EPE when accompanied by compatible clinical signs. Commercially available antemortem tests for EPE include fecal L. intracellularis-specific PCR and the serum immunoperoxidase monolayer assay (IPMA), both of which have been adapted from their use in pigs where the tests are highly specific; sensitivity is reported to be high for the IPMA\textsuperscript{37,53} but variable for fecal PCR.\textsuperscript{32,34}

Though the epidemiology of EPE is uncertain, it is believed that transmission occurs through the ingestion of L. intracellularis-contaminated fecal material from wild or domestic animals.\textsuperscript{46} While most cases of EPE typically occur in the fall and early winter,\textsuperscript{11,19} the reason for this seasonal predilection has not yet been determined. One possibility is the known susceptibility of weanlings to this infection and weaning typically occurs during this time of year.\textsuperscript{11} Another possibility would be that the environmental conditions at that time favor transmission by increasing exposure burdens.
Previous work looking at *L. intracellularis*-specific antibodies demonstrated seroprevalences ranging from 33.8-45.5% on two California farms\textsuperscript{97} and up to 60% on a Kentucky farm,\textsuperscript{19} utilizing the IPMA method. While these studies have focused on individual farms, none have determined the seroprevalence of *L. intracellularis* within a larger region. Detection of *L. intracellularis*-specific antibodies using enzyme-linked immunosorbent assays have been developed for the pig and rabbit.\textsuperscript{98-101} Here we adapted a porcine ELISA\textsuperscript{99} and used this ELISA method to determine seroprevalences on Thoroughbreds farms located in central Kentucky.
Materials and Methods

Farm selection and information gathering

A total of 25 Thoroughbred farms were recruited for participation in this study using various methods, including a presentation to the local farm managers club as well as emails and phone calls to local farm managers. The only requirements for inclusion were that the farm population of horses be 75% or greater Thoroughbreds and that the farm be located within a 25 mile radius of Lexington, Kentucky. Consent for inclusion of privately-owned horses was obtained from the farm managers, who were authorized to act on the behalf of any private owners.

After inclusion in the study, farms were asked to provide information regarding EPE cases in the preceding three years leading up to the study period. These data were used to classify farms as having presumptive cases of EPE, suspected cases of EPE, or no recent cases of EPE. Farms were considered to have had presumptive EPE cases if they had one or more weanlings with clinical signs compatible with EPE, hypoproteinemia and hypoalbuminemia, and either a concurrent positive fecal PCR or serum IPMA titer result (1:60 or greater) in the preceding three years. For the purpose of this study, compatible clinical signs for EPE were anorexia, fever, lethargy, depression, dependent edema, rapid weight loss, colic, and diarrhea. Farms were considered to have had suspected EPE cases if they had one or more weanlings with signs compatible with EPE, but neither a positive fecal PCR nor positive serum IPMA titer result (1:60 or greater). Farms were considered to have had no recent history of EPE if they experienced no horses with clinical signs of EPE within the preceding three years.
After the conclusion of the study, all participating farms were requested to complete a questionnaire regarding EPE cases on the farm during the study period. Based on this information, farms were classified for the study period into the three categories as above.

**Study Period**

The study began August 16\(^{th}\), 2010 and was to conclude January 5\(^{th}\), 2011. For some farms, an optional sample collection period from January 31\(^{st}\) to February 2\(^{nd}\), 2011 was included in the study due to a large increase in the number of seropositive samples in January. Samples were collected once during a three day period every four weeks from the farms.

**Horses**

While some farms housed non-Thoroughbred horses, only Thoroughbred horses were included in the study. All horses in the study were born during the 2010 foaling season and were weaned by November 2010. Farms were requested to provide a list of all 2010 horses present on the farm in early-August 2010. If a farm had fifteen or fewer 2010 horses at this time, all horses were included in the study. For those farms with 16 or more horses present in early-August, 15 horses were chosen randomly to participate (except for farm 19, from which all 16 horses were included in the study at the farm’s request). Randomization was achieved by assigning each horse a sequential number and utilizing a random number generator\(^{A}\) to select the 15 horses for inclusion in the study.

\(^{A}\) Microsoft Excel, Microsoft Corporation, Redman, WA
Each horse included in the study was assigned a unique three-digit identification number for ease of results tracking, as well as maintaining anonymity.

**Sample collection and handling**

Once every four weeks, a 10ml sample of whole blood was collected into individually labeled, sterile, red-top 10ml blood tubes via jugular venipuncture. Samples were submitted and held at centralized laboratories at 4°C for less than 24hrs before they were collected and transported to the Maxwell H. Gluck Equine Research Center at the University of Kentucky. Upon arrival, samples were immediately centrifuged at 800xg for 10 min. Serum was transferred to tubes labeled with the month and the unique three-digit code assigned to its horse and frozen at -20°C until analyzed.

For various reasons, including public sale, private sale, or a horse being returned to its owner, a number of blood samples were omitted from the study. As such, any horses that left the study prior to November were completely excluded from the data set. Horses remained in the study even if one or more samples were not collected after October.

Serum samples obtained from six *Lawsonia intracellularis*-challenged weanlings and 15 uninfected controls were included for the validation of the ELISA, with information regarding the challenge reported elsewhere.\(^\text{10}\)

**Lawsonia intracellularis-specific immunoperoxidase monolayer assay**

The IPMA method for determining *L. intracellularis*-specific antibody titers was performed as previously described.\(^\text{37}\)
Purification of *L. intracellularis*

Porcine-origin *L. intracellularis* was obtained from cell culture and purified using DEAE column chromatography which allowed the bacterium to be eluted as a whole organism. The presence of *L. intracellularis* in the eluate was verified microscopically using Gimenez stain, in addition to PCR for *L. intracellularis*, as previously described. Additionally, no other bacteria were detected following aerobic and anaerobic culture, as well as Gram staining. The pooled purified bacterial eluate with sodium azide was kept refrigerated (4°C). Quantification of the purified, whole-bacterium was performed using the BCA method.

*Lawsonia intracellularis*-specific enzyme-linked immunosorbent assay

The ELISA was based on a previously described method with respect to starting concentrations of reagents and a checkerboard titration scheme, as described elsewhere. Factors influencing background were minimized using previously described methods. From this, it was found that serum dilutions of 1:100 produced the most consistent results with minimal background. Further, based on this approach, the optimum concentration of antigen was 2.5µg/mL and the use of a polyvinyl alcohol block was superior to 5 or 10% skim milk.

The ELISA plates wells were coated with 2.5µg/ml of purified *L. intracellularis* in carbonate buffer, then covered with Parafilm-M and allowed to sit overnight at 4°C.

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B Immunolon 1b Flat Bottom Microtiter Plates, Thermo Scientific, Rochester, NY  
C Sigma Aldrich, St. Louis, MO
After plates incubated overnight, they were washed three times with phosphate-buffered saline with 0.05% Tween-20 (PBST) using an ELISA plate washer. The coated plates were then blotted dry before adding 200µl/well of blocking buffer (polyvinyl alcohol 1% (w/v) in distilled water) for two hours at room temperature. After blocking, the plates were washed three times, as above. Sera were diluted at 1:100 in blocking buffer and added (100 µl) to duplicate wells. Plates were then incubated at room temperature for 1 hour. Duplicate, serially diluted (1:60 through 1:3840) serum samples from a weanling exhibiting clinical signs compatible with EPE, including anorexia, weight loss, and dependent edema, were used to generate a standard curve. This weanling tested positive repeatedly at 1:1920 using the IPMA method. Negative control samples included serum from repeatedly L. intracellularis antibody-negative weanlings along with a duplicate sample of 100µl fetal equine serum diluted 1:100. After 1 hour incubation with diluted test and control sera, the plate was washed three times and 100µl of murine anti-horse IgG (1:4000) conjugated with horseradish peroxidase (HRP) was added to each well. The plate was then incubated in the dark for 1 hour at room temperature before being washed three times. To each well was then added 100µl of 3,3’,5,5’-tetramethylbenzidine (TMB) solution for two minutes before the reaction was stopped with TMB Stop solution. Absorbance at 450nm was read within 5 minutes using an ELISA.

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D Bennis Flexible Packaging, Neehah, WI
E Sigma Aldrich, St. Louis, MO
F MW 96/384, Beckman Coulter, Brea, CA
G Mowiol 6-98, Sigma Aldrich, St. Louis, MO
H Sigma Aldrich, St. Louis, MO
I SureBlue™ TMB Microwell Peroxidase Substrate, KPL, Gaithersburg, MD
J KP, Gaithersburg, MD
plate reader\(^K\). Results from the test sera were converted to an ELISA unit (EU) utilizing a linear trend line from the standard curve generated from each plate. A coefficient of determination (\(r^2\)) of \(\geq 0.90\) was required for the plate to be considered valid.\(^98\)

A positive cut-off of 55EU or greater was utilized based on non-challenged weanlings having an average of 33EU’s and a standard deviation of 7EU’s;\(^10\) these samples repeatedly tested negative via IPMA. By setting the cut off at 55EU’s, this value is three standard deviation units above the negative control averages and represents the upper limit of a 99% confidence interval.

**Evaluation of assay repeatability**

Twenty-four samples were selected for the ELISA repeatability test. These represented a variety of negative (<55EU), low (55-119EU), mid (120-239EU), and high (\(\geq 240\)EU) samples. For intra-assay repeatability, three replicates of each sample were performed on the same plate. For inter-assay repeatability, three replicates of each sample were run on duplicate plates on different days. Coefficient of variation (\(CV = SD/\text{mean} \times 100\%\)) of the three replicates from each test were evaluated. In addition, \(CV\)’s of the standard curve ODs from each plate was evaluated.

**Data analysis**

Farms 8 and 9 were ultimately considered as one farm data set since the horses from these two farms were combined into one population once the foals were weaned. Likewise, Farms 22 and 23 were combined into one data set for the same reason.

\(^K\) Benchmark Plus, Bio-Rad, Philadelphia, PA
One-way analysis of variance (ANOVA) (Holm-Sidak method\(^1\)) was used to evaluate differences in ELISA unit values between farms categorized based on their past EPE status. Post hoc t-tests were performed to evaluate the differences between groups. Chi-square analyses were used to assess seroprevalence results. Calculated P values \(\leq 0.05\) were considered to be statistically significant.

\(^1\) SigmaStat, SPSS Inc., Chicago, IL
Results

In order to validate our ELISA, serum samples of varying EU’s were run in triplicate with an overall intra-assay CV of 6.73 and inter-assay CV of 9.60. For the standard curve, CV’s ranged from 2.08 to 7.69 with a mean of 4.85 and the most dilute sample (1:3840 dilution) had the highest CV. Additionally, ELISA analysis of serial serum samples from *L. intracellularis*-challenged weanlings demonstrated seroconversion on or before day 20 post-challenge with all weanlings obtaining EU’s > 120 and a maximum of 746 EU while all non-challenged weanlings remained below 55 EU’s. Appendix I is provided to illustrate the relationship between OD and EU values for the standard curve, which is run on every ELISA plate and used to calculate EU values of unknown samples.

Provided in Figure 2.1 are all EU results for non-challenged controls and all positive values from experimentally challenged weanlings, as well as clinically affected field cases from the study period (EU values shown are those found at the initial time of presentation). Clinically EPE affected weanlings demonstrated similar, and in some cases greater, serum antibody levels against *L. intracellularis* when compared with experimentally challenged weanlings. Figure 2.2 includes EU results from all horses in the present study divided into two groups; study horses that failed to seroconvert (<55EU) during the study and study horses that seroconverted (≥55EU) at any point during the study period but failed to show clinical signs of EPE. A number of the non-clinical, seropositive horses exhibited very high EUs.
Figure 2.1: ELISA Unit (EU) values for non-challenged weanling controls, *L. intracellularis*-challenged weanlings, and clinical EPE cases. Dashed bar represents 55EU, the cut-off for positive samples.
Figure 2.2: All study time points from those weanlings that remained seronegative (<55EU) for the entire study and those that seroconverted at any point (≥55EU) during the study period. Each column for the study horses represents an individual horse. Dashed bar represents 55EU.
Overall, a total of 337 horses were included in the seroprevalence data set as they were present on the farms through at least November 2010. Of these 337 horses, a total of 229 horses, or 68.0% of the study population, tested positive (≥55 EU’s) for *L. intracellularis*-specific antibodies via the ELISA at one or more time points. The monthly percentage of previously seronegative horses that seroconverted during a given month and the accruing totals are shown in Figure 2.3. Overall there was a steady increase in the number of seropositive horses over time with marked increases in monthly seroconversion during October 2010 and January 2011. As shown in Figure 2.4, the majority of the seropositive animals for a given month had EU values in the range of 55-119EU. Beginning in October, one sees an increase in the frequency of high antibody levels (>120 EU) paralleling the increase in seroconversions noted in Figure 2.3.

Serological results from individual farms including past EPE status of the farm, EPE status during the study period, and calculated seroprevalence for *L. intracellularis* are shown in Table 2.1. A total of 8 farms experienced presumptive cases of EPE during the study period and no farms had suspected cases. Seven of these farms had already been classified as recently experiencing presumptive cases of EPE and one had previously experienced suspected cases of EPE. Farms without a recent history of EPE experienced no cases during the study period. Calculated farm-specific seroprevalences ranged from 14.3% to 100%. Figure 2.5 shows the average monthly seroconversion rate grouped by recent EPE history. While those farms with a recent history of EPE (presumptive or suspected) had some horses seroconvert each month, the farms with no recent history of EPE (none) had several months where zero or only one horse seroconverted. Table 2.2 shows the overall average seroprevalence as well as the average
positive and maximum EU values obtained from farms grouped by recent EPE status. Those farms with a presumptive history of EPE had significantly (p<0.001) higher average seroprevalences compared to those with suspected or no recent cases of EPE. Additionally, the average EU (p=0.079) and average maximum EU values (p=0.056) were found to be lower on farms with no recent history of EPE cases compared to the other groups. Moreover, the range of maximum EU values is much smaller for the farms with no recent EPE cases.
Figure 2.3: The percentage of previously seronegative horses that seroconverted (55EU or greater) during a given month as well as the cumulative number of seropositive horses.
Figure 2.4: The monthly distribution of positive ELISA results reported as ranges of EU’s.
Table 2.1. Seroprevalence by farm according to previous EPE history and EPE status during the study period. Farm-specific seroprevalences ranged from 14.3\% to 100\%.

<table>
<thead>
<tr>
<th>Farm</th>
<th>History of Previous EPE Cases</th>
<th>2010 Foaling Season EPE Cases</th>
<th>Total ELISA Positive Horses</th>
<th>Total # of Horses Sampled</th>
<th>Seroprevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No</td>
<td>No</td>
<td>11</td>
<td>15</td>
<td>73.3%</td>
</tr>
<tr>
<td>2</td>
<td>Suspected</td>
<td>No</td>
<td>3</td>
<td>15</td>
<td>20.0%</td>
</tr>
<tr>
<td>3</td>
<td>Presumptive</td>
<td>Presumptive</td>
<td>9</td>
<td>9</td>
<td>100%</td>
</tr>
<tr>
<td>4</td>
<td>Presumptive</td>
<td>No</td>
<td>7</td>
<td>12</td>
<td>58.3%</td>
</tr>
<tr>
<td>5</td>
<td>Presumptive</td>
<td>No</td>
<td>10</td>
<td>15</td>
<td>66.7%</td>
</tr>
<tr>
<td>6</td>
<td>Presumptive</td>
<td>Presumptive</td>
<td>9</td>
<td>10</td>
<td>90.0%</td>
</tr>
<tr>
<td>7</td>
<td>No</td>
<td>No</td>
<td>2</td>
<td>14</td>
<td>14.3%</td>
</tr>
<tr>
<td>8 &amp; 9</td>
<td>Presumptive</td>
<td>No</td>
<td>16</td>
<td>26</td>
<td>61.5%</td>
</tr>
<tr>
<td>10</td>
<td>Presumptive</td>
<td>Presumptive</td>
<td>9</td>
<td>15</td>
<td>60.0%</td>
</tr>
<tr>
<td>11</td>
<td>No</td>
<td>No</td>
<td>10</td>
<td>15</td>
<td>66.7%</td>
</tr>
<tr>
<td>12</td>
<td>Presumptive</td>
<td>No</td>
<td>5</td>
<td>9</td>
<td>55.6%</td>
</tr>
<tr>
<td>13</td>
<td>Suspected</td>
<td>Presumptive</td>
<td>13</td>
<td>15</td>
<td>86.7%</td>
</tr>
<tr>
<td>14</td>
<td>Presumptive</td>
<td>No</td>
<td>9</td>
<td>15</td>
<td>60.0%</td>
</tr>
<tr>
<td>15</td>
<td>Suspected</td>
<td>No</td>
<td>9</td>
<td>15</td>
<td>60.0%</td>
</tr>
<tr>
<td>16</td>
<td>Presumptive</td>
<td>Presumptive</td>
<td>11</td>
<td>11</td>
<td>100%</td>
</tr>
<tr>
<td>17</td>
<td>Presumptive</td>
<td>Presumptive</td>
<td>14</td>
<td>15</td>
<td>93.3%</td>
</tr>
<tr>
<td>18</td>
<td>Presumptive</td>
<td>No</td>
<td>15</td>
<td>15</td>
<td>100%</td>
</tr>
<tr>
<td>19</td>
<td>Presumptive</td>
<td>No</td>
<td>12</td>
<td>16</td>
<td>75.0%</td>
</tr>
<tr>
<td>20</td>
<td>No</td>
<td>No</td>
<td>2</td>
<td>7</td>
<td>28.6%</td>
</tr>
<tr>
<td>21</td>
<td>Presumptive</td>
<td>Presumptive</td>
<td>11</td>
<td>14</td>
<td>78.6%</td>
</tr>
<tr>
<td>22 &amp; 23</td>
<td>Presumptive</td>
<td>Presumptive</td>
<td>24</td>
<td>29</td>
<td>82.8%</td>
</tr>
<tr>
<td>24</td>
<td>No</td>
<td>No</td>
<td>10</td>
<td>15</td>
<td>66.7%</td>
</tr>
<tr>
<td>25</td>
<td>No</td>
<td>No</td>
<td>8</td>
<td>15</td>
<td>53.3%</td>
</tr>
</tbody>
</table>
Figure 2.5: Monthly seroconversions for groups (presumptive, suspected, none) of farms based on recent EPE history.
Table 2.2. Average seroprevalence, positive EU’s, and maximum EU’s grouped by EPE history.

<table>
<thead>
<tr>
<th>Previous EPE Cases</th>
<th>Average Seroprevalence</th>
<th>Average Positive EU</th>
<th>Average Max EU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presumptive</td>
<td>76.3%* (55.6-100%)</td>
<td>228.1 (85.5-378.9)</td>
<td>1683.4 (193.8-4312.3)</td>
</tr>
<tr>
<td>Suspected</td>
<td>55.6% (20-86.7%)</td>
<td>218.0 (59.7-326.2)</td>
<td>1604.6 (62.2-3319.4)</td>
</tr>
<tr>
<td>None</td>
<td>53.1% (14.3-73.3%)</td>
<td>123.9# (59.5-175.6)</td>
<td>453.9## (55.6-904.1)</td>
</tr>
</tbody>
</table>

*p<0.05; #p<0.079; ##p<0.056
Discussion

Here we described the use of an ELISA method to characterize the serological response of Thoroughbred weanlings to *L. intracellularis* on central Kentucky farms. This ELISA used column chromatography purified, whole *L. intracellularis*, thus optimizing reproducibility, as evidenced by an overall intra-assay CV of 6.73 and inter-assay CV of 9.60. Using this method, farm-specific seroprevalences ranged from 14.3 – 100%. As such, these results are comparable with a past screening study in the central Kentucky region which used the IPMA method and showed an EPE endemic farm to have a seroprevalence of approximately 60% while a non-endemic farm had a seroprevalence of 17%.19

Not surprisingly, seroprevalences corresponded well with the past history of EPE cases on the farm. Significant difference between groups based on recent EPE history was seen with respect to average seroprevalence. Additionally, farms with no recent clinical cases of EPE had both lower average EU and average maximum EU values for their positive samples. One possible explanation is that farms with no history of EPE cases likely had lower environmental burdens of *L. intracellularis* resulting in fewer horses being exposed to the bacterium (lower seroprevalence) and less antigenic stimulation per exposure (lower EU values). Evidence for this assertion is provided by the non-endemic farm from a previous study,19 which is also represented in the current study (Farm 15). While originally considered non-endemic, the farm has since reported suspected cases of EPE and was re-classified for the purpose of this study. Accordingly, the farm’s seroprevalence was found to be 60% with an average positive EU of 326.2 and
a maximum EU of 3319.4. Given that the epidemiology of EPE remains poorly defined, these data begin to indicate that relative burdens of *L. intracellularis* in the environment may explain why certain farms have an endemic problem with EPE.

Monitoring of weanlings by the enrolled farms ultimately identified 8 farms with presumptive cases of EPE during the study period from August 2010 through February 2011. Perhaps not surprisingly, these 8 farms represented only those with prior presumptive or suspected cases of EPE (7 and 1, respectively). While definitive antemortem diagnosis of EPE remains controversial, elevated *L. intracellularis*-specific antibody levels are commonly seen in clinically affected horses. These presumptive cases likewise exhibited an elevated antibody response, as was also detected in the *L. intracellularis*-challenged weanlings, using this ELISA.

While previous reports from the central Kentucky region suggest a single peak of exposure occurring during late-Fall/early-Winter, we provide new evidence of bimodal exposure occurring both in the fall and winter. These results correspond directly with anecdotal evidence indicating a large increase in the number of cases of EPE in this area during the months of January and February 2011. The reason for this increased incidence of EPE has yet to be elucidated. Possible explanations include changes in weather patterns, changes in management, and increased exposure to the unknown reservoir, which could include clinically or subclinically affected horses. With respect to these possibilities, it should be noted that the summer and fall of 2010 experienced less rainfall when compared with average amounts for the region. Additionally, there were lower daily temperatures starting in November with increased amounts of both rain and snow, beyond what is normal for central Kentucky during the early winter months.
While the ELISA method affords ease of use and reliability, the establishment of a cut-off value to separate negative and positive samples is critical. The current positive cut-off (≥55EU) utilized the average EU plus three standard deviations from non-challenged weanlings, reported elsewhere, and differentiated between known seropositive and seronegative horses. Twelve field cases evaluated using the ELISA demonstrated high levels of *L. intracellularis*-specific antibodies, equaling or surpassing those EUs detected in experimentally challenged weanlings. Also noted were several non-clinically affected weanlings with markedly elevated EU values. These could be indicative of a successful immunological response to infection or a subclinical case that was not detected by the farm; both situations were observed in the previous challenge study. As such, these findings provide further evidence that the new ELISA successfully detects antibodies to *L. intracellularis* in clinical and non-clinical horses.

There were two inherent limitations with the design of this study. First, there was an over representation of farms with presumptive or suspected cases of EPE in the data set. This likely reflected the fact that those farms with a prior history of what they consider a frustrating disease were more willing to participate and provide data. Additionally, the stigma associated with this disease in central Kentucky may have contributed to the large number of farms that declined to participate due to fears that their status with respect to *L. intracellularis* and EPE would become public. As such, the overall seroprevalence of 68% should not be considered representative of the equine population in this region without further, randomized sampling. Nevertheless, 20% of the horses surveyed came from farms with no recent history of EPE and we reported both farm-specific and group-specific seroprevalences. The other limitation and potential
source for error revolves around the possibility of cross-reaction of this ELISA with other bacteria. Phylogenetic studies have found that while *L. intracellularis* appears to be a member of unique pathogens, more recent work has shown *L. intracellularis* shares similarities with some rickettsial families. As such, potential cross-reactivity between other potential organisms cannot be excluded without further testing. However, work to validate the ELISA from which the method reported here was adapted failed to detect any cross-reactivity between *L. intracellularis* and related bacterial species.

By screening a large population of central Kentucky Thoroughbreds using a newly validated and equine-adapted ELISA, a high seroprevalence for *L. intracellularis*-specific antibodies was detected with variable farm-specific seroprevalences. Previous history of EPE on the farms was associated with significant differences in average seroprevalence indicating lower levels of exposure are present on farms with no history of EPE. Additionally, a bimodal, seasonal distribution of exposure was documented. The high farm-specific seroprevalences and bimodal distribution of exposure to *L. intracellularis* were unexpected and suggest that farms with a previous history of EPE remain at risk due to heightened exposure levels beyond early winter, as has been suggested previously.
CHAPTER THREE:

Determining the role of passively-acquired antibodies in infection and immunity to

*Lawsonia intracellularis*

**Summary**

Much like the other proliferative enteropathies caused by *Lawsonia intracellularis*, equine proliferative enteropathy typically affects weanlings and is rarely seen in older horses. Multiple hypotheses to explain age-based susceptibility to develop *L. intracellularis* include the decline of passively-acquired antibodies; this has never been examined in horses. A total of 369 mare and foal pairs from 15 central Kentucky Thoroughbred farms were ultimately used in this study. Serum samples were collected from mares and foals within 48 hours of parturition, and then monthly from foals through February of their yearling year. *L. intracellularis*-specific antibody levels were determined using an ELISA previously validated for use in horses. No effect of passively acquired antibodies on the occurrence of presumptive EPE or suspected infection with *L. intracellularis* was noted. In total, 5.3% and 6.3% of seropositive horses developed presumptive EPE or suspected infection with the bacterium, respectively. Using multiple logistic regression, it was found that colts were at a significantly greater risk than fillies of developing presumptive EPE or a combination of presumptive EPE or suspected infection with the bacterium. Additionally, those horses that were weaned in September or later months were at a significantly lower risk of developing a combination of either presumptive EPE or suspected infection. A geographical difference in the timing of exposure to the bacterium was identified, although no reason for this difference was
elucidated. This is the first study to show that passively-acquired antibodies to *L. intracellularis* do not have an effect on the occurrence of EPE or infection with the bacterium. A number of novel findings, including identification of the disease rate amongst exposed horses, warrant additional work as they may help to identify potential risk factors for *L. intracellularis* exposure and/or the reservoir host(s) of the bacterium.
Lawsonia intracellularis is an obligate, intracellular, gram-negative curved rod that invades intestinal crypt cells, primarily in the distal small intestine, and causes proliferation of the intestinal epithelium. This proliferation leads to thickening of the intestinal mucosa and the clinical signs associated with proliferative enteropathy. While initially described as a pathogen of pigs, L. intracellularis is now viewed as an emerging cause of proliferative enteropathy in a variety of mammalian species, including horses. In the horse, L. intracellularis infection results in equine proliferative enteropathy (EPE) which has been reported worldwide. Affected horses suffer weight loss and some ultimately succumb to the disease despite aggressive treatment.

Equine proliferative enteropathy typically affects weanlings and is rarely seen in older horses. Diagnosis and preventative measures for EPE are poorly defined while clinical signs in infected horses are non-specific and include anorexia, fever, lethargy, depression, and dependent edema, with colic and diarrhea infrequently seen. Ultrasonographic thickening of the small intestinal wall (>3mm) in weanlings, although not reliably seen, is highly suggestive of EPE. Antemortem diagnosis of EPE revolves around clinical signs, ultrasonographic findings, and evidence of hypoproteinemia and hypoalbuminemia, as well as results from commercially available diagnostic tests. The presence of hypoalbuminemia may be one of the best, most rapid, and inexpensive tests for EPE in weanlings with other compatible clinical signs. Commercially available antemortem tests include fecal polymerase chain reaction and serum serology using the
immunoperoxidase monolayer assay (IPMA). While definitive diagnosis of EPE can only be made at necropsy and relies upon the immunohistochemical (IHC) detection of the organism in characteristic intestinal lesions, IPMA has been widely used as an antemortem diagnostic tool for cases of EPE. The IPMA utilizes a commercially available antigen coated plate and a HRP-detection method which requires only an inverted microscope for analysis; however, this method is highly subjective and dependent on operator experience. Enzyme-linked immunosorbent assays (ELISA) are a more reliable and sensitive test that are readily automated and free of operator interpretation. ELISAs are frequently used for the detection of antibodies to specific pathogens and we have recently validated an \textit{L. intracellularis}-specific ELISA for use in horses.

Multiple hypotheses for the predilection of \textit{L. intracellularis} for weanlings exist, including the stress of weaning and the decline of maternal antibodies. While the nature of the immune response to \textit{L. intracellularis} remains ill defined, IgG antibodies have been implicated in protection against both porcine and equine PE. Past work has shown that passively acquired antibodies specific to \textit{L. intracellularis} were detectable by the IPMA method for 1-3 months post-parturition. Given the aforementioned limitations of the IPMA method, this approach may fail to completely define the true duration and quantity of the passively acquired antibodies. Our previous work has shown the incidence of \textit{L. intracellularis}-seropositive weanlings in the central Kentucky region approaches 68%, with some farms experiencing 100% weanling seroconversion. This high incidence provides the opportunity to follow groups of foals with varying levels of
passively-acquired antibodies against *L. intracellularis* in order to determine the role these antibodies play in protection from infection and EPE.

Very little is known regarding control of *L. intracellularis* exposure or other preventative measures for EPE as risk factors for this disease remain unknown. While there is interest in the use of a vaccine to prevent this disease,\textsuperscript{88} no product is currently licensed for use in horses nor are the protective immunological mechanisms against EPE defined. If passively-acquired antibodies are protective against the development of EPE, new preventative measures such as maternal vaccination or administration of *L. intracellularis* hyperimmune plasma to foals may be warranted as a means of decreasing the incidence of clinical disease. Here, we have examined the role that passively-acquired antibodies to *L. intracellularis* play in immunity and infection using a large scale, prospective field study.
Materials and Methods

Study Farms and Horses

Fifteen Thoroughbred farms in central Kentucky were enrolled in this study which took place from January 2012 through February 2013. The only requirements for inclusion were that the farm population of horses be 75% or greater Thoroughbreds and that the farm be located within a 25 mile radius of Lexington, Kentucky. Preference for inclusion was given to those farms with a documented history of EPE. Approval to conduct this study was obtained from the University of Kentucky's Institutional Animal Care and Use Committee and forms documenting informed consent for inclusion of privately-owned horses was obtained from the farm managers or farm veterinarians, whom were authorized to act on the behalf of private owners.

Immediately prior to the study's initiation, one farm elected to withdraw its participation (Farm 10) while Farm 16 was added as a portion of the foals originally located on Farm 4 were relocated to Farm 16. Farms 13 and 14 were ultimately regarded as the same farm as they were part of a complex of farms and, after weaning, study horses were comingled together.

Sample Collection, Processing, and Storage

Within 48hrs of parturition, whole blood samples (7 mls of peripheral blood collected via aseptic venipuncture from the jugular vein) were collected from the mare and newborn foal and allowed to settle in a refrigerator at 4°C. Samples were individually labeled with regard to the mare/foal’s name, as well as the date the sample
was collected. If foals received hyperimmune plasma (i.e. *Rhodococcus equi*) or high IgG plasma for failure of passive transfer, the farms were requested to obtain the blood sample after plasma had been administered in an effort to accurately represent *L. intracellularis*-specific antibodies obtained from both colostrum and the hyperimmune plasma. Treatment with these products was noted for later analysis.

After the initial collection time point, samples were subsequently collected approximately every four weeks only from the foals starting at 4 weeks of age through the middle of July, as described above. Samples were stored at 4°C on the farm until returned to the Maxwell H. Gluck Equine Research Center at the University of Kentucky for centrifugation (800g x 10min) followed by freezing of serum at -20°C until all samples collected through July from mare/foal pairs could be run concurrently (see below).

Starting at the end of July, all study horses (ranging in age from two months to six months) had blood samples collected during the same three day period every four weeks to ensure that all horses, irrespective of the farm, were sampled during the same time period. Samples were submitted by the farms and held at centralized laboratories at 4°C for less than 24hrs before they were collected and transported to the Gluck Equine Research Center. Upon arrival, samples were immediately centrifuged at 800xg for 10 min and held at 4°C for no more than 4 days before all samples were analyzed as below.

**Detection of *L. intracellularis*-specific antibodies**

Serological status in regards to *L. intracellularis* was determined using the ELISA method as previously described (see also Chapter 2),$^{20}$ except that plates were blocked
with polyvinyl alcohol 1% (w/v) in distilled water for one hour instead of two hours. Results from the tested sera were converted to an ELISA unit (EU) utilizing a linear regression line from a standard curve generated on each plate. A positive cut-off of 55EU or greater was utilized based on previous work.10

**Data Obtained from Farms**

Farms were asked to provide a range of information about each study horse, including sex, date of birth, post-foaling IgG result, whether hyperimmune or high IgG plasma was administered, whether the foal was treated for *Rhodococcus equi*, weaning date, and any other pertinent comments.

**Total Protein and Albumin Determination**

Total protein levels were determined via refractometry for all seropositive samples. For those samples with total protein levels below 5.3 g/dL, albumin levels were obtained using an ACE Alera (Alfa-Wassermann, West Caldwell, NJ) at the University of Kentucky’s Veterinary Diagnostic Laboratory. Albumin levels below 3.2 g/dL were considered to be low. During each sampling period from September to February (those periods in which seropositive horses were present), between 12-20 seronegative samples were randomly chosen as negative controls for total protein levels while a subset of those samples were used as albumin negative controls, all for comparison to total protein and albumin results from the other groups (see below). Additionally, forty seropositive samples with total protein levels at or above 5.3 g/dL were randomly chosen for albumin determination and comparison between the other groups.
Classification of Study Horse EPE Status

At the end of the study, horses were placed into one of four categories to describe their *L. intracellularis*/EPE status following the typical *L. intracellularis* "season" of October to February: presumptive EPE, suspect *L. intracellularis*-infected, seropositive, or non-exposed. Based on our previous work,\textsuperscript{20} presumptive EPE affected horses were those that experienced concurrent clinical signs compatible with EPE, moderate to severe hypoproteinemia (<4.5 g/dL) and hypoalbuminemia (<2.5 g/dL), and a positive *L. intracellularis*-specific ELISA result. For the purpose of this study, compatible clinical signs for EPE were anorexia, fever, lethargy, depression, dependent edema, rapid weight loss, colic, and diarrhea. Given that definitive diagnosis of EPE can only be made at necropsy and relies upon the immunohistochemical (IHC) detection of the organism in characteristic intestinal lesions,\textsuperscript{31} all clinical diagnoses of EPE were considered presumptive. Suspect *L. intracellularis*-infected horses were those with mild hypoproteinemia (4.5-5.2 g/dL) and hypoalbuminemia (2.5-3.1 g/dL) plus a positive *L. intracellularis*-specific ELISA result without concurrent clinical signs compatible with EPE. *L. intracellularis*-infected horses can experience a transient decrease in total protein and albumin levels along with a positive *L. intracellularis*-specific ELISA result in the absence of other clinical signs.\textsuperscript{10,39} However, since there was no demonstration of the presence of *L. intracellularis* (either via fecal PCR or necropsy sampling), these cases are referred to as "suspect *L. intracellularis*-infected". Seropositive horses were those with only a positive *L. intracellularis*-specific ELISA result (excluding passively-acquired *L. intracellularis* antibodies) and no other indication of *L. intracellularis*
infection. Horses with no detectable *L. intracellularis*-specific antibodies for the entirety of the study (excluding passively-acquired antibodies) were classified as seronegative.

**Data Analysis**

Logistic regression analysis was used to identify significant effects of different variables on EPE/*L. intracellularis* status including, but not limited to initial EU value, use of any hyperimmune plasma, *Rhodococcus equi* infection, and weaning month. One-way analysis of variance (ANOVA) was used to detect significant differences between seropositive groups (presumptive EPE, suspected infected, and seropositive-only) with respect to age at weaning, age at seroconversion, and time from weaning to seroconversion, as well as differences in the age at weaning between seronegative and seropositive horses. One-way ANOVA was also used to detect total protein and albumin differences between classification groups. Additionally, survival analysis (Kaplan-Meier) was used to determine the effect of initial serological status of the foal on time to seroconversion. To determine the effect of passively-acquired antibodies on seroconversion rates in the study population, analysis was performed using proportional hazards regression analysis (Cox PH regression) with maternal and initial foal antibody EU values as variables and seroconversion as the event. Calculated P values ≤0.05 were considered to be statistically significant.
Results

A total of 453 mare and foal pairs were initially enrolled in the study, from which initial, post-partum samples were collected. From these pairs, 253 mares were seronegative (<55EU) and 200 (44.2%) were seropositive post-parturition (55-1297 EU) while 281 foals were seronegative and 172 (38.0%) were seropositive (55-2552.6 EU). Average positive EU values for mare and foal post-partum samples were 135.5 and 164.7 EU, respectively. Interestingly, 41 foals were seropositive at parturition while samples from their mares were seronegative; these values ranged from 55.2 EU to 155.7 EU. Of these 41 foals, 35 had received Rhodococcus equi hyperimmune plasma. A variety of R. equi hyperimmune plasmas given to study foals were tested for L. intracellularis-specific antibodies and found to vary greatly; an average EU value of 130 EU was noted, although samples ranged from negative (<55EU) to a maximum of 919 EU. Passively-acquired antibodies to L. intracellularis above the 55EU cutoff were not detected in foals beyond the third collection (2 months of age), although two foals from the same farm (Farm 14) appeared to seroconvert during April and May (data not shown) as their mare and prior samples had been negative.

From the initial 453 mare and foal pairs, a total of 369 study horses were present through at least October 2012 and included in the final statistical analysis. Information regarding individual farms with respect to the number of study horses present through at least October, the number of presumptive EPE cases and suspect infected horses, and farm-specific seroprevalence from August 2012 to February 2013 is included in Table 3.1. Also included in Table 3.1, if available, is the previous year’s seroprevalence for
Table 3.1: Pertinent information regarding individual farms including the study-period seroprevalence and previous year's seroprevalence, if available.

<table>
<thead>
<tr>
<th>Farm Number</th>
<th>Initial # of Study Horses</th>
<th># of Study Horses Present Through At Least October</th>
<th># of Presumptive EPE Cases</th>
<th># of Suspect, <em>L. intracellularis</em> Cases</th>
<th>Study Period Seroprevalence</th>
<th>Previous Year's Seroprevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>50.0%</td>
<td>60.0%</td>
</tr>
<tr>
<td>2</td>
<td>128</td>
<td>92</td>
<td>4</td>
<td>2</td>
<td>55.4%</td>
<td>56.7%</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>11.8%</td>
<td>27.8%</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>19</td>
<td>0</td>
<td>2</td>
<td>68.0%</td>
<td>71.9%</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>70.0%</td>
<td>20.0%</td>
</tr>
<tr>
<td>6</td>
<td>39</td>
<td>38</td>
<td>2</td>
<td>4</td>
<td>73.7%</td>
<td>23.6%</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>25.0%</td>
<td>75.0%</td>
</tr>
<tr>
<td>8</td>
<td>28</td>
<td>28</td>
<td>1</td>
<td>0</td>
<td>52.9%</td>
<td>28.1%</td>
</tr>
<tr>
<td>9</td>
<td>17</td>
<td>16</td>
<td>2</td>
<td>1</td>
<td>77.8%</td>
<td>72.2%</td>
</tr>
<tr>
<td>11</td>
<td>10</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>36.4%</td>
<td>57.1%</td>
</tr>
<tr>
<td>12</td>
<td>11</td>
<td>10</td>
<td>0</td>
<td>1</td>
<td>80.0%</td>
<td>33.3%</td>
</tr>
<tr>
<td>13 &amp; 14</td>
<td>109</td>
<td>89</td>
<td>1</td>
<td>0</td>
<td>36.4%</td>
<td>N/A</td>
</tr>
<tr>
<td>15</td>
<td>21</td>
<td>18</td>
<td>0</td>
<td>2</td>
<td>72.7%</td>
<td>N/A</td>
</tr>
<tr>
<td>16</td>
<td>16</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>75.0%</td>
<td>N/A</td>
</tr>
</tbody>
</table>

1 Samples collected early October to January 2012  
2 Samples collected late October to January 2012  
3 Samples collected late November to January 2012  
4 One presumptive EPE case occurred in March 2013 and another in April- both were included in the data set  
5 The presumptive case of EPE was euthanized and a necropsy could not be performed in a timely manner  
6 Farm had at least one case of presumptive EPE in a non-study horse
each farm, which was collected with methods similar to as above from September 2011 to January 2012.

The combined seroprevalence for all study horses was 56.4% (208 seropositive horses) while farm-specific seroprevalences ranged from 11.8% to 80% (Table 3.1). Figure 3.1 shows the monthly percentage of previously seronegative horses that seroconverted during a given month where peak seroconversion rates were noted in October and January. Additionally, the cumulative percentage of all seropositive study horses during a given month is also included on Figure 3.1. Seroconversion data from each of the previous three years (2010-11, 2011-12, and 2012-13), using approximate dates for comparison, are provided in Figure 3.2. Figure 3.3 shows a representation of first-time seroconversions from the current study by each farm's geographic region and Figure 3.4 is provided to demonstrate the respective geographic regions.

During the study, a total of 11 horses developed presumptive EPE (3.0% of all study horses and 5.3% of seropositive horses) while an additional 13 developed suspected \textit{L. intracellularis} infection (3.5% of all study horses and 6.3% of seropositive horses). Two horses from Farm 2 developed presumptive EPE outside of the scheduled serum screening period (one in March 2013 and another in April 2013) but were included in the presumptive EPE data set as they met the criteria for inclusion. Tables 3.2 and 3.3 contain signalment and other pertinent information for the presumptive EPE and suspect infected horses, respectively.

Total protein and albumin values for the four classification groups were analyzed using one-way ANOVA with significant differences noted for total protein and albumin
Figure 3.1: Monthly seroconversion rate and the monthly total percentage of seropositive horses.
Figure 3.2: Regional seroconversion data from 2010-11, 2011-12, and 2012-13. Dates are approximate.
Figure 3.3: 2012-13 seroconversion data based on each farm's geographical location.
Figure 3.4: Approximations of the geographical categories used in Figure 3.3. The map has been modified from Google Earth.
Table 3.2: Signalment and other pertinent information from study horses with presumptive EPE.

<table>
<thead>
<tr>
<th>Horse ID</th>
<th>Farm</th>
<th>DOB</th>
<th>Sex</th>
<th>IgG</th>
<th>High-IgG Plasma</th>
<th>R. equi Plasma</th>
<th>R. equi Treatment</th>
<th>Weaning Month</th>
<th>Mare EU Value</th>
<th>Initial Foal EU Value</th>
<th>EPE Date</th>
<th>Total Protein (g/dL)</th>
<th>Albumin (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>075</td>
<td>2</td>
<td>3/10/12</td>
<td>Colt</td>
<td>&gt;800</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>August</td>
<td>Neg</td>
<td>Neg</td>
<td>12/29/12</td>
<td>3.6</td>
<td>2.5*</td>
</tr>
<tr>
<td>109</td>
<td>2</td>
<td>2/25/12</td>
<td>Colt</td>
<td>&gt;800</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>July</td>
<td>83</td>
<td>82.6</td>
<td>3/18/13</td>
<td>3</td>
<td>1.6</td>
</tr>
<tr>
<td>111</td>
<td>2</td>
<td>3/29/12</td>
<td>Filly</td>
<td>&gt;800</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>August</td>
<td>Neg</td>
<td>Neg</td>
<td>2/8/13</td>
<td>3.8</td>
<td>2.1</td>
</tr>
<tr>
<td>137</td>
<td>2</td>
<td>3/24/12</td>
<td>Colt</td>
<td>&gt;800</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>August</td>
<td>Neg</td>
<td>Neg</td>
<td>4/3/13</td>
<td>3</td>
<td>1.2</td>
</tr>
<tr>
<td>249</td>
<td>6</td>
<td>4/9/12</td>
<td>Colt</td>
<td>Not Reported</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Sept</td>
<td>Neg</td>
<td>Neg</td>
<td>10/16/12</td>
<td>3.2</td>
<td>2.3</td>
</tr>
<tr>
<td>253</td>
<td>6</td>
<td>3/1/12</td>
<td>Filly</td>
<td>&gt;800</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>July</td>
<td>Neg</td>
<td>Neg</td>
<td>10/15/12</td>
<td>2.6</td>
<td>1.4</td>
</tr>
<tr>
<td>359</td>
<td>8</td>
<td>2/8/12</td>
<td>Colt</td>
<td>&gt;800</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>July</td>
<td>92.2</td>
<td>309</td>
<td>12/31/12</td>
<td>2.5</td>
<td>1.1</td>
</tr>
<tr>
<td>366</td>
<td>9</td>
<td>4/12/12</td>
<td>Colt</td>
<td>&gt;800</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>August</td>
<td>Neg</td>
<td>Neg</td>
<td>10/8/12</td>
<td>3.7</td>
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<td>371</td>
<td>9</td>
<td>5/3/12</td>
<td>Colt</td>
<td>&gt;800</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>August</td>
<td>Neg</td>
<td>Neg</td>
<td>10/21/12</td>
<td>3.8</td>
<td>2</td>
</tr>
<tr>
<td>596</td>
<td>11</td>
<td>2/3/12</td>
<td>Colt</td>
<td>&gt;800</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>July</td>
<td>89.4</td>
<td>59.6</td>
<td>10/15/12</td>
<td>3.6</td>
<td>1.7</td>
</tr>
<tr>
<td>505</td>
<td>14</td>
<td>5/27/12</td>
<td>Colt</td>
<td>400-800</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Sept</td>
<td>Neg</td>
<td>Neg</td>
<td>1/27/12</td>
<td>2.6</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*No albumin result was reported immediately after clinical signs were noted. The value reported here was recorded 2 weeks after the onset of clinical signs and not included in data analysis.
Table 3.3: Signalment and other pertinent information from study horses with suspected *L. intracellularis* infection.

<table>
<thead>
<tr>
<th>Horse ID</th>
<th>Farm</th>
<th>DOB</th>
<th>Sex</th>
<th>IgG</th>
<th>High-IgG</th>
<th>R. equi Plasma</th>
<th>R. equi Treatment</th>
<th>Weaning Month</th>
<th>Mare EU Value</th>
<th>Initial Foal EU Value</th>
<th>Date of Diagnosis</th>
<th>Total Protein (g/dL)</th>
<th>Albumin (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>060</td>
<td>2</td>
<td>4/17/12</td>
<td>Colt</td>
<td>&gt;800</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>October</td>
<td>95.3</td>
<td>Neg</td>
<td>1/14/13</td>
<td>4.6</td>
<td>2.8</td>
</tr>
<tr>
<td>101</td>
<td>2</td>
<td>1/16/12</td>
<td>Colt</td>
<td>&gt;800</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>June</td>
<td>96.2</td>
<td>146</td>
<td>2/11/13</td>
<td>5</td>
<td>2.9</td>
</tr>
<tr>
<td>225</td>
<td>4</td>
<td>2/13/12</td>
<td>Filly</td>
<td>400-800</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>August</td>
<td>58.5</td>
<td>Neg</td>
<td>12/19/12</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>230</td>
<td>4</td>
<td>2/14/12</td>
<td>Colt</td>
<td>&gt;800</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>June</td>
<td>59.8</td>
<td>67.7</td>
<td>12/19/12</td>
<td>5.2</td>
<td>3.1</td>
</tr>
<tr>
<td>255</td>
<td>6</td>
<td>3/11/12</td>
<td>Filly</td>
<td>&gt;800</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>August</td>
<td>Neg</td>
<td>Neg</td>
<td>9/25/12</td>
<td>4.6</td>
<td>2.8</td>
</tr>
<tr>
<td>265</td>
<td>6</td>
<td>2/28/12</td>
<td>Filly</td>
<td>&gt;800</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>July</td>
<td>Neg</td>
<td>Neg</td>
<td>10/23/12</td>
<td>5</td>
<td>2.9</td>
</tr>
<tr>
<td>276</td>
<td>6</td>
<td>2/8/12</td>
<td>Colt</td>
<td>&gt;800</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>June</td>
<td>Neg</td>
<td>Neg</td>
<td>10/23/12</td>
<td>5.2</td>
<td>2.9</td>
</tr>
<tr>
<td>282</td>
<td>6</td>
<td>4/10/12</td>
<td>Colt</td>
<td>&gt;800</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>August</td>
<td>61.7</td>
<td>159</td>
<td>12/19/12</td>
<td>5.2</td>
<td>2.9</td>
</tr>
<tr>
<td>375</td>
<td>9</td>
<td>2/8/12</td>
<td>Colt</td>
<td>&gt;800</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>May</td>
<td>Neg</td>
<td>Neg</td>
<td>10/23/12</td>
<td>5.1</td>
<td>2.7</td>
</tr>
<tr>
<td>399</td>
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<td>3/9/12</td>
<td>Filly</td>
<td>&gt;800</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>August</td>
<td>Neg</td>
<td>Neg</td>
<td>10/23/12</td>
<td>5</td>
<td>3.1</td>
</tr>
<tr>
<td>420</td>
<td>12</td>
<td>3/29/12</td>
<td>Colt</td>
<td>Not Reported</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>August</td>
<td>Neg</td>
<td>Neg</td>
<td>10/23/12</td>
<td>4.8</td>
<td>2.3</td>
</tr>
<tr>
<td>588</td>
<td>15</td>
<td>2/6/12</td>
<td>Colt</td>
<td>&gt;800</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>July</td>
<td>106</td>
<td>123</td>
<td>10/23/12</td>
<td>4.8</td>
<td>2.8</td>
</tr>
<tr>
<td>589</td>
<td>15</td>
<td>2/14/12</td>
<td>Colt</td>
<td>400-800</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>July</td>
<td>Neg</td>
<td>Neg</td>
<td>10/23/12</td>
<td>5</td>
<td>3.1</td>
</tr>
</tbody>
</table>
between all of the groups except seronegative and seropositive (Figures 3.5 and 3.6). Table 3.4 provides values for each group, including minimum and maximum values observed, as well as the 95% confidence interval for each group. While there was an overlap in the confidence intervals between suspect infected and seronegative horses, there were no seronegative horses that had values for both total protein and albumin outside the calculated confidence interval, nor were there any outside the reference range used for group classification (TP <5.3 g/dL and albumin <3.2 g/dL).

One-way ANOVA was used to examine the different seropositive groups (presumptive EPE, suspect infected, and seropositive-only) for differences in ages at weaning and seroconversion, as well as the amount of time elapsed between weaning to seroconversion. Table 3.5 has values for each group, as well as an average of the three groups with respect to time from weaning to seroconversion and age at seroconversion. No significant differences were noted between the three groups; however, there was a significant difference (p<0.001) between the mean age at weaning between seronegative and all seropositive horses (152 days vs. 144 days, respectively). The earliest detectable exposure to *L. intracellularis* occurred 8 days prior to weaning and the latest at 258 days after weaning while the overall average of time from weaning to seroconversion between the three groups was 125 days. The youngest horse to seroconvert during the fall and winter months was 150 days old and the oldest 392 days old while the average age at seroconversion was 178 days (approximately 6.5 months).

The effect of passively-acquired antibodies on time to seroconversion in study horses was examined using a Kaplan-Meier curve (Figure 3.7) and no differences were noted between those foals that were seropositive versus seronegative immediately after
Figure 3.5: Total protein data from study horses based on their categorization.
Figure 3.6: Albumin data from study horses based on their categorization.
Table 3.4: Minimum, maximum, and calculated 95% confidence intervals for total protein and albumin based on study horse categorization. Units are g/dL.

<table>
<thead>
<tr>
<th>TOTAL PROTEIN</th>
<th>Reported</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minimum</td>
<td>Maximum</td>
</tr>
<tr>
<td>Presumptive EPE</td>
<td>2.5</td>
<td>4</td>
</tr>
<tr>
<td>Suspect <em>L</em> Infection</td>
<td>4.6</td>
<td>5.2</td>
</tr>
<tr>
<td>Seronegative</td>
<td>5.2</td>
<td>7</td>
</tr>
<tr>
<td>Seropositive</td>
<td>5.2</td>
<td>6.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ALBUMIN</th>
<th>Reported</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minimum</td>
<td>Maximum</td>
</tr>
<tr>
<td>Presumptive EPE</td>
<td>1.1</td>
<td>2.3</td>
</tr>
<tr>
<td>Suspect <em>L</em> Infection</td>
<td>2.3</td>
<td>3.1</td>
</tr>
<tr>
<td>Seronegative</td>
<td>3.1</td>
<td>3.8</td>
</tr>
<tr>
<td>Seropositive</td>
<td>2.9</td>
<td>3.8</td>
</tr>
</tbody>
</table>
Table 3.5: Ages (in days) for the various horse classification categories.

<table>
<thead>
<tr>
<th></th>
<th>Presumptive EPE</th>
<th>Suspect, <em>L. intracellularis</em> infection</th>
<th>Seropositive Only¹</th>
<th>Average of All Seropositive Horses</th>
<th>Seronegative Horses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Age at Weaning</td>
<td>144</td>
<td>145</td>
<td>144</td>
<td>144</td>
<td>152</td>
</tr>
<tr>
<td>Median Age at Weaning</td>
<td>149</td>
<td>147</td>
<td>144</td>
<td>145</td>
<td>152</td>
</tr>
<tr>
<td>Standard Deviation Age at Weaning</td>
<td>19</td>
<td>23</td>
<td>21</td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td>Minimum Age at Weaning</td>
<td>103</td>
<td>83</td>
<td>59</td>
<td>59</td>
<td>109</td>
</tr>
<tr>
<td>Maximum Age at Weaning</td>
<td>164</td>
<td>177</td>
<td>213</td>
<td>213</td>
<td>213</td>
</tr>
<tr>
<td>Average Time from Weaning to Seroconversion</td>
<td>134</td>
<td>118</td>
<td>122</td>
<td>125</td>
<td>N/A</td>
</tr>
<tr>
<td>Median Time from Weaning to Seroconversion</td>
<td>144</td>
<td>102</td>
<td>118</td>
<td>122</td>
<td>N/A</td>
</tr>
<tr>
<td>Standard Deviation Time from Weaning to Seroconversion</td>
<td>67</td>
<td>52</td>
<td>49</td>
<td>57</td>
<td>N/A</td>
</tr>
<tr>
<td>Minimum Time from Weaning to Seroconversion</td>
<td>32</td>
<td>54</td>
<td>-8</td>
<td>-8</td>
<td>N/A</td>
</tr>
<tr>
<td>Maximum Time from Weaning to Seroconversion</td>
<td>234</td>
<td>240</td>
<td>258</td>
<td>258</td>
<td>N/A</td>
</tr>
<tr>
<td>Average Age at Seroconversion</td>
<td>277</td>
<td>263</td>
<td>269</td>
<td>266</td>
<td>N/A</td>
</tr>
<tr>
<td>Median Age at Seroconversion</td>
<td>262</td>
<td>257</td>
<td>267</td>
<td>263</td>
<td>N/A</td>
</tr>
<tr>
<td>Standard Deviation Age at Seroconversion</td>
<td>75</td>
<td>50</td>
<td>52</td>
<td>56</td>
<td>N/A</td>
</tr>
<tr>
<td>Minimum Age at Seroconversion</td>
<td>172</td>
<td>197</td>
<td>150</td>
<td>150</td>
<td>N/A</td>
</tr>
<tr>
<td>Maximum Age at Seroconversion</td>
<td>387</td>
<td>392</td>
<td>392</td>
<td>392</td>
<td>N/A</td>
</tr>
</tbody>
</table>

¹ Does not include two foals from Farm 14 that may have been exposed during April and May
Figure 3.7: Survival analysis of seroconversion time-point based on the foal's post-partum antibody status. Time-points are 1= July, 2=August, 3=September, 4=October, 5=November, 6=December, 7=January, and 8=February.
birth. Further, Cox proportional hazards analysis revealed no significant effect of either post-partum maternal or foal antibody status on the timing of seroconversion (data not shown).

Multiple logistic regression analysis of data obtained for each study horse was performed using the occurrence of presumptive EPE, suspected *L. intracellularis* infection, and a combination of presumptive EPE or suspected *L. intracellularis* infection as the dependent variables. P-value results and odds ratios calculated from the analysis are included in Table 3.6. Of the parameters analyzed, colts were at a significantly greater risk for the development of presumptive EPE (p=0.038, odds ratio 5.299) or a combination of presumptive EPE or suspected infection (p=0.006, odds ratio 3.861). There was a trend (p=0.086, odds ratio 2.955) for colts to be at greater risk of developing suspect *L. intracellularis* infection. Additionally, those horses that were weaned in September or later months were at a significantly lower risk of developing a combination of either presumptive EPE or suspected infection (p=0.047, odds ratio 0.281). Thirty-eight study foals were treated for suspected *Rhodococcus equi* infection during the study, and while the use of hyperimmune *R. equi* plasma did not affect the occurrence of EPE, there was a trend for *R. equi* treatment to have a positive effect on the occurrence of suspected *L. intracellularis* infection (p=0.076, odds ratio 3.69).
Table 3.6: Odds ratios calculated from multiple logistic regression analysis. The parameter in () was analyzed as the positive response.

<table>
<thead>
<tr>
<th>LOGISTIC REGRESSION ODDS RATIO</th>
<th>Presumptive EPE</th>
<th>Suspect, <em>L. intracellularis</em> infection</th>
<th>Both Presumptive EPE &amp; Suspected <em>L. intracellularis</em> Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (Colt)</td>
<td>5.299**</td>
<td>2.955*</td>
<td>3.861***</td>
</tr>
<tr>
<td><em>R. equi</em> Plasma (Yes)</td>
<td>0.466</td>
<td>0.804</td>
<td>0.602</td>
</tr>
<tr>
<td>High IgG Plasma (Yes)</td>
<td>2.298</td>
<td>0</td>
<td>0.89</td>
</tr>
<tr>
<td><em>R. equi</em> Treatment (Yes)</td>
<td>0</td>
<td>3.69*</td>
<td>1.393</td>
</tr>
<tr>
<td>Weaning Month (Sep-Nov vs. May-Aug)</td>
<td>0.444</td>
<td>0.168*</td>
<td>0.281**</td>
</tr>
<tr>
<td>Mare EU value at Parturition (Positive)</td>
<td>0.608</td>
<td>2.067</td>
<td>1.159</td>
</tr>
<tr>
<td>Foal EU value at Parturition (Positive)</td>
<td>0.666</td>
<td>0.402</td>
<td>0.507</td>
</tr>
</tbody>
</table>

* p<0.1, **p<0.05, ***p<0.01
Discussion

Recognition of a necrotizing form of equine proliferative enteropathy,\(^9\) as well as previous work suggesting a significant economic impact of EPE,\(^{11}\) emphasizes the importance of understanding the principles of infection and immunity to \textit{L. intracellularis} in horses. Despite the decline of passively-acquired antibodies (especially maternally derived antibodies) as a possible explanation for the susceptibility of weanlings to \textit{L. intracellularis} infection, very little work in any species has examined this possibility. Here, relying on a large field study with naturally occurring cases, we have examined the effect of passively-acquired antibodies and found that they do not have a significant effect on the occurrence of EPE or suspected \textit{L. intracellularis} infection. Further, we have identified and documented an accurate disease attack rate for EPE and shown that weanling/yearling age does not appear to be a risk factor for the occurrence of clinical EPE or \textit{L. intracellularis} infection.

While only three of the eleven (27.3\%) presumptive EPE cases and four of the thirteen (30.8\%) suspected \textit{L. intracellularis} infections had detectable, passively-acquired \textit{L. intracellularis}-specific antibodies following birth, these proportions were similar to the total percentage of seropositive foals following birth (38\%). The overall percentage of mares that were seropositive at parturition (44.2\%) was lower than had been expected, especially given that \textit{L. intracellularis} appears to have become a relatively ubiquitous organism on many farms in central Kentucky and previous work by others has found seropositive rates in adult horses from 55-90\%\(^{19,20,107,108}\). One potential confounding factor in this study was the prevalent use of \textit{Rhodococcus equi} hyperimmune plasma on
many of the farms. We believe that this plasma, which contained variable levels of \( L.\ intracellularis \)-specific antibodies, may have affected the initial post-partum foal antibody levels. In fact, 35 of the 41 foals with seropositive post-partum samples while their mare's corresponding sample was seronegative received \( R.\ equi \) hyperimmune plasma. Of the six remaining foals with positive values, their mare's \( L.\ intracellularis \) antibody levels likely fell slightly below the cutoff (55EU) and, thus, were reported as negative. While data analysis failed to demonstrate an effect of the \( R.\ equi \) plasma on the occurrence of EPE, we opted to refer to the \( L.\ intracellularis \)-specific antibodies that were measured post-partum as passively acquired to account for those that were potentially obtained through hyperimmune plasma administration, rather than just from colostrum intake.

Gender was found to have a significant effect on the occurrence of EPE, with colts being significantly more likely to develop presumptive EPE or a combination of presumptive EPE or suspected infection. Out of the 369 study horses present in that data analysis, 164 were colts, 177 were fillies, and 28 were not reported. It is important to note that while data analysis indicated an increased risk for colts, fillies also developed both presumptive EPE and suspected \( L.\ intracellularis \) infections during the study and there have been numerous case reports of EPE in fillies as well.\(^9,11,31,109\) There was a trend \((p=0.076)\) for those horses that were treated as foals for \( R.\ equi \) to be at greater risk \((\text{odds ratio } 3.69)\) of developing suspect infections than those horses not treated for \( R.\ equi \). Likewise, there were no presumptive EPE cases that were treated for \( R.\ equi \) as foals. While a larger sample set is needed to examine this further, it is possible that prior infection with \( R.\ equi \) may have some protective effects against \( L.\ intracellularis \)
infection given that they are both intracellular bacteria and likely require a competent interferon-gamma response by the immune system to clear the infection (see Chapter 5 below). Prior *R. equi* infection may prime the foal's immature immune system such that, when challenged with *L. intracellularis*, the immune system is capable of recognizing the intracellular bacterium and responding appropriately. Further study into the possibility of cross-protection, using either naturally occurring field cases or experimentally challenged horses, is warranted.

Interestingly, when looking for an effect of weaning date on EPE occurrence, those horses weaned between September and November were at a significantly lower risk of developing combined presumptive EPE and suspected *L. intracellularis* infection when compared to those horses weaned between May and August. The reason for this difference is not immediately clear and testing over additional years is needed to identify whether this is a recurring predilection or a one-time occurrence. One possible explanation could be that mares provide an unknown protective effect for their foals. By not being weaned until during or after an exposure period (such as what was seen in this study during October), the late-weaned foals may be shielded from exposure.

While clinical EPE is exceedingly rare in nursing foals, it appears as though several study horses were exposed to the bacterium prior to weaning since it takes approximately 14 days for seroconversion to occur following exposure; in addition to the two foals that seroconverted during April and May while still on their mares, one seropositive horse seroconverted 8 days prior to weaning while two other horses seroconverted 9 and 10 days after weaning (data not shown). With prior work suggesting that horse-to-horse transmission of *L. intracellularis* is possible, as well as the suggestion that sows may
serve as a reservoir for the bacterium, \(^{59}\) mares may play a role in the epidemiology of EPE, a possibility that is explored in Chapter 4.

Given that there were no significant differences in age at weaning and seroconversion or in the time from weaning to seroconversion between the three seropositive groups, it would appear that age does not play a factor in the development of EPE or \(L. \text{intracellularis}\) infection when exposed to the bacterium. While it was found that seronegative horses were significantly older (by 8 days) at weaning than seropositive horses, there is no readily apparent explanation for this finding other than what has been mentioned above. It seems unlikely that an additional 8 days with its mare would provide complete protection from detectable \(L. \text{intracellularis}\) exposure in the months following weaning, however, a large, risk-factor based study would be needed to examine this beyond the scope of what has been presented here.

We previously reported a regional seroprevalence of 68\%,\(^{20}\) while in the study reported here the overall seroprevalence was 56.4\%. Given the preference to include those farms with prior EPE, neither of these rates are indicative of the overall seroprevalence rate in central Kentucky; instead, farm-specific seroprevalence levels allow for a better examination of \(L. \text{intracellularis}\) exposure. To that end, the farm-specific seroprevalences reported here, which ranged from 11.8-80\%, were similar to those we have reported earlier where values ranged from 14-100\%.\(^{20}\) Interestingly, the bimodal distribution of exposure noted in the previous study (2010-11) was again noted here (2012-13), with peak exposures in October and January, while only a single peak of exposure for the entire central Kentucky region was seen in 2011-12. Upon examining the location of the current study's farms, it is evident that there is only a single peak of
exposure for each region in a given season. Potential reasons for the regional differences include management practices, vaccination schedules, reservoir and/or wildlife population changes/fluctuations, and weather patterns. For those farms with seroprevalence data available from the prior year, the change in seroprevalence was varied, with some farms experiencing large increases or decreases, while others remained static; no immediate reasons for these changes were evident, however, this study was not designed to examine for differences between farms. In-depth examination of the potential reasons behind the regional and year-to-year farm exposure differences might help to identify potential risk factors for *L. intracellularis* exposure and/or the reservoir host(s) of the bacterium.

The fall and winter months (October through February) continue to be the primary time during which exposure to *L. intracellularis* is detected and when the majority of EPE cases occur in central Kentucky. However, the occurrence of EPE cases beyond February,\textsuperscript{20} including the March and April 2013 cases reported here, suggest that this range should be extended. Further, the apparent seroconversion of two nursing foals in April and May of 2012, while only a fraction of the entire study population, would also seem to support this assertion.

Hypoproteinemia with hypoalbuminemia has long been recognized as a common occurrence with EPE and is unique amongst the species *L. intracellularis* infects. This upset in total protein and albumin levels is what leads to dependent edema, one of the non-specific but common findings with EPE cases. Prior to data analysis and based on our clinical experience, we set the ranges listed above (TP: <4.5g/dL and 4.5-5.2 g/dL, albumin: <2.5g/dL and 2.5-3.1 g/dL) for presumptive EPE cases and suspect *L.*
intracellularis-infected horses, respectively. Not surprisingly, total protein and albumin were significantly different between the various groups, except for those differences between seronegative and seropositive-only horses. Further, the values that we set prior to analysis held up fairly well when compared to the calculated 95% confidence interval values. Although variations in sample analysis will exist between methods and laboratories, the references ranges we have provided for presumptive EPE (TP <4.5 g/dL and albumin <2.5 g/dL) and suspected infection (TP 4.5-5.2 g/dL and albumin 2.5-3.1 g/dL) cases appear to provide a good starting point for the classification of EPE cases. While there were several seronegative and seropositive horses with either total protein or albumin values below the reference ranges used here, none of them had concurrent hypoproteinemia and hypoalbuminemia, stressing the importance of evaluating both parameters in tandem. There can be several other causes of low total protein and albumin in young horses, and given that total protein and/or albumin have been targeted as a cheap and effective means of screening for EPE (including weekly screening of total protein by one farm in this study), it remains important to follow-up abnormal values with serologic screening prior to initiating treatment for L. intracellularis infection.

A point of discussion for several years has been how infrequently clinical disease occurs in weanling and yearling horses exposed to L. intracellularis. While this study sought to examine some of the potential risk factors for EPE, another goal was to determine the disease and infection rate among exposed horses. Traditionally, estimates of 5-10% of clinical EPE in seropositive horses have been used, but this assumption has never been proven in a large scale, field study before. Much like the overall seroprevalence we reported above, the attack rates amongst the entire study population
should be used with caution as the preferential inclusion of EPE endemic farms likely introduced a sampling bias. Among *L. intracellularis*-exposed horses, we found a clinical EPE attack rate (using the presumptive EPE cases) of 5.3% and an infection attack rate (using cases with suspected infections) of 6.3%; combined, there was an overall attack rate of 11.6% among seropositive horses. Current experimental *L. intracellularis* challenge models in horses induce clinical EPE at a rate of 33% or higher, suggesting that these models fail to mimic natural exposure to the bacterium and likely over-expose horses to elicit disease. With this newly documented attack rate, it should be possible to refine the experimental challenge models used to induce EPE, likely by decreasing the challenge doses, such that they more closely mimic natural disease.

By showing that passively-acquired antibodies specific for *L. intracellularis* failed to have a significant, protective effect against the occurrence of EPE, we have eliminated a potential explanation for the unique predisposition of young horses to EPE. Given that passively-acquired IgG is short-lived, IgG is the predominant immunoglobulin in colostrum and hyperimmune plasma products, and *L. intracellularis* infection is not a systemic infection but rather a localized, mucosal infection, it is not surprising that no significant effect of passively-acquired antibodies was noted in this study. Other potential reasons for weanling predisposition to EPE continue to include management changes associated with weaning and the post-weaning period (including stress), loss of maternally-derived nutrition and immunological factors (i.e. IgA), and the immune response to intracellular infection. Based on the findings presented here, age does not appear to have an effect on EPE occurrence amongst seropositive horses while age at
weaning might have an effect on seroconversion. Colts were found to be significantly more at risk for the development of EPE and *L. intracellularis* infection, however, fillies do account for many clinical cases of EPE. The identification of geographical exposure differences within a relatively small area is a novel finding that warrants further exploration as the reason(s) behind this difference may provide the first clues towards understanding the epidemiology of *L. intracellularis* infections on horse farms. Even though the disease rate demonstrated here (approximately 11% of seropositive horses) is relatively low compared to other equine diseases, the growing economic and welfare aspects of EPE suggest that it is a disease which should not be ignored.
CHAPTER FOUR:

Determining whether mares play a role in the epidemiology of *Lawsonia intracellularis* and equine proliferative enteropathy on an endemic farm

Summary

The epidemiology of equine proliferative enteropathy is poorly characterized, although it is known that a large number of animal species are capable of shedding *Lawsonia intracellularis* in feces. Recently it has been suggested that horse-to-horse transmission of the bacterium may be possible, although no work has examined the role mares may play in the epidemiology of EPE. The goal of this study was to utilize serological screening to detect exposure to *L. intracellularis*. Serum samples were used to determine whether mares show signs of exposure to the bacterium prior to their foals and whether seropositive mares were more or less likely to have seropositive foals/weanlings. Ultimately, 95 mares and 91 foals from a central Kentucky Thoroughbred farm with endemic EPE were utilized for this study and all foals were weaned by the end of October. Serum samples were analyzed using an equine-validated ELISA for *L. intracellularis*. Chi-square analysis found that those mares with 5 or more seropositive months through October had a significantly higher number of foals/weanlings with evidence of exposure to the bacterium and these foals were 5.667 times more likely to be seropositive from July 2012 through January 2013 of their yearling year than those foals from mares with fewer than 5 seropositive months through October. There was no effect of mare sero-status on the occurrence of presumptive EPE or suspected *L. intracellularis* infection in their offspring. While this study found that
previously seronegative mares and weanlings experienced similar seroconversion events in December and January, a role for mares in the epidemiology of EPE or spread of *L. intracellularis* could not be ruled out based on the results of this study.
Introduction

_Lawsonia intracellularis_ is the causative agent of equine proliferative enteropathy (EPE), a disease typically seen in weanlings and young yearling horses during the fall and early winter months. The most common clinical signs observed with EPE include anorexia, fever, depression, dependent edema, colic, and diarrhea. Diagnosis revolves around the presence of clinical signs, clinicopathologic disturbances including hypoproteinemia and hypoalbuminemia, and thickened small intestinal segments detected via abdominal ultrasound. Additional antemortem tests for the detection of _L. intracellularis_ infection include analysis for presence of the pathogen shed in feces by use of an _L. intracellularis_-specific PCR assay and analysis of serum samples for bacterium-specific antibody detection.

With respect to the epidemiology of _L. intracellularis_, a fecal-oral route of infection is suspected, and what little else is known has been primarily discovered through research with porcine PE and extrapolated into other species. In addition to horses, pigs, and hamsters, several studies have revealed the presence of _L. intracellularis_ a variety of species, including blacktailed jackrabbits, cottontailed rabbits, cats, striped skunks, Virginia opossums, coyotes, guinea pigs, mice, rats, hamsters, hedgehogs, ferrets, rabbits, wild pigs, dogs, foxes, calves, wolves, deer, ostriches, emus, monkeys, and giraffes. Thus far, attempts to incriminate any of these species in the spread or maintenance of _L. intracellularis_ within susceptible populations have only been suggestive and not proven conclusively; this includes a recent study in which weanling horses were challenged with feces from _L. intracellularis_-infected rabbits. In that
study, the challenged horses failed to develop EPE, however they did seroconvert following challenge. Work with porcine PE has, however, implicated subclinically affected pigs in the spread of the bacterium in swine operations and recent work with EPE has suggested a role for clinically affected horses. Further, some have suggested the potential for sows to harbor and transmit the bacterium to their offspring, an aspect of which has never been examined with EPE. Given that most Thoroughbred foals are co-housed in stalls with their respective mares for multiple hours per day, as well as the propensity for foals to exhibit coprophagic tendencies, it seems tenable that mares could act as asymptomatic shedders of the bacterium and expose their foals to \textit{L. intracellularis} prior to weaning.

The goal of this study was to utilize monthly serum sampling of mares and foals on an EPE endemic farm to detect exposure to \textit{L. intracellularis} and determine whether mares show signs of exposure to the bacterium prior to their foals. Additionally, we sought to determine whether seropositive mares were more or less likely to have seropositive foals/weanlings. Should we show that mares may play a role in the exposure of foals/weanlings to \textit{L. intracellularis}, this finding would warrant further exploration with regards to the epidemiology of EPE, as well as examination of potential steps for mitigation of this exposure as a means of decreasing equine proliferative enteropathy.
Materials and Methods

Study Farms and Horses

A Thoroughbred farm in central Kentucky, located within 20 miles of the University of Kentucky, was enrolled in this study, which took place from January 2012 through January 2013. The farm was considered endemic for EPE as it experienced multiple (3+) cases of clinical EPE in each of at least the three prior years. Approval to conduct this study was obtained from the University of Kentucky's Institutional Animal Care and Use Committee and forms documenting informed consent for inclusion of privately-owned horses were obtained from the farm veterinarian, who was authorized to act on behalf of private owners.

Sample Collection, Processing, and Storage

Within 48hrs of parturition, whole blood samples (7 mls of peripheral blood collected via aseptic venipuncture from the jugular vein) were collected from the mare and newborn foal and allowed to settle in a refrigerator at 4°C. Samples were labeled with the mare/foal’s name and the date of collection. After collection, samples were stored at 4°C on the farm until returned to the Maxwell H. Gluck Equine Research Center at the University of Kentucky for centrifugation (800g x 10min) followed by freezing of serum at -20°C.

After the initial collection time point, samples were subsequently collected approximately every four weeks from each mare and foal through the middle of July, as described above. Starting at the end of July, all study horses (mares and foals) had blood
samples collected during the same three day period every four weeks to ensure that all horses were sampled during the same time period. These samples were transported to the Gluck Equine Research Center and immediately centrifuged at 800xg for 10 min. Foal/weanling samples were held at 4°C for no more than 4 days before all samples were analyzed each month, as below, while mare samples were frozen at -20°C. After the final collection of blood samples in January, samples from each mare were thawed and run concurrently on the same plate using an \textit{L. intracellularis}-specific ELISA (see below).

\textbf{Detection of \textit{L. intracellularis}-specific antibodies}

Serological status in regards to \textit{L. intracellularis} was determined using the ELISA method as previously described (see also Chapter 2),\textsuperscript{20} except that plates were blocked with polyvinyl alcohol 1\% (w/v) in distilled water for one hour instead of two hours. Results from the tested sera were converted to an ELISA unit (EU) utilizing a linear regression line from the standard curve generated from each plate. A positive cut-off of 55EU or greater was utilized based on previous work.\textsuperscript{10}

\textbf{Classification of Study Horse EPE Status}

At the end of the study, horses were placed into four categories to describe their \textit{L. intracellularis}/EPE status following the typical \textit{L. intracellularis} "season" of October to February: presumptive EPE, suspect \textit{L. intracellularis}-infected, seropositive, or non-exposed. Based on our previous work,\textsuperscript{20} presumptive EPE affected horses were those that experienced concurrent clinical signs compatible with EPE, moderate to severe hypoproteinemia (<4.5g/dL) and hypoalbuminemia (<2.5g/dL), and a positive \textit{L.
intracellularis-specific ELISA result. For the purpose of this study, compatible clinical signs for EPE were anorexia, fever, lethargy, depression, dependent edema, rapid weight loss, colic, and diarrhea. Given that definitive diagnosis of EPE can only be made at necropsy and relies upon the immunohistochemical (IHC) detection of the organism in characteristic intestinal lesions, all clinical diagnoses of EPE were considered presumptive. Suspect L. intracellularis-infected horses were those with mild hypoproteinemina (4.5-5.2 g/dL) and hypoalbuminemia (2.5-3.1 g/dL) plus a positive L. intracellularis-specific ELISA result without concurrent clinical signs compatible with EPE. L. intracellularis-infected horses can experience a transient decrease in total protein and albumin levels along with a positive L. intracellularis-specific ELISA result in the absence of other clinical signs. However, since there was no demonstration of the presence of L. intracellularis (either via fecal PCR or necropsy sampling), these cases are referred to as "suspect L. intracellularis-infected". Seropositive horses were those with only a positive L. intracellularis-specific ELISA result (excluding passively-acquired L. intracellularis antibodies) and no other indication of L. intracellularis infection. Horses with no detectable L. intracellularis-specific antibodies for the entirety of the study (excluding passively-acquired antibodies) were classified as seronegative.

Data Analysis

Fisher's exact test was used to determine whether mare serological status had an effect on the occurrence of EPE while Chi-square and risk factor analysis were used to determine whether the number of seropositive months a mare experienced had a significant effect on seroconversion in her foal. Additionally, survival analysis (Kaplan-
Meier) was used to detect an effect of mare serological status on foal seroconversion. Calculated P values ≤0.05 were considered to be statistically significant.
Results

Initially, 130 mare and foal pairs were enrolled in this study, however only 95 mares and 91 foals were ultimately included in the final data analysis as they were present on the farm from parturition through at least October. Of the 95 mares, 42 (44.2%) were seropositive (≥55EU) at parturition, which ranged from January through May, while a total of 67 mares (70.5%) were seropositive at some point during the study. There was an overall decline in the percentage of mares that were seropositive for *L. intracellularis*-specific antibodies over the course of the study from January 2012 to January 2013 (Figure 4.1). The total number of positive samples varied, with 29.5% of mares remaining seronegative across all of their samples while a total of 46.3% of mares were seropositive for six or more months during the study (Table 4.1). The average mare seropositive ELISA value was 108.1EU while the maximum during the study was 1106.3EU.

To allow for easy comparison of antibody levels between mares, mare EU values were standardized to a fold-change scale by dividing each month's value by the first result that was reported for that mare; if the first recorded result was negative, a numerical value of 54.9 EU was assigned while all negative values were disregarded when averaging positive EU fold changes. Figure 4.2 demonstrates the monthly average fold changes that were noted amongst the mare samples (a fold change of 1 is equal to no change). Also included on Figure 4.2 are the first-time seroconversions for mares and weanlings which were used as an indicator of exposure to the bacterium. Mares had to have been
Figure 4.1: Total percentage of study mares that were seropositive during a given month and the average monthly positive EU value.
Table 4.1: The number and percentage of study mares that were either seronegative for the entirety of the study or seropositive for a range of months.

<table>
<thead>
<tr>
<th></th>
<th>Negative</th>
<th>Positive for &lt;3 months</th>
<th>Positive for 3-5 months</th>
<th>Positive for 6-9 months</th>
<th>Positive for 10+ months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Mares</td>
<td>28</td>
<td>17</td>
<td>6</td>
<td>31</td>
<td>13</td>
</tr>
<tr>
<td>Total Percentage</td>
<td>29.5%</td>
<td>17.9%</td>
<td>6.3%</td>
<td>32.6%</td>
<td>13.7%</td>
</tr>
</tbody>
</table>
Figure 4.2: Mare EU fold changes by month, as well as mare and foal seroconversion percentages.
seronegative since the beginning of the study and for the two following samplings for a subsequent positive sample to be considered seroconversion resulting from exposure to *L. intracellularis*. Peak fold changes amongst previously seropositive mares were noted in June to August, November, and January while small peaks in mare seroconversion were noted in July and October, with a large peak in mare seroconversion seen in January. Small increases in foal/weanling seroconversion occurred in June and July (0.8% of foal population) and November (1%) while large increases in seroconversion occurred in December and January. All study foals were weaned by the November sample collection.

Chi-square analysis determined that those mares with 5 or more seropositive months through October had a significantly higher number of foals/weanlings with evidence of exposure to the bacterium between July and January (p<0.001). Further, foals/weanlings from mares with 5 or more seropositive months through October were found to be 5.667 times more likely to be seropositive from July through January than those from mares with fewer than 5 seropositive months through October. No significant effect of mares with 5 or more seropositive months through October was noted on the occurrence of presumptive EPE or suspected *L. intracellularis* infection. Based on survival analysis, no significant difference in the time to seroconversion was noted between foals/weanlings from mares that were seropositive versus seronegative from parturition through October (Figure 4.3).
Figure 4.3: Survival analysis of the time to foal seroconversion based on whether the foal's mare was seropositive or seronegative through October. Time-points are 1=July, 2=August, 3=September, 4=October, 5=November, 6=December, and 7=January.
Discussion

Amongst the many aspects of *Lawsonia intracellularis* infections that have been researched in the years since the bacterium's discovery, maintenance within a population and spread from animal to animal remains one of the most poorly characterized. Here we have utilized an ELISA to screen mares and their foals for exposure to *L. intracellularis* and examined for a potential effect of seropositive mares on their foals with respect to seroconversion or the occurrence of clinical EPE/*L. intracellularis* infection. Based on results of this study, we were unable to rule out mares as possible sources of *L. intracellularis* infection for their foals.

Based on the endemic EPE status of the farm used in this study (Farm 21 in Chapter 2 and Farm 2 in Chapter 3), as well as work by others that has shown 99% of adults to be seropositive for *L. intracellularis* antibodies,\textsuperscript{108} the fact that only 70.5% of the mares reported here were seropositive during the study was surprising. Regardless, the reporting of high *L. intracellularis* exposure in adult horses continues to suggest regular exposure of horses, regardless of age, to the bacterium, as well as the potential for adults to serve as asymptomatic carriers/shedders of *L. intracellularis*. Further, finding 46.3% of the mare study population to be seropositive for 6 or more months while 13.7% was seropositive for 10 or more months may indicate either regular exposure or prolonged exposure to the bacterium, such as would be seen in a carrier state. EPE has long been known to be a disease of young horses and, while the ELISA used in this study utilizes a cut-off value validated for use in the at risk population,\textsuperscript{10,20} it is reasonable to expect exposure of adults to the bacterium on farms with affected young horses. Given
that we reported seronegative mares and weanlings towards the end of the study that seroconverted during the same time period there appears to be no reason to suspect that the method and cut-off are not valid for use in adult horses. To our knowledge, this is the first year-long study to serologically follow mares for exposure to *L. intracellularis*. As such, this was a pilot study and further sero-screening accompanied by regular fecal PCR testing of persistently seropositive mares would be useful to further help identify the role that mares may play in the spread of *L. intracellularis* on EPE endemic farms.

The steady decline in the overall percentage of study mares that were seropositive over the 13 month period of the study was unexpected. Based on work with younger horses, an increase in the overall number of seropositive mares during the fall and winter months was anticipated. Instead, only a small increase in seropositive mares was noted in December while a larger peak was noted between July and August. The peak during the summer is mirrored by a fold-change increase between June and August, as well as a small increase in first-time mare seroconversions during July and August. The combination of these findings would seem to suggest that mares may be exposed to *L. intracellularis* outside of the "normal" exposure period. While additional data from subsequent years is needed to confirm these findings, this could have a profound impact on our understanding of EPE epidemiology.

Supporting the theory that mares play a role in spreading *L. intracellularis*, we found that mares with five or more seropositive months through October (the last month in which foals were weaned) had foals/weanlings that were significantly more likely to exhibit evidence of exposure to *L. intracellularis* while mare sero-status had no effect on the occurrence of presumptive EPE or suspected *L. intracellularis* infection. Based on
experimental challenge studies in horses, a 14-21 day incubation period has been proposed for EPE with seropositive titers typically detected approximately 14 days after exposure.\textsuperscript{10,39} However, as presented in Chapter 3, the rate of naturally occurring EPE cases and \textit{L. intracellularis} infections amongst exposed horses is significantly lower (~11\% vs. 33-66\%) than is seen with experimental EPE models.\textsuperscript{10,39,57} This difference likely originates from the large challenge doses that are used to induce clinical disease in experimental settings. As such, it is feasible that naturally occurring cases are induced by lower doses, which may exhibit prolonged incubation and seronegative periods. If this were to be true, mares could be seeding their foal's environment with the bacterium, leading to detectable seroconversion several months after weaning. However, the large increase in seroconversions that occurred between December and January in both mares and weanlings, as well as the large increase in mare EU fold changes during that same time period would seem to suggest that mares and weanlings were exposed at the same time, making it unlikely mares are exposing their foals to \textit{L. intracellularis}. Further, the lack of an effect of mare seropositivity on the time period during which her foal/weanling seroconverted also suggests mares may not play a role in exposing young horses to the bacterium.

The apparent exposure of mares to \textit{L. intracellularis} during the summer months, without a concurrent, substantive event in the foal population, may suggest that mares are actually providing protection to their foals prior to weaning. The most likely mechanism by which mares would protect their foals would be through immunoglobulin A ingested by the foal in mare's milk. IgA has been suggested to play a significant role in immunity to \textit{L. intracellularis} in pigs given its propensity to accumulate in enterocytes within close
proximity to the bacterium, as well as the production of mucosal *L. intracellularis*-specific IgA following experimental infection.\textsuperscript{89,112} Given that IgA is present within mare's milk at high concentrations and is complexed with a secretory component that provides increased resistance to digestion within the gastrointestinal tract,\textsuperscript{113} milk-derived IgA could confer immunity to *L. intracellularis* while foals are nursing. Further, cessation of milk intake at weaning and beyond would lead to loss of this immunity, placing the weanlings at risk for the development of EPE. It seems plausible that IgA could play a significant role in immunity to the bacterium in horses and may warrant further examination.

This study set out to determine whether mares play a role in the spread and maintenance of *Lawsonia intracellularis* on EPE endemic farms and, based on the data, we were unable to rule-out a role for mares. Further, foals/weanlings from repeatedly seropositive mares (5 or more seropositive months from parturition to October 2012) were significantly more likely to exhibit signs of exposure to the bacterium as detected by an *L. intracellularis*-specific ELISA. This finding would seem to implicate mares in the spread of the bacterium, however, there was no effect of mare sero-status on the occurrence of presumptive EPE or suspected *L. intracellularis* infection in their foals. Additional work is needed to replicate naturally-occurring clinical EPE cases to determine whether the incubation and/or seronegative period is longer than 14-21 days, as has been reported previously with large-dose, experimental models. Interestingly, there appeared to be a protective effect of mares on nursing foals during the summer as there was evidence of mare seroconversion to the bacterium with very little concurrent
corresponding seroconversion in foals. One hypothesis for this protective effect could be milk-derived IgA.
CHAPTER FIVE:

Characterization of the interferon gamma response to *Lawsonia intracellularis* using an equine proliferative enteropathy challenge model

**Summary**

*Lawsonia intracellularis* is the etiological agent of infectious intestinal hyperplasia for which several clinical diseases have been described including proliferative enteropathy (PE), intestinal adenomatosis, and ileitis. While initially recognized as the causative agent of PE in pigs, *L. intracellularis* is now viewed as an emerging cause of intestinal hyperplasia in a wide range of mammalian species, including horses. Equine proliferative enteropathy (EPE) has been reported worldwide though definitive diagnosis is difficult and the epidemiology of the disease remains poorly understood. Weanlings and short yearlings, in particular, appear to be most at risk for infection, though the reasons for their particular susceptibility is unknown. Using an infectious challenge model for EPE, we demonstrate that EPE, as in porcine proliferative enteropathy, can exhibit three clinical forms; classical, subclinical and acute. Out of six pony weanlings, one developed signs of classic EPE, one developed acute EPE, and two developed subclinical EPE. Attempts to induce pharmacological stress through the use of dexamethasone failed to have any effect on outcome. Peripheral blood cells collected from those weanlings that developed clinical EPE exhibited decreased expression of interferon-gamma (IFN-γ) following *in vitro* stimulation with *L. intracellularis*. By contrast, those weanlings that did not develop clinical disease generated a robust IFN-γ
response. These results indicate IFN-\(\gamma\) likely plays a significant role in protection from
disease caused by \(L. \) intracellularis in the equid.

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Convention.

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Horohov DW. Characterization of the interferon gamma response to Lawsonia
intracellularis using an equine proliferative enteropathy challenge (EPE) model. *Vet
Introduction

*Lawsonia intracellularis*, an obligate intracellular, gram-negative rod, is the causative agent of proliferative enteropathy.\(^1\) While initially described as a pathogen of pigs,\(^4\) *L. intracellularis* is now viewed as an emerging cause of proliferative enteropathy in a variety of mammalian species,\(^91,92\) including horses.\(^14,93-95\) In the horse, *L. intracellularis* infection results in equine proliferative enteropathy (EPE) which has been reported worldwide.\(^15,25\) The bacterium infects the crypt epithelial cells primarily of the distal small intestine, causing hyperplasia and proliferation of the infected cells.\(^114\)

Several possible mechanisms for *L. intracellularis* pathogenicity have been proposed including perturbations in cell cycle processes of crypt epithelia; alterations of factors involved in the differentiation of normally immature and proliferative crypt cells; disturbances of relevant growth factor receptors; and inhibition of apoptotic cell death in the crypt.\(^40,41\)

Typical clinical signs of EPE include anorexia, fever, lethargy, depression, dependent edema, intermittent colic, and diarrhea. A common diagnostic test used by many practitioners is abdominal ultrasonography where a small intestinal wall thickness of >3mm can be considered pathognomonic for EPE when accompanied by compatible clinical signs; however, false negative results can occur in some cases of EPE.\(^11\) The presence of hypoproteinemia caused by hypoalbuminemia is also highly suggestive of EPE in weanlings.\(^11\) Definitive diagnosis of EPE is based on the presence of histologic changes and immunohistochemistry of small intestinal sections obtained at necropsy. Available commercial antemortem tests for EPE consist of fecal *L. intracellularis-*
specific PCR and the serum immunoperoxidase monolayer assay (IPMA). Both of these
tests are adapted from their use in pigs where the tests are highly specific. However,
sensitivity is quite variable for the fecal PCR assay.\textsuperscript{32,34,115} By contrast, sensitivity is
reported to be high for the IPMA,\textsuperscript{53} though the method is cumbersome, requiring visual
interpretation of the samples. Recently, we have described an \textit{L. intracellularis}-specific
ELISA for the detection of serum antibodies.\textsuperscript{20}

Cases of EPE are typically seen in weanling horses.\textsuperscript{11,18,94-96} While the
epidemiology of \textit{L. intracellularis} is poorly defined, a fecal-oral route of infection is
suspected.\textsuperscript{46} Several hypotheses exist as for the reason of this age predilection for EPE,
which include a decline in maternal antibodies (see Chapter 3), management changes that
occur during the weaning period, the stress of weaning and changing barns and/or
pastures, as well as implementation of new vaccination and deworming practices.\textsuperscript{11,18}
Additionally, the underdeveloped cell-mediated immunity in younger horses may not be
as capable of combating \textit{L. intracellularis} compared with adult horses.\textsuperscript{11}

The immunological aspects of EPE remain undefined and recent work has
focused on the antibody response of horses to \textit{L. intracellularis}.\textsuperscript{88} However, results from
other animal models indicate that cell-mediated immune mechanisms likely play a pivotal
role in resistance to this infection.\textsuperscript{54,76} Descriptive immunocytological studies of
intestinal tissue sections of pigs have shown evidence of cell-mediated immune response
to \textit{L. intracellularis}.\textsuperscript{4} Oral vaccination of pigs with an avirulent live strain of \textit{L}.\textsuperscript{intracellularis}, which is associated with protection from disease,\textsuperscript{54} results in \textit{Lawsonia}-
specific IFN-\(\gamma\) expression by PBMC.\textsuperscript{70} Thus, it appears likely that IFN-\(\gamma\) and other cell-
mediated immune mechanisms would play a central role in the horse’s immune response to this bacterium.

Unlike porcine proliferative enteropathy, there had been no established experimental infection model for *L. intracellularis* in the equid until recently. The pig infection model has facilitated studies looking at a variety of issues, including vaccine development, comparison of available diagnostic tests, creation of additional diagnostic tests, and evaluation of feed and feed additives on PPE. The lack of an experimental infection model in the horse has left a gap in our knowledge of EPE, with a large portion of our understanding of the disease adapted from swine research. The purpose of this study was to experimentally induce equine proliferative enteropathy in weanlings using a recently isolated and cultivated equine-origin *L. intracellularis* strain. Clinical disease was confirmed using available, routine diagnostic techniques, as well as post-mortem findings. Additionally, we identify a potential role for IFN-γ in protection from clinical EPE.
Materials and Methods

Horses

Six nursing pony foals between 16 and 19 weeks of age were selected based on their close age grouping from the University of Kentucky’s research herd where mares and foals were maintained on pasture. Prior to the study start (day -7), all six foals were determined to be healthy on physical exam by a licensed veterinarian with complete blood count, fibrinogen, and serum biochemistry profiles within normal ranges. At no time during the study, or in the week prior to challenge, were antimicrobials administered. The study was approved by the University of Kentucky’s Biosafety and Institutional Animal Care and Use Committees.

Experimental design

The day prior to challenge (day -1), the foals were weaned and immediately transported to the Maxwell H. Gluck Equine Research Center where each weanling was housed separately in an indoor stall for the remainder of the study. Weanlings were allowed ad libitum water and mixed grass hay. Additionally, each weanling received ¼ quart of sweet feed grain twice a day starting on day +2 as a means of detecting partial or total anorexia. Each foal received omeprazole\textsuperscript{M} (4mg/kg, q 24h, PO) from day -7 through day +2. An additional fifteen pony foals of approximately the same age remained at the farm for the duration of the challenge study serving as serological controls only and were not euthanized or necropsied at the end of the study.

\textsuperscript{M} Gastrogard\textsuperscript{®}, Merial, Duluth, GA
**Immune suppression assessment**

Immune suppression was induced using physiologic stress (weaning) and pharmacologic methods. The first involved weaning the foals on the day prior to challenge. Recent work in our laboratory has shown that the stress of weaning is significant enough to cause a decrease in cell-mediated immunity. Additionally, three of the six weanlings (1, 3, and 5) were randomly assigned to be administered dexamethasone at 0.25mg/kg, q 24 h, IV on day -1, 0.125mg/kg, q 24 h, IV on days 0 and +1, and 0.04mg/kg, q 24 h, IV on day +2. To assess immune suppression, peripheral blood (~3.0 mls) was collected directly into Paxgene tubes prior to weaning and daily afterwards for the determination of nascent expression of IFN-γ mRNA. Total RNA was isolated from the Paxgene tubes according to the manufacturer’s directions and the RNA converted to cDNA using avian leukosis virus reverse transcriptase. Cytokine-specific cDNA was amplified using real-time PCR and primers and probes specific for IFN-γ. Beta-glucuronidase was used as the housekeeping gene for RNA normalization. The efficiency of the PCR reaction was confirmed using LinReg. Results are expressed as relative quantities with the average of pretreatment blood samples used as the calibrator.

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N Dexium ™, Bimeda, Le Sueur, MN
O Qiagen Inc, Valencia, CA
Live-cultured Lawsonia intracellularis and challenge

Low passage, equine-origin *L. intracellularis* was grown by GBI Laboratories\(^p\), using previously described methods.\(^6,39\) Using the direct-count method,\(^37\) the number of organisms was found to be $4.45 \times 10^8$/ml. The inoculum was stored at -80°C until the day prior to challenge (day -1), when the entire sample of *L. intracellularis* was placed into a 4°C refrigerator to thaw slowly overnight. On day 0, 45mL of live *L. intracellularis* was placed into six individual 60cc catheter-tipped syringes. The six pony weanlings were then challenged with the equine-origin *L. intracellularis* (2x$10^{10}$ organisms/weanling) via nasogastric intubation. No weanlings were sedated for this process and feed was not withheld pre- or post-inoculation.

Monitoring of physical health

The six weanlings were monitored daily for pyrexia (>101.5°F), anorexia, colic, dependent edema, and diarrhea. Additionally, each study weanling was weighed and an abdominal ultrasound was performed weekly.

Antemortem diagnostic testing

On days -16 and -7 all six foals, as well as controls, tested negative for the antibodies to *L. intracellularis* via ELISA. Testing involved collecting 7ml of whole blood via jugular venipuncture into sterile 7 ml plain blood tubes. Blood was allowed to clot for 1-2 hours, after which time the tubes were centrifuged at 800xg for 10min. 1ml of serum was removed and placed into individual 1.5ml tubes that were labeled with the

\(^p\) Fergus Falls, MN
horses’ ID number and date of collection. Samples were frozen at -20°C until analyzed via ELISA. The method for this ELISA is provided in Chapter 2.

Fecal samples were collected from each weanling from day 0 through day +6 and biweekly thereafter. Each sample was stored in a separate, labeled 50ml conical tube at -20°C until a batch of samples was shipped overnight in cooler boxes to the University of California, Davis for real-time PCR detection of the aspartate ammonia lyase (aspA) gene of *L. intracellularis* as previously described.46 Biweekly blood samples were collected and submitted for total protein (ref: 5.3-7.5 g/dL) and albumin (ref: 3.0-4.0 g/dL) levels. Serum total protein and albumin levels were obtained using an ACE Alera at the University of Kentucky’s Veterinary Diagnostic Laboratory.

**In vitro stimulation of peripheral blood, RNA isolation, storage, processing, and analysis**

Green-top, lithium heparin blood tubes were collected from each weanling via jugular venipuncture on days -7, 0, +7, +14, +21, and +28 (when available). To each of the three tubes was aseptically added nothing (“control”), 10µl phorbol 12-myristate 13-acetate\(^Q\) (PMA; 25 ng/ml) and ionomycin (1 mM; Sigma), or 250µL modified-live *L. intracellularis* vaccine\(^R\). Tubes were then placed into a 37°C humidified incubator for 24 hours. After 24 hour incubation, 2.5 mL of blood was removed from each tube and placed into a corresponding PaxGene tube. Total RNA isolation and subsequent processing were completed per the manufacturer’s directions.

\(^Q\) Sigma Aldrich  
\(^R\) Enterisol ® Ileitis, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO
Euthanasia and post-mortem evaluation

All six weanlings were to be humanely euthanized at the end of the four week study (day +29); however, the severity of clinical disease in two of the weanlings (1 and 4) necessitated their euthanasia on day +21. Immediately following euthanasia, all six weanlings had full necropsies performed by boarded and board-eligible pathologists at the University of Kentucky’s Veterinary Diagnostic Laboratory. Mucosal scrapings from each weanling’s ileum were sent to the University of California, Davis for L. intracellularis-specific PCR testing, as above. Immunohistochemistry was utilized to detect the presence of L. intracellularis in the intestinal tract. The presence of the challenge strain of L. intracellularis obtained from necropsy samples was confirmed through the use of variable number tandem repeat (VNTR) analysis, as previously described.122

Immunohistochemistry

Tissue sections were stained using the EnVision+ Dual Link System-HRP immunohistochemical staining technique. Briefly, paraffin embedded tissue sections were placed on electrostatically charged glass slides, deparaffinized with xylene, and rehydrated. Endogenous hydrogen peroxide was quenched by applying a 3% hydrogen peroxide solution for 10 minutes. Slides were then rinsed with deionized water 3 times. Antigens were retrieved by adding 0.05% protease K to the tissues for 2 minutes, and then slides were re-rinsed an additional 3 times with deionized water. A 1:500 dilution of

\(^8\) Dako
L. intracellularis-specific monoclonal antibody\textsuperscript{T} was added to the slides for 45 minutes after which slides were rinsed with Tris-buffered-saline (TBS). A polymer conjugated with secondary antibodies was then applied to the tissue, allowed to incubate for 30 minutes, and then washed with TBS. Slides were incubated for 5 minutes with a substrate-chromogen solution and then were cover slipped. Tissues were finally analyzed for the presence or absence of L. intracellularis specific staining by light microscope. Negative controls consisted of both a negative reagent control and negative tissue control. The negative reagent control was performed by the procedure listed above with the exception that the primary monoclonal L. intracellularis antibody was substituted with a universal negative control mouse antibody (product #N1698, Dako North America, Inc.). Ileum from a single age-matched foal, which was euthanized for neurologic disease, was used as the negative tissue control.

**Case Definition**

After the conclusion of the study, weanlings were classified into three different categories (clinically affected, subclinically affected, and non-affected) based on the severity of observed clinical signs, the severity of clinicopathologic abnormalities, consistent fecal shedding of L. intracellularis as determined by fecal PCR for the aspA gene (as described above), the presence of gross or histologic changes consistent with EPE (pronounced rugation of the ileum and/or mucosal hyperplasia), and the presence or absence of L. intracellularis organisms in the distal small intestine as determined by immunohistochemistry and/or PCR for the organism on mucosal scraping samples. Due

\textsuperscript{T} Provided by Dr. Jeremy Kroll, Boehringer Ingelheim Vetmedica
to the shortened nature of this study, ELISA unit values were not considered when categorizing the weanlings. Weanlings were considered to be clinically affected with EPE when they experienced all of the following: overt clinical signs compatible with EPE, moderate to severe hypoproteinemia and hypoalbuminemia (less than 5.0 g/dl and 2.5 g/dl, respectively), more than two consecutive fecal samples PCR positive for *L. intracellularis*, and gross ileal mucosal proliferation with confirmed presence of *L. intracellularis* organisms either by IHC or PCR. Overt clinical signs were defined as the presence of two or more clinical signs compatible with EPE (anorexia, lethargy, depression, dependent edema, fever, colic, and diarrhea). Weanlings were considered to be subclinically affected if they did not experience all of the criteria listed for clinically affected weanlings. Non-affected weanlings were defined as those with none of the criteria listed for clinically affected weanlings.

**Data analysis**

Two-way repeated measures-analysis of variance (RM-ANOVAs) (Holm-Sidak method\(^U\)) was used to evaluate IFN-\(\gamma\) gene expression. Post hoc t-tests were performed to evaluate the differences between groups at each time point. Calculated P values ≤0.05 were considered to be statistically significant.

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\(^U\) SigmaStat, SPSS Inc., Chicago, IL
Results

The effect of weaning and dexamethasone treatment on IFN-γ production

Due to treatment randomization, the dexamethasone-treated foals had higher nascent IFN-γ mRNA expression prior to treatment; nevertheless, weaning significantly suppressed IFN-γ mRNA expression in both groups (Figure 5.1). This suppression was further exacerbated by treatment with dexamethasone on days 1 and 6.

Clinical signs, clinicopathologic changes, and necropsy findings

None of the control ponies demonstrated overt clinical signs compatible with EPE during the study or the two month period following the conclusion of the study. Overall, out of the six weanlings that were challenged with *L. intracellularis*, four weanlings (1, 2, 3, and 4) were ultimately diagnosed with EPE based on the combination of clinical signs, clinicopathologic findings, histologic changes, or PCR results (Table 5.1). Treatment with dexamethasone had no effect on clinical outcome as each of the treated ponies fell into one of the outcome groups (clinical, subclinical, and non-affected).

Weanling #1 exhibited signs of classic EPE including hypoproteinemia (Figure 5.2) and hypoalbuminemia (Figure 5.3). For welfare reasons, this weanling was euthanized on day 21. Histological examination at necropsy revealed elongated ileal villi and blunted, frequently branching crypts. Weanling #4 was the other clinically affected weanling; this weanling’s condition declined acutely leading to euthanasia on day 21. On necropsy, the ileum and distal jejunum were mildly to moderately proliferative and
Figure 5.1: Weaning and dexamethasone treatment suppressed nascent IFN-γ mRNA expression. Results are expressed as relative quantity (RQ) with the average of the day -7 samples used as the calibrator and time points significantly different (p<0.02) from d-7 are denoted with “#”. Significant differences (p<0.05) between treated and non-treated weanlings at each time point are designated “*”.

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Table 5.1: Gender, clinical signs, serum total protein and albumin levels, fecal PCR status, and ultrasonographic findings for each horse and the first day that the finding occurred, if applicable.

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<td>Y, D17</td>
<td>Y, 101.7F, D21</td>
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<td>Y, D16</td>
<td>Y, D13</td>
<td>Y, 5mm, D20</td>
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<td>Mild</td>
<td>+</td>
<td>+</td>
<td>Clinical</td>
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<td>Y, 104.1F, D20*</td>
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<td>Y, D20</td>
<td>Y, D20</td>
<td>Y, D13</td>
<td>None</td>
<td>Mild/Mod</td>
<td>Mild/Mod</td>
<td>+</td>
<td>-</td>
<td>Clinical</td>
</tr>
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<td>Y, D23</td>
<td>Y, D23</td>
<td>Y, D16</td>
<td>Y, 5mm, D20</td>
<td>Mild</td>
<td>Mod</td>
<td>+</td>
<td>-</td>
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<tr>
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<td>None</td>
<td>None</td>
<td>Y, D29</td>
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<td>No</td>
<td>None</td>
<td>Mild/Mod</td>
<td>Mod</td>
<td>+</td>
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<td>Subclinical</td>
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<td>No</td>
<td>None</td>
<td>None</td>
<td>Y, D16</td>
<td>No</td>
<td>No</td>
<td>None</td>
<td>Min/Mild</td>
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<td>-</td>
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<tr>
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<td>No</td>
<td>None</td>
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<td>Y, D23</td>
<td>No</td>
<td>No</td>
<td>None</td>
<td>Mild</td>
<td>Mild</td>
<td>-</td>
<td>-</td>
<td>Non-affected</td>
</tr>
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Y=Yes, D= Day, TP= total protein, Alb= albumin, U/S= ultrasound, IHC= immunohistochemistry, Min= minimal, Mod= moderate

Horses are ordered based on clinical status

**"Clinical EPE Status" refers to those ultimately classified as clinically affected, subclinically affected, or non-affected

** Mildly febrile on D-1- attributed to stress of relocation and weaning

*** Treated with dexamethasone

Low TP ≤5.3 mg/dL

Low Albumin <3.0 mg/dL

Febrile >101.5F

Normal SI size ≤3mm
Figure 5.2: Serum total protein levels in *L. intracellularis* challenged weanlings.
Figure 5.3: Serum albumin levels in *L. intracellularis* challenged weanlings.
rugated. Histologically, areas of mucosal necrosis and crypt herniation with branching were noted, as well as hyperplasia of the crypt epithelium (Figure 5.4). Additionally, both IHC (Figure 5.5) and mucosal scrapings PCR were positive for the bacterium. Subsequent testing for other causes of severe, acute necrotizing enteritis, including *Salmonella* sp. (both direct and enrichment broth culture), *Clostridium perfringens*, *C. difficile*, and *Neorickettsia risticii* was negative. Both weanlings #1 & 4 experienced weight loss during the course of the study, compared with the other four weanlings (Figure 5.6).

Weanlings #2 & 3 were diagnosed with subclinical EPE based on the absence of all of the required criteria to be classified as clinically affected. Weanling #2 demonstrated a mild, transient decrease in total protein and albumin levels beginning on day 23 (Figures 5.2 and 5.3), in contrast to the more profound decrease seen in the clinically affected group. While this weanling did test fecal PCR positive for *L. intracellularis* on days 16 and 20 it was consistently a weak positive. No *L. intracellularis* organisms were detected by IHC; however, the bacteria were detected by mucosal scrapings PCR. By comparison, weanling #3 demonstrated no signs of EPE and no feces tested PCR positive for *aspA*. However, the ileal mucosa was mildly to moderately proliferative and exhibited moderate rugation. Additionally, IHC examination of the distal ileum revealed positive staining for *L. intracellularis* within the enterocytes and mucosal scrapings PCR returned a positive result. While weanlings #2 and 3 had net weight gains during the study, their weight gain was less than that achieved by weanlings #5 and 6 (Figure 5.6).
Figures 5.4 a & b: Representative photomicrographs of ileal sections from an age-matched control and infected weanlings. (a) Normal small intestinal histology (mag=10x), (b) Ileal histologic section from weanling #4, showing marked epithelial hyperplasia as well as branching of the crypts (mag=10x).
Figures 5.5 a & b: Immunohistochemistry detection of *L. intracellularis*. (a) Negative control small intestine (mag=40x), (b) Representative staining of ileal section from weanling #4 showing positive staining in the crypt epithelial cells for *L. intracellularis*. (mag=40x)
Figure 5.6: Net percent weight change for each weanling.
Neither weanling #5 nor weanling #6 demonstrated any signs of EPE; additionally, feces from both weanlings tested PCR negative for *L. intracellularis*. On necropsy, both weanlings had minimal to mild ileal mucosal proliferation with mild rugation of the distal ileum. No *L. intracellularis* organisms were detected in either weanling using IHC or PCR of the mucosal scrapings. Both of these weanlings achieved the highest weight gains over the course of the study (Figure 5.6).

**Lawsonia intracellularis-specific ELISA**

The average *L. intracellularis*-specific ELISA value for the 15 serological control ponies was 33EU, with a standard deviation of 7EU with a maximum ELISA of 49EU and a minimum of 22EU. The control ponies remained ELISA negative through the last day of the study. Based on these controls, an ELISA value \( \geq 55\text{EU} \) (serological control mean plus 3 standard deviation units) was considered positive. Of the two clinically affected weanlings, weanling #1’s first positive ELISA test (56 EU) occurred on day 6 and the values continued to increase until the day of euthanasia, day 21 (Table 5.2). In comparison, weanling #4’s first positive ELISA value was on day 20. From the two subclinically affected weanlings, weanling #2’s first positive ELISA value was first detected on day 20 and increased through day 29, while weanling #3’s initial positive value was detected on day 13 and increased slowly until the end of the study. Both non-affected weanlings seroconverted on day 13 and remained seropositive through the end of the study (day 29).
Table 5.2: ELISA unit values for each horse in relationship to the day of challenge with *L. intracellularis*. Positive samples are denoted with a *. NS= No Sample

<table>
<thead>
<tr>
<th>Horse #</th>
<th>Day -7</th>
<th>Day 0</th>
<th>Day 6</th>
<th>Day 13</th>
<th>Day 20</th>
<th>Day 21</th>
<th>Day 27</th>
<th>Day 29</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>31</td>
<td>36</td>
<td>*56</td>
<td>*106</td>
<td>NS</td>
<td>*394</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>#2</td>
<td>39</td>
<td>30</td>
<td>44</td>
<td>32</td>
<td>*538</td>
<td>NS</td>
<td>*425</td>
<td>*746</td>
</tr>
<tr>
<td>#3</td>
<td>36</td>
<td>34</td>
<td>37</td>
<td>*141</td>
<td>*235</td>
<td>NS</td>
<td>*267</td>
<td>*452</td>
</tr>
<tr>
<td>#4</td>
<td>38</td>
<td>32</td>
<td>34</td>
<td>52</td>
<td>*368</td>
<td>*400</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>#5</td>
<td>27</td>
<td>26</td>
<td>27</td>
<td>49</td>
<td>*198</td>
<td>NS</td>
<td>*148</td>
<td>*186</td>
</tr>
<tr>
<td>#6</td>
<td>22</td>
<td>34</td>
<td>22</td>
<td>27</td>
<td>*241</td>
<td>NS</td>
<td>*202</td>
<td>*227</td>
</tr>
</tbody>
</table>
Interferon-gamma mRNA expression data in response to whole blood stimulation with *L. intracellularis* are presented in Figure 5.7. The clinically affected weanlings produced significantly (p<0.001) less IFN-γ mRNA following antigen stimulation than the other weanlings on day 20. A similar, though non-significant, difference was also seen on day 13 post-challenge. There were no significant differences between the groups in terms of their IFN-γ mRNA expression in response to PMA-ionomycin (data not shown).
Figure 5.7: *L. intracellularis*-induced IFN-γ production by peripheral blood cells. Results are expressed as relative quantity (RQ) with unstimulated control samples used as the calibrator.
Discussion

As an emerging disease of horses, the development of an effective and reliable challenge model for equine proliferative enteropathy is critical to further our understanding of this disease. This is particularly true with respect to developing new and improved diagnostics, as well as understanding the pathogenesis and immunology of EPE. These are the first results published from a *Lawsonia intracellularis* challenge in equids with infection confirmed by postmortem examination, demonstrating that equine proliferative enteropathy can exhibit three clinical forms. As such, one weanling demonstrated signs of classical EPE, another showed clinical signs and histological changes compatible with acute PPE ("acute EPE"), and two others exhibited signs of subclinical disease. The remaining two weanlings seroconverted, but exhibited no clinical or pathological signs indicating that they successfully cleared their infections.

With respect to porcine proliferative enteropathy, three different forms have also been identified: 1.) chronic, 2.) subclinical, and 3.) acute. In the chronic form, the most common signs are diarrhea, rough hair coat, decreased feed consumption, and reduced weight gain. In subclinically infected pigs, the most common clinical sign is poor weight gain, with younger pigs typically affected. The acute form of PPE usually affects older pigs, with severe intestinal hemorrhage, fever, and sudden death being the most common clinical signs, though necrotizing enteritis can be seen with cases of acute porcine proliferative enteropathy.

This is the first study to document successful induction of EPE confirmed by postmortem findings. Possible reasons for the success of this model include the large
inoculation dose used for each horse (4.45×10^8/ml or 2×10^{10} organisms per pony), as well as the use of pony weanlings rather than horse weanlings. This led to a larger concentration of *L. intracellularis* organisms/kg of body weight than if horse weanlings had been used. In pigs, an infectious dose of 10^4-10^6 has been suggested as infective. While the natural infectious dose needed to induce EPE in the horse is unknown, their larger body size, greater intestinal volume, and differences in the gastro-intestinal environment could account for the higher dose requirement. Additionally, it is also possible that natural infection occurs through repeated exposure to lower doses rather than a single, large bolus dose. Since all of the weanlings seroconverted in this study, it seems likely that our challenge dose was sufficient to cause infection. Corticosteroids were unlikely to have contributed to disease induction in this study since of the four affected weanlings, two were treated with dexamethasone and two were not. A more likely reason for the success of this model is that challenge coincided with the weaning period and the associated stress likely impacted cell-mediated immunity. Indeed, all foals exhibited depressed nascent IFN-γ mRNA expression in response to weaning. Dexamethasone treatment further diminished the IFN-γ response, yet there was no effect of this added suppression on clinical outcome. Either weaning alone provided sufficient reduction of cellular immunity for the infection to develop or depression of nascent IFN-γ mRNA expression played little role in the susceptibility of the weanlings to infection.

Results from other animal models indicate that cell-mediated immune mechanisms likely play a pivotal role in resistance to *L. intracellularis* infection. Descriptive immunocytological studies of intestinal tissue sections of pigs have shown evidence of cell-mediated immune response to *L. intracellularis*. Peripheral blood
mononuclear cells from infected pigs exhibit increased production of *L. intracellularis*-induced IFN-γ post challenge.\textsuperscript{70} Similarly, studies in mouse models have identified a significant role for IFN-γ in protective immunity to *L. intracellularis*.\textsuperscript{74,76} Genetic mapping studies in horses suggest a similar role for IFN-γ,\textsuperscript{127} though no direct demonstration has yet been provided. Here we show an association between the IFN-γ response following *in vitro* stimulation of peripheral blood with *L. intracellularis* and clinical outcome. Peripheral blood from the two weanlings that succumbed to the challenge infection produced significantly less IFN-γ compared to the surviving weanlings after incubation with *L. intracellularis*. Thus, it appears likely that IFN-γ and other cell-mediated immune mechanisms play a central role in the horse’s response to this bacterium.

The precise contribution of cell-mediated immunity to protection against EPE is also somewhat unclear. Since *L. intracellularis* replicates in the intestinal epithelium it could induce a cellular cytotoxic immune response against the infected cells, as is the case for other intracellular microorganisms.\textsuperscript{76} However, *L. intracellularis* infection are generally associated with modest cellular infiltration,\textsuperscript{74} therefore a direct effect of IFN-γ on cellular proliferation is thought to be the likely mechanism of action.\textsuperscript{74,76} A similar mechanism of action is seen in other intracellular infections, as well.\textsuperscript{128,129} Oral vaccination of pigs with an avirulent live vaccine of *L. intracellularis* is associated with protection from disease\textsuperscript{54} and the induction of IFN-γ by *L. intracellularis*-stimulated PBMC.\textsuperscript{89} The robust IFN-γ recall response we observed in those weanlings that were protected from clinical infection indicates a similar protective role for this cytokine in EPE.
CHAPTER SIX:

Conclusions and Future Research Directions

As an emerging disease of horses, there are many answered questions that revolve around equine proliferative enteropathy. Unfortunately, because of the relative rarity with which EPE occurs outside of central Kentucky, and even amongst exposed horses for that matter, all but the most basic of studies into EPE have yet to be performed. The research presented within this dissertation utilized the tremendous resource central Kentucky Thoroughbred farms provide to offer additional insight into why EPE occurs sporadically despite high exposure rates.

*All farms in central Kentucky experience exposure to L. intracellularis and previous EPE suggests future risk*

Perhaps one of the most surprising findings from this research came from Chapter 2, in which all farms, regardless of their prior EPE history, had evidence of weanling/yearling exposure to *L. intracellularis*. Given the other finding from that chapter in which prior history of EPE was associated with an increased seroprevalence rate, as well as a likelihood of developing disease in subsequent years, possible explanations for the differences between the groups would be *L. intracellularis* strain pathogenicity, exposure burdens, or a combination of the two. Titration challenge studies, much like those that have been performed in pigs, would help to elucidate whether there are differences in disease occurrence and antibody levels between dosages while experimental challenge with different strains of the bacterium would show whether
there truly are strain differences. Given that there is currently only one known isolated strain of equine-origin \textit{L. intracellularis} available for use, this second part is currently not possible. An alternative might include regular collection of fecal material from the at-risk population followed by genetic typing\textsuperscript{122} of the bacterium to look for similarities and differences on farms with endemic versus no EPE.

\textit{Passively-acquired antibodies to \textit{L. intracellularis} do not protect against EPE}

It is not surprising, given the enteric location of \textit{L. intracellularis} infection, that passively-acquired and systemically circulating antibodies do not have a protective role against EPE, despite a recent suggestion to the contrary in pigs.\textsuperscript{68} A larger number of EPE cases would have helped to strengthen these conclusions especially given the lower-than-expected number of mares that were seropositive at parturition. However, given that the rates of disease and infection and foal seropositivity following parturition were similar, there is little reason to question the findings presented here.

\textit{Disease "attack'' rates of \textasciitilde{11\%} can be expected in \textit{L. intracellularis}-exposed horses}

Traditionally, estimates of 5-10\% of seropositive horses developing clinical EPE have been used, but this assumption has never been proven in a large scale, field study before. With \textit{L. intracellularis}-exposed horses, we found a clinical EPE attack rate (using the presumptive EPE cases) of 5.3\% and an infection attack rate (using cases with suspected infections) of 6.3\%; combined, the overall attack rate was 11.6\% among seropositive horses. An accurate attack rate will allow for refinement of experimental
challenge models and testing of numerous hypotheses with respect to EPE epidemiology and disease susceptibility.

**Mares cannot be ruled out as playing a role in the epidemiology of EPE**

Given the amount of time that foals spend with their respective mares prior to weaning, as well as the relatively high number of mares that were seropositive for 6+ months (46.3%), mares must be regarded as a potential source of *L. intracellularis* for their foals. The data in this study did not eliminate mares as possible sources of the bacterium on an EPE-endemic farm. Since this was a pilot study, further serum screening accompanied by regular fecal PCR testing of persistently seropositive mares and all foals/weanlings on an endemic farm will be necessary to identify what role mares play in the spread of *L. intracellularis*. Because of the rarity with which nursing foals develop EPE, one of the possibilities is that mares, through milk-derived IgA, are protecting their foals from exposure and disease. This idea could be easily tested by weaning nursing foals and comparing their EPE susceptibility with that of nursing, non-weaned foals.

**IFN-γ plays a significant role in protection against the development of EPE**

This was the first study in any of the main target species to correlate *L. intracellularis*-induced IFN-γ production with disease protection. As has been mentioned earlier, IFN-γ seems the most likely effector of CMI in response to *L. intracellularis* infection and here we showed that failure to produce IFN-γ in response to infection may have ultimately led to the occurrence of EPE. Given that IFN-γ production increases with age in horses, one possible experiment would entail challenging neonates, foals,
weanlings, yearlings, and adults with *L. intracellularis*. It would then be possible to correlate their IFN-γ response *in vitro* to the bacterium with the presence of infection and/or disease as detected on necropsy. Likewise, with recent work from our lab showing that a dirty barn atmosphere is capable of stimulating an increase in IFN-γ+ lymphocytes, as well as work in pigs showing a markedly higher disease rate amongst challenged pigs, another experiment could examine the difference in disease rates between foals/weanlings raised only in pastures, a dirty barn, or a "clean" barn. Lastly, given the suggestion in Chapter 3 that prior *Rhodococcus equi* infection may protect against clinical EPE, a two-tiered challenge study (first, as foals with *R. equi*, followed by challenge, as weanlings, with *L. intracellularis*) would be useful to determine whether this relationship does exist and the potential mechanisms for it.

*Other directions*

As elucidated in Chapter 1, *L. intracellularis* appears to be capable of avoiding multiple arms of the immune system given the lack of an inflammatory response following infection. With our recent reporting of N-EPE cases, in addition to PHE cases, it is possible that these occur because the bacterium is detected via the CMI or innate immune system, or Tregs fail to down-regulate the localized immune response. Given that TGF-β is important for suppression of Th1 and Th2 function, the differentiation of inducible Tregs, and has been shown to increase following infection with *L. intracellularis*\(^68\), TGF-β knockout mice could possibly be used to examine its role in the pathology of *L. intracellularis* infection. If TGF-β is important in limiting overt
inflammation following infection with the bacterium, we would expect to see pathology similar to N-EPE or PHE in these mice.

This dissertation has provided both novel data as well as data to show that *L. intracellularis* infection and disease in horses is similar to what has been reported in other species. This all serves to provide the building blocks for future work into EPE from both epidemiology and immunology standpoints as a better understanding of *L. intracellularis* and its mechanisms of disease induction and immune evasion is necessary to develop strategies for combating and preventing equine proliferative enteropathy.
APPENDIX I:

Graph of Standard Curve Optical Density (OD) Values for Each *Lawsonia intracellularis*-Specific ELISA Unit Value

\[
y = 0.2776\ln(x) - 0.7838
\]

\[R^2 = 0.9922\]
APPENDIX II:

Acute Deterioration and Death with Necrotizing Enteritis Associated with *Lawsonia intracellularis* in Four Weanling Horses

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Cases

*Case 1:*

An 8-month-old Thoroughbred colt was evaluated in October 2010 with a less-than-one-day history of inappetence. Physical examination in the field revealed throat latch edema, depression, and a fever (103.8°F) (ref 99-101.5 °F). A complete blood count revealed leukocytosis (18.0 K/uL; ref 5.0-12.6 K/uL) with a relative neutropenia (49%; ref 55-65%) and lymphopenia (26%; ref 35-45%), as well as a toxic left shift (25% bands; ref 0-5%). Serum biochemistry abnormalities included hypoproteinemia (3.3 g/dL; ref 6-7.9g/dL), hypoalbuminemia (1.2 g/dL; ref 3.4-4.1 g/dL), and an increased BUN (45 mg/dL; ref 11-26 mg/dL), along with other abnormalities (Table A.1).
Table A.1. All available blood work results. Day 0= day initial signs of EPE were noted. Reference ranges are provided for each parameter in ( ).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Case #1 Day 0</th>
<th>Case #1 Day +2</th>
<th>Case #2 Day 0</th>
<th>Case #2 Day +1</th>
<th>Case #2 Day +3</th>
<th>Case #3 Day +3</th>
<th>Case #3 Day +4</th>
<th>Case #4 Day +1</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (5-12.6 K/ul)</td>
<td>18.0 (H)</td>
<td>35.4 (H)</td>
<td>6.2</td>
<td>1.6 (L)</td>
<td>31.5 (H)</td>
<td>42.4 (H)</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td>RBC (6.5-9.99 M/ul)</td>
<td>12.9 (H)</td>
<td>10.8 (H)</td>
<td>11.3 (H)</td>
<td>16.5 (H)</td>
<td>15.18 (H)</td>
<td>8.17</td>
<td>9.64</td>
<td></td>
</tr>
<tr>
<td>HGB (11-16 g/dL)</td>
<td>15.3</td>
<td>12.8</td>
<td>15.1</td>
<td>21 (H)</td>
<td>19.9 (H)</td>
<td>10.7 (L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCT (33-48%)</td>
<td>44.8%</td>
<td>39.2%</td>
<td>45%</td>
<td>63.9% (H)</td>
<td>50.4% (H)</td>
<td>27.4% (L)</td>
<td>34.9%</td>
<td></td>
</tr>
<tr>
<td>PLT (115-450 K/ul)</td>
<td>600 (H)</td>
<td>83 (L)</td>
<td>508 (H)</td>
<td>644 (H)</td>
<td>700 (H)</td>
<td>125</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neut (55-65%)</td>
<td>49% (L)</td>
<td>49% (L)</td>
<td>18% (L)</td>
<td>78% (H)</td>
<td>76% (H)</td>
<td>14 (L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bands (0-5%)</td>
<td>26% (H)</td>
<td>26% (H)</td>
<td>12% (H)</td>
<td>15% (H)</td>
<td>9% (H)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph (35-45%)</td>
<td>26% (L)</td>
<td>25% (L)</td>
<td>70% (H)</td>
<td>7% (L)</td>
<td>15% (L)</td>
<td>84 (H)</td>
<td></td>
<td></td>
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<tr>
<td>Mono (0-6%)</td>
<td>0% (L)</td>
<td>0% (L)</td>
<td>0% (L)</td>
<td>0% (H)</td>
<td>0% (H)</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na (133-140 mEq/L)</td>
<td>122 (L)</td>
<td>115 (L)</td>
<td>134 (L)</td>
<td>123 (L)</td>
<td>118 (L)</td>
<td>125 (L)</td>
<td>133</td>
<td></td>
</tr>
<tr>
<td>K (2.5-5 mEq/L)</td>
<td>3.4</td>
<td>3.2</td>
<td>3.9</td>
<td>3.3</td>
<td>3.3</td>
<td>4.4</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>Cl (97-105 mEq/L)</td>
<td>90 (L)</td>
<td>86 (L)</td>
<td>89 (L)</td>
<td>85 (L)</td>
<td>100</td>
<td>92 (L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO2 (25-32 mEq/L)</td>
<td>19 (L)</td>
<td>14 (L)</td>
<td>25 (L)</td>
<td>17 (L)</td>
<td>20 (L)</td>
<td>20 (L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anion Gap</td>
<td>13</td>
<td>15</td>
<td>12</td>
<td>17</td>
<td>13</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUN (11-26 mg/dL)</td>
<td>45 (H)</td>
<td>59 (H)</td>
<td>32 (H)</td>
<td>50 (H)</td>
<td>33 (H)</td>
<td>47 (H)</td>
<td>4.7 (L)</td>
<td></td>
</tr>
<tr>
<td>Creatinine (0.8-1.8 mg/dL)</td>
<td>1.3</td>
<td>1.8</td>
<td>1.2</td>
<td>2.4 (H)</td>
<td>1.4</td>
<td>1.9 (H)</td>
<td>0.7 (L)</td>
<td></td>
</tr>
<tr>
<td>Calcium (11.6-13.2 mg/dL)</td>
<td>9.1</td>
<td>12.6</td>
<td>9.6 (L)</td>
<td>8.5 (L)</td>
<td>8.0 (L)</td>
<td>8.2 (L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Protein (6-7.9 g/dL)</td>
<td>3.3</td>
<td>3.5 (L)</td>
<td>5.1 (L)</td>
<td>3.7 (L)</td>
<td>2.3 (L)</td>
<td>1.7 (L)</td>
<td>3.6 (L)</td>
<td></td>
</tr>
<tr>
<td>Albumin (3.4-4.1 g/dL)</td>
<td>1.2</td>
<td>1.1 (L)</td>
<td>2.8 (L)</td>
<td>2.5 (L)</td>
<td>1.5 (L)</td>
<td>0.9 (L)</td>
<td>0.6 (L)</td>
<td>1.8 (L)</td>
</tr>
</tbody>
</table>
*Lawsonia intracellularis*-induced equine proliferative enteropathy (EPE) was suspected because of the combination of hypoproteinemia, hypoalbuminemia, inappetence, and the autumn presentation. Treatment consisted of intravenous oxytetracycline\(^V\) (6.6 mg/kg IV q24hr), flunixin meglumine\(^W\) (1 mg/kg IV q12h), oral omeprazole\(^X\) (1 mg/kg PO q24hr), dexamethasone\(^Y\) (0.1 mg/kg IV q24hr), intravenous crystalloid fluids\(^Z\) (10 ml/kg IV bolus once), and intravenous colloids\(^AA\) (10 ml/kg IV bolus once). Despite treatment, the weanling was euthanized within 48 hours of presentation due to continued deterioration and signs of pulmonary disease characterized by nasal discharge, epistaxis, and tachypnea. Blood work submitted the morning of euthanasia revealed a worsening leukocytosis (35.4 x10\(^3\)/uL) with an unchanged differential, as well as continued hypoproteinemia (3.5 g/dL), hypoalbuminemia (1.1 g/dL), and an increased BUN (59 mg/dL).

**Case 2:**

An 8-month-old Thoroughbred filly from the same farm as case #1 presented in October 2011 with inappetence of less than 12-hours duration. Physical examination revealed fever (102.5°F) and an unthrifty appearance, including a poor hair coat. Examination of the serum albumin concentration at presentation revealed hypoalbuminemia (2.8 g/dL). A complete blood count the following morning revealed no significant abnormalities, but hypoproteinemia (5.1 g/dL) and a worsening

\(^V\) Liquamycin LA-200, Pfizer Animal Health, New York, NY  
\(^W\) Banamine, Merck Animal Health, Summit, NJ  
\(^X\) UlcerGard, Merial Ltd., Duluth, GA  
\(^Y\) Dexium, Bimeda, Le Sueur, MN  
\(^Z\) Normosol-R, Hospira, Lake Forest, IL  
\(^AA\) Hetastarch, Hospira, Lake Forest, IL
hypoalbuminemia (2.5g/dL) were seen on serum biochemistry; additionally, an L. intracellularis immunoperoxidase monolayer assay (IPMA) titer of ≥1:240 was reported. Other abnormalities were also noted (Table A.1). On the basis of the time of year, the presence of anorexia, hypoproteinemia, hypoalbuminemia, and the weanling’s positive IPMA titer, in addition to the farm’s previous history, a presumptive diagnosis of EPE was made. Treatment initially consisted of oral doxycyclineBB (10 mg/kg PO q12h) and flunixin meglumine (1 mg/kg IV q12h); however, due to the continued decline of the weanling, the doxycycline was discontinued and intravenous oxytetracycline (6.6 mg/kg IV q24hr) initiated two days after presentation.

The following morning (approximately 60 hours after initial signs were noted), the filly was depressed and lethargic, with a fever of 105.9°F. CBC revealed severe leukopenia (1.6 x10³/uL), neutropenia (18%), lymphocytosis (70%), and toxic left shift (12%). The serum biochemistry abnormalities included azotemia as well as a decline of serum total protein (3.7 g/dL) and albumin (1.5 g/dL)(Table S1). Concerns about the filly’s condition prompted referral for more intensive care. On arrival, the filly remained depressed and lethargic with a fever of 103.1°F. Additionally, the filly was tachycardic (78 bpm; ref 20-44 bpm) and tachypneic (48 bpm; ref 8-24 bpm). Abdominal ultrasound revealed thickened small intestinal wall (6mm; ref ≤3mm) and free fluid in the abdominal cavity. Abdominocentesis was performed, and fluid analysis revealed a WBC count of 77,500 cells/uL (90% neutrophils and 10% mononuclear cells) (ref 0.5 - 5.0x10³/uL WBC and 1:1 ratio of neutrophils:mononuclear cells) and total protein of 2.0 g/dL (ref

---

BB Hagyard Pharmacy, Lexington, KY
0.5-1.5 g/dL). In addition, a moderate number of short Gram-negative rods and rare Gram-positive cocci were noted, but no plant or fecal material was seen in the peritoneal fluid sample. Culture results of the abdominal fluid revealed *Actinobacillus equuli*. Within several hours of hospitalization, the filly became agitated, developed cyanotic mucous membranes, and exhibited agonal activity. The filly was euthanatized approximately 72 hours after initial presentation at the farm.

**Case 3:**

A 6-month-old Thoroughbred colt was evaluated on the farm in November 2011 due to a 72-hour history of lethargy. On presentation, the colt was lethargic, tachycardic (68 bpm), and febrile (103.5°F). Additionally, the colt had dark red/purple mucous membranes and delayed capillary refill time. The colt had developed diarrhea within the previous 12 hours, and severe throatlatch and preputial edema were noted. Immediately prior to referral to the McGee Medicine Center, treatment was initiated at the farm and included intravenous oxytetracycline (6.6 mg/kg IV q24hr), flunixin meglumine (1 mg/kg IV q12h), and synthetic colloids (10 ml/kg IV bolus once). A complete blood count indicated leukocytosis (31.5 x10^3/uL) with neutrophilia (78%), lymphopenia (7%), and a left shift (15%). Serum biochemistry abnormalities included hypoproteinemia (2.3 g/dL) and hypoalbuminemia (0.9 g/dL), along with other metabolic abnormalities (Table S1). Abdominal ultrasound demonstrated thickening of the small intestinal wall and a preliminary diagnosis of EPE was made.

Following referral, the colt remained febrile despite non-steroidal anti-inflammatory drug (NSAID) treatment; further, epistaxis developed, with progressively
increasing volumes of blood exiting both nostrils, ultimately leading to respiratory
distress. The respiratory distress was partially alleviated with the placement of a
nasotracheal tube. Spontaneous epistaxis continued and the colt also began passing
dark/bloody diarrhea and blood tinged urine. Because of welfare concerns in the face of
suspected disseminated intravascular coagulation (DIC), the colt was euthanized less than
96 hours after developing clinical signs. Blood work values collected prior to euthanasia
revealed an increased leukocytosis (42.4 x10³/uL), as well as a progressive
hypoproteinemia (1.7 g/dL) and hypoalbuminemia (0.6 g/dL) (Table S1).

Case 4:

A 6-month-old Thoroughbred colt presented in late October 2011 with a fever
(105°F) five hours prior to initial evaluation; the fever was immediately treated by the
farm with flunixin meglumine (1 mg/kg IV q12h). The weanling was small for its age
despite having received regular preventative health care and physical examination
revealed no other significant findings. A complete blood count revealed a normal white
blood cell count (7.7 x10³/dL) with a neutropenia (14%) and lymphocytosis (84%). The
serum biochemistry abnormalities included hypoproteinemia (3.6g/dL) and
hypoalbuminemia (1.8g/dL)(Table A.1). EPE was tentatively diagnosed, and treatment
was initiated with oxytetracycline¹ (10 mg/kg IV q24hr). At the time antimicrobial
treatment was initiated, the colt was eating and had normal vital sign values. Four hours
after examination, the colt developed peracute signs of shock and gastrointestinal crisis,
including tachycardia (108 bpm), tachypnea (40-50 bpm), dark purple mucous
membranes, and general signs of severe abdominal discomfort (rolling on the ground).
Despite emergency intravenous fluid resuscitative care consisting of IV crystalloids (7 ml/kg bolus) and colloids (2 ml/kg bolus), the weanling quickly became recumbent and died less than 45 minutes into the crisis and approximately 24 hours after initial presentation.

**Necropsy Findings:**

Necropsy findings involving the small intestine were similar in all 4 cases. Segments of the mucosa of jejunum, ileum, or both were thickened (cases #1-4), variably reddened (cases #1, 2, and 4), and corrugated (cases #2 and 4). Regions of ulceration and greenish-brown necrotic mucosal plaques were occasionally evident (case #3). Peyer’s patches were hemorrhagic (case #4), and the intestinal lumen contained a moderate amount of dilute reddish-brown fluid (cases #1, 3, and 4). The cecal and colonic walls were moderately edematous in cases #2 and 3. Additional lesions identified at necropsy included: pneumonia and gastric ulcers (case #1); cestodiasis, fibrinous peritonitis, and vegetative valvular endocarditis (case #2); multiple acute renal infarcts (case #3); and mesenteric lymphadenopathy (case #4).

*L. intracellularis* was associated with the small intestinal lesions in all 4 cases. *L. intracellularis* DNA was detected in ileal mucosal scrapings by PCR in all four cases. *L. intracellularis* specific immunohistochemistry was performed on two cases (#2 and 4) and directly identified the bacterium in the necrotic lesions of both cases.

Various bacteria were cultured from the intestines and systemic organs; culture results and tetracycline antibiograms, given that tetracycline-class drugs are typically the first antimicrobial administered to animals with suspected EPE in central Kentucky, are
presented in Table A.2. The gram positive cocci noted in the abdominal fluid sample of case #2 were not noted at necropsy. This could possibly be due to the administration of antimicrobials or the cocci were anaerobic and not cultured at necropsy.

Histologically, small intestinal lesions were morphologically diagnosed as severe subacute segmental necroulcerative enteritis (cases #1 and 4) or severe chronic segmental proliferative and necroulcerative enteritis (cases #2 and 3). All 4 cases exhibited regions of mucosal necrosis, erosion, and ulceration that were covered by mats of small amounts of fibrin, cell debris, and acidophilic proteinaceous material, low number of neutrophils and macrophages, and mixed populations of bacteria (Figures 3 and 4). The lamina propria and submucosa, subjacent to the ulcers, were edematous and infiltrated with low numbers of neutrophils, macrophages, and lymphocytes. Multifocal blood vessels contained fibrin thrombi. Additionally in cases #2 and 3, multifocal regions of the mucosa were proliferative and exhibited elongated villi, branched crypts, and crypt and glandular epithelial hyperplasia. Few crypts contained cell debris and acidophilic proteinaceous material. Tertiary findings included: necrotizing glomerulonephritis, hepatitis, and embolic pneumonia (case #1); vegetative valvular endocarditis and peritonitis (case #2); renal infarction (case #3); and renal glomerular thrombosis (case #4).
Table A.2. Bacteriology results obtained at necropsy. Includes the bacterial species, isolation location, and tetracycline-class antimicrobial sensitivity.

<table>
<thead>
<tr>
<th>Case #</th>
<th>Bacterium</th>
<th>Isolation Location</th>
<th>Tetraacycline/Doxycycline Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Streptococcus zooepidemicus</td>
<td>Lung</td>
<td>Susceptible</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>Liver</td>
<td>Moderately Susceptible</td>
</tr>
<tr>
<td></td>
<td>Actinobacillus equuli subsp equuli</td>
<td>Lung</td>
<td>Resistant</td>
</tr>
<tr>
<td>2</td>
<td>Actinobacillus equuli subsp equuli</td>
<td>Peritoneal cavity</td>
<td>Not Reported</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>Heart</td>
<td>Resistant</td>
</tr>
<tr>
<td>3</td>
<td>No pathogenic organisms isolated</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>4</td>
<td>E. coli</td>
<td>Small intestine</td>
<td>Resistant</td>
</tr>
</tbody>
</table>
Figure A.1: Ileum, age-matched control. Villi are distinct and of normal length. H&E (40X total magnification). Bar = 1mm
Figure A.2: Ileum, chronic EPE. Villi are segmentally blunted and fused. Crypts are hyperplastic, elongated, and multifocally branched. H&E (40X total magnification). Bar = 1mm
Figure A.3: Ileum, necrotizing EPE, case 2. Villi are shortened and the crypts are mildly hyperplastic. Superficial villar tips are necrotic and covered by small amounts cell debris. H&E (40X total magnification). Bar = 1mm
Figure A.4: Ileum, necrotizing EPE, case 2. Higher magnification of the necrotic villar tips. H&E (400X total magnification). Bar = 100um
Discussion

Proliferative enteropathies induced by *Lawsonia intracellularis* infections have been noted to occur in a variety of species worldwide. Most notable is *L. intracellularis* infection in pigs where financial losses from porcine proliferative enteropathy are significant. The majority of what is known with respect to *L. intracellularis* has been discovered from work in pigs and largely extrapolated to the horse. Porcine proliferative enteropathy exhibits several clinical entities, including the subclinical form in which affected pigs exhibit no clinical signs but shed the bacterium, the chronic form where affected pigs fail to grow as well as littermates, and the acute form (normally referred to as porcine hemorrhagic enteritis or PHE), which is often characterized by hemorrhagic diarrhea and acute death. While acute morbidity and mortality has long been recognized as a common clinical presentation of *Lawsonia intracellularis* infection in swine, this clinical presentation has never, to the authors’ knowledge, been described in multiple field cases of EPE. Here we have presented four cases detailing an acute clinical entity with necrotic enteritis, which we suggest be referred to as a necrotizing form of EPE (N-EPE). This designation serves to provide not only a pathologic description, but also an inference to the observed severity of clinical disease and rapid deterioration of the affected animal.

With EPE, clinical signs of anorexia, dependent edema secondary to hypoproteinemia and hypoalbuminemia, depression, lethargy, and weight loss are typically observed during the fall and winter months (August-February); fever, colic, and diarrhea are less frequently seen. Ultrasonographic detection of thickened
small intestinal walls in weanlings is highly suggestive of EPE, although the finding can be inconsistent.\textsuperscript{11} Clinical cases of EPE generally develop slowly over several days, but during an EPE challenge study one challenged weanling developed what we termed "acute EPE" given the rapid deterioration and necrotizing enteritis that was noted in the weanling.\textsuperscript{10} Since this was an experimental challenge study that likely failed to adequately replicate the field conditions in which \textit{L. intracellularis} infection is typically contracted, we surveyed local practitioners for other, "acute EPE" cases. During the span of thirteen months (October 2010-November 2011), the four cases detailed above were identified in the central Kentucky region. A literature review found that the first published description of EPE by Duhamel and Wheeldon\textsuperscript{13} recounted the acute deterioration (5 days) and death of a foal, similar to the 24-96 hour time courses of our cases, whereas a more recent report documented the occurrence of necrotizing enteritis associated with \textit{L. intracellularis} infection in one horse with a 10+ day history of EPE.\textsuperscript{130} Based on the short timeframe in which we selected cases, as well as the occasional report of various facets of the N-EPE clinical entity, it is highly likely that cases of N-EPE have occurred with regularity and simply not been recognized until now. Likewise, the previous case of "acute EPE" we reported\textsuperscript{10} should, instead, be considered a case of N-EPE based on the criteria listed above. Given that not all cases of N-EPE will expire as a result of EPE or secondary bacterial infections, practitioners may elect to diagnose surviving animals with "acute clinical EPE" and reserve N-EPE as a pathologic diagnosis.

In pigs, it has been suggested that \textit{Lawsonia} associated necrotic enteritis develops from inflammation and necrosis induced by secondary bacterial invaders.\textsuperscript{131} The clinical
signs observed with these four weanlings were highly unusual for EPE. Damage induced to the intestinal mucosal barrier by secondary pathogens likely predisposed these weanlings to endotoxemia or bacteremia and resulted in secondary systemic sequelae. This assertion appears to be supported by the presence of thickened mucosa associated with the necrotizing and ulcerative enteritis, suggesting that alimentary damage preceded bacterial translocation. The clinicopathologic, gross necropsy, and histologic findings in cases #1 and #2 suggest that the observed clinical signs resulted directly from secondary bacteremia. Interestingly, these two cases originated from the same farm but 12 months apart. Finding regular, yearly cases of EPE on certain farms has been reported previously, but the occurrence of two separate cases of N-EPE on the same farm in different years is a novel finding. This could suggest that the particular strain of \textit{L. intracellularis} on this farm is potentially more virulent and that this farm should monitor diligently for reoccurrence of N-EPE. Case #3 survived the longest after initial presentation (~96 hours); however, treatment for EPE was not started until approximately 72 hours post-presentation. This weanling appeared to develop fulminant disseminated intravascular coagulation after treatment was initiated, as demonstrated by the occurrence of uncontrolled epistaxis, hematuria, melena/hematochezia, and multiple renal infarcts. Possible causes for the development of DIC in this weanling include septicemia/bacteremia, endotoxemia, or a decrease in circulating antithrombin-3 due to hypoproteinemia. The last case, Case #4, survived for the shortest amount of time (<24 hours post-presentation) and was the only weanling to die spontaneously. Based on the renal glomerular tuft thrombosis, this weanling likely died due to cardiovascular compromise initiated by acute DIC.
Equine proliferative enteropathy has been a disease viewed as having a well-defined clinical presentation and a high treatment success rate when properly diagnosed. The cases presented here provide an important repudiation to this commonly held view in that, despite the correct preliminary diagnosis and appropriate treatment, all four of these weanlings succumbed to complications arising from EPE. This would suggest that treatment of N-EPE cases (those with signs of secondary bacterial infection and/or endotoxemia) should be treated aggressively with broad-spectrum antimicrobials to ensure coverage against both Gram-positive and Gram-negative bacteria, anti-thrombotics, and anti-endotoxic measures such as low-dose flunixin meglumine and/or intravenous polymyxin-B. Care must be taken, however, when administering NSAIDs and/or polymyxin-B due to their potential nephrotoxic effects in patients with hypovolemia or in which there is a predisposition for azotemia: additionally, NSAID use could exacerbate existing mucosal compromise. While no anaerobes were isolated from the cases presented above, practitioners should be aware of the possibility of secondary infections with anaerobic bacteria and consider metronidazole treatment if they believe it to be warranted.

More work is needed to understand the risk factors for the development of N-EPE, which could include environmental/husbandry differences, genetic polymorphisms, the infectious dose, *L. intracellularis* strain, concurrent disease, or animal’s immune status. Our previous work with an experimental challenge model seems to suggest that the host immune system likely plays the largest role in this type of clinical presentation as the infectious dose and strain used in that study was consistent across six challenged weanlings, yet only one developed N-EPE; however, based on the
occurrence of two N-EPE cases on the same farm in consecutive years, a significant role of bacterial strain cannot be ruled out. Regardless, practitioners must be cognizant of the necrotizing form of EPE as weanlings may fail to demonstrate the classic, chronic clinical signs associated with EPE (including weight loss and dependent edema) and/or require emergency treatment to prevent secondary complications such as septicemia, endotoxemia, and DIC, all of which could ultimately lead to acute death.
REFERENCES


VITA

Allen E. Page

Education and Post-D.V.M. Work Experience:

**Ph.D.** Veterinary Science, 2009-Present
Dissertation emphasis: *L. intracellularis* in horses
G.P.A. 3.72
University of Kentucky
Lexington, KY
Advisor: Dr. David Horohov

**Rotating Intern,** 2008-2009
In-house internal medicine and ambulatory
Hagyard Equine Medical Institute
Lexington, KY

**D.V.M.,** 2004-2008,
G.P.A. 3.83
University of CA, Davis
Davis, CA

**B.S.** Animal Science, 2000-2004
Equine Emphasis
University of CA, Davis
Davis, CA

**High School** Class of 2000
La Jolla Country Day School
La Jolla, CA

Honors and Awards

**2012** AAEP/EQUUS Foundation Research Fellow

**2009-Present** Morris Animal Foundation/Zoetis Veterinary Scholars Research Fellow

**2008** Phi Zeta, Lambda Chapter, Honor Society of Veterinary Medicine, School of Veterinary Medicine, University of California, Davis

Committees

**2013** Dean Search Committee, Student Representative, University of Kentucky, College of Agriculture, Food, and Environment

Manuscripts:


**Book Chapters:**


**Abstracts:**


**Local/State Presentations:**

**National Presentations:**

**International Presentations:**