Investigation of an emergent, tetracycline-resistant, abortifacient Campylobacter jejuni clone in a pregnant guinea pig model

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Investigation of an emergent, tetracycline-resistant, abortifacient *Campylobacter jejuni* clone in a pregnant guinea pig model

by

Eric Ryan Burrough

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

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This dissertation is dedicated to:

Brenda
Lauren
Olivia
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CHAPTER 1: GENERAL INTRODUCTION

Statement of the Problem

_Campylobacter_ spp. have been recognized as an important cause of sheep abortion for decades,\(^1\) and, historically, the most common isolates have been _C. fetus_ subsp. _fetus_, _C. jejuni_, and _C. coli_, with decreasing frequency, respectively.\(^1^-^5\) Epizootics of abortion often occur within affected flocks with up to 50% of the flock aborting,\(^1\) and, during these epizootics, two or even all three species of _Campylobacter_ have been recovered from the same flock.\(^1\) Additionally, abortion-associated isolates of both _C. fetus_ subsp. _fetus_ and _C. jejuni_ have traditionally shown antigenic and genetic heterogeneity across flocks and across lambing seasons.\(^3^-^8\)

Two recent analyses\(^9^-^10\) of ovine abortion-associated _Campylobacter_ isolates have revealed that _C. jejuni_ has replaced _C. fetus_ subsp. _fetus_ as the predominant species isolated, and, in one of these analyses,\(^10\) which surveyed isolates from multiple states and across multiple lambing seasons, 93% of the _C. jejuni_ isolates were of a single genetic clone (named clone SA for Sheep Abortion) and 100% were resistant to tetracycline. The emergence of a single clone of tetracycline-resistant _C. jejuni_ associated with the preponderance of abortions across multiple states and multiple lambing seasons reveals a dramatic shift in the epidemiology of ovine campylobacteriosis and suggests that a change in preventative strategies for controlling this disease may be warranted.

Much of what is known regarding the pathogenesis of _C. jejuni_ infection is based on its role as an intestinal pathogen of man; however, even in this regard, knowledge of
the molecular mechanisms employed by this bacterium lags behind that of other enteric pathogens.\textsuperscript{11} One significant impediment to the study of \textit{Campylobacter} pathogenesis is the lack of appropriate laboratory animal models that adequately replicate natural disease.\textsuperscript{11,12} While it is accepted that in the pathogenesis of ovine campylobacteriosis pathogenic \textit{Campylobacter} spp. must bind to and invade intestinal epithelia, survive the host immune response, and disseminate hematogenously to other organs;\textsuperscript{13} knowledge of the precise mechanisms underlying each of these requisite steps is vague at best, particularly with regard to dissemination and the apparent tropism of certain strains of \textit{C. jejuni} for the fetoplacental unit.

\textbf{Specific aims}

The overriding goal of the studies described herein was to enhance understanding of some of the potential pathologic mechanisms underlying abortion in sheep associated with the emergent, tetracycline-resistant clone SA of \textit{C. jejuni}. Our central hypothesis for this set of studies was that this particular clone is highly pathogenic with marked tropism for the fetoplacental unit and resistance to traditional prevention strategies. To address this hypothesis, the following specific aims were set forth: 1) develop an appropriate laboratory animal model of abortion following oral inoculation (Chapter 2) and use this model to 2) assess the expression of Toll-like receptors in infected versus uninfected placentas (Chapter 3), 3) compare the efficacy of commercially available vaccines and an experimental bacterin against challenge with the IA3902 isolate of clone SA (Chapter 4),
and 4) investigate the effects of different placental factors and blood and bile from pregnant animals on the growth and chemotaxis of \textit{C. jejuni} IA3902 (Chapter 5).

**Dissertation organization**

This dissertation is organized in the alternative format with six chapters. The first chapter (Chapter 1) consists of a general introduction and literature review. The four chapters that follow (Chapter 2 – 5) are comprised of four individual manuscripts, and are followed by a final chapter (Chapter 6) containing general conclusions and suggestions for future research. References appear at the end of each chapter. Two of the manuscripts have been published in or accepted for publication in the \textit{American Journal of Veterinary Research} (Chapter 2 and Chapter 4), one has been published in \textit{Veterinary Pathology} (Chapter 3), and the fourth manuscript has been submitted to \textit{Veterinary Pathology} (Chapter 5).

**Literature review**

\textbf{Campylobacter spp.: General characteristics and zoonotic risk}

\textit{Campylobacter} spp. are gram-negative, nonsporeforming rods with a characteristic curved, spiral or S-shaped morphology and darting motility.$^{14,15}$ Most species have a single polar unsheathed flagellum at one (monotrichous) or both (amphitrichous) ends of their cells.$^{15}$ Campylobacters are generally microaerophilic, nonsaccharolytic, and utilize amino acids and intermediates of the tricarboxylic acid
cycle for energy; however, recent evidence suggests certain strains of *C. jejuni* have the capacity to metabolize L-fucose. There are currently 17 species in the genus *Campylobacter* and they are widespread in nature and infect a broad range of hosts including birds, animals, and man. Originally classified in the genus *Vibrio*, campylobacters were later moved to their own genus based on differences in metabolism and DNA composition; as such, much of the early literature bears the former taxonomy and disease in animals was historically known as vibriosis. The predominant *Campylobacter* spp. of importance in veterinary medicine include: *C. fetus*, *C. jejuni*, and *C. coli*.

*C. fetus* grows between 25°C and 37°C, and includes two subtypes that are frequently implicated in animal abortions: *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus*. *C. fetus* subsp. *venerealis* is a venereal pathogen of cattle resulting in infertility and abortion, whereas *C. fetus* subsp. *fetus* causes contagious abortion in sheep and sporadic abortion in cattle, horses, and man.

*C. jejuni*, formerly classified as *C. fetus* subsp. *jejuni*, and the closely related *C. coli*, belong to what is known as the thermophilic group of campylobacters due to their optimal growth at a higher temperature, 42°C, than that of other species. The average prevalence of these two thermophilic campylobacters in the feces of domestic cattle, sheep, and pigs has been reported to range from 21.9% - 26.7% and from 18.3% - 36.6% while the average reported prevalence for poultry was 41.4%. *C. jejuni* is a well-recognized and important cause of contagious abortion in sheep and is also reported to cause abortion in cattle, goats, mink, and dogs; whereas *C. coli* has been
associated with abortion in sheep\textsuperscript{5} and man.\textsuperscript{30,31} As a zoonotic pathogen, \textit{C. jejuni} is best recognized as a cause of acute enteritis with profuse, watery diarrhea;\textsuperscript{32,33} however, human infection may also lead to a variety of extraintestinal diseases including abortion.\textsuperscript{21,34-37}

While poultry products are considered the predominant source in sporadic cases of human \textit{C. jejuni} infection,\textsuperscript{38-40} sheep and cattle intermittently shed \textit{Campylobacter} spp. throughout their lives, often in a seasonal pattern,\textsuperscript{41} and therefore their housing facilities and the associated surface and subsurface waters should be considered another potential source of human exposure. Of these two domestic ruminants, sheep would appear to be the most significant source of exposure as the average recovered concentration of thermophilic campylobacters from the feces of infected flocks was roughly 10-fold higher than that from infected cattle.\textsuperscript{23} Additionally, the average isolation rate of thermophilic campylobacters from small intestinal contents of lambs at slaughter has been reported as high as 91.7\%\textsuperscript{42} providing a significant source of potential carcass contamination. Not surprisingly, \textit{C. jejuni} was isolated from 71.3\% of sheep carcasses in one abattoir study,\textsuperscript{43} and surveys of retail meat products have reported isolation of \textit{C. jejuni} from 80\% of lamb liver samples\textsuperscript{44} and 6.5\% of commercial lamb and mutton.\textsuperscript{45} Finally, flies collected from pastures housing both sheep and cattle have been shown to carry viable \textit{Campylobacter} organisms, including both \textit{C. jejuni} and \textit{C. coli}, and may therefore serve as yet another potential source of transmittance from infected livestock to humans through contact with food and food preparation surfaces.\textsuperscript{46}
Ovine campylobacteriosis

Reproductive disease in sheep associated with infection by *Campylobacter* spp. has long been referred to as epizootic abortion given the classical occurrence of abortion outbreaks, or storms, affecting roughly 25% of the flock on average. Reproductive performance is one of the most significant factors affecting the profitability of small ruminant operations, and therefore such epizootics of abortion may lead to severe economic losses. *Campylobacter*-associated abortion often ranks as one of the top three infectious causes of ovine abortion in many parts of the world, and a 2001 survey indicated that *Campylobacter* spp. were the most commonly reported infectious cause of abortion in the United States during the three years preceding the survey. The incidence of abortion within a flock depends upon the number of ewes pregnant beyond the first month of gestation and the level of prior exposure to the infecting strain. Immunity after natural infection is achieved even if an animal does not abort during an epizootic. Such postinfection immunity has been demonstrated to last at least one year and is thought to last approximately two years on average; however, given the frequent heterogeneity of infectious species and strains associated with epizootics, the potential exists for *Campylobacter*-associated abortions in successive lambing seasons. Infection is acquired orally, most often following consumption of infected products of parturition; however, fecal contamination of water supplies is relatively common and increased fecal shedding has been observed in times of high abortion rates. Infectious *Campylobacter* spp. have also been recovered from flies captured in grazing areas and
from the feces of infected magpies, crows, and other birds revealing additional potential means of spread between farms.

The pathogenesis of ovine campylobacteriosis is thought to entail oral exposure and ingestion of contaminated material with subsequent intestinal colonization, mucosal invasion, transient bacteremia and placental localization. Gall bladder colonization by C. fetus has been reported following natural abortion, oral inoculation, and intravenous inoculation, and, in ewes intravenously inoculated, gall bladder colonization was often associated with concurrent intestinal colonization thus supporting the potential role of gall bladder colonization after bacteremia in the development of carrier ewes and overall disease epidemiology. Indeed, carrier ewes are likely an important potential source of disease spread as Campylobacter spp. can be recovered from small intestinal contents and bile of apparently healthy sheep at slaughter, and C. fetus recovered from the bile can readily induce abortion in ewes. In 1976, an Australian survey revealed potentially pathogenic campylobacters in the bile of 3.3% of sheep tested (33/1015) and 91% (30/33) of those isolates were identified as C. jejuni. Infection in ewes is predominantly intestinal, and involvement of the uterus is considered by some to be an accidental outcome of bacteremia in nonimmune pregnant ewes. In fact, a period of detectable bacteremia as short as 15 minutes is reportedly sufficient for establishing uterine infection in pregnant ewes. One pivotal step in the pathogenesis of Campylobacter-associated abortion is invasion of the intestinal epithelium allowing access to the blood stream and systemic spread. For C. fetus subsp. fetus, systemic infection is dependent on the expression of surface layer (S-layer)
proteins\textsuperscript{72} which block complement 3b (C3b) binding\textsuperscript{73} and thereby inhibit complement-mediated killing mechanisms; however, these proteins are not expressed by \textit{C. jejuni}.

Much of the research involving pathogenesis of \textit{C. jejuni} infection has focused on intestinal colonization as it relates to human disease. Human pathogenic isolates of \textit{C. jejuni} have been demonstrated to colonize the intestinal mucus layer,\textsuperscript{74} and a number of potential adhesions have been identified which may play a role in adherence to the mucosal epithelium, however, their role in disease is uncertain.\textsuperscript{11} Pathogenic isolates of \textit{C. jejuni} are generally considered invasive,\textsuperscript{11} and thought to employ a microtubule-dependent invasion system,\textsuperscript{75} whereby bacteria are taken into an endosomal vacuole and travel along microtubules to the perinuclear area.\textsuperscript{76} These internalized bacteria then undergo limited replication within intestinal cells\textsuperscript{76} and move within vacuoles\textsuperscript{77} to the basolateral membrane for exocytosis.\textsuperscript{75} The presence of functional flagella is a key factor in efficient colonization\textsuperscript{78,79} and cellular uptake of \textit{C. jejuni},\textsuperscript{80} while flagellar paralysis allows adherence but not invasion of eukaryotic cells.\textsuperscript{81} Many strains of \textit{C. jejuni}, as well as \textit{C. coli} and \textit{C. fetus}, produce cytolethal-distending toxin (CDT),\textsuperscript{82} a nuclease capable of inducing double strand breaks in DNA of both proliferating and nonproliferating host cells, thereby leading to cell cycle arrest or cell death.\textsuperscript{83} Such dead or dying cells within the mucosa may provide adequate disruption of the epithelial barrier to allow entry of bacteria into the lamina propria. Both the cellular invasion of \textit{C. jejuni} and the production of CDT alone induce IL-8 secretion by human intestinal cells \textit{in vitro},\textsuperscript{84} suggesting the induction of an active neutrophil response and local containment of infection; however, survival of \textit{C. jejuni} for at least 6 days has been demonstrated within
mononuclear phagocytes, revealing at least one potential method of bacterial survival and dissemination. Taken together, these findings provide a generalized understanding of the pathogenesis of septic abortion due to *Campylobacter* spp.; however, there are still key knowledge gaps in the mechanisms underlying each of the requisite steps in this process and the observed variation in virulence between *Campylobacter* species and strains.

Ovine campylobacteriosis manifests as a spectrum of clinical disease including late term abortion, stillbirth, premature birth, birth of weak lambs and occasionally metritis. Subclinical infection may also occur with infected ewes delivering clinically normal carrier lambs. Abortion generally occurs 7 to 25 days postexposure, and it is typical for one or two ewes to abort initially, followed by a rapid rise in abortion incidence 2 to 3 weeks later. Large numbers of organisms are generally present within aborted materials and are most readily recovered from the placenta, fetal stomach contents and fetal liver, respectively. Grossly there is placentitis characterized by swollen, pulpy cotyledons covered with exudate and an edematous chorionic stroma. Lesions are generally focused on the placentomes while the intercotyledonary spaces are less severely affected. Histologically there is arteriolitis, infiltration of predominantly histiocytes, and often high numbers of bacteria within trophoblasts and endothelial cells. Fetal lesions are variable and include nonspecific edema, suppurative bronchopneumonia, and occasionally random necrotizing hepatitis with a characteristic targetoid gross appearance.
As stated previously, the most common isolates from *Campylobacter*-associated ovine abortions have historically been *C. fetus* subsp. *fetus*, *C. jejuni*, and *C. coli*, with decreasing frequency, respectively;\textsuperscript{1,2} however, several recent studies have demonstrated a significant shift in the epidemiology of this disease with *C. jejuni* becoming increasingly more prevalent.\textsuperscript{2,8-10} In one study, not only was *C. jejuni* the most common isolate, but 93% of the isolates were of a single genetic clone, and 100% of the isolates were resistant to tetracycline.\textsuperscript{10} The emergence of a single, antibiotic-resistant genetic clone of *C. jejuni* associated with the preponderance of abortions from multiple farms in multiple states is intriguing as there has traditionally been significant antigenic and genetic heterogeneity among isolates.\textsuperscript{3,6-8,88} Additionally, as tetracyclines are the only class of antimicrobials approved for the treatment of campylobacteriosis in sheep,\textsuperscript{89} it seems possible that emergence of this particular strain may have at least in part been enhanced by chemoprophylaxis.

**Animal models of campylobacteriosis**

One major impediment to the study of *C. jejuni* pathogenesis has been a lack of appropriate animal models.\textsuperscript{11,12} *C. jejuni* is present in the feces of many domestic and wild avian and mammalian species\textsuperscript{90-101} but rarely causes the type of acute enteritis observed in humans.\textsuperscript{80} Nonhuman primates are likely the best candidate model for studying the pathogenesis of enteric campylobacteriosis\textsuperscript{12,102} but their use carries both financial and ethical challenges. Mustelids present another potential model for study of enteric campylobacteriosis as the development of acute enterocolitis has been demonstrated in both mink\textsuperscript{103} and ferrets;\textsuperscript{104-106} however, the availability of specific-
pathogen-free animals is limited and commercially available ferrets sold for research purposes often shed *C. jejuni* in their feces.\textsuperscript{107} *C. jejuni* is not a commensal organism of laboratory mice,\textsuperscript{12,108} and several studies have shown colonization following oral inoculation;\textsuperscript{108-110} however, colonized mice generally lack intestinal pathology.\textsuperscript{11} Interestingly, bacteremia has been observed in mice orally inoculated with both *C. jejuni*\textsuperscript{108,111} and *C. fetus*,\textsuperscript{112} suggesting that they, at the very least, may be an effective model for studying this aspect of the pathogenesis of systemic campylobacteriosis.

Research exploring the pathogenic mechanisms underlying ovine campylobacteriosis would ideally involve studies in the target species; however, the costs of animal housing, the frequency of intestinal and bile colonization in apparently healthy sheep,\textsuperscript{68,69} the high cost and low of availability of specific-pathogen-free animals, and lengthy gestation are significant impediments. Therefore, the smaller size, reduced costs of housing, and ease of obtaining *Campylobacter*-negative stock make laboratory animals a desirable model for studying specific aspects of disease pathogenesis. As reproductive failure is the predominant disease outcome in affected sheep, an appropriate animal model should closely replicate this aspect of the disease. Abortion due to *C. jejuni* has been reported in farmed mink\textsuperscript{28} and reproductive failure has been experimentally induced in ferrets and mink following both intravenous and oral inoculation with *C. jejuni*;\textsuperscript{113} however, as stated previously, the use of this model is limited by the availability of *Campylobacter*-negative animals.\textsuperscript{107}

The domestic guinea pig (*Cavia porcellus*) has a gestation length that varies from 59 to 72 days (mean 68)\textsuperscript{114} and gives birth to precocious young,\textsuperscript{115} making it closer to the
target species in reproductive behavior than many other laboratory rodents and thereby a desirable candidate for studying the pathogenesis of *Campylobacter*-associated abortion. Accordingly, the induction of abortion in pregnant guinea pigs after intraperitoneal (IP) inoculation has been used as a screening tool for virulence of *C. fetus*, *C. jejuni*, and *C. coli* isolates from both animals and man,\textsuperscript{116-119} and to compare efficacy of commercial and experimental *Campylobacter* vaccines.\textsuperscript{120-123} Pure cultures of *C. fetus* have been recovered from the uterine fluid of both pregnant and non-pregnant guinea pigs which were parenterally inoculated with the organism\textsuperscript{124} suggesting a tropism for this environment following bacteremia in this species, and *C. fetus* has been recovered in pure culture from the gall bladder of pregnant guinea pigs\textsuperscript{124,125} suggesting the potential for localization in this organ similar to what is observed in sheep. Guinea pigs aborting following IP challenge with *C. fetus* often have pronounced suppurative endometritis and uterine edema,\textsuperscript{126} and horizontal transmission of *C. fetus* has been observed between guinea pigs inoculated intravaginaally and uninoculated cagemates,\textsuperscript{125} further supporting the appropriateness of the guinea pig as a model for study of campylobacteriosis; however, these methods do not fully replicate the natural disease process as bacteremia is not the result of intestinal invasion with parenteral inoculation and an oral inoculation model would seem superior for study of all phases of disease pathogenesis.

Oral inoculation of guinea pigs has been attempted with human isolates of *C. fetus* and *C. jejuni*;\textsuperscript{119} however, abortion did not occur in any orally inoculated animals and bacteremia was only observed in one animal inoculated with *C. fetus* compared with much higher rates of abortion and bacteremia following IP inoculation with the same
strains. Abortion was observed in 22% (2/9) of guinea pigs following oral inoculation with a combined inoculum of both bovine and ovine origin* C. fetus* that had been subjected to five serial IP guinea pig passages to increase pathogenicity;* however, C. fetus* was not recovered from the uterus or fetal tissues of either aborting animal, and organisms with features consistent with C. fetus* were only observed in Gram stained smears of uterine contents from one of the aborting animals. As these previous attempts at oral challenge in guinea pigs did not employ a pure culture of an ovine-origin, abortion-derived isolate, it remains to be seen if known abortifacient strains would behave differently following oral inoculation in pregnant guinea pigs, and further support for the appropriateness of a guinea pig oral inoculation model comes from its successful employ in the study of disease due to Toxoplasma gondii,* another common ovine reproductive pathogen.

**Toll-like receptors and the maternofetal interface**

The junction of mother and fetus has often been described as an immunological paradox. While the endometrium must tolerate the implantation of a relatively allogenic placenta, it is paramount that immune surveillance be maintained against a broad spectrum of potential pathogens, and yet an overactive response may lead to a lack of fetal tolerance. Accordingly, the innate immune system provides the primary defense against infection at the maternofetal interface. Significant numbers of macrophages, natural killer (NK) cells and fewer γ/δ T cells have been described in the early decidua,* and these cells, along with the physical barrier imparted by the placenta, were traditionally thought to provide the essential innate immune functions necessary at the
site of implantation. Recent studies, however, have shown that trophoblasts and decidual cells directly participate in the innate immune response through their expression of conserved pathogen recognition receptors.\textsuperscript{131-133}

The Toll receptor was first identified in \textit{Drosophila} as a transmembrane receptor involved in the establishment of dorsoventral polarity in the developing embryo.\textsuperscript{134} Continued work with mutant flies revealed that those lacking components of the Toll-mediated pathway were highly susceptible to fungal and bacterial infections,\textsuperscript{135,136} thus demonstrating a link between the Toll-mediated pathway and regulation of immune responses in flies. As the signaling pathway of Toll in \textit{Drosophila} shares remarkable similarity to that of the mammalian interleukin-1 (IL-1) pathway,\textsuperscript{137} it was proposed that this might be a conserved signaling pathway, and, indeed, a mammalian homologue of the \textit{Drosophila} Toll protein was later identified\textsuperscript{138} and then further expanded into a family of proteins known as Toll-like receptors (TLRs).\textsuperscript{139-142}

TLRs are well-characterized pattern recognition receptors (PRRs) that each recognize specific, highly conserved pathogen-associated molecular patterns (PAMPs), play a key role in innate immune function, and have been extensively reviewed.\textsuperscript{143-146} In brief, there are 10 members of the TLR family (TLRs 1-10) that have been identified in cattle, swine, sheep, mice and humans, each sharing a high degree of genetic sequence similarity across species.\textsuperscript{147} TLRs can be grouped based on their location of cellular expression with TLRs 1, 2, 4, 5 and 6 being expressed at the cell surface, while TLRs 3, 7, 8, and 9 are expressed intracellularly.\textsuperscript{146} TLRs are expressed on both immune and non-immune cells, and most tissues express at least one TLR.\textsuperscript{148} Among the TLRs, TLR2 has
the widest range of known ligands including PAMPs from gram-positive bacteria, gram-negative bacteria, mycobacteria and mycoplasma,\textsuperscript{144} and this is likely made possible, at least in part, through its formation of heterodimers with either TLR1 or TLR6.\textsuperscript{149,150} Other well known PAMPS and their corresponding TLRs include lipopolysaccharide (LPS) and TLR4,\textsuperscript{151} flagellin and TLR5,\textsuperscript{152} and pathogen-associated nucleic acid patterns and TLRs 3, 7, 8, and 9.\textsuperscript{143}

While each TLR binds a unique set of PAMPs, most TLRs signal through a common intracellular pathway.\textsuperscript{144} With the exception of TLR3, all TLRs interact with myeloid differentiation factor 88 (MyD88) which recruits the IL-1 receptor associated kinase (IRAK).\textsuperscript{153} Once phosphorylated, IRAK associates with tumor necrosis factor receptor-associated factor 6 (TRAF6) and leads to downstream activation of c-Jun N-terminal kinase (JNK) and nuclear factor-kappa B (NF-κB).\textsuperscript{144} Activation of these transcription factors generally leads to the production of inflammatory cytokines such as TNF-α, IL-1β, and IL-6.\textsuperscript{154} Additionally, TLR-3 and TLR-4 have been shown to signal through a MyD88-independent pathway by their association with Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF)\textsuperscript{155} which, through TANK-binding kinase (TBK-1), activates the transcription factor interferon regulatory factor 3 (IRF-3).\textsuperscript{156} Activation of IRF-3 leads to the production of the type-I interferons, IFN-α and IFN-β.\textsuperscript{154}

Expression of mRNA for TLRs 1-10 has been demonstrated in human term placental tissues,\textsuperscript{148} and multiple immunohistochemical studies have revealed the expression of TLR2 and TLR4 protein in both first trimester and term placenta.\textsuperscript{133,157,158} Immunohistochemistry of first trimester placental tissues has further revealed that both
TLR2 and TLR4 are highly expressed in villous cytotrophoblasts and extravillous trophoblasts, but are absent in the syncytiotrophoblast, suggesting the early syncytiotrophoblast may serve solely as a barrier to infection and does not actively participate in the TLR response. Therefore, TLRs expressed in villous cytotrophoblasts will respond to a pathogen only once it has successfully penetrated the syncytiotrophoblast barrier. In contrast, studies of term placental tissue have revealed significant immunoreactivity for TLR2 and TLR4 in extravillous trophoblasts and the syncytiotrophoblast, but not in the villous cytotrophoblast cells. Another study, confined to TLR2, was consistent with this shift, demonstrating positive TLR2 immunoreactivity in all trophoblast layers of second trimester placentas except the syncytiotrophoblast, with a shift to positive syncytiotrophoblast immunoreactivity in the term placenta. Whether this shift in TLR distribution reflects a true developmental change in the placenta, or simply variability in experimental technique, remains to be seen; however, this phenomenon is intriguing when compared with the epidemiology of bacterial-induced adverse pregnancy responses at various points during gestation, particularly the number of pathogens that are associated with late term disease.

In a more detailed immunohistochemical analysis of TLR4 expression, cytoplasmic reactivity was observed in the syncytiotrophoblast of both first trimester and term placenta, with greater expression in the term tissues. Additionally, darker staining was observed at the maternal facing plasma membrane versus the basal membrane, and this polarized staining pattern suggests greater expression at the surface which is continuously exposed to maternal blood and supports the involvement of TLR4 in the
initiation of a rapid response to maternal bacterial infection, particularly late in gestation. Demonstration of constitutive expression of TLR4 in human extravillous trophoblast cells, regardless of gestational stage, suggests a role for these cells as part of the first line of defense against invading pathogens at the materno-fetal interface; a role further supported by the considerable over-expression of TLR4 in these cells during inflammatory states, such as preeclampsia, that are associated with high levels of TNF-α.

In vitro investigation of the response of trophoblast cells derived from different stages of gestation to binding of TLR2 and TLR4 with their respective ligands has revealed significant gestational-stage-dependent differences. For example, in human first trimester trophoblast cells, which lack TLR6, TLR2 ligation by either peptidoglycan or lipoteichoic acid triggers apoptosis rather than the classical cytokine upregulation observed in other tissues, and this TLR2-induced apoptosis is mediated by MyD88 recruitment of the Fas-associated protein with death domain (FADD) and the initiation of the caspase cascade. In contrast, term placental explants and first trimester trophoblast cells transfected to express TLR6 produce the proinflammatory cytokines IL-6 and IL-8 when exposed to known TLR2 ligands. As term placental cytotrophoblast cells have been shown to express TLR6, these findings suggest that when trophoblasts are exposed to certain TLR2 ligands the presence or absence of TLR2/TLR6 heterodimers may be key in determining whether the exposed trophoblasts secrete cytokines or undergo apoptosis, respectively. The response of trophoblast cells to TLR4 ligation by LPS appears more consistent throughout gestation as exposure of first
trimester trophoblast cells to LPS results in the production of high levels of the cytokines TNF-α and INF-γ,\textsuperscript{159} and exposure of term placental explants to LPS results in the production of the proinflammatory cytokines IL-6 and IL-8.\textsuperscript{157} While TLR4 ligation by LPS does not directly induce trophoblast apoptosis,\textsuperscript{159} the resultant high levels of TNF-α and INF-γ have been shown to increase the sensitivity of trophoblast cells to Fas-mediated apoptosis,\textsuperscript{166} and recent work with \textit{Chlamydia trachomatis} has revealed that TLR4 not only binds LPS, but also chlamydial heat shock proteins (cHSPs) and that cHSP60 binding in particular can trigger caspase-induced apoptosis in second trimester trophoblast cells.\textsuperscript{167} As such, TLR ligation in trophoblast cells appears to have the capacity to induce both apoptosis and cytokine release with the outcome dependent upon gestational stage and ligand composition.

Decidual cells express mRNA for TLRs 1-10,\textsuperscript{131} and protein expression has been observed for TLRs 1-6,\textsuperscript{168} however, there is limited work involving the effects of ligand binding in decidual cells,\textsuperscript{169} and the results are somewhat conflicting. In one study,\textsuperscript{131} exposure of human decidual cells to peptidoglycan, a known TLR2 ligand, failed to induce production of either IL-6 or IL-8, while exposure to LPS induced significant levels of IL-6 and modestly increased IL-8. In contrast, in this same study, exposure of decidual cells to either ligand induced nuclear translocation of NF-κB,\textsuperscript{131} a critical step in the TLR signaling process. Additionally, in a separate study,\textsuperscript{168} exposure of decidual cells to either LPS or peptidoglycan resulted in significantly increased levels of IL-8. Thus, it seems clear that LPS has the capacity to induce a proinflammatory cytokine
response in decidual cells by way of TLR4, while the effects of TLR2 ligation are less clear.

Previous studies have revealed that TLR2 and TLR4, but not TLR5 are capable of binding PAMPs of *Campylobacter* spp. Unlike many other flagellated bacteria, the flagellin produced by *C. jejuni* does not effectively bind TLR5,\(^{170}\) and both live and lysed *C. jejuni* fail to induce TLR5-dependent cytokine expression in human and chicken cells.\(^{171}\) *C. jejuni* and *C. coli* both induce IL-8 secretion by human intestinal epithelial cells in a TLR-mediated, MyD88-dependent manner requiring the presence of functional flagella and CDT,\(^{172}\) and the production of IL-6 by human intestinal epithelia has been described through the interaction of *C. jejuni* surface polysaccharides and TLR2.\(^{173}\) Interestingly, lysed *C. jejuni* cells are reported to be more effective than live bacteria in inducing TLR2 and TLR4 dependent cytokine responses in both chicken and human cells,\(^{171}\) suggesting that bacterial lysis may actually enhance the TLR-stimulative potential of *C. jejuni*. Activation of dendritic cells by *C. jejuni* is significantly reduced in the absence of TLR2, TLR4, MyD88 and TRIF indicating that all four components are important in maximal signaling of these cells following *C. jejuni* challenge.\(^{174}\) Lastly, *C. rectus* infection has been associated with increased expression of TLR4 protein in trophoblasts of the murine placental labyrinth.\(^{175}\)

**Vaccination in the prevention of ovine campylobacteriosis**

In the 1960s, Miller and Jensen first described the potential for control of ovine campylobacteriosis through vaccination.\(^{176,177}\) Administration of two subcutaneous doses prior to breeding of either live *C. fetus* organisms or a whole-cell, formalin-killed *C. fetus*
bacterin, resulted in 100% and 72% protection, respectively, against homologous oral challenge during the fifth month of gestation; whereas only 16% of the unvaccinated controls failed to abort.\textsuperscript{176} With the addition of mineral oil or aluminum as an adjuvant, the protection imparted by killed \textit{C. fetus} bacterins was enhanced to 100% and 96%, respectively, in the face of homologous oral challenge and required just a single dose of the bacterin;\textsuperscript{177} however, the level of protection fell to 14 - 59% against heterologous serotypes.\textsuperscript{178} Storz et al. expanded upon these findings by revealing that when two doses of a monovalent, aluminum adjuvanted, \textit{C. fetus} bacterin were administered 3 weeks apart, 75% of the flock remained protected against homologous challenge 41 months postvaccination.\textsuperscript{179} In light of this prolonged immunity, Storz et al. further showed that vaccinating only the replacement ewes within a herd, once at 18 months of age and once prior to breeding, was effective in preventing epizootics of abortion in the herd for up to five years.\textsuperscript{180}

The antigens of \textit{C. fetus} important for conferring resistance to disease in sheep may be capsular antigens, flagellar antigens, or both,\textsuperscript{181} and the intact bacterium is not required for the development of immunity as 100% protection from abortion has been reported in ewes administered two doses of a cell-disrupted, formolized, adjuvanted vaccine and later challenged with a homologous strain.\textsuperscript{182} There is also evidence that antibody to S-layer proteins is effective in preventing abortions due to \textit{C. fetus}, and that conserved regions within S-layer proteins may offer some cross protection between heterologous serotypes.\textsuperscript{183} In the evaluation of serologic responses of sheep to vaccination with three separate monovalent cell-disrupted bacterins, slight agglutination
titers to heterologous serotypes were observed, again suggesting that there may be some antigenic overlap between serotypes.\textsuperscript{184}

In efforts to maintain profitability, it is imperative that sheep producers keep fixed costs at a minimum. As such, the benefits of potential vaccines are often weighed against their cost and the potential risk for disease occurrence, and it is often desirable to administer vaccines as combination products. Indeed, \textit{Campylobacter} vaccines have been described containing multiple serotypes\textsuperscript{178,184} and strains,\textsuperscript{121,185} or in combination with the agent of enzootic abortion, \textit{Chlamydophila psittaci}.\textsuperscript{185-187} While vaccination can be effective in the prevention of ovine campylobacteriosis, it is by no means a panacea. Experimental studies have demonstrated inadequate protection, even in the face of homologous challenge, in ewes vaccinated with commercial bacterins of questionable efficacy,\textsuperscript{188} and in those vaccinated with experimental bacterins and subsequently challenged with heterologous serotypes.\textsuperscript{178} Additionally, field cases of \textit{Campylobacter}-associated abortion have been reported in flocks receiving commercial monovalent\textsuperscript{189} and bivalent\textsuperscript{9} vaccines, and there are reports of an increased incidence of isolation of non-vaccine strains from epizootics of abortion in New Zealand.\textsuperscript{190} Accordingly, no single vaccine will likely be protective against all abortifacient \textit{Campylobacter} spp., and continued efficacy testing is required to monitor the usefulness of a given vaccine.

Large scale efficacy testing of \textit{Campylobacter} vaccines in the target species would be cost prohibitive. As such, the pregnant guinea pig has been described as an effective laboratory animal model for testing the efficacy of commercial \textit{Campylobacter} vaccines intended for cattle\textsuperscript{120,121} and sheep,\textsuperscript{121} as well as experimental bacterins.\textsuperscript{122,123}
These efficacy studies have revealed that immunization with a monovalent bacterin of either *C. fetus* or *C. jejuni* is not cross protective against challenge with the opposite species, and that protection is lower when challenged with a heterologous strain of the same species. These findings provide further support for the use of multivalent vaccines in the prevention of ovine campylobacteriosis.

In an effort to develop an alternative to traditional vaccination strategies, a group of ewes was experimentally infected with *C. fetus* and later vaccinated with a single dose of a formalized, disrupted cell, homologous bacterin immediately after the onset of the first abortion within the group, and this treatment effectively reduced subsequent abortions in the remaining ewes. These findings were confirmed in field cases where treatment with two doses ten days apart was effective in curbing additional abortions within a flock if administered at the time of indicator abortion; however, no effect was observed if treatment was delayed until 2 weeks after the indicator event. These findings suggest that, if administered early, vaccination in the face of an epizootic may be an effective tactical strategy to control losses due to campylobacteriosis while minimizing costs.

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CHAPTER 2: PATHOGENICITY OF AN EMERGENT, OVINE ABORTIFACIENT CAMPYLOBACTER JEJUNI CLONE ORALLY INOCULATED INTO PREGNANT GUINEA PIGS

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Abstract

**Objective**—To compare pathogenicity of an emergent abortifacient *Campylobacter jejuni* (IA3902) with reference strains after oral inoculation in pregnant guinea pigs.

**Animals**—58 pregnant guinea pigs.

**Procedures**—Twelve animals were challenged IP with *Campylobacter jejuni* IA3902 along with 5 sham-inoculated controls to confirm abortifacient potential. Once pathogenicity was confirmed, challenge via oral inoculation was performed whereby 12
animals received IA3902, 12 received *C. jejuni* isolated from ovine feces (OF48), 12 received a fully sequenced human *C. jejuni* isolate (NCTC 11168), and 5 were sham-inoculated controls. After abortions, animals were euthanized and samples were collected for microbial culture, histologic examination, and immunohistochemical analysis.

**Results**—*C. jejuni* IA3902 induced abortion in all 12 animals following IP inoculation and 6 of 10 challenged orally. All 3 isolates colonized the intestine after oral inoculation, but only IA3902 induced abortion. There was evidence of systemic infection for both IA3902 and NCTC 11168; however, *C. jejuni* was only recovered from fetoplacental units of animals inoculated with IA3902. Immunohistochemical analysis localized *C. jejuni* IA3902 infection to subplacental trophoblasts, perivascular tissues, and phagocytes in the placental transitional zone.

**Conclusions and Clinical Relevance**—This study revealed that *C. jejuni* IA3902 is a unique, highly abortifacient strain with the ability to colonize the intestines, induce systemic infection, and cause abortion because of its affinity for the fetoplacental unit. Guinea pigs could be effectively used in the study of septic abortion after oral inoculation with this *Campylobacter* strain.

**Introduction**

Abortions in sheep as a result of campylobacteriosis are common throughout the world, with regional variability in the species isolated and abortion rate. In a 2001 survey that spanned 22 states and included 72.3% of US sheep producers,
campylobacteriosis was reported as the most common cause of infectious abortion in domestic flocks, with 53.7% of those reports confirmed by a veterinarian or diagnostic laboratory. Historically, *Campylobacter fetus* subsp. *fetus* was the predominant isolate in sheep with campylobacteriosis, whereas *Campylobacter jejuni* and *Campylobacter coli* accounted for a smaller percentage.3–6 However, 2 US studies7,8 have indicated a dramatic shift in this relationship, with *C. jejuni* becoming the predominant species causing abortion in sheep. Abortion-causing isolates of *C. fetus* subsp. *fetus* and *C. jejuni* have considerable heterogeneity.9–11 Analysis by use of pulsed-field gel electrophoresis has revealed a major shift in the distribution of isolates. An initial study8 of *C. jejuni* isolates from aborting ewes in Iowa flocks had a preponderance of a single clone despite recovery from multiple Iowa farms during multiple lambing seasons.8 Expansion of this evaluation confirmed that this single clone of tetracycline-resistant *C. jejuni* was the predominant *Campylobacter* strain isolated from abortions of sheep in South Dakota, Idaho, California, Oregon, and Nevada.8 These findings strongly suggest that a highly abortifacient clone of *C. jejuni* (named clone SA for Sheep Abortion) has emerged that is now a leading cause of abortion outbreaks in sheep in the United States.

Intraperitoneal inoculation of pregnant guinea pigs has been used as a method to evaluate the pathogenicity of *Campylobacter* spp.12,13 and to assess the efficacy of vaccines.14–16 Their small size, ease of housing, and relatively short duration of gestation make guinea pigs a desirable animal for use in studying *Campylobacter*-induced abortion, particularly when compared with the use of sheep or goats, which are the species most commonly affected in nature. However, an IP technique is not totally appropriate for use
in evaluating the pathogenesis of septic abortions attributable to \textit{C. jejuni} because it bypasses critical initial steps in the abortion process, including intestinal colonization and bacterial invasion. Currently, there is limited data regarding oral inoculation of \textit{C. jejuni} in guinea pigs. In another study,\textsuperscript{17} oral inoculation of 4 pathogenic isolates of \textit{C. jejuni} obtained from the intestines of humans failed to induce abortion in pregnant guinea pigs. We hypothesize that this was attributable to differences in virulence among \textit{C. jejuni} isolates and that highly abortifacient clinical isolates, such as \textit{C. jejuni} IA3902, should be effective in inducing abortion after oral inoculation.

In the study reported here, we compared \textit{C. jejuni} IA3902, a clinical isolate of clone SA, with an isolate obtained from the feces of sheep (\textit{C. jejuni} OF48) and an isolate obtained from humans (\textit{C. jejuni} NCTC 11168) to determine their ability to induce abortion after oral inoculation in pregnant guinea pigs. In all guinea pigs that aborted, immunohistochemical analysis was used to identify and localize organisms within placental tissues to provide insights into mechanisms involved in \textit{Campylobacter}-induced abortion.

\textbf{Materials and Methods}

\textbf{Animals}—Fifty-eight pregnant Hartley guinea pigs (approx. 2 to 3 weeks of gestation) were obtained from a commercial source\textsuperscript{a} for use in the study. Seventeen guinea pigs were used in a preliminary IP challenge experiment. Mean weight at time of arrival for these guinea pigs was 865 g. Subsequently, 41 pregnant guinea pigs were used in an oral challenge experiment. Mean weight at time of arrival for these guinea pigs was
797 g. At arrival, a rectal swab specimen was obtained from each guinea pig, placed in
transfer medium, and plated for Campylobacter culture.

Guinea pigs were housed in standard metal cages with wood chip bedding and fed
a pelleted commercial diet formulated for guinea pigs. Animals were housed in groups (4
or 5 guinea pigs/cage for the IP challenge exposure and 3 or 4 guinea pigs/cage for the
oral challenge exposure) and allowed to acclimate to their environment for 7 days prior to
inoculation. During this time, weights were recorded daily and guinea pigs were marked
with black hair dye to provide unique identification for each animal. For the oral
challenge exposure, treatment groups were housed in separate banks of cages, and strict
hygiene was used between handling of groups to prevent cross-contamination. All
procedures were approved by the Institutional Animal Care and Use Committee at Iowa
State University.

**Campylobacter strains**—*C. jejuni* isolates were used in challenge exposures. The
IA3902 isolate was cultured from an aborted ovine fetus. This tetracycline-resistant
strain of *C. jejuni* is the predominant genetic clone associated with abortion in sheep, as
determined by pulsed-field gel electrophoresis, multilocus sequence typing, and *cmp* gene
sequence typing.\(^8\) The OF48 isolate was cultured from feces of a sheep that did not have
clinical signs (abortion or diarrhea). The NCTC 11168 isolate\(^b\) is from humans and has
been completely sequenced.\(^18\) Fresh bacterial cultures were obtained following 24 hours
of growth on MH agar in anaerobic jars under microaerobic conditions (5% oxygen, 10%
carbon dioxide, and 85% nitrogen) at 42°C. These cultures were collected and placed in
MH broth, diluted to desired concentrations on the basis of optical density, and then used
as inocula in the challenge experiments. The final number of organisms in each suspension was determined by counting the number of viable CFUs.

**IP challenge exposure**—Two groups of guinea pigs were used in a preliminary experiment. The first group (which comprised 5 guinea pigs) served as sham-inoculated control animals and were inoculated IP with 1.0 mL of MH broth, whereas the second group (which comprised 12 guinea pigs) was inoculated IP with 1.0 mL of 1.5 X 10^5 CFUs of *C. jejuni* IA3902/mL of MH broth. This concentration was chosen on the basis of reports^12,13^ in which pregnant guinea pigs were challenge exposed with *C. jejuni* via IP inoculation. Guinea pigs in both groups were inoculated by use of 1-mL tuberculin syringes with a 26-gauge, 3/8-inch needle.

**Oral inoculation**—Four groups of guinea pigs were used in the oral challenge exposure experiment. The first group (n = 5 guinea pigs) served as sham-inoculated control animals and received 1.0 mL of MH broth. The second group (n = 12 guinea pigs) received 1.0 mL of 5.5 X 10^8 CFUs of *C. jejuni* OF48/mL of MH broth. The third group (n = 12 guinea pigs) received 1.0 mL of 5.9 X 10^8 CFUs of *C. jejuni* NCTC 11168/mL of MH broth. The fourth group (n = 12 guinea pigs) received 1.0 mL of 6.0 X 10^8 CFUs of *C. jejuni* IA3902/mL of MH broth. These doses were chosen to approximate those used in other experiments in which *C. jejuni* was administered orally to pregnant ferrets^19^ and guinea pigs.^17^ Guinea pigs in all 4 groups were inoculated orally via a curved, 18-gauge, 3-inch, stainless-steel feeding needle. Food was withheld from the guinea pigs for 12 hours prior to inoculation to diminish the amount of food retained in the oral cavity.
Monitoring, euthanasia, necropsy, and collection of samples—After inoculation, guinea pigs were weighed daily by use of a digital gram scale and were observed at least twice daily for signs of abortion or impending abortion. These signs included vaginal bleeding, expelled fetuses or fetal membranes, and sudden substantial weight loss (> 10% in 24 hours). Once a guinea pig had aborted or an impending abortion was identified, that animal was euthanized via IP injection of sodium pentobarbital (approx. 150 mg/kg) and immediately necropsied. All guinea pigs that had not aborted were euthanized 21 days after inoculation and necropsied.

Necropsy of guinea pigs involved inspection for gross lesions and collection of samples for bacterial culture and histologic examination. Rectal swab specimens for *Campylobacter* culture were obtained by use of sterile minitip cotton swabs immediately after euthanasia. Other samples harvested for *Campylobacter* culture included heart blood, bile, and uterine tissue from each dam and placenta, liver, and lung tissues from each fetus. Pooled fetal stomach contents were collected only during the oral challenge study. Heart blood, bile, and fetal stomach contents were collected by use of a sterile tuberculin syringe with a 26-gauge, 3/8-inch needle. Samples of uterine, placental, and pooled fetal liver and lung tissues were placed in separate sterile plastic bags (IP challenge) or separate sterile Petri plates (oral challenge). All samples were refrigerated until immediately prior to culture, with bacterial culture performed on most samples on the day of collection.

Samples collected for histologic examination included liver, gallbladder, and uterine tissues for each dam and placenta, liver, and lung tissues for each fetus. Samples
obtained for histologic examination were placed in neutral-buffered 10% formalin for 24 hours, embedded in paraffin, and processed routinely for H&E staining.

**Campylobacter culture and semiquantitative enumeration of C. jejuni from necropsy samples**—A few drops of each fluid sample (blood, bile, or fetal stomach contents) were placed directly onto culture media and streaked by use of sterile cotton swabs. Rectal swab specimens were streaked directly onto culture media. Uterine tissues, pooled fetal liver and lung tissues, and placental tissues were minced with a scalpel or scissors; then, swab specimens were obtained from the minced tissues by use of a sterile cotton swab and streaked onto culture media. All samples were spread onto MH agar containing a *Campylobacter* selective supplement (polymyxin B, rifampicin, trimethoprim, and cycloheximide) and a *Campylobacter* growth supplement (sodium pyruvate, sodium metabisulfite, and ferrous sulfate) and were incubated for 48 hours in anaerobic jars under microaerobic conditions at 42°C. After incubation, *Campylobacter*-like colonies on each plate were counted to determine the number of CFUs in each sample.

**Histologic scoring of uterine lesions**—Endometrial inflammation was evaluated by calculating the mean number of leukocytes in 10 hpfs (40X magnification). Uterine lesions were scored in each hpf by use of the following criteria: 0 = inflammatory infiltrate was not identified, 1 = < 10 leukocytes/hpf, 2 = 10 to 20 leukocytes/hpf, and 3 = > 20 leukocytes/hpf. An additional 0.5 points was added to the score when the inflammation extended into the underlying myometrium.
**Immunohistochemical analysis**—Placental samples were collected at necropsy and embedded in paraffin as described previously. Tissues were sectioned at a thickness of 3 μm, mounted on aminoalkylsilane-coated glass slides, and placed in an oven at 56°C for 2 hours. Sections were routinely deparaffinized in xylene and rehydrated in graded alcohol solutions and water baths. Endogenous peroxidase inhibition was achieved by immersion (2 immersions; 10 min/immersion) in baths of 3% H₂O₂ in water. Sections were incubated with 0.1% protease in a Tris buffer (pH, 7.6) at 37°C for 15 minutes. Slides were rinsed 3 times in PBS solution and then were placed in an automated cell staining system. To inhibit nonspecific binding, sections were incubated in 10% neutral goat serum at 22°C for 20 minutes. The primary antibody, which was directed against the major outer membrane protein of *C. jejuni*, was prepared as described in another study. This antibody was used at a dilution of 1:300; slides were incubated at 22°C for 60 minutes followed by rinsing in a bath of PBS solution for 5 minutes. A commercially available biotinylated secondary antibody was used at a dilution of 1:80; slides were incubated at 22°C for 15 minutes followed by rinsing in a bath of PBS solution for 5 minutes. Sections were then incubated with horse radish peroxidase–streptavidin conjugate at 22°C for 15 minutes followed by rinsing in a PBS bath for 5 minutes. The final reaction was developed by use of a commercial chromogen. Sections were rinsed and routinely counterstained with Shandon Harris hematoxylin and Scott’s tap water. Sections were dehydrated through graded alcohol and xylene solutions prior to mounting. Positive control samples were obtained from paraffin blocks of ovine placental tissues that had positive results when cultured for *C. jejuni* IA3902. Negative control samples
consisted of sections from culture-negative tissues obtained from nonaborting guinea pigs inoculated with *C. jejuni* IA3902 and from sham-inoculated control guinea pigs.

**Statistical analysis**—A commercial statistical software package was used to perform all tests. Chi-square analysis and Fisher’s exact test for binomial variables were employed when comparing abortion rate, incidence of necrotizing hepatitis, and incidence of necrosuppurative placentitis between groups, and a 1-way ANOVA was used to detect differences in mean uterine scores among groups. Results were considered significant at values of $P \leq 0.05$.

**Results**

**IP challenge exposure**—All rectal swab specimens collected before challenge exposure had negative results when cultured for *Campylobacter* spp. Ten of 12 guinea pigs inoculated with IA3902 aborted, with 8 guinea pigs aborting 2 or 3 days after inoculation and the remaining 2 guinea pigs aborting 8 days after inoculation. Necropsy revealed that the 2 guinea pigs that did not abort were not pregnant; 1 of these guinea pigs had a teratoma of the left ovary. For the control group, none of the 5 guinea pigs aborted, and necropsy revealed that all 5 were pregnant.

Calculation of the abortion rate excluded the 2 guinea pigs that were not pregnant. Thus, the abortion rate for guinea pigs inoculated IP with IA3902 (10/10) was significantly ($P < .001$) higher than the abortion rate for the control guinea pigs (0/5).

**Oral challenge exposure**—All rectal swab specimens collected before challenge exposure had negative results when cultured for *Campylobacter* spp. Six of 12 guinea
pigs inoculated with IA3902 aborted; 4 guinea pigs aborted between 4 and 6 days after inoculation, and the other 2 guinea pigs aborted between 7 and 10 days after inoculation. None of the guinea pigs in the remaining groups aborted. Necropsy revealed that 2 guinea pigs in each of the IA3902, NCTC 11168, and OF48 groups and 1 guinea pig in the control group were not pregnant; we determined that these guinea pigs had not been pregnant on the basis of a lack of appreciable weight gain during the study. These guinea pigs were excluded from the calculations for abortion rate.

Abortion rates differed significantly ($P < 0.001$) among groups (Table 1). The abortion rate for guinea pigs inoculated with IA3902 (6/10) was significantly ($P < 0.001$) higher than the rate for any of the other 3 groups (0/10, 0/10, and 0/4 for guinea pigs inoculated with NCTC 11168, OF48, or a sham inoculation, respectively). In addition, necropsy revealed that 1 of the 4 remaining pregnant guinea pigs from the IA3902 group had histologic and bacteriologic evidence of fetal infection.

Gross liver lesions consisted of multiple, variably sized, round, white foci; they were identified in 9 guinea pigs inoculated with IA3902 and 3 guinea pigs inoculated with NCTC 11168. The predominant histopathologic liver lesion was random, multifocal to coalescing hepatitis, which was detected in decreasing frequency in the IA3902 (11/12), NCTC 11168 (8/12), and OF48 (4/12) groups; this lesion was not detected in the control group (0/4). Inflammatory foci were variably sized, and the associated infiltrate differed depending on the interval between challenge exposure and necropsy. In guinea pigs that aborted within a few days after challenge exposure, inflammatory foci were primarily heterophilic. As the interval between challenge exposure and abortion
increased, the infiltrate became dominated by lymphocytes, plasma cells, and macrophages.

Incidence of multifocal random hepatitis differed significantly \((P < 0.01)\) among groups. The incidence of random hepatitis was significantly \((P < 0.01)\) higher in the IA3902 group, compared with the incidence for the OF48 and control groups; however, incidence did not differ significantly \((P > 0.1)\) from the NCTC 11168 group. Incidence of random hepatitis was significantly \((P = 0.021)\) higher in the NCTC 11168 group compared with the incidence for the control group, but did not differ significantly \((P > 0.1)\) from the OF48 group. Incidence of random hepatitis for the OF48 group did not differ significantly \((P = 0.182)\) from the incidence for the control group.

Mild periportal infiltrates of lymphocytes and plasma cells were detected in most of the samples from all groups (Table 1). Additionally, congestion or edema of the gall bladder were evident in 5 guinea pigs of the IA3902 group, 5 guinea pigs of the NCTC 11168 group, 3 guinea pigs of the OF48 group, and 1 guinea pig of the control group.

Uterine lesions were variable and consisted of suppurative endometritis, metritis, and hemorrhage of varying severity. Uterine edema was evident in guinea pigs from all the IA3902, NCTC 11168, and OF48 groups but was most severe in those from the IA3902 group. In general, uterine inflammatory scores were highest in guinea pigs inoculated with IA3902 (mean 2.3; range, 1.0 to 3.5), with lower scores in a few guinea pigs of the OF48 (mean 0.4; range, 1.0 to 2.5) and control (mean 0.5; range, 1.0 to 1.5) groups and no metritis detected in the NCTC 11168 group.
Mean uterine scores differed significantly ($P < 0.001$) among groups (Table 1). Mean uterine score for the IA3902 group was significantly ($P < 0.001$) higher than the mean uterine score of any of the other 3 groups, whereas the mean uterine scores for the NCTC 11168 and OF48 groups did not differ significantly ($P = .294$ and $P = 0.792$, respectively) from the mean uterine score for the control group.

Microscopic placental lesions consisted of a combination of hemorrhage, suppurative inflammation, and necrosis (Figure 1). Placental lesions were evident in 8 guinea pigs inoculated with IA3902 (including all 6 that aborted) and in 1 guinea pig inoculated with NCTC 11168. These changes were detected in both the maternal and fetal portions of the placenta, with severity of the lesions increasing with an increase in the number of days after inoculation.

The incidence of necrosuppurative placentitis differed significantly ($P < 0.001$) among groups (Table 1). Incidence of placentitis in the IA3902 group (8/10) was significantly ($P = 0.006$, $P = 0.001$, and $P = 0.015$, respectively) higher than the incidence for the NCTC 11168 (1/10), OF48 (0/10), and control (0/4) groups; whereas incidence for the NCTC 11168 and OF48 groups did not differ significantly ($P > 0.999$), compared with the incidence for the control group.

High numbers of *C. jejuni* were cultured from 7 of 10 placental samples, 6 of 10 fetal liver or lung samples, and 6 of 10 uterine samples in the IA3902 group. Moderate numbers of *C. jejuni* were cultured from 10 of 12 rectal swab specimens in the IA3902 group, and low numbers of *C. jejuni* were generally cultured from 5 of 12 heart blood samples, 4 of 9 fetal stomach contents samples, and 2 of 12 bile samples in the IA3902
group. Low to moderate numbers of *C. jejuni* were cultured from 9 of 12 rectal swab specimens from the NCTC 11168 group and 6 of 12 rectal swab specimens from the OF48 group. *C. jejuni* was not cultured from any other tissues in these groups or from any samples collected from the control group. The percentages of samples with positive results for culture of *C. jejuni* were summarized (Figure 2). The source and number of CFUs for bacterial cultures of guinea animals inoculated with IA3902 were also summarized (Table 2).

**Immunohistochemical analysis**—Immunohistochemical analysis for *C. jejuni* was performed on all available placental tissues from guinea pigs that aborted during the study. Staining for *C. jejuni* was not detected in any negative control samples or culture-negative aborted tissues. In guinea pigs inoculated IP with IA3902, staining for *C. jejuni* was evident in 5 of 6 culture-positive samples (placental tissues or fetal tissues when placental samples were not available for culture). In guinea pigs inoculated orally with IA3902, staining for *C. jejuni* was evident in 5 of 7 culture-positive samples. *C. jejuni* organisms were identified within the cytoplasm of subplacental trophoblasts, within phagocytes, and in extracellular spaces surrounding trophoblasts in areas of placentitis and necrosis (Figure 1). Organisms were most prevalent at the periphery of the subplacenta within the junctional zone. Multifocal intracellular organisms were also detected within the deeper portions of the endometrium and myometrium and surrounding the uteroplacental arteries; however, this distribution was limited to samples from guinea pigs that aborted at longer intervals after inoculation.
Discussion

Campylobacteriosis has been described as the most common cause of infectious abortion in sheep in the United States, and \textit{C. jejuni} has become the predominant species. Pulsed-field gel electrophoresis, multilocus sequence typing, and \textit{cmp} gene sequence typing of \textit{C. jejuni} isolates from multiple farms in multiple states during multiple lambing seasons have revealed that most of these isolates are of a single clone, which is represented by \textit{C. jejuni} IA3902. In the guinea pigs of the study reported here, we identified several unique features of \textit{C. jejuni} IA3902, when compared with those of a sheep commensal fecal isolate (\textit{C. jejuni} OF48) and a fully sequenced human isolate (\textit{C. jejuni} NCTC 11168).

\textit{C. jejuni} IA3902 was uniquely proficient in its ability to induce abortion. In the preliminary experiment with IP challenge exposure, which was intended to verify the abortifacient potential of this isolate, all of the pregnant guinea pigs aborted. After oral inoculation, \textit{C. jejuni} IA3902 was the only isolate that induced abortion (6/10 pregnant guinea pigs aborted). Had the study been extended a few additional days, the abortion rate after oral inoculation would likely have been even higher with IA3902 because placentitis and fetal infection were identified in 1 guinea pig that had not yet aborted when the study was terminated. This is in contrast to results of a study in which 4 pathogenic human isolates of \textit{C. jejuni} were evaluated in pregnant guinea pigs (abortion rates for those isolates ranged from 0% to 67% following IP inoculation and 0% following oral inoculation). To our knowledge, the study reported here is the first to reveal that oral inoculation of \textit{C. jejuni} can be effective in inducing abortion in pregnant guinea pigs.
guinea pigs. These results indicated that *C. jejuni* IA3902 was highly abortifacient and that pregnant guinea pigs were an effective method for evaluating the pathogenicity of this isolate for a natural route of infection (oral) or after IP inoculation.

The pathogenesis of *Campylobacter* spp. that induce abortion when acquired by a nonvenereal route of transmission entails oral exposure, intestinal colonization, bacterial invasion, bacteremia, and infection of the fetoplacental unit.\(^{1,21-23}\) Sheep appear to be at increased risk for developing campylobacteriosis because healthy sheep often harbor *Campylobacter* spp. within the intestines and gall bladder.\(^{1,24}\) In a recent study,\(^{25}\) *Campylobacter* spp. were identified in 49.5% of intestinal, gall bladder, and fecal samples obtained from healthy sheep. The pathogenic mechanisms used by *C. jejuni* in the evasion of the physical and immunologic barriers in the intestine have been extensively studied and have been described elsewhere.\(^{26,27}\) In the study reported here, semiquantitative cultures of fecal samples revealed that all 3 strains of *Campylobacter* organisms were capable of colonizing the intestinal tract after oral administration, with *C. jejuni* IA3902 cultured in the greatest number of samples (10/12 [83%]) collected during necropsy, followed by *C. jejuni* NCTC 11168 (9/12 [75%]) and OF48 (6/12 [50%]).

Once the intestines have been colonized, abortifacient *Campylobacter* spp. must breach the intestinal epithelium and induce bacteremia. Bacteremia attributable to *C. jejuni* has been described,\(^{28-30}\) and results of 1 study\(^{31}\) indicated that certain strains of *C. jejuni* have an enhanced ability to induce bacteremia. Because of the stress associated with blood collection and the limited blood volume that can be harvested from guinea pigs on a daily basis, the magnitude and duration of bacteremia was not directly assessed
in the present study, and an indirect gauge of systemic infection was used to estimate the incidence of bacteremia. Acute hepatitis has been reported as a sequela to bacteremia attributable to *C. jejuni* in humans, and liver biopsy specimens from a patient with acute hepatitis and concurrent enteritis attributable to *C. jejuni* revealed nonspecific reactive hepatitis with focal necrosis. Additionally, multifocal random hepatitis is a consistent necropsy finding secondary to bacteremia or septicemia for numerous organisms, including host-adapted *Salmonella* spp. such as *Salmonella enterica* serovar Dublin in cattle and *Salmonella enterica* serovar Choleraesuis in swine. In the study reported here, the identification of multifocal, random hepatitis was used as indirect evidence of systemic infection. This lesion was identified in 23 of 36 orally inoculated guinea pigs, including 11 of 12 guinea pigs inoculated with *C. jejuni* IA3902 and 8 of 12 guinea pigs inoculated with *C. jejuni* NCTC 11168. The significantly higher rate of hepatitis in both groups indicated that each strain likely induced a degree of bacteremia, and it therefore seems likely that the differences in abortion rate were attributable to a mechanism other than the ability of these organisms to breach the intestinal epithelium and induce bacteremia.

Predilection for the fetoplacental environment is a common attribute of abortifacient agents, and it appears that the greatest difference between *C. jejuni* IA3902 and the other evaluated isolates was its affinity for the fetoplacental unit. All 3 isolates were able to colonize the intestines, and there is evidence that both *C. jejuni* IA3902 and *C. jejuni* NCTC 11168 induced bacteremia after oral inoculation, yet only IA3902 appeared to be able to colonize the placenta, infect trophoblasts, and induce abortion.
Guinea pigs inoculated with *C. jejuni* IA3902 consistently developed endometritis and placentitis with high numbers of *C. jejuni* (> 1,000 CFUs) recovered from uterine, placental, and pooled fetal tissues; all of which provided evidence for an apparent tropism of IA3902 for the fetoplacental unit.

Analysis of our results indicated that immunohistochemical analysis was less sensitive than microbial culture as a means of detecting *C. jejuni* in tissues, which is consistent with results in other studies.\(^{35,36}\) However, immunohistochemical analysis was extremely useful in characterizing the distribution of organisms in placental tissues. *C. jejuni* IA3902 appeared to localize at the periphery of the subplacenta near its junction with the main placenta early during the course of infection and was evident within both inflammatory cells and syncytiotrophoblasts of the subplacenta. This localization appears logical because this is the site of placental vascular invasion\(^{37}\) and would be the first location reached during episodes of bacteremia. Additionally, guinea pigs infected for longer periods before they aborted often had immunohistochemical evidence of abundant pericellular and intracytoplasmic organisms associated with the periarterial trophoblasts in the decidua. These data suggested that trophoblasts of subplacental origin were preferentially infected, whereas those of the main placenta were spared. It is worth mentioning that there are considerable differences between the placentation of guinea pigs and ewes. Guinea pigs have a discoid, labyrinthine, hemochorial placenta with extensive trophoblast invasion of the decidua and uterine arterial walls.\(^{38,39}\) In contrast, sheep have a cotyledonary, epitheliochorial placenta and lack trophoblast invasion of uterine blood vessels.\(^{39}\) Although there are noticeable differences in placentation
between these 2 species, our immunohistochemical findings of organisms within trophoblasts, leukocytes, and perivascular tissues was consistent with that in another study\textsuperscript{40} of placentas from aborting sheep infected with \textit{C. jejuni}. Together, these findings suggest that pathogenic \textit{C. jejuni} strains are capable of infecting trophoblasts regardless of placentation type, and this provides further supporting evidence for the appropriateness of the use of pregnant guinea pigs as a method for evaluation of \textit{Campylobacter}-induced abortion. To the authors’ knowledge, the information reported here is the first description of the localization and distribution of \textit{Campylobacter} spp. within the placenta of aborting guinea pigs.

In the study reported here, we determined that \textit{C. jejuni} IA3902 was a unique, highly abortifacient strain with the ability to colonize the intestines, cause systemic infection, and induce abortion as a result of its affinity for the fetoplacental unit. Although we confirmed the highly pathogenic nature of IA3902 in pregnant guinea pigs, additional in vivo studies in sheep are required to determine the degree of virulence in pregnant ewes. \textit{C. jejuni} IA3902 appears to be a valuable strain for studying the pathogenesis of \textit{Campylobacter}-induced abortion. To facilitate the study of pathogenic mechanisms, the genome of IA3902 is being sequenced by our laboratory group, and we will compare it with a fully sequenced, nonabortifacient strain (NCTC 11168) to attempt to identify unique virulence traits of this abortifacient clone. Results also revealed that pregnant guinea pigs are useful for studying the pathogenesis of abortion attributable to this unique \textit{Campylobacter} strain. Pregnant guinea pigs have been used to assess the efficacy of vaccines against \textit{C. fetus},\textsuperscript{14-16} and, given the recent shift from \textit{C. fetus} to
predominantly *C. jejuni* causing abortions in sheep, there is an urgent need to explore the development of a vaccine against *C. jejuni* for the control of this emergent, highly pathogenic strain.

**Acknowledgements**

This work was supported by a grant from the Iowa Livestock Health Advisory Council. The authors thank Deborah Moore for assistance with the immunohistochemical analysis.

**Footnotes**

a. Elm Hill Labs, Chelmsford, MA.
b. ATCC 700819, American Type Culture Collection, Manassas, VA.
c. BBL CultureSwab, Becton-Dickinson Co, Franklin Lakes, NJ.
d. Whirl-Pak bags, Nasco, Fort Atkinson, WI.
g. OptiMaxPlus, BioGenex, San Ramon, CA.
h. MultiLink, BioGenex, San Ramon, CA.
i. Zymed, Invitrogen, Carlsbad, CA.
j. NovaRED, Vector, Burlingame, CA.
k. Thermo Scientific, Pittsburgh, PA.
l. SAS, version 9.1, SAS Institute Inc, Cary, NC.
References


Table 1. Summary of strain-related differences in abortion rate and histopathologic lesions following oral inoculation with *C. jejuni*

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Dose</th>
<th>Aborted*</th>
<th>Multifocal random hepatitis*</th>
<th>Necrosuppurative placentitis*</th>
<th>Mean uterine inflammatory score†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>MH broth only</td>
<td>0/4</td>
<td>0/5</td>
<td>0/4</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>OF48</td>
<td>5.5 x 10⁸ CFU</td>
<td>0/10</td>
<td>4/12</td>
<td>0/9‡</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>NCTC 11168</td>
<td>5.9 x 10⁸ CFU</td>
<td>0/10</td>
<td>8/12</td>
<td>1/10</td>
<td>0 ± 0.3</td>
</tr>
<tr>
<td>IA3902</td>
<td>6.0 x 10⁸ CFU</td>
<td>6/10</td>
<td>11/12</td>
<td>8/10</td>
<td>2.3 ± 0.3</td>
</tr>
</tbody>
</table>

*Represents the number of guinea pigs with this characteristic/number of guinea pigs inoculated. †Score ranged from 0 (inflammatory infiltrate not identified) to 3.5 (> 20 leukocytes/hpf and the inflammation extended into the underlying myometrium). ‡One guinea pig gave birth before the end of the study and a placental sample was not obtained.
Table 2. Sample source and semiquantitative recovery of *C. jejuni* cultured from samples obtained during necropsy of guinea pigs inoculated with IA3902

<table>
<thead>
<tr>
<th>Sample source</th>
<th>No. of culture-positive samples*</th>
<th>Low recovery (&lt; 50 CFUs)†</th>
<th>Moderate recovery (50 to 1,000 CFUs)‡</th>
<th>High recovery (&gt; 1,000 CFUs)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>5/12</td>
<td>3/5</td>
<td>2/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Bile</td>
<td>2/12</td>
<td>0/2</td>
<td>1/2</td>
<td>1/2</td>
</tr>
<tr>
<td>Feces</td>
<td>10/12</td>
<td>2/10</td>
<td>6/10</td>
<td>2/10</td>
</tr>
<tr>
<td>Fetal stomach contents (pooled)</td>
<td>4/8‡</td>
<td>2/4</td>
<td>1/4</td>
<td>1/4</td>
</tr>
<tr>
<td>Gravid uterus</td>
<td>6/10</td>
<td>0/6</td>
<td>1/6</td>
<td>5/6</td>
</tr>
<tr>
<td>Fetal liver or lung (pooled)</td>
<td>6/10</td>
<td>1/6</td>
<td>0/6</td>
<td>5/6</td>
</tr>
<tr>
<td>Placenta</td>
<td>7/10</td>
<td>0/7</td>
<td>0/7</td>
<td>7/7</td>
</tr>
</tbody>
</table>

*Values reported represent number of samples with positive culture results/number of samples tested. †Results reported represent number of samples within that category/number of samples with positive culture results. ‡Fetal stomach contents were only obtained for 8 of 10 pregnant guinea pigs. Pooled = Samples for all fetuses of a specific guinea pig were pooled for microbial culture.
Figure 1. Photomicrographs of sections of placental tissues obtained from guinea pigs inoculated with *C. jejuni* IA3902. A—Notice the necrosuppurative placentitis and hemorrhage at the junctional zone (JZ) between the subplacenta (SP) and main placenta (MP) and marked vasculitis of the adjacent uteroplacental artery (UA) H&E stain; bar = 200 μM. B—Photomicrograph of the same section of placental tissue in panel A after immunohistochemical staining for *C. jejuni*. Notice the *C. jejuni* organisms (red stain) located within inflammatory cells and in extracellular spaces in the junctional zone. Primary antibody was directed against the major outer membrane protein of *C. jejuni*, followed by a biotinylated secondary antibody and incubation with horseradish peroxidase–streptavidin conjugate and developed by use of a commercial chromogen;
counterstained with Shandon Harris hematoxylin and Scott’s tap water; bar = 200 μm.

C—Photomicrograph of a section of guinea pig placental tissue after immunohistochemical staining for C. jejuni. Notice the C. jejuni organisms (red stain) within the trophoblasts and syncytial streamers (SS) of the subplacenta. Bar = 50 μm.

D—Photomicrograph of a uteroplacental artery (UA) from a guinea pig that aborted 8 days after inoculation. Notice the marked vasculitis and abundant perivascular C. jejuni organisms (red stain). Immunohistochemical stain for C. jejuni; bar = 50 μM.
Figure 2. Percentage of samples with positive results when cultured for *C. jejuni*.

Samples were obtained during necropsy of guinea pigs after oral inoculation with *C. jejuni* strain IA3902, NCTC 11168, or OF48 or a sham inoculum. Necropsies were performed at the time of abortion or at 21 days after inoculation in guinea pigs that did not abort.
CHAPTER 3: EXPRESSION OF TOLL-LIKE RECEPTORS 2 AND 4 IN SUBPLACENTAL TROPHOBLASTS FROM GUINEA PIGS (Cavia porcellus) FOLLOWING INFECTION WITH CAMPYLOBACTER JEJUNI

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Abstract

Toll-like receptors (TLRs) 2 and 4 are well-characterized cell-surface receptors that recognize specific pathogen-associated molecular patterns and play an important role in pathogen recognition and activation of the innate immune system. Variable expression of TLR2 and TLR4 has been described in trophoblasts from both normal and diseased placentas, yet there are limited data regarding trophoblast TLR expression in response to specific placental pathogens, and TLR expression in the guinea pig placenta has not been described. The guinea pig is an effective model for Campylobacter-induced abortion of
small ruminants and we have previously shown by immunohistochemistry that
*Campylobacter jejuni* localizes within syncytiotrophoblasts of the guinea pig subplacenta.

The present study was designed to determine if the expression of either TLR2 or TLR4 would be affected in subplacental trophoblasts following infection with *C. jejuni*.

Immunohistochemistry for TLR2 and TLR4 was performed on placenta from guinea pigs that aborted following inoculation with *C. jejuni* and from sham-inoculated controls. Quantitative assessment of TLR expression was performed, and mean immunoreactivity for TLR2 was significantly higher in subplacental trophoblasts from animals that aborted compared with uninfected controls \((P = 0.0283)\) whereas TLR4 expression was not statistically different \((P = 0.5909)\). These results suggest that abortion in guinea pigs following infection with *C. jejuni* is associated with increased TLR2 expression in subplacental trophoblasts and may reveal a possible role for TLR2 in the pathogenesis of *Campylobacter*-induced abortion.

**Introduction**

*Campylobacter* spp. are a frequent cause of ovine abortion worldwide,\(^1\) and a recent USDA:APHIS survey reported that campylobacteriosis was the most common cause of infectious abortion in US flocks.\(^2\) Historically, *Campylobacter fetus* subsp. *fetus* has been the major isolate from cases of ovine campylobacteriosis.\(^3\) However, two recent studies have shown a marked shift in this relationship with *C. jejuni* becoming the predominant isolate.\(^4,5\) Various molecular typing methods including pulsed-field gel electrophoresis have further demonstrated that a single tetracycline-resistant *C. jejuni*
clone (SA clone for Sheep Abortion) has emerged as the predominant cause associated with ovine abortions in Iowa, South Dakota, Idaho, California, Oregon, and Nevada. Despite the relative frequency of campylobacteriosis, the exact mechanisms of Campylobacter-induced abortion in small ruminants are largely unknown, and the focus of the present study is to gain insight into potential mechanisms underlying the pathogenesis of this disease.

Toll-like receptors (TLRs) are a well-characterized family of pattern recognition receptors (PRRs) that play a key role in innate immune function and have been extensively reviewed. In brief, there are currently 13 members of the TLR family that have been identified in mice, of which (TLRs 1-10) have also been identified in cattle, swine, sheep, and humans, and TLR genes have a high degree of sequence similarity between species. TLRs can be grouped based upon the characteristics of their ligands and on the location of cellular expression. TLRs 1, 2, 4, 5 and 6 recognize specific pathogen-associated molecular patterns (PAMPs) such as triacyl lipopeptide (TLR1/TLR2), peptidoglycan (TLR2), lipopolysaccharide (TLR4), flagellin (TLR5), and diacyl lipopeptide (TLR6/TLR2), and are expressed on the cell surface; whereas TLRs 3, 7, 8 and 9 recognize pathogen-associated nucleic acid patterns and are expressed in intracellular compartments such as endosomes. TLR2 recognizes the most diverse group of ligands and this is made possible, at least in part, by the formation of heterodimers with TLR1 and TLR6. Members of the TLR family are expressed by a variety of cell types including those directly involved in host immunity, such as macrophages,
neutrophils, and dendritic cells and non-immune cells such as fibroblasts, endothelial cells and various epithelial cells.\(^6\)

The maternofetal interface has long been considered an immunological paradox as the endometrium must allow for implantation of the allogenic placenta while maintaining adequate immune surveillance against a diverse spectrum of potential pathogens.\(^12\) The role of PRRs in this process, particularly TLRs, has been an active area of research and the subject of a recent review.\(^13\) Immunohistochemistry has been used to characterize the expression of TLR2 and TLR4 in the various trophoblast layers from both normal and diseased human placentas;\(^14-17\) however, there are limited data regarding trophoblast TLR expression associated with specific placental pathogens and TLR expression in the guinea pig placenta has not been described. We have previously shown by IHC that \textit{C. jejuni} localizes within junctional zone trophoblasts of the guinea pig subplacenta,\(^18\) and we hypothesize that expression of one or more TLRs will be increased in subplacental trophoblasts following infection with \textit{C. jejuni} IA3902 (a clinical sheep abortion isolate belonging to the SA clone).

Previous studies have revealed that TLR2 and TLR4, but not TLR5 are capable of binding PAMPs of \textit{Campylobacter} spp. More specifically, the flagellin of \textit{C. jejuni} does not effectively bind TLR5,\(^19,20\) \textit{C. jejuni} and \textit{C. coli} induce IL-8 secretion by polarized human intestinal epithelial cells in a TLR-mediated manner,\(^21\) \textit{C. rectus} infection increases TLR4 expression in murine placentas,\(^22\) \textit{C. jejuni} surface polysaccharides induce IL-6 production in human intestinal epithelial cells via TLR2,\(^23\) and lysed \textit{C. jejuni} effectively activates both chicken and human TLR2 and TLR4.\(^24\) Accordingly, in
the study reported here, IHC was used to evaluate the expression of TLR2 and TLR4 in guinea pig subplacental trophoblasts following infection with \textit{C. jejuni} IA3902 in order to delineate the involvement of these TLRs in response to this specific placental pathogen.

\textbf{Materials and Methods}

\textbf{Sample collection}—Blocks of formalin-fixed, paraffin-embedded guinea pig placenta were obtained from animals used in previous experiments.\textsuperscript{18} In these experiments, animals were inoculated orally with \textit{C. jejuni} IA3902 at approximately 4 weeks of gestation with abortions occurring between 4 and 14 days post-inoculation. Aborted placentas (44 total) were obtained from 12 orally inoculated guinea pigs. Pooled placental tissues from each of these dams were culture positive for \textit{C. jejuni} indicating that at least one of the placental samples from each orally challenged guinea pig was infected. Control placentas (28 total) were obtained from 9 sham-inoculated animals at 21 days post-inoculation.

\textbf{Histologic evaluation}—H&E stained sections of each placenta were evaluated to determine the presence or absence of suppurative placentitis as defined by the presence of infiltrating heterophils within the junctional zone between the subplacenta and decidua.

\textbf{Immunohistochemistry}—Formalin-fixed, paraffin-embedded guinea pig placental samples were prepared for IHC as previously described.\textsuperscript{18} For antigen retrieval, sections were treated with 0.1\% protease in a Tris buffer (pH, 7.6) at 37\degree C for 10 minutes followed by 3 rinses in PBS solution prior to placement in an automated cell staining system (BioGenex, USA). Sections were then incubated in 10\% neutral goat serum at
22°C for 20 minutes to inhibit nonspecific binding. Primary antibodies consisted of commercially available, affinity purified rabbit-antihuman TLR2 (Rockland, USA) and affinity purified rabbit-antihuman TLR4 (Rockland, USA), and were used at dilutions of 1:500 and 1:300, respectively. Rabbit-antihuman TLR2 and TLR4 primary antibodies have been previously shown to be cross-reactive in guinea pig tissues. Slides were incubated at 22°C for 60 minutes (TLR2) or 120 minutes (TLR4) followed by rinsing in a bath of PBS solution for 5 minutes. The secondary antibody (BioGenex, USA), horse radish peroxidase–streptavidin conjugate (Invitrogen, USA), chromogen (Vector, USA), and counterstaining steps were performed as previously described. Positive control samples consisted of guinea pig spleen and lung tissues for evaluation of receptor expression on phagocytes. Negative controls were prepared from the same blocks and consisted of sections in which the primary antibody was omitted and sections in which normal (preimmune) rabbit serum was applied in place of the primary antibody.

IHC for C. jejuni was also performed on all aborted placentas as previously described.

**Image analysis**—IHC-stained sections were examined with an Olympus BX60 light microscope (Olympus, Japan) and RGB color digital images were recorded using an Olympus DP70 camera (Olympus, Japan) with 2040 x 1536 bit resolution and 24-bit depth. Three 40X magnification images were obtained for each subplacenta and included one image taken from each lateral aspect of the junctional zone and one from the area just below the central excavation. The recorded microscopic fields were oriented such that
subplacental trophoblasts filled as much of the field as possible while excluding any inflammatory exudate present in the junctional zone.

To quantify the level of TLR protein expression in subplacental trophoblasts, all digital images were analyzed using ImageJ image analysis software (NIH, USA) and a colour deconvolution (stain separation) plug-in implementing methods previously described. Using the plug-in, the red color (positive immunohistochemical staining) was isolated in each image and then converted to a black and white image and set to a common threshold. Thresholds of 175 and 200 were selected for TLR2 and TLR4, respectively. The percent area of each image exhibiting the selected threshold level of staining was then quantified. The mean percent stained area was then determined for the three images from each placenta, and the average staining of all placentas from each individual guinea pig was calculated and compared.

**Statistical analysis**—A commercial statistical software package (SAS 9.2, SAS Institute, USA) was used to perform independent samples t-tests for a difference in mean TLR protein expression between groups. Statistical significance was determined as \( P \leq 0.05 \).

**Results**

**Histologic findings in affected placentas**—Placentas from aborting guinea pigs were typically characterized by one or more of the following changes: congestion of the placental labyrinth, multifocal necrosis of the placental disc, and multifocal to coalescing necrosis of subplacental trophoblasts (Figure 1) that was often associated with
heterophilic infiltration. The placental junctional zone in at least one placenta from all (12/12) aborting guinea pigs was infiltrated with heterophils; however, not every placenta from each aborting animal was equally affected. This was most notable in guinea pigs with higher fetal numbers, as would be expected, and occasionally placentas from individual fetuses lacked histologic lesions. In total, 88.6% (39/44) of placentas from aborting guinea pigs had evidence of suppurative placentitis. IHC for *C. jejuni* revealed that 65.9% (29/44) of placentas from these aborting animals had direct evidence of bacterial infection within subplacental trophoblasts and phagocytes in the placental junctional zone, and 100% (29/29) of IHC-positive placentas had suppurative placentitis.

Placentas from sham-inoculated controls lacked significant lesions (Figure 2). Multifocally there were small areas in which subplacental trophoblasts appeared hypereosinophilic with pyknotic nuclei and often surrounded accumulations of scant to moderate amounts of eosinophilic cellular and karyorrhectic debris (necrosis) admixed with basophilic granular material (mineral); however, these areas were not associated with heterophilic infiltration and were generally limited to the lateral aspects of the junctional zone.

**Expression of TLR2**—IHC of aborted placentas revealed a moderate increase in TLR2 immunoreactivity within trophoblasts of the main placenta as compared with controls; however, strong positive immunoreactivity was a consistent feature of subplacental syncytiotrophoblast cells and syncytial islands within lacunae, whereas the cytotrophoblast layer lacked significant staining (Figure 3). In sham-inoculated control tissues, a low level of diffuse cytoplasmic immunoreactivity for TLR2 was evident in
trophoblasts of the interlobium of the main placenta, whereas the syncytiotrophoblast and cytotrophoblast cells of the subplacenta were generally unstained (Figure 4). Positive immunoreactivity was also observed in heterophils and macrophages within control tissues while negative controls were nonreactive (not shown).

**Expression of TLR4**—IHC of aborted placentas revealed moderate TLR4 immunoreactivity within trophoblasts of the main placenta and in the mesenchyme and surrounding vascular spaces of subplacental lamellae. A low level of positive immunoreactivity was an inconsistent feature of subplacental syncytiotrophoblast cells, while the adjacent cytotrophoblast layer lacked significant staining (Figure 5). IHC of sham-inoculated control tissues revealed scant cytoplasmic immunoreactivity for TLR4 in trophoblasts of the interlobium, moderate immunoreactivity within the mesenchyme and surrounding vascular spaces in subplacental lamellae, and minimal to no immunoreactivity within subplacental syncytiotrophoblast and cytotrophoblast cells (Figure 6). Strong positive immunoreactivity was also observed in heterophils and macrophages within control tissues while negative controls were nonreactive (not shown).

**Image analysis of subplacental immunoreactivity**—TLR2 immunoreactivity was significantly higher in subplacental trophoblasts from aborted placentas compared with non-aborted, sham-inoculated controls ($P = 0.0283$). The mean percent area corresponding to positive immunohistochemical staining for images obtained from each group are summarized (Figure 7). This difference was even more significant when comparing mean TLR2 immunoreactivity between controls and only those placentas with
suppurative placentitis and positive IHC for _C. jejuni_ \((P = 0.0108)\), whereas mean immunoreactivity of placentas with evidence suppurative placentitis but IHC-negative for _C. jejuni_ and those aborted placentas that lacked placentitis did not differ significantly from controls \((P = 0.7350 \text{ and } P = 0.0724, \text{ respectively})\). These results are summarized in Figure 8.

TLR4 immunoreactivity did not differ significantly between aborted placentas and controls \((P = 0.5909)\). The mean percent area corresponding to positive immunohistochemical staining for images obtained from each group are summarized (Figure 7). Additionally, mean TLR4 immunoreactivity did not differ significantly when comparing control placentas with only those placentas from aborting animals that had suppurative placentitis and positive IHC for _C. jejuni_ \((P = 0.9830)\), those with only suppurative placentitis \((P = 0.9321)\), or only those placentas from aborting guinea pigs that lacked placentitis \((P = 0.2380)\). These results are summarized in Figure 8.

**Discussion**

Campylobacteriosis is a well-recognized cause of small ruminant abortion worldwide; however, the exact molecular mechanisms involved in this process are not known. We have recently shown using IHC that, following oral inoculation, _C. jejuni_ IA3902 localizes within syncytiotrophoblast cells of the guinea pig subplacenta,\(^{18}\) and in the present study, 65.9\% (29/44) of placentas from infected guinea pigs had evidence of _C. jejuni_ antigen by IHC, consistent with the 71.4\% noted in our previous work. To
further explore potential mechanisms associated with *Campylobacter*-induced abortion, IHC for TLR2 and TLR4 was performed on these placental tissues.

The guinea pig placenta consists of a disc-like, labyrinthine chorioallantoic main placenta with separate subplacenta and yolk sac placenta. The subplacenta is unique to hystricognath rodents and its histological, histochemical and ultrastructural properties have been thoroughly described elsewhere. In brief, the subplacenta is separated from the main placenta by a thin layer of fetal mesenchyme, and the distal segments of the uteroplacental arteries enter the main placenta through the periphery of this junction. It is in this location that we consistently observe *C. jejuni* via IHC which seems logical as this is the site of placental vascular invasion and would be the first location reached during episodes of bacteremia. The subplacenta is composed of lamellae of mesenchyme which support a folded layer of cytotrophoblast cells that give rise to a syncytiotrophoblast layer along the decidual surface. As gestation progresses, syncytiotrophoblasts form extensive lacunae that contain abundant eosinophilic material and syncytial islands. The interface between the subplacenta and decidua is known as the junctional zone, and our current and previous immunohistochemical findings indicate that, in pregnant guinea pigs, *C. jejuni* IA3902 localizes within subplacental trophoblasts at the periphery of this junctional zone near the site of placental vascular invasion, while the main placenta is spared. For this reason, we focused our efforts in the present study on TLR expression within subplacental trophoblasts.

In the study reported here, we observed markedly increased immunoreactivity for TLR2 protein in the syncytiotrophoblast layer of the subplacenta from guinea pigs.
aborting following infection with *C. jejuni* versus sham-inoculated controls. This occurred without significant change in the underlying cytotrophoblast cells, and this is significant as the syncytiotrophoblast constitutes the frontal barrier of the placenta at the maternofetal interface. As the guinea pig generally has multiple offspring in each litter, it is likely that during any bacteremic event there may be an uneven distribution of organisms seeding fetoplacental units. As such, we further subdivided the placentas from aborting guinea pigs based upon the presence of positive IHC for *C. jejuni*, the presence placentitis without IHC for *C. jejuni*, and the absence of placentitis. Previous studies have shown that IHC is less sensitive than culture in detecting *Campylobacter* spp. within placental tissues,\(^{18,32}\) thus it is possible that all inflamed placentas were infected with *C. jejuni*, but that campylobacter numbers in IHC-negative inflamed placentas were substantially lower than IHC-positive placentas. Within the subdivided groups, placentas with placentitis and positive IHC for *C. jejuni* had significantly greater levels of TLR2 immunoreactivity when compared with controls, while the placentas from aborting animals that were inflamed but IHC-negative or that lacked placentitis did not differ from controls. These findings are intriguing and suggest an association between the presence of *C. jejuni* and increased expression of TLR2. While these results do not give information with regard to the exact mechanism of increased TLR2 expression, the presence of increased TLR2 protein suggests a potential role in the disease pathogenesis. A recent study in human intestinal epithelial cells has shown that *C. jejuni* surface polysaccharides induce IL-6 production via TLR2,\(^{23}\) and studies in first trimester human trophoblasts have revealed that TLR2 ligation induces increased production of IL-6 and
IL-8 as well as increased trophoblast apoptosis. The combined presence of suppurative placentitis and increased TLR2 expression in the infected tissues of this report is consistent with this type of pro-inflammatory response; however, further studies are required to determine if the increased expression of TLR2 is a direct effect of the organism or a downstream effect of placental inflammation, and to elucidate the exact cytokine response that occurs in guinea pig trophoblasts in response to *C. jejuni*.

In contrast to our findings with TLR2, TLR4 protein expression in subplacental trophoblasts did not differ significantly between controls and placentas from aborting animals overall or from any of the subdivided groups. These results suggest that altered TLR4 expression is not associated with *Campylobacter*-induced abortion in guinea pigs. While these results appear to conflict with the findings of increased TLR4 in murine placentas exposed to *Campylobacter rectus*, it is worth noting that there are significant differences between the two studies. First, unlike caviomorphs, mice lack a subplacenta and TLR4 IHC in the study by Arce, et al. (2009) assessed expression in trophoblasts from the placental labyrinth. Second, abortion was not the endpoint of the murine study so the level of TLR4 expression at the point of pregnancy termination was not assessed. Additionally, challenge with *C. rectus* in the murine study was designed to assess the placental response to chronic oral exposure to a periodontal pathogen, whereas the present study assessed the placental response to acute infection with a highly abortifacient *C. jejuni* strain. Finally, Arce, et al. (2009) only evaluated TLR4 so it remains to be seen what, if any, TLR2 response might occur in murine placentas following infection with *C. rectus*. It is also conceivable that both receptors may be
elevated at some point throughout the disease process as time-dependent interdependence between TLR4 and TLR2 has also been described.\textsuperscript{35} Limitations of this study include its retrospective nature and the lack of specific time matched control placentas for each aborted placenta. The presence of non-inflamed placentas within the pool of aborted tissues did, however, serve as an internal control, and, considering that the immunoreactivity of these tissues was similar to sham-inoculated control tissues, it appears that the constitutive expression of TLR2 and TLR4 in the guinea pig subplacenta does not vary significantly throughout the period studied. Additionally, the relevance of these findings to ovine campylobacteriosis is uncertain; however, a recent study of obesity induced placental inflammation in ewes revealed elevated mRNA expression for both TLR2 and TLR4 with a concurrent increase in proinflammatory cytokines,\textsuperscript{36} and these results support a role for TLRs in the response of ovine placenta to inflammatory stimuli.

In summary, we observed increased TLR2 immunoreactivity in subplacental syncytiotrophoblast cells from guinea pigs aborting following oral inoculation with \textit{C. jejuni} while TLR4 immunoreactivity was essentially unchanged, and that these changes were most prominent in syncytiotrophoblasts from placentas with direct evidence of \textit{C. jejuni} by IHC. Identification of these changes in subplacental syncytiotrophoblast cells is significant as these cells form the frontal barrier at the maternofetal interface and therefore suggest a role for TLRs in bacterial sensing and the subsequent inflammatory response generated during septic abortion due to \textit{C. jejuni}. To our knowledge, this is the first report of TLR expression in the guinea pig placenta and the first report to describe
trophoblast TLR expression associated with a specific, well-documented placental pathogen. These findings warrant further investigation to determine the exact role of TLR2 expression in the pathogenesis of *C. jejuni*-associated abortion.

**Acknowledgements**

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**References**


**Figure 1. Placenta; guinea pig infected with C. jejuni 1A3902.** The placental labyrinth (PL) is markedly congested and the subplacental lamellae (*) are undergoing degeneration and necrosis and beginning to separate from the main placenta. The central excavation (CE) is to the right. HE.

**Figure 2. Placenta; guinea pig, sham-inoculated control.** The placental labyrinth (PL), central excavation (CE), subplacental lamellae, and extensive lacunae (+) appear normal. HE.
Figure 3. Subplacenta; guinea pig infected with *C. jejuni* IA3902; IHC for TLR2.

Note the strong immunoreactivity of syncytiotrophoblasts (arrows) and syncytial islands within lacunae (arrowheads), while the subjacent cytotrophoblast layer and mesenchyme are relatively unstained (asterisks). NovaRED substrate and Mayer’s hematoxylin counterstain.

Figure 4. Subplacenta; guinea pig, sham-inoculated control; IHC for TLR2. Note the overall lack of immunoreactivity compared with Figure 3, particularly within the syncytiotrophoblast cells lining the subplacental lamellae (arrows) and syncytial islands within lacunae (arrowheads). The subjacent cytotrophoblast layer and mesenchyme are also unstained (asterisks). NovaRED substrate and Mayer’s hematoxylin counterstain.
Figure 5. Subplacenta; guinea pig infected with *C. jejuni* IA3902; IHC for TLR4. Note the low level of specific immunoreactivity overall, with the strongest staining within the mesenchyme and surrounding vascular spaces within subplacental lamellae (asterisks). Syncytiotrophoblast cells (arrows) and syncytial islands (arrowheads) are unstained. NovaRED substrate and Mayer’s hematoxylin counterstain.

Figure 6. Subplacenta; guinea pig, sham-inoculated control; IHC for TLR4. Note the low level of immunoreactivity overall, with moderate staining within the mesenchyme of subplacental lamellae (asterisks). Syncytiotrophoblasts (arrows) and syncytial islands (arrowheads) are unstained. NovaRED substrate and Mayer’s hematoxylin counterstain.
Figure 7. Percent area of subplacental photomicrographs corresponding to positive staining for TLR2 or TLR4 per 40X field (mean ± SEM). Values represent mean immunoreactivity for all placentas from aborting guinea pigs (n=12; 44 placentas total) and non-aborted controls (n=9; 28 placentas total). * = Values differ significantly ($P = 0.0283$).
Figure 8. Percent area of subplacental photomicrographs corresponding to positive staining for TLR2 or TLR4 per 40X field (mean ± SEM). Values represent mean immunoreactivity for all aborted placentas IHC+ for *C. jejuni* (n=9; 30 placentas total), aborted placentas with placentitis but IHC- for *C. jejuni* (n=5; 9 placentas total), aborted placentas without placentitis (n=3; 5 placentas total) and non-aborted controls (n=9; 28 placentas total). * = Value differs significantly from controls (*P* = 0.0108).
CHAPTER 4: COMPARISON OF TWO COMMERCIAL OVINE CAMPYLOBACTER VACCINES AND AN EXPERIMENTAL BACTERIN IN A GUINEA PIG MODEL

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Abstract

Objective—To compare efficacy of two commercial ovine Campylobacter vaccines and an experimental bacterin in guinea pigs following IP inoculation with \textit{C. jejuni} IA3902.

Animals—51 female Hartley guinea pigs.

Procedures—Pregnant and non-pregnant animals were randomly assigned to 1 of 4 treatment groups and administered 2 subcutaneous doses 14 days apart of commercial vaccine A (n = 13), commercial vaccine B (n = 12), an experimental bacterin prepared
from the challenge strain (n = 12), or a sham vaccine (n = 14). 10 days later, animals were challenged IP with *C. jejuni* IA3902. 48 hours post-challenge, animals were euthanized, a complete necropsy was performed, and blood and tissue samples were obtained for culture.

**Results**—Administration of vaccine B or the experimental bacterin significantly reduced 48 hour infection rates versus sham-vaccinated controls (*P* < 0.001 and *P* = 0.0162, respectively), whereas those administered vaccine A did not differ from controls (*P* > 0.999). A significantly reduced 48 hour infection rate was associated with administration of vaccine B independent of pregnancy status.

**Conclusions and Clinical Relevance**—Administration of vaccine B significantly reduced infection in guinea pigs challenged with *C. jejuni* IA3902 similar to a homologous bacterin. These results suggest vaccine B or an autogenous product may be effective in controlling ovine campylobacteriosis due to this emergent abortifacient strain. Additionally, culturing blood, liver, bile and uterus in non-pregnant guinea pigs 48 hours post-inoculation may be a useful screening tool for comparing efficacy of *C. jejuni* vaccines.

**Introduction**

*Campylobacter* spp. are a well-recognized and notable cause of ovine abortion worldwide with abortion rates of 5-50% reported in affected flocks.¹ A recent national survey ranked campylobacteriosis first among infectious causes of abortion in domestic sheep.² Two recent studies³,⁴ have demonstrated that *Campylobacter jejuni* is the
predominant species responsible for Campylobacter-associated sheep abortion in the United States, and molecular typing techniques have further revealed that within these C. jejuni a single tetracycline-resistant clone (named SA clone for Sheep Abortion) has emerged as the predominant cause of ovine abortion in Iowa, South Dakota, Idaho, California, Oregon, and Nevada. This new, emerging clone is significant in that Campylobacter spp. isolated from ovine abortions in different regions and during different lambing seasons traditionally exhibit marked genetic and antigenic heterogeneity.

The effectiveness of vaccination for the prevention of ovine campylobacteriosis has been well-documented; however, experimental studies have also revealed inadequate protection to homologous challenge in ewes vaccinated with commercial vaccines of questionable efficacy and have demonstrated inadequate cross protection in those vaccinated and later challenged with heterologous serotypes. Additionally, Campylobacter spp. abortions have been reported in field cases from flocks receiving commercial monovalent and bivalent vaccines. Accordingly, no single vaccine will likely be protective against all abortifacient Campylobacter spp., and continual efficacy testing is imperative to ensure the ongoing usefulness of a given vaccine. The pregnant guinea pig is an effective model for screening abortifacient Campylobacter spp., and has been described for testing the efficacy of commercial Campylobacter vaccines intended for cattle and sheep, as well as experimental bacterins. These efficacy studies have further demonstrated that immunization with a monovalent bacterin of either Campylobacter fetus subsp. fetus or C. jejuni is not cross protective against challenge
with the opposite species, and that protection is lower when challenged with a heterologous strain of the same species. These findings strongly support the need for polyvalent vaccines in the prevention of ovine campylobacteriosis.

In the United States, there are currently two commercially available, ovine-labeled polyvalent *Campylobacter* vaccines, each containing both *C. fetus* subsp. *fetus* and *C. jejuni* strains. Given the emergence of *C. jejuni* clone SA as the predominant *Campylobacter* sp. recovered from sheep abortions across multiple states, we hypothesize that one or both of these vaccines may not be protective against this highly-abortifacient strain. Previous studies have assessed the prevention of abortion in pregnant guinea pigs following IP challenge as the evaluation endpoint for *Campylobacter* vaccine efficacy; however, given the increased cost associated with acquisition of pregnant animals and the housing costs associated with a multi-week observational study, we sought to determine if a mixed population of pregnant and non-pregnant females would be an adequate, cost-effective model for screening *Campylobacter* vaccines. As bacteremia is a requisite step in the pathogenesis of non-venereal *Campylobacter*-induced abortion, the presence of blood and tissue infection in non-pregnant animals should provide an indication of the potential for abortion. In our previous work, we observed abortions in pregnant guinea pigs as early as 48 hours following IP inoculation with *C. jejuni* IA3902 (a clinical abortion isolate belonging to the SA clone), and organisms were recoverable from the blood, bile, uterus and fetoplacental units at that time. Accordingly, in the study reported here, blood and tissue colonization at 48 hours was selected as the evaluation endpoint to assess the efficacy of
two commercially available ovine campylobacteriosis vaccines and an experimental homologous bacterin in eliminating infection following IP challenge with *C. jejuni* IA3902 in both pregnant and non-pregnant guinea pigs.

**Materials and Methods**

**Humane animal care and use** – All procedures described herein were approved by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University, Ames, IA.

**Animals** – 51 female Hartley guinea pigs with an average weight of 879 g were obtained from a commercial source. All animals were recently bred but pregnancy could not be guaranteed for animals purchased at this stage of gestation. Upon arrival, a rectal swab was obtained from each guinea pig, placed in transfer medium, and plated for *Campylobacter* culture as described below. Guinea pigs were randomly assigned into four treatment groups (n = 14, n = 13, n = 12, n = 12), housed in individual cages with wood chip bedding, and fed a commercial pelleted guinea pig diet ad libitum. Each guinea pig received the first subcutaneous dose of their respective vaccine treatment within 24 hours of arrival, and received a second subcutaneous dose 14 days later. Ten days after receiving the second vaccination, guinea pigs were challenged IP with *C. jejuni* IA3902.

**Pregnancy determination** – Abdominal palpation was performed to screen for pregnancy approximately 14 days after arrival. Pregnancy status was confirmed by abdominal ultrasound 2 to 5 days prior to inoculation.
**Vaccines** – Vaccine A\(^a\) was a commercial, polyvalent product labeled for prevention of campylobacteriosis in sheep via two 5 ml subcutaneous doses. For the purposes of the present study, the dose was reduced to 1 ml and administered subcutaneously in the interscapular region. Vaccine B\(^b\) was a commercial, polyvalent product labeled for prevention of ovine campylobacteriosis via two 2 ml subcutaneous doses. To keep the effective dose the same as that used for Vaccine A (20% of the labeled dose), the dose was reduced to 0.4 ml and administered subcutaneously in the interscapular region. The commercial products each contained both *C. fetus* subsp. *fetus* and *C. jejuni* strains in an aluminum hydroxide adjuvant. Accordingly, aluminum hydroxide gel\(^d\) was used in preparing the homologous bacterin for this experiment. For preparation of the bacterin, a frozen stock of *C. jejuni* IA3902 was grown microaerobically on Mueller-Hinton (MH) agar for 48 hours at 42°C, then subpassaged on MH agar for an additional 24 hours at 42°C under the same atmospheric conditions. Lawns of *C. jejuni* were harvested in phosphate buffered saline (PBS) directly from plates by washing and then pelleted by centrifugation (15 min at 7,000g). The pellet was then washed and resuspended in 10 ml of PBS containing 0.3% formalin. This suspension was centrifuged again (30 min at 7,000g), the supernatant discarded, and the wash process repeated two additional times. The final pellet was weighed and resuspended in sterile PBS to achieve a final concentration of 5 mg of *C. jejuni* cells per ml. This final concentration was selected based upon a previous report\(^{22}\) comparing antigen dose and various adjuvant types in which this concentration provided 93% protection against homologous challenge. A few drops of the diluted cells were streaked
on MH agar or added to enrichment broth, both of which were then placed in a microaerophilic environment to check efficacy of formalin killing. Once killing was verified, sterile aluminum hydroxide gel was added to the diluted cell suspension to achieve a final concentration of 0.2 mg Al(OH)$_3$/ml. Higher concentrations of aluminum hydroxide adjuvant have been used in previous *Campylobacter* vaccine studies; however, lower concentrations have been reported to be effective with experimental *Bordetella* and *Salmonella* vaccines and a lower concentration was therefore selected to decrease the risk of adverse reactions. A hemacytometer was used to quantify the number of particles per ml for each preparation. The approximate concentrations for the three preparations were 6.29 X $10^7$ per ml for vaccine A, 5.03 X $10^8$ per ml for vaccine B, and 8.83 X $10^7$ particles per ml for the experimental bacterin. The roughly ten-fold higher concentration of particles per ml in vaccine B was further support for its administration at a reduced dose compared with the other treatments. The experimental bacterin was administered as a 1 ml subcutaneous dose in the interscapular region. A sham vaccine was prepared containing 0.2 mg/ml of aluminum hydroxide in sterile PBS and was administered as a 1 ml subcutaneous dose in the interscapular region.

**Challenge-exposure inocula** – Fresh bacterial cultures of *C. jejuni* IA3902 were obtained following 24 hours of growth on MH agar in anaerobic jars under microaerobic conditions (5% O$_2$, 10% CO$_2$, 85% N$_2$) at 42ºC. These cultures were collected in MH broth, diluted to desired concentrations based on optical density (OD$_{600}$) to give approximately 1 X $10^6$ CFU/ml, and then used as inocula in the challenge experiments. The final number of organisms in each suspension was determined by viable colony-
forming unit (CFU) counting. From our extensive experience in the laboratory, we expect that 24-hour grown (mid-logarithmic phase) *C. jejuni* cultures would yield approximately $1 \times 10^7$ CFU/ml when adjusted to OD$_{600}$:0.1.

**Animal inoculation** – 10 days after receiving their second vaccination, all animals were inoculated IP with 1 ml of $7.5 \times 10^5$ to $1.0 \times 10^6$ CFU/ml MH broth culture of *C. jejuni* IA3902. This concentration was chosen based upon results from our previous work, and a report describing IP challenge with *C. jejuni* in vaccinated pregnant guinea pigs.

**Monitoring, euthanasia and necropsy sampling** – Once inoculated, guinea pigs were observed at least twice daily for signs of abortion or impending abortion. These signs include vaginal bleeding, presence of expelled fetuses or visible fetal membranes. All animals were euthanized 48 hours post-inoculation via IP injection of sodium pentobarbital (approximately 150 mg/kg) and necropsied immediately. At necropsy, animals were inspected for gross lesions and samples were taken for bacterial culture, serology and histopathology. Samples harvested for *Campylobacter* isolation included heart blood, liver, bile, uterus, and all placental units. Samples of liver, gallbladder, uterus and placenta were taken for histopathology and placed in 10% neutral buffered formalin for 24 hours prior to paraffin embedding and routine processing for hematoxylin and eosin (H&E) staining.

**Isolation and semi-quantitative enumeration of *C. jejuni* from necropsy samples** – Heart blood and bile were collected and transported using sterile needles and syringes. Liver, uterus, and placenta were placed in separate sterile Petri plates for
transport prior to culture. All samples were kept refrigerated until immediately prior to
culture and were cultured on the day of collection. Fluid samples (blood and bile) were
placed directly onto culture media and streaked using sterile cotton swabs. Tissue
samples (liver, uterus, placenta) were minced with a scalpel/scissors, swabbed with a
sterile cotton swab, and streaked onto the culture media. All samples were spread onto
MH agar containing a *Campylobacter* selective supplement\(^e\) (polymyxin B, rifampicin,
trimethoprim and cycloheximide) and a *Campylobacter* growth supplement\(^f\) (sodium
pyruvate, sodium metabisulphite, and ferrous sulphate) and were incubated for 48 hours
in anaerobic jars under microaerobic conditions at 42ºC. Following incubation,
*Campylobacter*-like colonies on each plate were counted to determine the CFUs in each
sample.

**Enzyme-linked immunosorbent assay (ELISA)** – ELISA was used to determine
the level of *C. jejuni*-specific IgG antibodies in guinea pig sera. Microtiter plates\(^g\) were
first coated with 100 µl of whole membrane components (approx. 60 ng/well) of *C. jejuni
IA3902* in coating buffer (sodium carbonate; pH 9.6) overnight at 25ºC. Then, plates
were incubated with a blocking buffer (PBS containing 2 % milk, 2 % bovine serum
albumin, and 0.1 % Tween-20) at 37ºC for 1 hr. Serum samples were diluted in the
blocking buffer to 1:100, and then 100 µl of each dilution was added to individual wells.
Duplicate wells were used for each sample. After incubation at 25ºC for 2 hr, the plates
were washed 3 times with the wash buffer (PBS containing 0.1% Tween-20). Polyclonal,
horseradish peroxidase (HRP) labeled goat-anti-guinea pig IgG\(^h\) was diluted to 1:1000 in
the blocking buffer and added to the wells (100 µl/well). After 2 hr incubation at 25ºC,
the plates were washed three times with the wash buffer before the HRP substrate was added. OD values of individual wells were measured using an ELISA reader at 405 nm.

**Statistical analysis** – A commercial statistical software package was used to perform all analyses. Fisher’s exact test for binomial variables was employed when comparing the presence or absence of positive *Campylobacter* culture from samples of blood, liver, bile, uterus and placenta. A 1-way ANOVA was used to detect differences in ELISA results obtained from sera and *P* values are reported after Tukey adjustment for multiple comparisons.

**Results**

*Campylobacter* spp. were not isolated from any of the pre-challenge rectal swabs. One sham-vaccinated guinea pig aborted approximately 36 hours post-inoculation with *C. jejuni* IA3902 and was euthanized and necropsied immediately. All remaining guinea pigs were euthanized 48 hours post-inoculation at which time clinical signs of impending abortion were not observed.

**Gross lesions** – At necropsy, gross lesions were limited to the liver and consisted of random, pinpoint to 2 mm diameter white foci scattered throughout the parenchyma. These lesions were noted in 10/14 sham-vaccinated animals, 11/13 animals receiving vaccine A, 2/12 animals receiving vaccine B, and 2/12 animals receiving the experimental bacterin.

**Histologic lesions** – Suppurative placentitis was detected in the junctional zone between the subplacenta and decidua in all but one placental sample with positive culture
results for *C. jejuni* (7/8) and in no culture negative placental samples (0/11).

Additionally, multifocal hemorrhage was present in the junctional zone of some affected placentas. Random multifocal suppurative hepatitis was a consistent finding in animals receiving a sham vaccination or vaccine A. Lesions were most frequent and of the greatest severity in the group receiving vaccine A. Rare, small suppurative infiltrates were observed in a few animals receiving vaccine B or the experimental bacterin.

**Campylobacter culture** – *C. jejuni* was recovered from at least one sample (blood, bile, liver, uterus or placenta) in 26 of 51 animals. Of these 26 animals, 8 were pregnant and 18 were non-pregnant. The frequency of positive blood and tissue cultures are summarized by treatment group and by pregnancy status within treatment groups in Table 1. When compared with controls, animals receiving vaccine B had significantly lower recovery of *Campylobacter* from blood (*P* = 0.0171), bile (*P* = 0.0171), liver (*P* = 0.0017), uterus (*P* = 0.0425), and placenta (*P* = 0.0476). Animals receiving the homologous bacterin had significantly lower recovery from the liver and bile (*P* = 0.0472 and 0.0171, respectively) versus controls. Recovery rates for animals receiving vaccine A did not differ from controls (*P* > 0.25, all samples). When comparing treatment groups by pregnancy status, the distribution of recovery rates was similar to that observed in each group overall; however, statistical significance was often not maintained given the small number of samples in certain subsets.

For all animals with demonstrable tissue infection, the quantity of *Campylobacter* recovered from each sample type is summarized in Table 2. Overall, *Campylobacter* was recovered in the highest numbers from the placenta of pregnant animals (8/8 with > 1000
CFUs) and bile of non-pregnant animals (11/18 with > 1000 CFUs). While the recovery rates were generally low (< 50 CFUs), the majority of infected animals (23/26) had positive liver culture independent of pregnancy status. For all samples except bile, organisms were recovered in greater quantity from pregnant animals.

**ELISA results** – Vaccine A, vaccine B and the experimental bacterin each induced a significant level of *Campylobacter*-specific antibody relative to sham vaccination ($P < 0.0001$, $P = 0.0033$, and $P < 0.0001$, respectively). The mean OD values for each group are summarized in Figure 1. The mean OD for vaccine A and the experimental bacterin did not differ significantly ($P = 0.7961$); however, the mean OD for vaccine B was significantly lower than both the experimental bacterin and vaccine A ($P = 0.0058$ and $P = 0.0408$, respectively).

**Vaccine efficacy (elimination of detectable infection)** – An animal was considered to have eliminated infection at 48 hours if all tested tissues were culture-negative for *Campylobacter*. The percentage of animals from each treatment group in which infection was eliminated is summarized in Figure 2. Vaccine B yielded the highest overall percentage with 11/12 eliminating infection, and this differed significantly ($P = 0.000479$) from sham-vaccinated controls where only 3/14 were culture-negative for *Campylobacter*. A significantly greater percent free of infection was observed with vaccine B when comparing the subgroups of pregnant ($P = 0.0333$) or non-pregnant ($P = 0.0256$) animals as well. The overall clearance rate for animals receiving the homologous bacterin was 9/12 which differed significantly from controls ($P = 0.0162$); however, this significance was not maintained when comparing pregnant ($P = 0.1667$) or
non-pregnant \((P = 0.1058)\) animals separately. There was no significant difference in the elimination percentage overall or by pregnancy status when comparing animals receiving vaccine A versus sham-vaccinated controls \((P > 0.999\) for all tests).

**Discussion**

Comparative vaccine efficacy studies against emergent pathogens and novel pathogen strains are vital for veterinary practitioners and producers to help guide product selection during the development of disease management strategies. Unfortunately, the cost and logistics of rapidly assessing vaccine efficacy in the target species is often prohibitive. Ideally, *Campylobacter* vaccine efficacy studies would be undertaken in sheep; however, high seroprevalence for *Campylobacter* spp., the high carriage rate of *Campylobacter* spp. in the intestine and bile of healthy sheep\(^{27,28}\), the high cost of *Campylobacter*-negative SPF sheep, and a lengthy gestation are significant impediments to the assessment of multiple vaccine treatments in pregnant ewes. The goals of the present study were to design and evaluate a more manageable model for screening *Campylobacter* vaccines, and to employ this model in the assessment of the potential efficacy of 2 commercially available ovine-labeled *Campylobacter* vaccines\(^{a,b}\) against an emergent *C. jejuni* strain.

The small size, reduced housing costs, and ease of obtaining *Campylobacter*-negative animals make guinea pigs a desirable model for evaluating the efficacy of *Campylobacter* vaccines. In previous studies,\(^{20-23}\) the prevention of abortion in pregnant guinea pigs following IP challenge has been the evaluation endpoint; however, if animals
are to receive two doses of vaccine prior to inoculation, they need to be purchased at a
time point prior to that at which pregnancy can be confirmed, or a large breeding colony
needs to be established. In either situation, it is unlikely that 100% of vaccinated animals
will be pregnant or will successfully become pregnant and would therefore be unusable if
abortion is the sole evaluation endpoint. Additionally, the lengthy post-inoculation
observation period (up to 21 days) and any unused non-pregnant animals further inflate
the overall cost of previously described model systems. We have shown that pregnant
guinea pigs are highly susceptible to \textit{C. jejuni} IA3902,\textsuperscript{19} and that high numbers of
organisms are potentially recoverable from blood and other non-reproductive tissues
(liver and bile) in addition to fetoplacental units as early as 48 hours post-inoculation.
Thus, the present study was designed to evaluate the utility of a mixed population of both
pregnant and non-pregnant guinea pigs in screening for \textit{Campylobacter} vaccine efficacy,
a process that has not previously been described.

In contrast to venereally acquired campylobacteriosis in cattle, sheep are more
likely to acquire \textit{Campylobacter} spp. organisms by the oral route with subsequent
bacteremia and seeding of fetoplacental units by abortifacient strains. As such, resolution
of bacteremia following intraperitoneal challenge was selected as a means of screening
the efficacy of ovine campylobacteriosis vaccines. In the study reported here, elimination
of infection was considerably higher in animals receiving vaccine B (11/12 [92%]) or the
experimental homologous bacterin (9/12 [75%]) versus those receiving vaccine A (2/13
[15%]) or a sham-vaccination (3/14 [21%]). This relative efficacy was maintained for
each treatment independent of pregnancy status. The efficacy of vaccine B was
statistically greater than that of the sham control treatment when analyzed overall or between subsets of pregnant and non-pregnant animals indicating a clear relationship between this product and resolution of infection with *C. jejuni* IA3902. The homologous bacterin was slightly less efficacious than vaccine B and statistical significance versus controls was evident when comparing all animals treated; however, a statistically significant difference was not maintained when comparing pregnant subsets due to the low number of animals in each subset. Administration of vaccine A failed to significantly reduce infection 48 hours after inoculation with IA3902 in any form of analysis.

ELISA revealed that both commercial products and the experimental bacterin induced a significant level of *Campylobacter*-specific antibody relative to sham-vaccinated controls. This provides additional evidence of a difference in efficacy between the three treatments with regard to resolution of infection with *C. jejuni* IA3902, as the specificity of the antibodies in each group appears to differ despite a similar quantitative antibody response. Vaccine B had a lower mean OD than either vaccine A or the experimental bacterin yet was associated with the highest level of efficacy based on resolution of infection. While it is tempting to infer this as evidence of antibody quality over quantity, it is possible that a greater percentage of antibodies from vaccine B were consumed during the resolution of infection resulting in a lower OD in the 48 hour sera. Further study with preinoculation serology is necessary to fully evaluate the quantitative difference in antibody level between treatments; however, observing the greatest resolution of infection in the group with the lowest mean OD suggests that each
treatment produced an adequate quantity of antibody and therefore makes vaccination failure an unlikely source of the observed differences between groups.

*C. jejuni* was recovered from blood, bile, liver, and uterus in both pregnant and non-pregnant guinea pigs 48 hours post-inoculation, revealing that non-pregnant guinea pigs experience a similar overall tissue distribution relative to pregnant animals following IP inoculation with this organism. In non-pregnant animals, the greatest relative quantity of *C. jejuni* was recovered from the bile, whereas in pregnant animals the placenta was the tissue associated with highest rates of recovery with considerably less recovery from the bile. Previous studies have shown that *C. jejuni* is attracted to the mucin component of bile, and that certain bile acids induce virulence gene expression in *C. jejuni*, thus, identifying *C. jejuni* in the bile is not surprising. However, the differential predilection for the bile in non-pregnant guinea pigs versus the placenta in pregnant animals observed in our current study is intriguing given that a recent report revealed *C. jejuni* is driven by energy taxis and seeks conditions most favorable for growth. The results of this present study may therefore suggest the presence of a placental factor more chemoattractive than bile that drives the placental tropism observed with this organism. Further study involving the tropic effects of individual placental factors is necessary to further elucidate the nature of placental tropism with *C. jejuni*.

In summary, the present study demonstrates that the evaluation of blood and tissue infection in both pregnant and non-pregnant guinea pigs 48 hours after IP inoculation can effectively be used to screen potential *Campylobacter* vaccines in a shorter, more cost-effective manner than that previously described. Using this model, we
have shown that administration of two doses of vaccine B to both pregnant and non-
pregnant guinea pigs significantly reduced infection following IP inoculation with *C. jejuni* IA3902 comparable to that of a homologous bacterin while administration of vaccine A was ineffective despite inducing a significant level of *Campylobacter*-specific antibody. This underscores the importance of efficacy testing against emergent pathogenic strains and provides further evidence of inadequate cross protection between heterologous *C. jejuni* strains. These findings also suggest the presence of common protective antigens shared by the strains included in vaccine B and the challenge strain IA3902. Further studies such as immunoblotting with representative sera from the different groups may provide clues to this observation. As *C. jejuni* IA3902 has recently been described as the predominant ovine abortion-associated isolate in multiple states,\(^4\) protection against this emergent strain should be an essential component of effective campylobacteriosis disease management programs, and the added fact that IA3902 is highly resistant to tetracycline increases the importance of vaccination in disease prevention. Given the remarkably high percentage of guinea pigs receiving vaccine B (11/12 [92%]) that were free of infection 48 hours post-inoculation with a quantity of *C. jejuni* IA3902 previously shown to induce abortion in 100% of inoculated animals,\(^19\) it is likely that vaccine B would be the most effective currently available commercial vaccine for controlling ovine campylobacteriosis due to this specific strain; however, these findings do not necessarily reflect the effectiveness of either commercial product against the broad spectrum of *Campylobacter* spp. sheep may encounter in the field. Additionally, the high level of efficacy observed with the experimental homologous
bacterin in this study suggests that in vaccinated flocks experiencing abortion due to an unspecified strain of *Campylobacter*, an autogenous product may be an appropriate consideration.

**Acknowledgments**

This study was supported by the Iowa Livestock Health Advisory Council [grant number 109-05-66] and the Iowa State University CVM Summer Scholars Research Program.

**Footnotes**

a. *Campylobacter fetus* bacterin (serial #1369B), Colorado Serum, Denver, CO  
b. *Campylobacter fetus-jejuni* bacterin (serial #06-251), Hygieia Laboratories, Woodland, CA  
c. Elm Hill Labs, Chelmsford, MA  
d. Aluminum hydroxide gel (13mg/ml), Sigma-Aldrich, St. Louis, MO  
e. Preston *Campylobacter* selective supplement, Oxoid Ltd, Cambridge, England  
g. Nunc-Immune plate, Nunc, Denmark  
h. Anti-Guinea Pig IgG (H+L), peroxidase labeled, KPL, Gaithersburg, MD  
i. ABTS 2-Component Microwell Peroxidase Substrate, KPL, Gaithersburg, MD  
j. FLUOstar Omega Microplate Reader, BMG Labtech Inc, Durham, NC  
k. SAS 9.2, SAS Institute, Cary, NC
References


Table 1. Summary of vaccination-related differences in blood and tissue cultures 48 hours after intraperitoneal inoculation with *C. jejuni*

<table>
<thead>
<tr>
<th>Vaccine type</th>
<th>Blood</th>
<th>Bile</th>
<th>Liver</th>
<th>Uterus</th>
<th>Placenta</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All animals</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>6/14</td>
<td>6/13†</td>
<td>10/14</td>
<td>5/14</td>
<td>3/3</td>
</tr>
<tr>
<td>Vaccine A</td>
<td>4/13</td>
<td>9/13</td>
<td>9/13</td>
<td>8/13</td>
<td>2/2</td>
</tr>
<tr>
<td>Vaccine B</td>
<td>0/12*</td>
<td>0/12*</td>
<td>1/12**</td>
<td>0/12*</td>
<td>1/7*</td>
</tr>
<tr>
<td>Bacterin</td>
<td>1/12</td>
<td>0/12*</td>
<td>3/12*</td>
<td>2/12</td>
<td>2/7</td>
</tr>
<tr>
<td><strong>Pregnant animals only</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>2/3</td>
<td>0/2†</td>
<td>2/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>Vaccine A</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td>Vaccine B</td>
<td>0/7</td>
<td>0/7</td>
<td>1/7</td>
<td>0/7**</td>
<td>1/7*</td>
</tr>
<tr>
<td>Bacterin</td>
<td>1/7</td>
<td>0/7</td>
<td>2/7</td>
<td>2/7</td>
<td>2/7</td>
</tr>
<tr>
<td><strong>Non-pregnant animals only</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
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<td>6/11</td>
<td>8/11</td>
<td>2/11</td>
<td></td>
</tr>
<tr>
<td>Vaccine A</td>
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<td>7/11</td>
<td>7/11</td>
<td>6/11</td>
<td></td>
</tr>
<tr>
<td>Vaccine B</td>
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<td>0/5</td>
<td>0/5*</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>Bacterin</td>
<td>0/5</td>
<td>0/5</td>
<td>1/5</td>
<td>0/5</td>
<td></td>
</tr>
</tbody>
</table>

Values reported represent the number of samples with positive culture results/number of samples tested.
†A bile sample was unavailable from one guinea pig in this group. *Values vary significantly from sham-vaccinated controls (P < 0.05). **Values vary significantly from sham-vaccinated controls (P < 0.01).
Table 2. Semiquantitative recovery of *C. jejuni* from necropsy samples obtained from animals not protected by vaccination against intraperitoneal challenge with IA3902

<table>
<thead>
<tr>
<th>Sample source</th>
<th>Quantity of organisms recovered*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood</td>
</tr>
<tr>
<td><strong>Pregnant animals</strong></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>+++</td>
</tr>
<tr>
<td>Sham</td>
<td>-</td>
</tr>
<tr>
<td>Sham</td>
<td>+++</td>
</tr>
<tr>
<td>Vaccine A</td>
<td>+++</td>
</tr>
<tr>
<td>Vaccine A</td>
<td>+++</td>
</tr>
<tr>
<td>Vaccine B</td>
<td>-</td>
</tr>
<tr>
<td>Bacterin</td>
<td>+</td>
</tr>
<tr>
<td>Bacterin</td>
<td>-</td>
</tr>
<tr>
<td><strong>Non-pregnant animals</strong></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>-</td>
</tr>
<tr>
<td>Sham</td>
<td>+</td>
</tr>
<tr>
<td>Sham</td>
<td>+</td>
</tr>
<tr>
<td>Sham</td>
<td>+</td>
</tr>
<tr>
<td>Sham</td>
<td>-</td>
</tr>
<tr>
<td>Sham</td>
<td>-</td>
</tr>
<tr>
<td>Vaccine A</td>
<td>-</td>
</tr>
<tr>
<td>Vaccine A</td>
<td>+</td>
</tr>
<tr>
<td>Vaccine A</td>
<td>-</td>
</tr>
<tr>
<td>Vaccine A</td>
<td>+</td>
</tr>
<tr>
<td>Vaccine A</td>
<td>-</td>
</tr>
<tr>
<td>Vaccine A</td>
<td>-</td>
</tr>
<tr>
<td>Bacterin</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total # positive</strong></td>
<td>11/26</td>
</tr>
</tbody>
</table>

* - = no growth; + = low growth (< 50 CFUs); ++ = moderate growth (50 – 1000 CFUs); +++ = high growth (> 1000 CFUs); † = no sample available.
Figure 1. Summary of serum antibody levels by treatment group (mean ± SEM).

Antibody level represents the mean optical density (OD) as determined by ELISA on serum samples obtained from guinea pigs at necropsy 48 hours after IP inoculation with C. jejuni IA3902. **Values vary significantly from sham-vaccinated controls ($P < 0.01$). ***Values vary significantly from sham-vaccinated controls ($P < 0.001$).
Figure 2. **Summary of vaccine efficacy by treatment group.** Percent free of infection represents the number of animals with negative *Campylobacter* culture results in all tissues sampled/total number of animals sampled. Samples were obtained from guinea pigs at necropsy 48 hours after IP inoculation with *C. jejuni* IA3902. *Values vary significantly from sham-vaccinated controls (P < 0.05).** Values vary significantly from sham-vaccinated controls (P < 0.01).
CHAPTER 5: SPATIAL DISTRIBUTION OF PUTATIVE GROWTH FACTORS IN THE GUINEA PIG PLACENTA AND THE EFFECTS OF THESE FACTORS, PLASMA, AND BILE ON THE GROWTH AND CHEMOTAXIS OF CAMPYLOBACTER JEJUNI

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Abstract

In the United States, a single clone of *Campylobacter jejuni* (named clone SA for Sheep Abortion) has recently been identified as a predominant cause of ovine abortion across multiple states, spanning multiple lambing seasons. We have previously described the pregnant guinea pig as a model for studying this clone, and have demonstrated that this organism has a marked predilection for the subplacenta while sparing the placental disc in this species. In the study described here, we compare the effects of subplacental and placental factors as well as bile and plasma from pregnant and non-pregnant guinea pigs on the growth and chemotaxis of *C. jejuni* IA3902, a field isolate of clone SA,
versus a reference strain (NCTC 11168). Both strains grew better in subplacental tissue extracts at 24 hours; however, only IA3902 maintained this enhancement at 48 hours. Histochemistry and lectin histochemistry were used to localize mucin, iron, and L-fucose within the placental unit. These individual factors along with progesterone and estradiol were evaluated for effects on growth and chemotaxis of *C. jejuni*. Mucin, iron and L-fucose were growth promoting while L-fucose was also chemotactic for both strains. Progesterone, estradiol and pregnant guinea pig plasma did not affect growth or chemotaxis, and no difference was observed when bile from pregnant and non-pregnant animals was compared. These findings demonstrate specific factors within the guinea pig placental unit that drive chemotaxis and enhance growth of *C. jejuni*, shedding light on potential mechanisms underlying the fetoplacental tropism observed with this strain.

**Introduction**

Campylobacteriosis is a significant cause of ovine abortion worldwide with abortion rates ranging from 5-50% in affected flocks.\(^1\) In a 2001 survey by the United States Department of Agriculture, campylobacteriosis ranked first among infectious causes of abortion in domestic sheep.\(^2\) Recent studies have revealed that *Campylobacter jejuni* has become the predominant species associated with ovine abortions in the United States,\(^3,4\) and further analysis using pulsed-field gel electrophoresis has demonstrated that within these *C. jejuni* isolates a single tetracycline-resistant clone (named clone SA for Sheep Abortion) has emerged as the predominant cause of ovine abortion in Iowa, South Dakota, Idaho, California, Oregon, and Nevada.\(^4\) The identification of this single clone
recovered from abortions in different regions and during different lambing seasons is significant in that *Campylobacter* spp. traditionally exhibit marked genetic and antigenic heterogeneity.\(^3,5-7\)

The chemotactic behavior of *C. jejuni* has been described in response to specific chemical elements, with strong chemoattraction observed towards mucin, L-fucose, and bile.\(^8\) Additionally, it has been shown that not only is *C. jejuni* drawn toward specific environmental stimuli, but that these stimuli also support its growth and therefore this response is a form of energy taxis driving the organism toward optimal conditions for growth.\(^9\) To the authors’ knowledge, the effects of the sex hormones progesterone and estradiol on the growth and chemotaxis of *C. jejuni* has not been described; however, significant *in vitro* growth enhancement in the presence of both chemicals has been reported with *Campylobacter rectus*,\(^10\) and no effect was noted in the presence of progesterone with *Campylobacter fetus* subsp. *venerealis*.\(^11\)

We have previously demonstrated the high virulence of clone SA using pregnant guinea pigs as a model for studying abortion following oral inoculation with *C. jejuni* IA3902, a field isolate of clone SA, which induced abortion at a rate of 60%; whereas oral inoculation with *C. jejuni* NCTC 11168 or a sheep fecal isolate did not induce abortion in any inoculated animals.\(^12\) In this previous study,\(^12\) *C. jejuni* IA3902 was recovered from the blood, bile, feces, uterus and placenta of aborting animals, while NCTC 11168 and the sheep fecal isolate were only recovered from the feces. This finding strongly indicates that IA3902 is highly invasive and causes systemic infection in pregnant animals. Using immunohistochemistry, we have localized *C. jejuni* organisms
primarily within subplacental trophoblasts, the lateral aspects of the subplacental/decidual junctional zone, and surrounding maternal arteries in the decidua while the main placenta was spared, suggesting that *C. jejuni* IA3902 is differentially localized in the placental unit. Despite these advances in understanding of the pathogenesis of clone SA, the factors influencing its pathogenicity and differential localization in infected tissues are still unknown. The specific aims of this current study were fourfold: 1) to compare the growth and chemotaxis of IA3902 versus NCTC 11168 in tissue extracts from the guinea pig subplacenta and placenta, 2) to characterize the spatial distribution of putative growth promoting factors in various regions of the guinea pig placental unit, 3) to evaluate the effects of these potential factors on the growth and chemotaxis of IA3902 and NCTC 11168 in vitro, and 4) to compare the effects of pregnant versus non-pregnant guinea pig plasma and bile on the growth and chemotaxis of both strains. These studies were undertaken in an effort to shed light on potential mechanisms that may underlay the fetoplacental tropism and spatial localization of *C. jejuni* IA3902 during septic abortion in guinea pigs.

**Materials and Methods**

**Humane animal care and use**—All procedures described herein were approved by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University, Ames, IA  50011.

**Animals**—20 female Hartley guinea pigs were obtained from a commercial source (Elm Hill Labs, Chelmsford, MA). Upon arrival, 10 of the animals were pregnant
at approximately 35 days of gestation and 10 were non-pregnant. Pregnancy was confirmed by abdominal palpation. Guinea pigs were group housed, provided wood chip bedding, fed a commercial pelleted guinea pig diet ad libitum, and allowed to acclimate for 5 days.

**Euthanasia and necropsy sampling**—All animals were euthanized 5 days after arrival via IP injection of sodium pentobarbital (approximately 150 mg/kg) and necropsied immediately. At necropsy, pregnant animals were placed in dorsal recumbency, the abdominal hair was clipped and the skin was aseptically prepared with povidone scrub and alcohol. A sterile surgical drape was placed over the prepared abdominal skin and sterile surgical instruments were used to incise the abdomen and exteriorize the gravid uterus. The entire uterus was removed intact and carefully transferred to a laminar flow hood before further processing. Once under the hood, the uterus was incised with a sterile surgical blade and each placental disc along with its associated subplacenta was carefully removed as a unit. Each placental unit was then cut in half longitudinally and one part was placed in 10% neutral buffered formalin while the other half was prepared for use in the in vitro growth and taxis assays. In preparation for these assays, careful aseptic technique was used to separate the subplacenta from the placental disc using sterile curved surgical scissors. The separated subplacenta and placental disc were placed into separate sterile specimen bags (Whirl-pak, Nasco, USA) and then pooled for pairs of guinea pigs to yield a total of 5 samples for analysis. For both pregnant and non-pregnant animals, heart blood and bile were collected using sterile needles and syringes, blood samples were placed in anticoagulant tubes, and plasma was
separated for analysis. Due to small sample volumes, plasma samples were also pooled for each pair of guinea pigs (5 samples total) and bile samples were pooled for groups of two or three guinea pigs (4 samples total). Placental samples taken for histopathology remained in neutral buffered formalin for 24 hours and were then embedded in paraffin and processed routinely for histochemical staining.

**Histology and histochemistry**—Serial sections from each placenta were cut to 5 µm and stained with Perl’s iron stain, Alcian blue pH 2.5, and the periodic acid-Schiff reaction with and without diastase pretreatment. Sections were evaluated to determine the presence or absence of material with staining characteristics consistent with iron, acid mucin, and neutral mucin within the different aspects of the placental unit (placental disc, visceral yolk sac placenta, parietal yolk sac placenta, subplacenta, and decidual junctional zone). Each area of each placenta was then quantified subjectively based on the amount of positive staining noted in a random 40X field [negative (-) if no staining was observed, low (+) if < 10% of the field stained positively, moderate (++) if 10 – 25% staining was observed, and abundant (+++) if > 25% of the field stained positive]. Staining was also evaluated for distribution within each tissue type and classified as intracellular or extracellular and multifocal or diffuse.

**Lectin histochemistry**—Serial sections from each placenta were cut to 3 µm, placed on aminoalkylsilane coated glass slides and placed in a 56°C oven for 2 hours. Sections were routinely deparaffinized in xylene and rehydrated in graded alcohol and water baths. To inhibit endogenous peroxidase, sections were immersed (2 immersions; 10 min/immersion) in 3% hydrogen peroxide in water. For antigen unmasking, sections
were treated with Tris-EDTA (pH 9.0) in a stream bath for 20 minutes, allowed to cool to room temperature, and then rinsed three times in PBS prior to placement in an automated cell staining system (BioGenex, USA). Lectins employed consisted of commercially available biotinylated *Ulex europaeus* agglutinin I (UEA-I [Vector, USA]) and biotinylated *Lotus tetragonolobus* lectin (LTA [Vector, USA]) applied to sections at 20 µg/ml and incubated at 22°C for 30 minutes followed by rinsing in a bath of PBS solution for 5 minutes. Lectin binding was visualized using a commercial kit (Vectastain Elite ABC, Vector, USA) and chromogen (NovaRED, Vector, USA) per manufacturer instructions and the sections were then counterstained with hematoxylin and mounted routinely. Specificity of lectin binding was confirmed by preabsorbing lectins in 250 mM of L-fucose prior to application to serial sections. Complete elimination of binding was observed after preabsorption with UEA-I and complete to markedly reduced binding was observed with LTA. Negative controls were prepared from serial sections in which the lectin was omitted and the dilution buffer was applied in its place. Sections were evaluated to determine the presence or absence of binding within the different aspects of the placental unit (placental disc, visceral yolk sac placenta, parietal yolk sac placenta, subplacenta, and decidual junctional zone). Lectin binding in each area of each placenta was then quantified subjectively based on the distribution and intensity of positive staining in a random 40X field [negative (-) if no staining was observed, low (+) if mild scattered cytoplasmic staining was observed, moderate (++) if mild to moderate cytoplasmic and multifocal apical membrane staining was observed, and abundant (+++) if...
if moderate to marked cytoplasmic and strong diffuse apical membrane staining was observed.

**Growth assays**—Subplacental and placental tissues were placed in PBS (10% w/v), crushed mechanically via hand, centrifuged at 1000g for 5 minutes to remove large tissue fragments, and the resultant supernatants were collected for use in the growth assays. Plasma samples were heat-treated at 56°C for 30 minutes prior to use to inactivate complement and bile samples were used directly. For all biological samples, 100 µl from an overnight culture of *C. jejuni* (grown on MH agar under microaerophilic conditions at 42°C) that had been adjusted to an *OD*$_{600}$ of 0.1 (approximately 1 x $10^7$ CFU/ml) in PBS, was inoculated into separate culture tubes containing 2 ml of the biological sample to be tested. At 24 and 48 hours, 100 µl of each inoculated sample was then plated onto MH agar plates at different dilutions to determine the number of colony-forming units (CFUs) for each sample.

Commercially available mucin (hog gastric), L-fucose, progesterone, estradiol (Sigma, USA) and iron were obtained for use as substrates in the growth assays. Progesterone and estradiol were dissolved in DMSO:PBS (1:4), while other chemicals were dissolved in PBS. Mucin was autoclaved at 121°C for 15 minutes prior to use and all other chemicals were filter sterilized using a 0.45 µm filter. Each substrate was added to minimum essential medium (MEM α [cat. #41061-029], Invitrogen, USA) to obtain the following solutions separately: mucin (0.1%); iron (40 µM); L-fucose (0.05 M) + iron (40 µM); progesterone (50 ng/ml) + iron (40 µM); and estradiol (50 ng/ml) + iron (40 µM). For all prepared solutions, 10 ml of each was inoculated with 100 µl of *OD*$_{600}$
of 0.1 C. jejuni cells in MEM Alpha (resulting in an initial OD$_{600}$ of approximately 0.001) and incubated under microaerophilic conditions at 42°C. At 24, 48, and 72 hours, tubes containing each inoculum were shaken at 130 rpm and the OD$_{600}$ value was determined using a spectrometer.

**Chemotaxis assays**—A fresh overnight culture of each *Campylobacter jejuni* strain (IA3902 and NCTC 11168) on Mueller-Hinton (MH) agar plates was harvested in MH broth, pelleted by centrifugation at 5000g for 5 minutes, and then resuspended in PBS with the bacterial concentration adjusted spectrophotometrically to approximately 4 x 10$^9$ CFU/ml with temperate PBS soft agar (0.4%) as described by others.$^8,9$ 12 ml of bacterial suspension was poured into a 90 mm sterile Petri dish and 6 mm sterile filter discs (Whatman) saturated with approximately 50 µl of test solution were placed on the solidified bacterial suspension. Solutions tested included subplacental and placental extracts (10% and 50% solutions as prepared in the growth assays), undiluted plasma, undiluted heat inactivated plasma, undiluted non-pregnant bile, pregnant bile (25%, 50%, and 100%), iron (0.01 M in double distilled water), mucin (0.1% and 1.0% solutions autoclaved at 70°C for 30 minutes), L-fucose (0.1 M in double distilled water), progesterone (50 ng/ml in 1:4 DMSO:PBS), and estradiol (50 ng/ml in 1:4 DMSO:PBS). After 4 hours of incubation at 42°C under microaerophilic conditions, chemotactic activity was examined over a light source. Bacterial accumulation around filter discs or zones of clearing were interpreted as zones of attraction or repulsion, respectively. Deoxycholic acid (0.1 M) was used as a control for chemical repulsion based on results
from a previous study.\textsuperscript{9} For mucin, chemotaxis was also measured using the hard-agar plug assay as described by others.\textsuperscript{8}

**Statistical analysis**—A commercial statistical software package (JMP 8.0.2, SAS Institute Inc., USA) was used to perform all analyses. Independent samples t-tests were utilized to detect differences in growth rates by strain in the tissue homogenate assays, and by pregnancy status in the bile assays. One-way ANOVA was used to detect differences in growth rates between multiple substrates with $P$ values reported after Tukey adjustment for multiple comparisons. Fisher’s exact test for binomial variables was employed when comparing the presence or absence of positive *Campylobacter* culture by pregnancy status in the plasma assays.

**Results**

**Histology and histochemistry**—In sections stained with the periodic acid-Schiff reaction following diastase pretreatment, abundant PAS positive material, compatible with neutral mucin, was present within subplacental lacunae (Figure 1a) while the associated lamellae did not stain. Moderate to abundant PAS positive, diastase resistant extracellular material was also identified throughout the junctional zone with frequent marked perivascular accumulation. A low level of diffuse apical cytoplasmic staining was noted in the visceral yolk sac endoderm while the parietal yolk sac and placental disc did not stain. These results are summarized in Table 1.

Staining with Alcian blue pH 2.5 revealed moderate amounts of globular extracellular material within subplacental lacunae (Figure 1b) consistent with acid mucin.
Similar material was present in moderate amounts within the junctional zone, most often along the lateral aspect and frequently surrounding the maternal arteries. There was a low level of positive staining in the apical cytoplasm of cells lining vascular channels in the placental labyrinth and along the apical membrane of the parietal yolk sac endoderm. Cells of the visceral yolk sac endoderm were characterized by diffuse, strongly positive apical cytoplasmic and membrane staining. These results are summarized in Table 1.

Perl’s staining revealed low to moderate amounts of scattered intracellular and extracellular iron in the placental disc along the junction of the interlobium and the labyrinth (Figure 2) and surrounding large maternal blood lacunae. Lesser amounts of scattered intracellular and extracellular iron were noted along the basal and less often lateral margins of the junctional zone. No iron was noted in the subplacenta or either layers of the yolk sac in the sections evaluated. These results are summarized in Table 1.

**Lectin histochemistry**—For the two lectins tested (UEA-I and LTA), significant binding was only observed in the visceral yolk sac endoderm. In sections treated with UEA-I, marked diffuse apical membrane binding was present in the endodermal cells in all sections (Figure 3a) with lesser amounts of granular cytoplasmic staining noted occasionally. The underlying mesenchymal cells did not stain. Treatment with LTA revealed moderate amounts of similar apical membrane binding in multifocal, individualized endodermal cells and moderate amounts of granular cytoplasmic staining (Figure 3b). For both lectins, the membrane binding was completely inhibited following preincubation of the lectin with L-fucose solution. Neither lectin reacted with vascular
endothelium in any of the sections evaluated, consistent with previous studies in guinea pigs and other rodents. The lectin binding results are summarized in Table 1.

Growth assays—None of the biological or chemical preparations yielded microbial growth when plated on MH agar incubated at 37°C aerobically, indicating that the aseptic techniques employed during sample preparation had prevented microbial contamination that would otherwise have confounded interpretation of the results. In the majority of the pooled placental tissues tested, higher CFU counts were noted for both strains of *C. jejuni* (IA3902 and NCTC 11168) in the subplacental tissues versus placental disc material at 24 hours. Interestingly, at 48 hours, this enhanced growth in subplacental tissues was maintained only for IA3902, whereas NCTC 11168 began to grow better in the placental disc material and at this time point the growth multiples for each strain differed significantly (*P* = 0.0397). The mean growth multiples for both strains at both time points are shown in Figure 4.

At 24 hours, the incidence of bacterial survival was higher in non-pregnant plasma versus pregnant plasma for both strains; however, statistical significance was not attained in the analysis by strain due to low sample numbers associated with sample pooling. CFU counts were generally low overall, suggesting an antimicrobial effect of the plasma. Analyzing the incidence of bacterial survival at 24 hours without subdividing by strain revealed a significantly increased incidence in non-pregnant versus pregnant plasma (*P* = 0.0076), and these results are summarized in Figure 5. At 48 hours, the incidence of bacterial survival did not differ by pregnancy status for either strain as organisms were not recovered from the majority of samples at this time point.
Due to the small volume of the bile samples, only IA3902 was tested in all samples and CFU counts did not differ significantly by pregnancy status at either 24 or 48 hours ($P = 0.5350$ and $P = 0.3844$, respectively). Mean CFU counts per ml for pregnant versus non-pregnant bile at 24 hours were $7.8 \times 10^3$ and $5.5 \times 10^3$, respectively. Three samples of bile were available for testing NCTC 11168, two pregnant and one non-pregnant, and similar CFU counts were observed as with IA3902; however, statistical analysis was not performed given the low number of samples.

Growth of \textit{C. jejuni} in the presence of mucin, iron and L-fucose was compared to that in MEM control. At 24 hours, growth of IA3902 in the presence of mucin was significantly greater than that in MEM control, L-fucose, and iron ($P < 0.0001$, $P = 0.0026$ and $P = 0.0074$, respectively); whereas growth of NCTC 11168 in mucin was only significantly greater than that of the control ($P = 0.0051$). Growth of IA3902 in the presence of iron at 24 hours was also significantly greater than that in controls ($P = 0.0270$), while growth of NCTC 11168 was not significantly different ($P = 0.4350$). At 48 hours, growth in the presence of L-fucose was significantly greater than that in all other substrates for both IA3902 and NCTC 11168 ($P < 0.0001$, all comparisons). Additionally, growth in the presence of both iron and mucin was significantly greater than that in controls for both \textit{C. jejuni} strains at this time point ($P < 0.0001$, all comparisons). At 72 hours, growth in the presence of all three substrates was again significantly greater than that in MEM control for both \textit{C. jejuni} strains ($P < 0.0001$, all comparisons), and growth of NCTC 11168 in the presence of L-fucose was greater than that in iron or mucin ($P < 0.0001$, $P = 0.0003$, respectively). The mean growth rate in the
presence of each substrate and at each time point is summarized for both IA3902 and NCTC 11168 in Figure 6a and Figure 6b, respectively.

The growth of *C. jejuni* in the presence of estradiol + iron, progesterone + iron, and iron alone was compared to that in MEM + DMSO control. At 24 hours, growth in the presence of all three substrates was significantly greater than that in MEM + DMSO control for both IA3902 (*P* < 0.001, all comparisons) and NCTC 11168 (*P* < 0.02, all comparisons); however, there was no difference between growth in iron alone and iron with the addition of either hormone for either of the *C. jejuni* strains (*P* > 0.85, all comparisons). At 48 and 72 hours, the results for both *C. jejuni* strains were similar to the 24 hour time point with growth in all three test substrates significantly higher than that in MEM + DMSO control (*P* < 0.001, all comparisons), and no significant difference observed between any of the iron or hormone combinations for either IA3902 (*P* > 0.13, all comparisons) or NCTC 11168 (*P* > 0.80, all comparisons). The mean growth rates in the presence of each substrate and at each time point are summarized for both IA3902 and NCTC 11168 in Figure 7a and Figure 7b, respectively.

**Chemotaxis assays**—The results of the filter disc chemotaxis assays are summarized in Table 2. No significant differences were noted between strains in response to any of the tested biological or commercial chemical samples. The only tested chemical with positive chemoattraction was L-fucose (Fig 8a); while bile, regardless of pregnancy status, was strongly chemorepellent (Fig 8b). For both strains, the diameters of the chemorepulsion zones were concentration dependent where larger diameters correlated to more concentrated bile. For the 25%, 50% and 100% bile solutions from
pregnant animals, the respective zones of repulsion measured 11mm, 14mm and 17mm for strain IA3902, and 11mm, 13mm, and 15.5mm for strain NCTC 11168. No effect was observed with mucin in the chemical-in-plug assay for either strain.

**Discussion**

The pathogenesis of ovine campylobacteriosis is complex and includes intestinal invasion, evasion of the host immune response, hematogenous dissemination, and fetoplacental colonization, which ultimately culminates in abortion of the fetus with recovery of Campylobacter in large numbers from expelled placentas. Affected flocks often experience waves of abortions involving 23.2% of the flock on average. The epidemiology of this disease is further complicated by the high prevalence rate of C. jejuni in domestic flocks, high numbers of healthy sheep with carriage in the bile, and the marked heterogeneity of strains encountered. Recently, a single clone of C. jejuni has been recognized as the predominant cause of Campylobacter-associated abortions in the United States, and we have previously demonstrated that the pregnant guinea pig is an effective model for studying this highly abortifacient clone. To the authors’ knowledge, factors that may drive the fetoplacental tropism of C. jejuni have not been previously described in any species.

The guinea pig placenta consists of a discoid, labyrinthine main placenta with a separate yolk sac placenta and subplacenta. The subplacenta, a feature unique to histrichognath rodents, is composed of extensive lamellae of cytotrophoblasts lined by a layer of syncytiotrophoblast with large extensively vacuolated lacunae.
subplacenta forms the frontal barrier at the maternofetal interface and penetrates the decidua forming the junctional zone. The subplacenta is separated from the main placenta by a layer of fetal mesenchyme, the lateral aspects of which are penetrated by the maternal arteries as they enter the placental disc. We have previously shown by immunohistochemistry that *C. jejuni* localizes within this junctional zone and within subplacental trophoblasts following oral infection, a seemingly appropriate location as this is the site of maternal vascular invasion. In mid to late gestation, the placenta becomes a major source of progesterone production in guinea pigs, with hormone synthesis occurring in the spongy zone syncytiotrophoblast of the placental disc. The yolk sac placenta is inverted in the guinea pig, with its visceral endoderm facing the uterine lumen and in direct contact with the endometrium. The functions of this inverted yolk sac include receptor-mediated endocytosis, passage of maternal antibodies, and absorption, degradation and transfer of maternal serum proteins.

In the study reported here, we compared the effects of whole tissue extracts from two separate regions of the guinea pig placenta, as well as various subcomponents within each region, on the growth and chemotaxis of two strains of *C. jejuni*, IA3902 and NCTC 11168. These two strains were previously demonstrated to have markedly different abortive capacity following oral inoculation. Both strains responded similarly to all *in vitro* tests with the exception of the 48 hour growth assay in whole tissue extracts. While both strains grew to higher numbers in the subplacental extracts at 24 hours, only IA3902 maintained this preferential growth at 48 hours suggesting a potential difference between the two strains in their ability to grow within various regions of the guinea pig placenta.
This is significant in that our previous immunohistochemical studies have shown that IA3902 colonizes the subplacenta and junctional zone while the main placenta is spared.

Previously published studies on the growth and chemotaxis of *Campylobacter* spp. provided potential target compounds to evaluate within the placenta, including mucin, L-fucose, iron, estrogen and progesterone.\(^8,10,23,24\) In an attempt to further explain the fetoplacental tropism we have observed with IA3902, we performed histochemical techniques to localize these potential growth promoting substances within the guinea pig placenta. Experimental studies have revealed that both hog gastric and bovine gall bladder mucin support the growth of *C. jejuni*.\(^8\) Accordingly, we have identified moderate to abundant amounts of both acid and neutral mucin within subplacental lacunae and the junctional zone, consistent with our previous immunohistochemical localization of *C. jejuni* in tissues from aborting guinea pigs,\(^12\) while the main placenta contained only scant amounts of acid mucin. Previous studies have demonstrated that *C. jejuni* has the capacity to utilize iron from host sources,\(^23\) and acquisition of iron appears important in the pathogenesis of *Campylobacter* infection as reduced growth of *C. jejuni* has been demonstrated in low-iron environments.\(^24\) In the study reported here, low to moderate amounts of scattered intracellular and extracellular iron were observed in both the junctional zone and the placental disc.

The results of the 24 hour growth assays revealed that mucin significantly enhanced growth of both strains of *C. jejuni* versus controls and that for IA3902 this growth was also significantly greater than that in the presence of iron or L-fucose. Additionally, growth in the presence of iron at 24 hours was significantly greater than
that of controls for IA3902. Enhanced growth in the presence of iron and mucin versus MEM control was evident for both strains at the 48 and 72 hour time points. The demonstration of strongly growth promoting substances in the same location where we have previously identified *C. jejuni* by immunohistochemistry suggests energy taxis as an underlying mechanism for this localization rather than simple dissemination subsequent to bacteremia, and this is consistent with previous studies whereby *C. jejuni* has been shown to undergo energy taxis toward conditions most favorable for growth.\(^9\) L-fucose was also strongly growth promoting, particularly at the 48 hour time point for both strains tested. This finding is intriguing as *C. jejuni* has been shown to utilize L-fucose as a growth substrate in a strain-specific manner, and both IA3902 and NCTC 11168 harbor the operon responsible for this utilization.\(^25\) Our lectin binding assays revealed L-fucose compatible residues only in the visceral yolk sac placenta, a site where we have not yet identified *C. jejuni* immunohistochemically; however, the yolk sac placenta was not specifically evaluated in our previous work and initial organism localization in this region of the placenta cannot be definitively ruled out.

Bacterial survival in plasma and bile from both pregnant and non-pregnant guinea pigs was compared to determine if a pregnancy related factor present in these fluids, such as the hormones progesterone and estradiol, might enhance the growth of either strain of *C. jejuni*. For both strains, the incidence of bacterial survival was higher in non-pregnant versus pregnant plasma at 24 hours, and neither strain showed enhanced growth in the presence of either progesterone or estradiol in the individual factor assays suggesting that these hormones do not stimulate the growth of either strain. This is in contrast to a
previous report of enhanced growth in the presence of both hormones with *C. rectus*,\textsuperscript{10} and in agreement with previous findings of no growth enhancement in the presence of progesterone with *C. fetus* subsp. *venerealis*.\textsuperscript{11} Both strains survived well in the presence of bile regardless of pregnancy status, again suggesting that soluble factors of pregnancy in the guinea pig and their metabolites do not directly enhance the growth of either *C. jejuni* IA3902 or NCTC 11168. These findings are of particular note with regard to progesterone, as pregnant guinea pigs have markedly elevated midgestational plasma progesterone levels, with a roughly fifty-sixfold increase versus non-pregnant controls.\textsuperscript{26}

*C. jejuni* responds to its environment, undergoing chemotaxis toward a variety of specific chemical substrates\textsuperscript{8} and ultimately toward areas that will enhance its growth.\textsuperscript{9} We were particularly interested in the potential chemotactic effects of progesterone as both guinea pigs and sheep have increasing placental synthesis of progesterone as pregnancy advances\textsuperscript{20,27} which might serve to explain the late gestational incidence of ovine campylobacteriosis and the marked tropism of this organism for the placenta; however, we did not observe chemotaxis of either strain toward progesterone, pregnant plasma, or extracts from the placental disc, suggesting that neither strain responds directly to the presence of progesterone. We did observe marked chemotaxis of both strains toward L-fucose, consistent with a previous study involving two different strains of *C. jejuni*.\textsuperscript{8} This finding is significant in that our lectin binding assays detected the presence of both UEA-I and LTA reactive residues along the apical membrane of the visceral yolk sac placenta, consistent with α-L-fucose containing surface glycans.\textsuperscript{28} The apical surface of the visceral yolk sac epithelium is in direct contact with the
endometrium as gestation progresses\textsuperscript{17} and, as the yolk sac is thought to serve in maternofetal exchange,\textsuperscript{21} it may serve as another potential entry point for \textit{C. jejuni} into the guinea pig placenta during episodes of bacteremia. At the very least, the presence of abundant L-fucose residues within the fetoplacental unit suggests that chemotaxis towards this particular chemical may play a role in the fetoplacental tropism in this species.

Previous chemotaxis studies involving \textit{C. jejuni} have revealed chemoattraction towards several types of mucin\textsuperscript{8,9,29} which we could not confirm in the present study using swine gastric mucin in either the saturated filter disc or hard-agar plug procedure. While chemotaxis of \textit{C. jejuni} toward hog gastric mucin has been demonstrated previously using a hard-agar plug procedure,\textsuperscript{8} the strains tested in that particular study did not include either IA3902 or NCTC 11168, raising the possibility that strain-related differences in chemotaxis may occur. Additionally, two recent studies have raised concern about the potential for false positive results in chemical-in-plug chemotaxis assays, as apparent chemotaxis has been observed in motility deficient mutant strains of \textit{C. jejuni}\textsuperscript{30} and other bacteria.\textsuperscript{31}

Gall bladder colonization and fecal shedding may be important in the maintenance of \textit{Campylobacter} spp. in an animal population. In previous studies in our laboratory, IA3902 has been isolated from the bile of both pregnant and non-pregnant guinea pigs, and \textit{C. jejuni} has been recovered from the bile of healthy sheep at slaughter.\textsuperscript{16,32,33} Interestingly, in the present study, bile, regardless of pregnancy status, was strongly chemorepellent for both IA3902 and NCTC 11168, and the diameter of the
zones of chemorepulsion was concentration dependent where larger diameters correlated with more concentrated bile. This is in contrast to a previously published study where diluted bovine and chicken bile were described as chemoattractive for *C. jejuni* strains of chicken fecal origin; however, in the same study, the residual bile components after removal of the mucin fraction were strongly chemorepellent for all tested strains. Taken together these findings would suggest that the observed chemorepulsive effect of bile in the present study is due either to differences in the composition (e.g. mucin) of guinea pig bile relative to that of bovine or chicken origin, or potentially due to strain-specific responses of *C. jejuni*.

In summary, we have demonstrated the presence of specific elements within the guinea pig placenta that enhance growth (iron, mucin, L-fucose) and drive chemotaxis (L-fucose) of *C. jejuni*. Furthermore, we have identified two of these elements (iron, mucin) within the subplacenta and the decidua interface, consistent with our previous immunohistochemical localization of *C. jejuni* in these same areas in aborted tissues following inoculation with *C. jejuni* IA3902. These findings suggest a potential underlying mechanism for the marked placental tropism we have observed with this strain; however, as we observed similar *in vitro* effects with both a known abortifacient strain (IA3902) and a reference strain (NCTC 11168) that did not induce abortion following oral inoculation, differences in response to particular placental or pregnancy-associated factors alone do not appear to be responsible for the differing abortifacient potential of these two strains. Further work is required to better elucidate mechanisms underlying the marked difference in abortive capacity observed between *Campylobacter*
strains following oral inoculation including intestinal invasive capacity, virulence gene expression, level of bacteremia, and evasion of the host immune response. While we have utilized a guinea pig model to identify potential host factors that may underlie the extensive fetoplacental tropism observed in vivo, additional studies are required to determine if similar factors are present in the late gestational ovine placenta as this is the target species and the time during which the preponderance of Campylobacter-associated abortion occurs.

Acknowledgments

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References


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### Table 1 – Summary of histochemistry and lectin histochemistry findings

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Subjective quantification of staining*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neutral mucin</td>
</tr>
<tr>
<td>Placental disc</td>
<td>-</td>
</tr>
<tr>
<td>Visceral yolk sac placenta</td>
<td>++</td>
</tr>
<tr>
<td>Parietal yolk sac placenta</td>
<td>+</td>
</tr>
<tr>
<td>Subplacental lacunae</td>
<td>+++</td>
</tr>
<tr>
<td>Junctional zone</td>
<td>+++</td>
</tr>
</tbody>
</table>

Values reported represent the average of all evaluated placental materials from 10 guinea pigs tested.

* - = negative; + = low; ++ = moderate; +++ = abundant.
‡ = multifocal staining; † = apical membrane staining.
Table 2 – Summary of chemotactic responses of *C. jejuni* by strain

<table>
<thead>
<tr>
<th>Test sample</th>
<th>Taxis response*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subplacenta (10%, 50%)</td>
<td>0,0</td>
</tr>
<tr>
<td>Placental disc (10%, 50%)</td>
<td>0,0</td>
</tr>
<tr>
<td>Mucin, porcine stomach (0.1%, 1%)</td>
<td>0,0</td>
</tr>
<tr>
<td>L-Fucose (0.1M)</td>
<td>+</td>
</tr>
<tr>
<td>Iron ([Fe_2(SO_4)_3]) (0.01M)</td>
<td>0</td>
</tr>
<tr>
<td>Plasma (pregnant, heat inactivated)</td>
<td>0</td>
</tr>
<tr>
<td>Plasma (pregnant, untreated)</td>
<td>0</td>
</tr>
<tr>
<td>Progesterone (50ng/ml)</td>
<td>0</td>
</tr>
<tr>
<td>Estradiol (50ng/ml)</td>
<td>0</td>
</tr>
<tr>
<td>Bile, pregnant (25%, 50%, 100%)</td>
<td>-</td>
</tr>
<tr>
<td>Bile, non-pregnant (100%)</td>
<td>-</td>
</tr>
<tr>
<td>Deoxycholic acid (0.1M)</td>
<td>-</td>
</tr>
</tbody>
</table>

* + = attraction; 0 = no response; - = repulsion
Figure 1. Subplacenta; guinea pig; histochemistry for mucoprotein. (a) Subplacental lacunae (*) and junctional zone (^) containing abundant PAS+ material. Periodic acid-Schiff reaction. (b) Subplacental lacunae (*) containing moderate amounts of acid mucin and moderate to abundant amounts within the junctional zone (^) and surrounding maternal blood vessels (arrow). Alcian blue, pH 2.5.
Figure 2. Placenta; guinea pig; histochemistry for iron. Moderate amounts of iron (blue staining) are present within the placental disc along the junction of the interlobium and the labyrinth. The parietal yolk sac placenta is at left. Perl’s iron stain.

Figure 3. Visceral yolk sac placenta; guinea pig; lectin histochemistry for L-fucose. (a) Peroxidase staining of Ulex europaeus agglutinin I (UEA-I) binding sites. Endodermal cells have abundant diffuse apical membrane labeling with scant cytoplasmic reactivity while the underlying mesenchyme is non-reactive. Harris’ hematoxylin counterstain. (b) Peroxidase staining of Lotus tetragonolobus lectin (LTA) binding sites. Multifocal and often individualized endodermal cells (arrowheads) have abundant diffuse apical membrane labeling while the majority of endodermal cells have scant to moderate cytoplasmic reactivity and the underlying mesenchyme is non-reactive. Harris’ hematoxylin counterstain.
Figure 4. Summary of mean growth multiples in placental tissues at 24 and 48 hours. Values represent the mean growth multiple (CFUs in subplacental extracts / CFUs in placental disc extracts) for *C. jejuni* strains IA3902 and NCTC 11168. At 48 hours, values differed significantly (* = P = 0.0397) indicating greater growth of strain IA3902 versus NCTC 11168 in subplacental extracts at this time point.
Figure 5. Summary of the incidence of bacterial survival at 24 hours in pregnant versus non-pregnant plasma. Shaded areas of the graphs represent the proportion of samples from which bacteria were recovered (incidence of bacterial survival) for both *C. jejuni* IA3902 and NCTC 11168 combined. The incidence of survival in non-pregnant plasma was significantly greater (*P* = 0.0076).
Figure 6. Summary of mean growth rates (OD$_{600}$) of *C. jejuni* in the presence of individual placental factors at 24, 48 and 72 hours. (a) Growth of IA3902 was significantly higher in the presence of mucin than all other factors at 24 hours, while growth in iron was also greater than MEM control. At 48 hours, growth of IA3902 was significantly higher in the presence of L-fucose than all other factors and growth in iron and mucin was also greater than that in MEM control. At 72 hours, growth of IA3902 was significantly higher in all three test substrates relative to MEM control. (b) Growth of NCTC 11168 was significantly higher in the presence of mucin than that in MEM control at 24 hours. At 48 hours, growth of NCTC 11168 was significantly higher in the presence of L-fucose than all other factors and growth in iron and mucin was also greater than that in MEM control. At 72 hours, growth of NCTC 11168 was significantly higher in all three test substrates relative to MEM control, and growth in the presence of L-fucose was also greater than that in mucin or iron.
Figure 7. Summary of mean growth rates (OD\textsubscript{600}) of \emph{C. jejuni} in the presence of individual plasma factors at 24, 48 and 72 hours. (a) At all time points, growth of IA3902 in the presence of all three iron containing treatments was significantly greater than that in MEM + DMSO control; however, no differences were observed with the addition of either progesterone or estradiol. (b) At all time points, growth of NCTC 11168 in the presence of all three iron containing treatments was significantly greater than that in MEM + DMSO control; however, no differences were observed with the addition of either progesterone or estradiol.
Figure 8. Chemotaxis assays with *C. jejuni* suspended in PBS soft agar. Filter discs saturated with test chemicals were placed onto solidified bacterial suspension and migrations zones measured after 4 hours of incubation at 42°C. (a) Strain NCTC 11168 showing chemoattraction toward a filter disc saturated with 0.1M L-fucose solution. (b) Strain IA3902 showing strong chemorepulsion away from a filter disc saturated with undiluted pregnant guinea pig bile.
CHAPTER 6: GENERAL CONCLUSIONS

General Discussion

Ovine campylobacteriosis is frequently listed as one of the top three causes of sheep abortion worldwide,\textsuperscript{1-4} and has recently been identified as the leading cause of infectious abortion in the United States.\textsuperscript{5} Additionally, within campylobacters recovered from field cases of sheep abortion, a single clone of \textit{Campylobacter jejuni}, named clone SA, has emerged accounting for 93\% of the isolates from multiple states and spanning multiple lambing seasons.\textsuperscript{6} The central hypothesis for the studies described in this dissertation was that clone SA of \textit{C. jejuni} is highly pathogenic with resistance to traditional prevention strategies and marked tropism for the fetoplacental unit. The work in this dissertation supports our central hypothesis in four ways as data generated from these studies: 1) confirm the highly pathogenic nature of \textit{C. jejuni} clone SA in a pregnant guinea pig model, 2) describe alterations in expression of TLRs in trophoblasts from guinea pigs infected with IA3902, a field isolate of clone SA, 3) reveal variability in the efficacy of commercially available \textit{Campylobacter} vaccines in preventing infection of guinea pigs with IA3902, and 4) characterize the spatial distribution of specific factors within the guinea pig placenta that promote growth and drive chemotaxis of \textit{C. jejuni} IA3902.

Oral inoculation of pregnant guinea pigs is an effective model to study the pathogenesis of abortion due to \textit{C. jejuni} IA3902
Although intraperitoneal inoculation of pregnant guinea pigs has been described as a model for assessing virulence of *Campylobacter* spp., this type of inoculation does not fully replicate disease caused by abortifacient campylobacters where oral exposure is the natural route. A previous report describing oral inoculation of human-origin *Campylobacter* spp. into pregnant guinea pigs failed to induce abortion; however, we hypothesized that highly abortifacient campylobacters of ovine-origin, such as *C. jejuni* clone SA, would be effective in inducing abortion after oral inoculation in guinea pigs. In Chapter 2, we demonstrated that oral inoculation of *C. jejuni* IA3902, a field isolate of clone SA, into pregnant guinea pigs was indeed effective in reproducing disease similar to that observed in sheep with the induction of abortion in 60% (6/10) of inoculated animals and a 70% (7/10) fetoplacental infection rate. Additionally, we demonstrated that oral inoculation of pregnant guinea pigs with a reference strain (*C. jejuni* NCTC 11168) and a commensal strain from sheep feces resulted in a high level of intestinal colonization at 21 days postinoculation but did not induce abortion or result in fetoplacental infection in any of the inoculated animals (0/20). Not only did oral inoculation with IA3902 induce abortion, but our microscopic and immunohistochemical evaluations revealed suppurative placentitis, maternal vasculitis, and numerous organisms within subplacental trophoblasts and phagocytic cells, consistent with the microscopic appearance following experimental infection with *C. jejuni* in sheep. These results suggest that oral inoculation in pregnant guinea pigs will be a valuable model system for future investigations of pathogenesis, assessing virulence of field isolates, and development of therapeutic compounds and vaccines.
In the guinea pig, infection of subplacental trophoblasts with \textit{C. jejuni} IA3902 is associated with increased expression of TLR2

The innate immune system plays a key role in defense against pathogens at the maternofetal interface and the function of PRRs at this interface has been the subject of much recent research.\textsuperscript{12} TLRs are the most widely studied of the PRRs, and there have been several recent reports documenting alterations in their expression at the protein level in both pathologic and nonpathologic placentas;\textsuperscript{13-16} however, data regarding TLR responses to specific placental pathogens are limited. In Chapter 3, we sought to determine if alterations in the expression of TLR2 or TLR4 would occur in subplacental trophoblasts from guinea pigs orally inoculated with \textit{C. jejuni} IA3902. In guinea pigs, the subplacental syncytiotrophoblast forms the frontal barrier between mother and fetus, and, as such, would be the first layer of the placenta exposed to potential pathogens arising from the endometrium, and, as maternal blood vessels enter the placental disc at the periphery of its junction with the subplacenta,\textsuperscript{17} the syncytiotrophoblast in this location would be the first exposed during episodes of bacteremia. Using a combination of routine microscopy and immunohistochemistry on serial sections, we demonstrated significantly increased expression of TLR2 protein in syncytiotrophoblasts from placentas with suppurative placentitis and concurrent infection with \textit{C. jejuni}; whereas immunoreactivity for TLR2 in syncytiotrophoblasts of inflamed but uninfected placentas and TLR4 expression in all infected or inflamed placentas was not significantly different versus sham-inoculated controls. These results suggest that the presence of \textit{C. jejuni} organisms was associated with the observed increase in TLR2 expression, and provide
support for future research to determine the exact role of TLR2 expression in the pathogenesis of *Campylobacter*-associated septic abortion.

**Commercially available, ovine-labeled *Campylobacter* vaccines are variably efficacious in preventing infection with *C. jejuni* IA3902 in guinea pigs**

While vaccination for the prevention of ovine campylobacteriosis was first described over 50 years ago, its usefulness is somewhat hindered by the heterogeneity of campylobacters associated with epizootics of abortion and the lack of cross protection between various serotypes, species and strains. Accordingly, we hypothesized that the emergence of a single clone of *C. jejuni* isolated from the preponderance of sheep abortions in the United States may be due to selective pressure imparted by resistance of this clone to traditional prevention strategies including vaccination.

In Chapter 4, using a guinea pig model, we demonstrated that one of two commercially available, ovine-labeled multivalent *Campylobacter* vaccines was entirely ineffective in preventing systemic infection in intraperitoneally inoculated guinea pigs 48 hours post inoculation, while a second commercially available vaccine was highly efficacious and comparable to a homologous bacterin in resolving infection in inoculated animals. These results suggest that at least one commercially available vaccine may be ineffective in preventing disease due to IA3902 in sheep, and that a homologous bacterin may be an appropriate consideration in flocks experiencing disease. Additionally, we demonstrated, for the first time, that resolution of infection in both pregnant and non-pregnant guinea pigs 48 hours post inoculation was an effective tool for screening
vaccine efficacy. As the use of non-pregnant animals provides a significant cost savings in early efficacy studies where large numbers of animals are required, these results provide the framework for an ideal model system that will be of significant benefit for future Campylobacter vaccine studies.

The guinea pig placental unit contains components that drive chemotaxis and support growth of C. jejuni IA3902

Previous studies have revealed that mucin and iron support the growth of C. jejuni\(^ {27,28}\) and that the organism is driven by metabolism-dependent energy taxis toward optimal chemical conditions for growth.\(^ {29}\) While C. jejuni has been traditionally deemed nonsaccharolytic, recent research has revealed that certain strains have the capacity to metabolize L-fucose.\(^ {30}\) In Chapter 2, our immunohistochemical analysis of placentas from guinea pigs orally inoculated with IA3902 revealed numerous C. jejuni organisms within subplacental trophoblasts and phagocytic cells at the periphery of the junctional zone. Given this consistent localization, we hypothesized that, in guinea pigs, the subplacenta and junctional zone contain factors which promote growth and drive taxis of C. jejuni IA3902 and that these factors are less prevalent or absent in other areas of the placenta.

In Chapter 5, we demonstrated that two strains of C. jejuni, IA3902 and NCTC 11168, grew more efficiently in subplacental tissue extracts than main placental extracts at 24 hours, and that only IA3902 maintained this enhanced growth in subplacental extracts at 48 hours. We then used routine histochemistry and lectin histochemistry to characterize the spatial localization of acid and neutral mucins, iron, and L-fucose within
the guinea pig placental unit. These analyses revealed that the subplacenta and junctional zone contained acid mucin, neutral mucin, and iron; whereas the main placenta contained only iron and scant amounts of acid mucin. L-fucose containing surface glycans were only observed in the visceral yolk sac placenta. Using select individual chemical factors in vitro we revealed that both strains of C. jejuni exhibited enhanced growth in the presence of mucin and iron at 24, 48, and 72 hours, and in the presence of L-fucose at 48 and 72 hours. Our in vitro chemotaxis studies revealed that both strains experience strong chemoattraction to L-fucose and chemorepulsion to bile regardless of pregnancy status. In summary, these results support our hypothesis that the subplacenta and junctional zone contain factors that support the growth of C. jejuni, and that these factors are more prevalent in this location than in the main placenta. Additionally, there is an abundance of a strongly chemoattractive and growth promoting substance in the visceral yolk sac placenta which may also play a role in the fetoplacental tropism we have observed with this organism. The lack of an observable difference in behavior in the majority of our in vitro assays between a highly abortifacient strain (IA3902) and a strain previously demonstrated not to induce abortion following oral inoculation in pregnant guinea pigs (NCTC 11168; Chapter 2) suggests that energy taxis alone is not responsible for the observed differences in vivo, and should help focus future studies on other aspects of pathogenesis such as intestinal epithelial invasion, microbial gene expression, and evasion of the host immune response.
Recommendations for Future Research

These studies have revealed that oral inoculation of pregnant guinea pigs is an effective model for studying the pathogenesis of Campylobacter-associated septic abortion as it closely replicates the disease observed in sheep. We have identified three strains of C. jejuni that exhibit markedly different pathogenicity in this model system and provide a framework for future pathogenesis studies. There appear to be specific factors within the guinea pig placental unit that provide adequate resources for Campylobacter growth, and, while this helps to explain the effectiveness of C. jejuni as a placental pathogen in this species, it is not sufficient to explain our observed differences in overt disease caused by the various Campylobacter strains. We observed intestinal colonization with all three species, thus it seems likely that the observed differences in pathogenicity lie early in the disease process and future studies aimed at these early steps seem warranted, such as differences in intestinal epithelial invasive capacity and the ability of different strains to evade the host immune response. As Campylobacter spp., including C. jejuni, are often present in the gall bladder and intestines of apparently healthy sheep, differences in microbial gene expression may also underlay differences in pathogenic behavior and may be an important area of future study.

Our studies also revealed that, similar to observations in sheep, pathogenic campylobacters readily colonize the guinea pig gall bladder and are recoverable from the bile. Gall bladder colonization and intermittent shedding may be a key event in the epidemiology of disease within infected flocks, and studies determining the spatial localization of organisms within the gall bladder epithelium and mucus layer as well as
those involving microbial gene expression in the presence of bile from both pregnant and non-pregnant animals would be of value in elucidating the behavior of these organisms within the bile and the role of this colonization in the overall pathogenesis of ovine campylobacteriosis.

While we observed increased expression of TLR2 protein in subplacental syncytiotrophoblasts from guinea pigs infected with IA3902, the exact role of this expression in the overall pathogenesis of Campylobacter-associated septic abortion remains to be determined. Further study involving the quantification of inflammatory cytokine responses, such as IL-6, IL-8 and TNF-α, in fetal fluids and placental tissues from guinea pigs infected with IA3902, as well as quantification of apoptosis in placental trophoblasts, may give additional information regarding the potential role of TLRs in this disease. Additionally, comparing these effects to those occurring in response to the administration of exogenous TLR2 and TLR4 agonists, TLR2 and TLR4 antagonists, and infection with IA3902 in TLR knock out animals would all be of value in further delineating the potential role of these receptors in the pathogenesis of adverse pregnancy outcomes secondary to sepsis with C. jejuni.

References


APPENDIX A: PERMISSIONS

Monday 7 February 2011

Eric R. Burrough, DVM, Dipl. ACVP
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Dear Dr. Burrough:

Permission is granted to include in your dissertation, the content of your paper previously published in the American Journal of Veterinary Research (AJVR). We grant permission to you to include the paper in the print and electronic versions.

Pathogenicity of an emergent, ovine abortifacient Campylobacter jejuni clone orally inoculated into pregnant guinea pigs

Eric R. Burrough, Orhan Sahin, Paul J. Plummer, Qijing Zhang, Michael J. Yaeger
American Journal of Veterinary Research (AJVR), 2009;70:1269-1276.

Thank you for your commitment to veterinary medicine.

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