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Investigation of a mouse and a sheep model to study pathogenic mechanisms of Campylobacter jejuni fetoplacental infection

Victoria Dale Lashley

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Investigation of a mouse and a sheep model to study pathogenic mechanisms of *Campylobacter jejuni* fetoplacental infection

by

Victoria Dale Lashley

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

Major: Veterinary Pathology

Program of Study Committee:
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Iowa State University

Ames, Iowa

2016

Copyright © Victoria Dale Lashley, 2016. All rights reserved.
This dissertation is dedicated to:

My parents, Ruthven and Barbara
My children, Ivana and Yadavendra
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CHAPTER 1: GENERAL INTRODUCTION

Statement of the Problem

*Campylobacter* species colonize an extensive range of hosts including wildlife, pet and farm animals, are a frequent cause of gastrointestinal disease and are a zoonotic risk for humans.\(^{53}\) Evidence suggests that over the past decade there has been an increasing incidence of campylobacteriosis globally.\(^{46}\) A fast expanding, hypervirulent clone of *Campylobacter jejuni* (*C. jejuni*) has emerged, with the ability to cross the intestinal mucosa, enter the systemic circulation and cause abortion.\(^{89}\) This clone, *C. jejuni* clone SA (SA for Sheep Abortion), is now the primary agent of ovine abortion in the US, producing significant losses in sheep enterprises and has been documented to cause sporadic cases and outbreaks of gastroenteritis in humans.\(^{70,90}\)

Despite the increasing incidence of *C. jejuni* infection, the significant medical and economic consequences that can occur and the fact that the genome sequence of the organism is available, the cellular and molecular pathogenic mechanisms of disease are poorly understood.\(^{73}\) This is largely because there is a lack of suitable animal models that reliably and consistently replicate the pathophysiological and immunological responses in the gastrointestinal and reproductive systems of the infected host. The absence of appropriate laboratory models capable of consistently replicating the natural disease remains a significant impediment to the study of the reproductive pathology and abortion inducing capabilities of this new clone. A thorough and complete understanding of how and where *C. jejuni* localizes within the fetoplacental unit and knowledge of the precise
mechanism by which it produces fetoplacental compromise and abortion is currently unknown.

Specific aims

The ultimate aim of this research was to advance knowledge of the mechanisms of C. jejuni clone SA abortion. The hypotheses were that 1) Laboratory mice could be used as an animal model for C. jejuni-induced reproductive pathology studies (Chapter 2); 2) Cytokines have an integral role in the abortion process and there is increased cytokine production from the murine fetoplacental tissue post infection with C. jejuni IA3902 (Chapter 3); 3) Oral, intravenous (IV) and intraperitoneal (IP) inoculation of pregnant sheep with C. jejuni will result in abortion (Chapter 4); 4) Apoptosis is a pathogenic mechanism of C. jejuni clone SA-induced abortion and indicators of apoptosis can be detected in the infected fetoplacental unit (Chapter 5) and 5) Toll-like receptor 4 (TLR4) and transcription of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) expression would be increased following ovine placental infection with C. jejuni clone SA (Chapter 5).

To test these hypotheses four studies were conducted. In the first study, we set out to examine whether pregnant mice represent a suitable model in which to investigate the reproductive consequences of C. jejuni infection. Two strains of mice were challenged with C. jejuni clone SA via two routes (oral and IP) and gross and histopathological effects were determined. In the second study, the expression of six cytokines from C. jejuni infected murine placenta was measured to determine the role that cytokines may play in C. jejuni-induced reproductive disease. In a third study, which involved a pilot
and a second larger study, the pathology of *C. jejuni* clone SA (administered via the oral, IV and IP routes) on pregnant sheep was investigated, in an attempt to establish a reliable pregnant sheep model for *C. jejuni* clone SA abortion. In a final study, ovine placenta confirmed infected via microbial culture and immunohistochemistry (IHC) for the major outer membrane protein (MOMP), was assessed for the presence of apoptosis via a commercial assay and caspase 3 production as well as NFκB and TLR4 via IHC. Studies of apoptosis and presence of caspase 3 on infected ovine placenta were conducted to determine if these processes and enzymes played a role in abortion caused by the pathogen. As a component of the final study and to improve the understanding of the pathophysiology of *C. jejuni* clone SA we established the spatial localization of the organism in infected ovine placenta along with the presence of previously described trophic factors including mucin, iron and L-fucose.

**Dissertation organization**

The dissertation is composed of six chapters and organized in the alternative journal paper format. Chapter 1 is the introduction chapter. The subsequent four chapters (Chapter 2-5) are four individual manuscripts with the paper’s title as the main chapter heading. Dr. Victoria Lashley, the PhD candidate, was the primary author of each manuscript. Chapter 6 is the final chapter with conclusions and considerations for future studies. References are located at the end of each chapter.
Literature review

**C. jejuni: Epidemiology and significance**

The genus *Campylobacter* is currently composed of 26 species, 2 provisional species and 9 sub-species.\(^{46}\) *Campylobacter* species are Gram-negative spiral, rod or curved bacteria without a flagellum, with a single polar flagellum or bipolar flagella depending on the species and are non-spore forming, approximately 0.2 to 0.8 by 0.5 to 5 microns.\(^{53,84}\) *C. jejuni* is composed of two subspecies that differ significantly in their ubiquity and ecology, *C. jejuni* subsp. *doylei* for which there are no known animal hosts and *C. jejuni* subsp. *jejuni* which is normally referred to as simply *C. jejuni*.\(^{48}\)

*C. jejuni* is the most frequent cause of gastroenteritis in people in the developed world and despite measures to reduce intestinal colonization in poultry flocks and subsequent human infection, the incidence of infection has remained high.\(^{78}\) The US Centers for Disease Control and Prevention estimates that over 1.3 million people in the United States are infected every year with *C. jejuni*, resulting in 76 deaths annually.\(^{73}\) Gastroenteritis caused by *Campylobacter* spp. in humans can arise from handling raw chicken meat or eating inadequately cooked chicken, having contact with pets and drinking or swimming in contaminated water.\(^{33}\) Interestingly, the organism can asymptomatically inhabit the gastrointestinal system of a large number of animal hosts, from production and companion animals to reptiles, wild birds and mammals and all these animals can serve as reservoirs of infection for humans.\(^{28}\) Even healthy dogs, particularly dogs under 2 of age years, shed *Campylobacter* spp. in their feces with *C. jejuni* being the second most frequent isolate detected.\(^{37}\)
Commercial agricultural livestock operations provide unique ecological niches for a wide variety of *Campylobacter* genotypes.\(^8^5\) Production and livestock management systems appear to influence the selection of certain genotypes of *C. jejuni*. Research has demonstrated that the widespread use of the antibiotic tetracycline for the control of abortions in sheep has facilitated the selection of the highly pathogenic, tetracycline-resistant, SA clone, at least in the US.\(^9^1\) Ovine gallbladder and intestinal colonization likely contribute to the occurrence of carrier ewes in the epidemiology of the disease on sheep production systems.\(^1^3,^1^6\)

Multilocus sequence typing (MLST) and whole genome sequencing (WGS) are techniques that have enabled a deeper understanding of *C. jejuni* epidemiology and genetic evolution in influencing phenotypic variation of *C. jejuni* populations.\(^2^6,^4^5\) Colonization of multiple host species in divergent ecological niches can result in rapid adaptation of the genome of *C. jejuni* primarily by frame-shift mutations that alter gene expression or by horizontal gene transfer (HGT) which allows *C. jejuni* to acquire new genes from other bacteria.\(^7^4\) Research is discovering the pathogen in previously undocumented environments and species. Anthropic transfer of *C. jejuni* to sentinel marine wildlife species, namely, grey seal pups with histologic evidence of disease underscores the inherently complicated environmental, microbial and host relationships that exist with this pathogen.\(^9\)

The fact that the same organism can cause disease in different systems in different hosts definitely adds more complexity to the mix. The tetracycline resistant *C. jejuni* capable of causing abortion in sheep is genetically identical to isolates from human cases of gastroenteritis.\(^8^6\) People with gastroenteritis confirmed to be caused by this isolate had
a history of contact with cattle and farm environments. Further, while wild birds have been proven to contribute to a seasonal pattern of human campylobacteriosis, associations with wildlife may be more difficult to investigate in abortion epizootics on sheep livestock operations.

The organism has developed mechanisms to survive in the environment including the ability to enter a viable but non culturable (VBNC) state where it transforms from a motile spiral form to a coccoid form when subject to unfavorable conditions of low nutrient availability or suboptimal temperatures. Another mechanism is the formation of a biofilm, which in this instance is a composite of *Campylobacter* micro colonies bound together by an extracellular matrix in an attempt to form a less hostile microenvironment. *C. jejuni* has been isolated from aquatic biofilms with survival times of up to 42 days.

Recently, *C. jejuni* was categorized as a major antimicrobial resistant threat due to the increase in fluoroquinolone resistance and resistance to macrolide antibiotics which were previously the main drugs of choice for treatment. Antibiotic resistance, which is a global health challenge, has been caused by antibiotic overuse in the human population and in animal production systems for treatment, growth promoting effects and other off-label uses. *Campylobacter sp.* display a number of antibiotic resistance mechanisms including alteration of the DNA gyrase target for fluoroquinolones; reduced membrane permeability due to MOMP and drug efflux via CmeABC for macrolides and acquisition of the tetracycline resistant gene (*tetO*) for resistance against tetracyclines. *Campylobacter* acquire these resistance determinants by mutation and horizontal gene transfer (HGT) mediated by transduction, transformation and conjugation.
In order to cause enteric disease, the organism must enter the gastrointestinal tract, survive the harsh acidic conditions of the stomach, colonize and multiply in the distal ileum and colon by using various motility, adhesion, cell invasion and cytotoxin production capabilities to overcome host defenses and cause diarrhea.\(^{24}\) It was reported that \textit{C. jejuni}'s outer membrane proteins caused avian lymphoblast apoptosis suggesting that this was a pathogenic mechanism used by the organism.\(^{93}\) The ability of \textit{C. jejuni} to activate apoptotic pathways in other cells and the role of this process in the pathogenesis of gastrointestinal and reproductive disease is unknown. Additionally, other possible mechanisms of reproductive tract infection and abortion induction by \textit{C. jejuni} have not been fully elucidated and further research is urgently needed in this area.

\textbf{C. jejuni as a reproductive pathogen and cause of abortion}

The normal flora of the vaginal canal in domestic animals does not include \textit{C. jejuni}.\(^{40,51}\) The organism can be harbored and shed intermittently in the feces of a wide range of wild and domestic animals and birds.\(^{66}\) \textit{C. jejuni} is a frequent isolate in infectious abortion cases in ruminants, mainly in sheep causing ‘epizootic abortion’ and to some degree in cattle and goats.\(^{69}\) In sheep, \textit{Campylobacter} generally causes late-term abortions with minimal clinical signs in the ewe prior to abortion.\(^{35}\) Infection is acquired via the ingestion of feed sources contaminated by feces or the products of abortion.\(^{30,72,76}\) The ovine gastrointestinal tract is then colonized usually without diarrhea but with bacteremia in susceptible animals and subsequent infection of the uterus and placenta.\(^{12}\) Since the organism is highly contagious, most pregnant ewes in the flock get infected
through ewes that abort during the current outbreak.\textsuperscript{56} Infected ewes that abort generally acquire immunity as abortions are generally not observed with subsequent infection.\textsuperscript{59}

\textit{C. jejuni} abortions have been reported in 19\% of 120 and 10\% of 108 beef cattle herds in Canada.\textsuperscript{83} In that outbreak, cows were reported to have had placental retention and weight loss. \textit{C. jejuni} was cultured in large numbers from infected bovine placenta and fetal tissues and this was accompanied by histologic changes of necrotizing, suppurative placentitis and fetal bronchopneumonia.\textsuperscript{83, 88} \textit{C. jejuni} is also a documented cause of abortion in goats. There was diarrhea before or concurrent with abortions in 5/21 goats from which \textit{C. jejuni} was cultured from stomach content and various tissues from the aborted fetuses.\textsuperscript{7}

\textit{C. jejuni} caused an outbreak of abortion (18\%) on a Canadian mink ranch with 29\% of the breeding females also failing to conceive.\textsuperscript{39} In that outbreak, the organism was cultured from the liver of aborted fetuses, maternal feces and tissues including placenta, uterus and spleen. Abortion has been reported in experimentally infected ferrets which could not be prevented by acquired immunity from previous infection.\textsuperscript{10}

\textit{C. jejuni} was isolated from canine placenta, fetal lung and liver and was reported as the cause of death in two adult female Bulldogs after fetoplacental infection with abortion and neonatal death.\textsuperscript{69} The organism was also isolated in from the profuse, odorless hemorrhagic, vaginal fluid from three German Shepherd bitches after late-term abortions.\textsuperscript{62} The pathogenesis of abortion in pregnant dogs has not been determined but may have a similar mechanism as in sheep as the organism can be carried
asymptomatically in the intestine of canines and is not an inhabitant of the vaginal flora of these species. 17,62

In humans, *C. jejuni* infections during pregnancy have resulted in abortion, stillbirth, prematurity and neonatal sepsis.75 There has been some question as to whether *C. jejuni* as a placental pathogen in humans arises from ascending infection due to the presence of chorioamnionitis25 or to septicemia as in other species.57,1 For the most part, the reproductive pathology in humans and sheep are similar with foci of neutrophilic infiltration, peri-villous fibrin deposition with placental necrosis and eventual fetal death.76

Pregnant guinea pigs abort after experimental infection with *C. fetus, C. jejuni* and *C. coli* and the pregnant guinea pig model was identified as a suitable and practical model for *Campylobacter* pathogenicity studies, regardless of the host of origin of the isolates.77 *C. jejuni* IA3902 is a representative isolate of clone SA and was determined to be a unique, highly abortifacient strain with the ability to colonize the intestines, cause systemic infection and induce abortion in pregnant guinea pigs due to its affinity for the fetoplacental unit.18 This highly pathogenic, tetracycline resistant clone has become the predominant cause of sheep abortions in the United States.91 The emergent strain is a zoonotic agent and retains its ability to cause gastroenteritis in humans in addition to producing significant economic losses for sheep producers.90 MOMP is the most abundant membrane protein in *C. jejuni* and functions as both a porin and an adhesin; is encoded by the *porA* gene, which has recently been determined to undergo specific amino acid substitutions in loop4 and it is this feature which is responsible for the systemic infection, hypervirulence and abortion inducing characteristics of *C. jejuni* clone SA.89
Animal models for the study of reproductive *Campylobacter sp* infection

Enteritis caused by *C. jejuni* has been reported in young macaques, weaning-age ferrets, dogs, cats, pigs and chickens, rodents, and rabbits have been experimentally inoculated by various routes with *C. jejuni*. The animal model that most closely resembles gastrointestinal infection in humans is the oral infection model of nonhuman primates. Intestinal colonization has been established in a number of laboratory animals challenged with *C. jejuni* including infant mice, puppies and piglets and in these species, infection is usually chronic and almost always asymptomatic with intermittent excretion of the organism.

Despite the high prevalence of infection with *C. jejuni*, its significant medical and economic consequences, and knowledge of its genome sequence, the molecular and cellular mechanisms involved in the pathogenesis of this disease are not well understood due to a paucity of robust experimental animal models that mimic natural infection. Replicating clinical symptoms of Campylobacteriosis in animal models have proved difficult when the animal model is different from the human host, when these models become cost prohibitive or when results are inconsistent. Acute enterocolitis is the typical outcome after infection with *Campylobacter* spp. in most mammalian species while meningitis, endocarditis and arthritis occasionally occur. In ruminants, reproductive pathology, specifically infertility and abortion, are the primary concerns.

Cellular alterations from trophoblastic invasion, bacterial products and products of the host inflammatory response during infection of the fetoplacental unit with *C. jejuni* clone SA likely all contribute to the reproductive pathology and abortion that
results. In the guinea pig placenta, a single layer of trophoblasts separates the fetal from the maternal circulation. As a result, infection of trophoblasts by C. jejuni from maternal vessels may be the path through which the organism can gain access across the fetoplacental barrier. Similarly, there is only one layer of trophoblasts between the maternal blood and fetal vessels in the human placenta (labyrinthine hemomonochorial). On the contrary, the murine placenta is hemotrichorial with three layers of trophoblasts between the maternal blood and fetal vessels.

By the proposed natural oral route of infection, C. jejuni would have had to cross the intestinal barrier as well as the fetoplacental barrier to cause eventual fetal infection. While some intestinal pathogens disrupt tight junctions and others actively cross enterocytes to cause gastrointestinal infection, the exact mechanism by which pathogens cross the placental barrier and infect the fetus are often elusive.

In pregnant guinea pigs, C. jejuni IA3902 spares the main placenta and is present within subplacental trophoblasts at the circumference of the junctional zone close to the site of placental vascular invasion. The pregnant guinea pig model is used in vaccination efficacy studies and to screen for virulence of C. coli, C. fetus and C. jejuni. Additionally, abortion in guinea pigs is used as an indicator of Campylobacter pathogenicity. Currently, no other model has been able to replace the pregnant guinea pig for reproductive studies with C. jejuni due to the ease of reproducibility of abortion, ease of handling and housing of the animal, ability to use various routes of administration along with the short gestation of the guinea pig.
The ovine placenta is chorioallantoic, adeciduate, cotyledonary, villous and with an interhemal barrier described as synepitheliochorial. The susceptibility or resistance of the barrier structure of the ovine placenta to bacterial infection is unknown. Gross lesions reported in sheep experimentally infected with \textit{C. jejuni} include abortion, stillbirth, uterine prolapse and retained placenta with histological lesions of necrosuppurative placentitis and/or endometritis.\textsuperscript{35,71} Published reports of experimental studies in sheep are limited and a fully reproducible ovine model has not been comprehensively described. The associated housing costs, high frequency of natural gallbladder and gastrointestinal colonization, challenges associated with securing specific-pathogen-free or naïve pregnant ewes and the relatively long gestation in these animals are some of the major obstacles hindering research advancement in the target species.

\textbf{\textit{C. jejuni, cytokines, apoptosis, TLR4 and NFκB}}

Cytokines are small proteins that regulate cells by binding to receptors present on target cells.\textsuperscript{82} Receptor binding triggers intracellular signaling, altered gene expression, and the production of other cytokines, surface receptors or feedback inhibition.\textsuperscript{43} Cytokines function locally and systemically and are classified as chemokines, lymphokines, or interleukins based on target action, cell of secretion or function.\textsuperscript{64} They regulate physiologic and pathologic processes involved in innate and acquired immunity, inflammatory responses and hematopoiesis and are produced by many types of cells.\textsuperscript{49} While cytokines are generally not stored intracellularly, their levels may be amplified, attenuated or they may initiate the secretion of other mediators, especially when there is
inflammation within the affected organ or tissue. Analysis of cytokine profiles can aid investigations into host-pathogen interactions, bacterial persistence and bacterial clearance from infected hosts.

Central to the ability of *C. jejuni* to cause gastrointestinal disease is that it induces the production of pro-inflammatory cytokines. Cells produce cytokines when *C. jejuni* binds to lipoprotein JlpA, a *C. jejuni* surface protein, which interacts with host cell heat shock protein, Hsp90, to activate NFκB via mitogen-activated protein (MAP) kinases, ERK and p38 intracellularly. Additionally, the dysregulation of pro-inflammatory and anti-inflammatory cytokines post infection with *C. jejuni* may cause increased susceptibility to enteritis.

Systemic infection of *C. jejuni* in BALB/c mice was associated with the initial production of pro-inflammatory cytokines, TNF, IL1 and IL2 and subsequent production of anti-inflammatory cytokines IL4 and IL10 in the spleen and large intestine. In that same study, it was found that once the systemic infection had been cleared, the cytokine response was predominantly anti-inflammatory and associated with the production of IL4 and IL10 in the liver and small intestine. Interestingly, even mucin expression in reproductive tract epithelia may vary in response to cytokine influences. TNFα, IL4 and IL13 can upregulate mucin expression via increased MUC2 transcription through NFκB activation. *C. jejuni* is chemotactic for and has enhanced growth in the presence of mucin. Therefore the cytokines produced as a result of *C. jejuni* infection are capable of enhancing the growth of the pathogen within the fetoplacental unit.
Trophoblasts produce cytokines and altered cytokine profiles are characteristic of numerous disorders of gestation. Murine abortion was induced after *Listeria monocytogenes* infection significantly upregulated trophoblast pro-inflammatory cytokine production. Sheep placenta infected with *C. jejuni* SA had increased expression of cytokine genes which were likely involved in inflammation and necrosis. The cytokines produced from a *C. jejuni* infected sheep placenta and the role of these cytokines in the pathophysiologic processes of the associated reproductive pathology and abortion are currently unknown.

TLRs are pattern recognition receptors that recognize specific, highly conserved pathogen-associated molecular patterns and play a key role in innate immune defense. TLRs in mammals function to detect specific components of bacterial, fungal and viral pathogens. TLR4 is essential for detecting lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria like *C. jejuni*. LPS can elicit potent immuno-stimulatory activity by excessive activation of monocytes and macrophages leading to endotoxic shock. TLR4 is present in placenta and is highly expressed in trophoblasts. Intrauterine infection has been linked to increased trophoblast apoptosis and it was demonstrated that TLR mediated apoptosis was partly due to the activation of caspase 3. Trophoblasts recognize and respond to pathogens through the expression of TLRs. Since *C. jejuni* infects and resides in trophoblasts, the TLR-mediated trophoblast response could significantly direct pregnancy outcome in a *C. jejuni* infected placenta.

The roles of altered pro-inflammatory and anti-inflammatory cytokines, the process of apoptosis, the activation of TLR4 and production of NFκB in *C. jejuni*
infection in the reproductive tract and specifically the infected fetoplacental unit require further investigation in order to determine the potential significance of these in the pathophysiology of abortion induced by the pathogen.

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CHAPTER 2: INVESTIGATING THE SUITABILITY OF A LABORATORY MOUSE MODEL TO STUDY THE PATHOGENESIS OF ABORTIFACIENT CAMPYLOBACTER JEUNI

Modified from a paper to be submitted to Veterinary Pathology

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Abstract

The aim of this study was to assess whether pregnant mice represent a useful model to study the reproductive pathology of Campylobacter jejuni (C. jejuni) IA3902 using the endpoint of positive microbial culture of the organism from the fetoplacental unit. Pregnant BALB/c and CD-1 mice (14 days gestation), were inoculated orally with 0.5 ml (1x10⁹ colony forming units CFU/ml) and intraperitoneally (IP) with 0.1 ml
(1x10^9 CFU/ml) of *C. jejuni* IA3902. The organism was recovered by microbial culture from the fetoplacental unit in 10 out of 10 (10/10) (100%) BALB/c and 10/10 (100%) CD-1, IP inoculated pregnant mice and in 2/7 (29%) BALB/c and 3/8 (38%) CD-1 orally inoculated pregnant mice. Gross reproductive pathology included necrohuppurative placentitis, fetal resorption, intrauterine fetal death, dead (stillborn) pups and maternal multifocal hepatitis. Histological fetoplacental changes consisted of locally extensive neutrophilic and necrotizing placentitis with intraleisonal bacterial colonies of *C. jejuni*; ulcerative endometritis and random multifocal hepatitis with rare cholecystitis in the dam. Immunohistochemistry for the major outer membrane protein (MOMP) of *C. jejuni* revealed moderate to large numbers of the organism at the periphery of the placental discs within trophoblasts and extracellularly with invasion into the placental disc largely via the vascular network. The organism is trophic for neutral mucin, iron and L-fucose within the murine placenta. *C. jejuni* IA3902 has affinity for the murine reproductive tract, specifically the fetoplacental unit, where it results in a necrotizing placentitis with positive microbial recovery after both IP and oral challenge in BALB/c and CD-1 pregnant mice.

**Keywords**

*Campylobacter jejuni*, histopathology, immunohistochemistry, laboratory mice, placentitis, reproductive pathology
Introduction

*C. jejuni* is a small (0.2-0.9 µm wide and 0.2-5.0 µm long), spiral, Gram-negative bacteria, principally known as a zoonotic pathogen. The organism is non-spore forming, but vigorously motile by means of a single polar flagellum at one or both ends, it’s most studied virulence determinant. *Campylobacter* related infections are leading causes of foodborne bacterial disease in humans and reproductive loss in sheep with abortion rates that range from 5% to 50% in infected flocks. *C. jejuni* colonizes the gastrointestinal tract of a wide range of animals that act as reservoirs of infection by fecal shedding. Insights into the molecular mechanisms of disease and immunopathogenesis have been severely limited due to the absence of convenient murine infection models.

Historically, murine models of *C. jejuni* infection have been limited by sporadic colonization and/or absence of clinical disease. *C. jejuni* resistance in mice is likely influenced by continuous active competition from resident intestinal microbiota that outcompete *C. jejuni* for survival and tolerance of the murine immune system to *C. jejuni*. A murine model for the study of *C. jejuni* abortion is needed to reduce research costs compared to the commonly used guinea pig. Additionally, the reproductive system of the laboratory mouse has been extensively studied. Mice have a hemochorial placenta similar to humans, a short gestation period, defined genetics and their immune system has been well characterized and is similar to that of humans. The mouse model has proven useful for the study of the pathogenesis of reproductive disease due to *Chlamydomphila abortus* and vaccine related investigations and a pregnant mouse model has been characterized for the study of bovine *Tritrichomonas fetus* infection. Murine models
have been established for human reproductive research as a mouse model for preeclampsia has been described.²

Several studies have demonstrated that abortion can be reliably produced in guinea pigs following either oral or intraperitoneal inoculation of C. jejuni.⁶,⁷ Despite routine recovery of C. jejuni from naturally aborted ovine fetuses, there has been limited success reproducing abortion in experimentally infected pregnant sheep, the species in which reproductive loss is of greatest concern. There have been no published reports of the reproductive pathology of C. jejuni in mice, which, because of their size, ease of handling and availability of various reagents, are the most commonly used laboratory research animal model. The mouse model has been used by some researchers to study other aspects of Campylobacteriosis and it has been demonstrated that bacteremia is a very common and consistent finding, which is an essential step in the pathogenesis of abortion.³⁰ Mice have also been used in several vaccination studies,¹⁷,²¹ including those against reproductive bacterial pathogens.¹²,¹⁴ The purpose of this study was to investigate whether mice represent a useful research model to assess the reproductive pathogenicity of C. jejuni.

**Materials and Methods**

Mice: 30 BALB/c mice (24 females and 6 males) and 30 CD-1 mice (24 females and 6 males), 6-8 weeks old were sourced from a commercial supplier and acclimated for 5 days. Mice were kept in isolator cages in groups of 4 in an infectious disease containment room with autoclaved wood chip bedding and fed a commercial pelleted
mouse diet and water ad libitum. Four female mice were group housed with a male of the same species for 14 days. On day 15, female mice were bled (0.1-0.2 ml blood collected from the saphenous vein using a 25G needle), inoculated with *C. jejuni* IA3902 and housed in individually identifiable cages for observation twice daily for one week. There were 4 control mice per strain. All mice were humanely euthanized one week post inoculation with 0.1-0.2 ml ketamine/xylazine solution (80/16 mg/kg) via intraperitoneal injection using a 25 G needle followed by exsanguination. At necropsy, cardiac blood was collected for serum testing. Uterus, placenta, fetal tissues and maternal liver were collected for histopathology and blood, uterine contents and cecal swabs were collected for microbiology.

*C. jejuni* IA3902 inoculum: 0.5ml (1x10^9 CFU/ml) was administered once, orally and 0.1ml (1x10^9 CFU/ml) was administered once IP.

Experimental design

The reproductive pathology of *C. jejuni* IA3902, a clinical isolate of clone SA, was assessed after either oral or intraperitoneal inoculation in two strains of mice. Sixty, 7-8 week old mice, 30 outbred CD-1 (24 female and 6 male) and 30 inbred BALB/c mice (24 female and 6 male) were purchased from a commercial source. They were acclimated for 5 days before use. Four female mice were group housed with a male of the same species for 14 days for breeding. On day 15, all study animals were bled and inoculated with abortifacient *C. jejuni* IA3902. 0.1-0.2 mls of blood was collected from the lateral saphenous vein using a 25 G needle. Mice were placed head first into a 50 ml centrifuge
tube with a 1 cm diameter whole in the conical end for restraint which allowed access to the animals rear legs, the hair was shaved from the lateral aspect of the leg using a small electric clipper, petroleum jelly was lightly rubbed onto the skin, the vein was gently pricked with the needle and blood was collected by capillary action into a hematocrit tube. After collection a sterile gauze pad was gently pressed over the puncture site to stop bleeding. Ten female mice of each strain were inoculated orally after a 12 hour long feed withdrawal. Mice were lightly anesthetized via gaseous anesthesia in an anesthetic chamber with 2 % isoflurane and then quickly removed, and inoculated orally with 0.5 mls of C. jejuni IA3902 at 10^9 CFU/ml, via gastric lavage using a stainless steel, ball-tipped 24 gauge (G) 1.0 inch animal feeding tube. Ten female mice of each strain were inoculated IP. Each mouse was briefly placed on its back with its head tilted slightly downward and 0.1 ml of C. jejuni IA3902 at 10^9 CFU/ml injected IP using a 25G needle. After inoculation female mice were immediately housed in individually identifiable cages for observation twice daily for one week. For each group, there were 4 control (non-inoculated) female mice per strain also housed individually.

Over the subsequent week, all mice were monitored twice daily for clinical signs of inactivity, unresponsiveness, ruffled hair coat, diarrhea, vaginal discharge and abortion (fetal loss). All mice were humanely euthanized one week post inoculation. For euthanasia, mice were rendered completely unconscious using 100-200 µl IP Ketamine/Xylazine (80/16 mg/kg) administered IP with the mouse briefly placed on its back with head tilted slightly downward. This produced deep anesthesia and was followed by exsanguination, after the mouse became unconscious, as determined by loss
of righting reflex (body movement) and/or unresponsiveness to noxious stimuli and in

At the time of necropsy, the following samples were collected: heart blood for
serum collection and bacterial culture, cecal swab, uterus and uterine contents (placenta
and fetus) for bacteriology and maternal liver with gallbladder, uterus and uterine
contents for histopathology. The study was approved by Institutional Animal Care and
Use (IACUC), protocol 5-15-8018-M and fully compliant the regulations of the
Laboratory Animal Resources (LAR) group of Iowa State University (ISU), at whose
facility the study was undertaken. Appropriate personal protective equipment was worn
when mice were inoculated, samples collected and necropsy performed both in the
animal treatment and in the necropsy rooms.

Necropsy, microbial culture and histopathology

All mice were subjected to a complete necropsy. At necropsy, all gross lesions
were noted and samples collected for microbial culture and histological examination.
Cecal swabs were obtained using sterile minitip cotton swabs with transport media
immediately after euthanasia. Uterus, placenta and fetal tissue were harvested in sterile
petri dishes for *Campylobacter* culture. All samples for microbial culture were
refrigerated immediately upon collection and plated on the day of collection. Rectal
swabs and blood were directly streaked onto culture media. Uterus and fetoplacental
tissue were minced, swabbed and streaked onto Mueller-Hinton (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). Plates were incubated for 48 hours in anaerobic jars under micro aerobic conditions at 42 °C. *Campylobacter* colonies were counted on each plate to determine the number of colony forming units (CFU) in each sample.

Uterus, fetoplacental unit, placenta, liver and gallbladder were examined histologically. Tissues were fixed in 10% neutral buffered formalin for 24 hours. Preserved tissues were paraffin embedded and 5-7 micron sections were mounted on slides and routinely stained with hematoxylin and eosin.

**Neutrophil scoring of reproductive lesions**

The mean number of neutrophils present in five fields (40X magnification) was assessed for each of four locations in the reproductive tissue including, the periphery and parenchyma of the placental disc, the uterine wall and uterine lumen. Neutrophil counts were scored for each location using the following criteria (1= <10 neutrophils/hpf; 2=11-25 neutrophils/hpf; 3=26-50 neutrophils/hpf and 4= >51 neutrophils/hpf).

**Histochemistry**

**Lectin**

Three micron (3 µm) serial sections were set on aminoalkylsilane-coated glass slides, baked at 56 °C for 2 hours and 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% hydrogen peroxide (H$_2$O$_2$) in water. Antigen was unmasked by treating sections with Tris-EDTA (pH 9.0) in a stream bath for 20 minutes, cooled to room temperature, and rinsed 3 times in phosphate buffered saline (PBS) prior to placement in an automated cell staining system (BioGenex, US). The lectin used was the commercially available biotinylated *Lotus tetragonolobus* lectin agglutinin I (LTA; Vector, US) applied to sections at 20 mg/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) and chromogen (NovaRED, Vector) as per the manufacturer’s instructions; the sections were then counterstained with hematoxylin and mounted routinely. Specificity of lectin binding was confirmed by preabsorbing lectins in 250mM of L-fucose prior to application to serial sections. Negative controls were prepared from serial sections with the lectin omitted and replaced with dilution buffer. Lectin binding was subjectively quantified in the fetoplacental unit, uterus and yolk sac (visceral and parietal layers) as low/absent, moderate or abundant in random 40X magnification fields and classified as to whether staining was cytoplasmic or membranous.

**Special stains**

Perls’ iron stain, Alcian blue pH 2.5 and the Periodic acid-schiff (PAS) reaction without diastase pretreatment were performed on twelve sections randomly drawn from each of the groups along with one control mouse slide. Serial sections were cut to 5 µm, stained and assessed to determine the presence of absence of material with characteristics
consistent with iron, acid mucin and neutral mucin within the fetoplacental unit, yolk sac (visceral and parietal layers) and uterine wall. Staining was subjectively classified as low/absent, moderate or abundant, intracellular, extracellular, multifocal or diffuse.

Immunohistochemistry

Placental discs collected at necropsy were processed, embedded and sectioned at 3 microns, mounted on aminoalkylsilane-coated glass slides and baked for 2 hours at 56 °C. Slides were deparaffinized routinely in xylene and rehydrated in graded alcohol solutions and water. Inhibition of endogenous peroxidase was via 2 immersions for 10 mins each in 3% H$_2$O$_2$ in water. Slides were incubated for 15 minutes at 37 °C in 0.1% protease in Tris buffer of pH 7.6, then rinsed 3 times in PBS solution and stained automatically. Nonspecific binding was inhibited by incubation with 10% neutral goat serum at 22 °C for 20 minutes. The primary antibody was directed against the MOMP of C. jejuni, as described by Zhang et al$^{32}$ at a dilution of 1:300 and incubated at 22 °C for 60 minutes and then rinsed in PBS for 5 minutes. A commercially available biotinylated secondary antibody was used at a dilution of 1:80 and incubated at 22 °C for 15 minutes then rinsed in PBS for 5 minutes. Slides were then incubated with horse radish peroxidase-streptavidin conjugate at 22 °C for 15 minutes and rinsed in PBS for 5 minutes. The final reaction used a commercial chromogen. Slides were rinsed and routinely counterstained with Shandon Harris hematoxylin and Scott’s tap water. Slides were dehydrated through graded alcohol and xylene solutions prior to mounting. Positive controls were sourced from paraffin blocks of ovine placenta that was positive for
*C. jejuni* IA3902 by microbial culture. Negative controls were from un-inoculated mice in the study.

**Statistical analysis**

A commercial statistical software package, SAS 9.4 version was used to perform a nonparametric Kruskal-Wallis test to detect differences in mean neutrophil scores among inoculation routes (BALB/c Oral, BALB/c IP, CD-1 Oral, CD-1 IP and control) and for different locations (placenta periphery and parenchyma, uterine lumen and wall) in mice with *C. jejuni* isolated from placenta and uterus by microbial culture. Secondly, the same nonparametric Kruskal-Wallis test was performed to test if different placental culture levels (level 0=no CFU, level 1=<50 CFU, level 2=50-1000 CFU, level 3=TNTC) impacted neutrophil scores at the placental periphery and in the placental parenchyma, and if different uterine culture levels impacted neutrophil scores for the uterine lumen and uterine wall. Results were considered significant at values of p ≤ 0.05.

**Results**

There were 14 mice from which there was high microbial recovery (TNTC) of *C. jejuni* IA3902 from the fetoplacental discs, including 3 orally inoculated mice (1 BALB/c and 2 CD-1) and 11 IP inoculated mice (4 BALB/c and 7 CD-1) (Table 1). For mice with infected fetoplacental discs, while there did not appear to be any obvious effect on litter size or crown-rump length as compared to control mice, there was a significant decrease
in the birth of live young with 93% of mice with infected fetoplacental discs failing to produce live young.

Gross reproductive pathology (Table 2) ranged from the birth of term dead pups (stillborn mice found in cage), intrauterine fetal death (tan/white mid-sized conceptus) and placentitis (edematous and hemorrhagic, yellow or green placental discs) (Figure 1) and fetal resorption (placental disc without macroscopic evidence of a subjacent fetus) (Figure 2).

At necropsy, gross evidence of fetoplacental infection was found only in animals from which *C. jejuni* IA3902 was isolated in moderate and high counts from the fetoplacental disc, with 100% correlation between the presence of gross reproductive pathology at necropsy and the microbial recovery of moderate to high counts of *C. jejuni* IA3902. When there was high recovery (TNTC) of *C. jejuni* from the fetoplacental discs, BALB/c mice inoculated IP displayed all four gross changes at necropsy including dead pups, dead fetuses, fetal resorption and placentitis. Both BALB/c and CD-1 mice demonstrated gross evidence of placentitis after intraperitoneal inoculation with the BALB/c strain having placentitis after oral inoculation with high recovery of the organism from infected fetoplacental tissue. One CD-1 mouse had intrauterine fetal death with moderate recovery (50-1000 CFU) of *C. jejuni* from the fetoplacental disc following oral inoculation. Control mice of both strains did not have dead pups, dead fetuses, and gross or histological evidence of fetal resorption or placentitis.
There were rare, 1 mm wide white foci on the liver of two BALB/c mice, from which the organism was positively identified by culture. One of these mice was inoculated IP (>1000 CFU) and the other was orally inoculated (50-1000 CFU).

Microbial culture Results

*C. jejuni* IA3902 was cultured from cecal swabs at necropsy from all challenged mice except 2 CD-1 mice, (one was orally inoculated and the other was inoculated IP). The organism was cultured more often and in much higher numbers from the placenta than from the free uterine wall, irrespective of strain of mouse or route of administration. The organism had an affinity for the murine fetoplacental unit as it could be cultured from the fetoplacental unit of every single mouse (all 10 BALB/c and all 10 CD-1 mice) inoculated intraperitoneally. Heavy bacterial growth (>1000 CFU or TNTC) from the fetoplacental disc was detected in CD-1 mice twice as often as in BALB/c mice. After oral inoculation in both mouse strains recovery of *C jejuni* IA3902 from the fetoplacental unit was possible in 2/7 BALB/c mice (1 at < 50 CFU and 1 at >1000 CFU) and 3/8 CD-1 mice (1 at 50-1000 CFU at 2 at >1000 CFU) (Table 3).

Histopathology, histochemistry and immunohistochemistry

Histologically, the uterine lesions of *C. jejuni* IA3902 inoculated pregnant mice consisted of multifocal areas neutrophilic infiltration, endometritis, endometrial ulceration, necrosis and occasional hemorrhage. There was a neutrophilic placentitis with
moderate to large numbers of neutrophils disrupting cellular integrity primarily along the periphery of the placental disc. Within these foci at the periphery of the infected placental discs, there were variable amounts of necrotic cellular debris and hemorrhage with occasional bacterial colonies 150-250 microns in diameter. 13/14 mice that had high recovery (TNTC) of *C. jejuni* IA3902 by microbial culture from the fetoplacental discs also had increased numbers of neutrophils within the periphery of the fetoplacental disc (neutrophilic placentitis) (Table 3). There were variable numbers of neutrophils within the uterine lumen and endometrium of the 4 non‐pregnant mice (3 BALB/c mice and 1 CD-1 mouse that were all orally inoculated) with rare ulcerative endometritis. *C. jejuni* was not cultured from the uterus of these mice.

Inoculated mice that gave birth prior to the study termination and from which *C. jejuni* was isolated had moderate to large numbers of neutrophils present within the submucosa and endometrial glands and erythrocytes within the uterine lumen. Postpartum control mice had low numbers of neutrophils and erythrocytes within the uterine lumen and submucosa.

There was a significant difference in neutrophil scores between inoculation routes for the uterine lumen (p value 0.0159), where neutrophil scores from BALB/c mice inoculated IP, which had the highest mean score (34.15), were significantly higher than the controls, which had the lowest mean neutrophil score (18.00). Also there was a significant difference among inoculation routes for neutrophil scores in the uterine wall (p value 0.0057), where BALB/c inoculated orally had significantly higher mean neutrophil score (31.80) compared to the control which had the lowest mean neutrophil score (10.50).
Culture of higher levels of *C. jejuni* from tissues was typically associated with a more pronounced inflammatory response as measured by neutrophil scores. The mean neutrophil score at the placental periphery (10.85) was significantly higher (p value 0.0049) when numerous *Campylobacter* (level 3) were isolated from the fetoplacental disc (Table 4) when compared to the mean neutrophil score (3.00) when no organisms were isolated (level 0). Similarly, neutrophil scores for the uterine lumen at microbial culture level 1 (score 18.0, p value 0.0028) and microbial culture level 2 (score 14.0, p value 0.0028), and the uterine wall at microbial culture level 3 (16.25, p value 0.0089) were significantly higher than neutrophils scores at these locations with microbial culture level 0 had mean neutrophil score 5.0 in the uterine lumen and 5.5 within the uterine wall.

The spatial distribution of mucin, iron and L-fucose within the murine placental unit and adjacent tissue was assessed for all murine fetoplacental discs (Table 5). In sections stained with PAS, which stains neutral mucins, there was moderate to abundant globular staining in the placental disc and yolk sac, within the cytoplasm of trophoblasts and yolk sac cells (intracellularly) and extracellularly (Figure 3). Alcian blue staining, which stains acid mucins, was absent to low. Iron was present multifocally within the parenchyma of the placental disc, mainly within vascular sinusoids (Figure 4). Lectin histochemistry for LTA, demonstrated moderate to abundant, multifocal, cytoplasmic and membrane staining of cells of the yolk sac, uterine mucosa and extracellular staining at the periphery of the placental disc (Figure 5).

Histologically, the white foci on the liver of the two BALB/c mice, one inoculated IP and the other inoculated orally, presented as random multifocal necrotizing hepatitis
and mild lymphoplasmacytic cholangitis. Rarely, there were increased numbers of mixed inflammatory cells within sinusoids and in the submucosa and the mucosa of the gallbladder (cholecystitis).

Immunohistochemistry for MOMP was performed on five placental samples, one sample drawn from each of the four groups in which positively identified by microbial culture and one control. Staining for *C. jejuni* was completely absent in the control slide, but was positively identified within each of the four murine fetoplacental discs from which high numbers of *C. jejuni* were isolated. Positive staining was present within the cytoplasm of trophoblasts mainly at the periphery of the placental disc, within neutrophils and extracellularly in vascular sinusoids of severely affected placental discs (Figure 6).

**Discussion**

Several animal models for intestinal Campylobacteriosis have been investigated including an oral ferret, piglet, rabbit, chick/avian and SCID mouse model, while the pregnant guinea pig has been the primary model for reproductive studies. In spite of over 30 years of research, there is no consensus on a widely acceptable animal model for human intestinal and reproductive Campylobacteriosis research. Mice are the most commonly used animal to study genital chlamydial infections with two established murine models in which the course of disease and outcome can be varied depending on the mouse strain, inoculating dose, age of the mice and levels of reproductive hormones.
There are several reasons why establishing a murine model for reproductive studies with *C. jejuni* IA3902 would be useful. These include the fact that mice are the lowest species on the phylogenetic tree that can be used appropriately to investigate the viability of a reproductive model and both the murine and human placenta is discoid and hemochorial, which supports a similar pathogenesis in reproductive disease studies. Murine models have already been defined for various infectious agents and vaccine studies and bacteremia has already been demonstrated to be a common and consistent finding with Campylobacteriosis. Murine studies would also be ideal for elucidating the genetic factors involved in host defense against *C. jejuni* infection as there are murine models with defined deletions for components of innate and adaptive immunity. Additionally, experiments in pregnant mice have demonstrated that *Campylobacter sp.* can translocate from a distant site of infection to the placenta to induce adverse effects on the fetus and placenta.

There was a significant decrease in the birth of live young from mice with *C. jejuni* infected placentas as 93% of mice with heavily infected fetoplacental discs failing to produce live young. There was also a high correlation between the presence of gross reproductive pathology at necropsy and microbial recovery of moderate to high counts of *C. jejuni* IA3902. This is similar to what has been demonstrated in guinea pigs. Abortions, while not observed in this study, occurred in 6/12 pregnant guinea pigs within 4 and 10 days post oral inoculation with *C. jejuni* IA3902. The short gestation in mice (18-22 days), dose and time of inoculation and the relatively short interval between challenge and necropsy may have precluded abortions from being observed in this study. Stillbirths have been reported as part of the reproductive pathology associated with *C.
jejuni IA3902 infection in sheep. This was also a finding in infected mice in this study but has not been reported in experimentally infected guinea pigs. Fetal resorption has been reported in pregnant mice after administration of lipopolysaccharide (LPS) and was seen in this study.

In two BALB/c mice, there was random multifocal hepatitis grossly and histologically with microbial recovery of the organism from hepatic tissue, in addition to cholecystitis. Multifocal hepatitis has also been reported with pregnant guinea pigs inoculated with C. jejuni IA3902, and in both species is likely associated with the bacteremia that precedes fetoplacental infection.

Exudation of leukocytes is a feature of the reproductive tract infection with Campylobacter. In this study, there was a statistically significant neutrophilic infiltrate within the endometrial wall (endometritis). This is in agreement with sheep studies using this pathogen, where histopathological lesions included suppurative necrotizing placentitis and/or endometritis and also in agreement with guinea pig studies where uterine lesions, though variable, consisted of suppurative endometritis, metritis and hemorrhage.

There were variable numbers of neutrophils (scores between 2 to 4) accompanied by rare ulcerative endometritis within the endometrium of challenged mice that were not pregnant at study termination. The organism was not recovered by culture from this location in these animals one week post inoculation, at necropsy. In comparison, in non-pregnant guinea pigs, the organism was cultured at low levels (<50 CFU) from the uterus in 2/11 (18%) animals two days post inoculation. It is notable that in the guinea pig
study all animals were challenged IP, the study was terminated at a much shorter interval of 48 hours post challenge and only low bacterial counts were detected in only 2 guinea pigs. Based on histologic changes, it appears that in mice, as in guinea pigs, *C. jejuni* is trophic for the uterus even in non-pregnant animals.

Within the *C. jejuni* infected fetoplacental unit, there was a neutrophilic placentitis with moderate to large numbers of neutrophils disrupting cellular integrity primarily along the periphery of the placental disc with necrotic cellular debris and hemorrhage and bacterial colonies 150-250 microns in diameter. Neutrophilic placentitis, a feature of the reproductive tract infection in pregnant guinea pigs, was also a consistent and reliable feature in infected fetoplacental tissue irrespective of mouse strain or route of infection. High neutrophil scores correlated with microbial recovery from heavily infected fetoplacental discs, as 12/13 mice (92 %) from which *C. jejuni* IA3902 was recovered in high counts (TN TC), neutrophils scores were 2 or higher. There was a significant difference among placental microbial culture levels for neutrophil scores for the placental periphery (p value 0.0049) where level 3 (TN TC microbial culture) had a higher mean neutrophil score than level 0 (control with no microbial growth).

The proposed pathogenesis for *Campylobacter* abortion is oral ingestion followed by intestinal colonization and translocation, bacteremia, and infection of the fetoplacental unit. In both strains of mice, *C. jejuni* IA3902 could be isolated from the blood, uterus and fetoplacental unit after oral inoculation which supports the suitability of the model as it demonstrates replication of the natural disease with bacteremia resulting from intestinal invasion with subsequent infection of the fetoplacental tissue. However, in both strains of mice evaluated, the percentage of animals with demonstrable fetoplacental
infection following oral inoculation (29% for BALB/c and 38% for CD-1) was considerably lower than is typically observed in guinea pigs following oral challenge (70%). Though less desirable for the study of the overall pathogenesis of *Campylobacter*-induced reproductive disease, IP inoculation resulted in more consistent fetoplacental infection with accompanying gross and histologic lesions. Similar to findings in guinea pigs, there was high correlation with fetoplacental infection and gross and histological lesions in *C. jejuni* IA3902 infected murine reproductive tissue.

The organism was identified via immunohistochemistry for the major outer membrane protein (MOMP) within the cytoplasm of trophoblasts and infected neutrophils located in necrotic foci at the periphery of the fetoplacental unit, in the uterine lumen and the endometrial submucosal tissue including the vasculature, and within giant trophoblasts at the periphery of infected the fetoplacental units. Extracellularly, *C. jejuni* was located in and around necrotic foci at the periphery of fetoplacental units and within the vasculature of the most severely affected fetoplacental tissue/placental disc and uterus. This is partly in agreement with cellular investigations of *C. jejuni* which supports a transcellular translocation. Similarly, there is agreement with findings from pregnant guinea pig studies, where *C. jejuni* IA3902 was identified within the cytoplasm of trophoblasts, within phagocytes, in extracellular spaces surrounding trophoblasts in areas of placentitis and necrosis and occasionally surrounding uteroplacental arteries. The presence of *C. jejuni* within murine trophoblasts and vasculature of the fetoplacental unit is also consistent with what has been described in sheep where the organism has been detected within trophoblasts and blood vessels of infected ovine placenta. In that same study, Hedstrom proposed trophoblast necrosis
with release of the organism to the chorionic villi and vasculature led to subsequent vascular dissemination to the fetus.

There was strong staining for neutral mucin (PAS) in locations of the fetoplacental unit where *C. jejuni* was located but no staining with Alcian blue (acid mucin) in these same tissues. Similarly, there was strong staining for L-fucose (lectin) and iron (Perls’ iron) in regions where there was heavy colonization by *C. jejuni*. This indicates that the organism has an affinity for areas of the placental discs where there is abundant neutral mucin (at the periphery of the placental discs both within the cytoplasm of trophoblasts and extracellularly), iron (within vascular sinusoids of placental discs) and L-fucose (cytoplasmic and membrane staining of cells and extracellularly at the periphery of placental discs). Previous work has found that mucin, iron and L-fucose are trophic factors for *C. jejuni* IA3902 and the findings in this murine study is consistent with and supports data from guinea pig studies.\(^8\)

The two main limitations of this study were the relatively small number of pregnant mice used and the slightly variable gestation lengths. Two strains of mice challenged by two different routes were evaluated in this study to assess the mouse as a model for *C. jejuni*-induced reproductive disease. From these results, BALB/c mice may be an appropriate murine model for studying the reproductive tract pathology of *C. jejuni* IA3902, as this strain displayed a wider range of gross lesions including fetal resorption and dead pups. That being said, CD-1 mice had 70% high microbial recovery from infected fetoplacental discs and may be an alternate consideration as a murine model. IP and oral inoculation routes remain viable options for future studies as the organism could be recovered by microbial culture from the fetoplacental discs and there were statistically
significant inflammatory responses in the reproductive tract using these 2 inoculation routes in BALB/c and CD-1 mice. A higher inoculum e.g. 1 ml of *C. jejuni* IA3902 at $10^9$ CFU/ml is recommended for oral challenge.

Currently, the most commonly used model to assess the reproductive consequences of *C. jejuni* infection is the guinea pig, where IP inoculation of IA3902 resulted in fetoplacental infection, abortion, and demonstrable metritis and placentitis in 100% of challenged animals. After oral challenge in pregnant guinea pigs, 7/10 placental samples had high numbers of *C. jejuni*, with each animal from which *C. jejuni* was isolated consistently demonstrating metritis and placentitis. In this study, *C. jejuni* was recovered from the fetoplacental unit following oral inoculation in only 2/7 BALB/c and 3/8 CD-1 mice, which was relatively low, yet there was 100% recovery of the organism from the fetoplacental disc of IP-inoculated animals from both the BALB/c and CD-1 groups. Since the outcomes following IP inoculation were similar, this disparity in results between guinea pigs and mice following oral inoculation is likely associated with either the ability of the organism to colonize the intestine or translocate across the mouse intestine leading to bacteremia.

There was 95% isolation of *C. jejuni* from the feces at necropsy in both strains of mice following oral or IP challenge, which suggests similar widespread intestinal colonization regardless of strain of mouse. Most mice had moderate counts (50-1000 CFU) of the bacteria in the feces one week after inoculation though BALB/c inoculated IP had high counts (>1000 CFU). So although bacteremia in mice following oral inoculation has been demonstrated, the main problem with the murine model using oral challenge may lie with establishing sufficient colonization in the gut to facilitate adequate
translocation to the bloodstream. Murine intestinal colonization may be improved by using germ-free mice, high challenge doses, mice with humanized microbiota or with vancomycin pre-treated mice. Future studies could also investigate the reproductive pathology of *C. jejuni* IA3902 on Sigirr deficient (−/−) mice. These mice are deficient in Single IgG IL1 Related Receptor (SIGIRR) and have been proposed as a model for *C. jejuni* pathogenesis as they develop significant intestinal inflammation in response to colonization by *C. jejuni*. The phenomenal rise of *C. jejuni* IA3902 as the major zoonotic abortifacient pathogen in the sheep industry in the United States, combined with its tetracycline resistance and lack of an effective currently approved chemotherapeutic agent, or consistently effective vaccines makes the agent a significant force to be reckoned with and underscores the value of establishing appropriate animal models to facilitate much needed research. Results of this initial study indicate that the murine model can be further developed to facilitate reproductive studies with *C. jejuni*.

**Conclusion**

The murine model demonstrated significant potential for future study of the reproductive pathology of *C. jejuni* infection. 100% fetoplacental infection was established in BALB/c and CD-1 mice following IP challenge. There was a relatively low rate (33% of challenged animals) of fetoplacental infection following oral inoculation with *C. jejuni* IA3902. This was the main shortcoming of the murine model investigated in this study compared to the rate (70%) of fetoplacental infection following oral
inoculation in the guinea pig model. Trophic factors for *C. jejuni* are present in the murine fetoplacental unit, including L-fucose, mucin and iron in locations where the organism was confirmed by MOMP IHC, which is similar to reports describing these same trophic factors in the guinea pig placenta. In the placental disc, *C. jejuni* was present within the cytoplasm of trophoblasts and neutrophils in necrotic foci at the periphery of infected fetoplacental units and extracellularly, within and adjacent to necrotic foci at the periphery of fetoplacental units and within the vasculature of the most severely affected fetoplacental tissue/placental disc. In the uterus, *C. jejuni* was present within neutrophils in the uterine lumen and the endometrial submucosal tissue including the vasculature and extracellularly within the vasculature of the most severely affected uteri. Histologically, placentitis and endometritis were consistently demonstrated in *C. jejuni* infected murine reproductive tissues. *C. jejuni* causes a locally extensive neutrophilic placentitis with intraleional bacterial colonies, supported by high recovery (>1000 CFU) of the organism by microbial culture, conclusively meeting the endpoint criteria in this study.
References


18 Newell DG: Animal models of Campylobacter jejuni colonization and disease and the lessons to be learned from similar Helicobacter pylori models. *Journal of Applied Microbiology* 2001:90(S6):57S-67S.


Table 1. Number of pregnant mice, litter size, crown rump length and number of mice producing live young for mice from which there was high microbial recovery (TNTC) of *C. jejuni* IA3902 from the fetoplacental disc.

<table>
<thead>
<tr>
<th>Strain and route of inoculation</th>
<th>Number of pregnant mice with high recovery of <em>C. jejuni</em> IA3902 from the fetoplacental disc</th>
<th>Ave. number of young per litter</th>
<th>Ave. crown-rump length (cm) at necropsy</th>
<th>Number of mice with live young</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c Oral</td>
<td>1</td>
<td>6</td>
<td>3</td>
<td>0/1</td>
</tr>
<tr>
<td>CD-1 Oral</td>
<td>2</td>
<td>13.5</td>
<td>1</td>
<td>0/2</td>
</tr>
<tr>
<td>BALB/c IP</td>
<td>4</td>
<td>5.3</td>
<td>2.5</td>
<td>1/4</td>
</tr>
<tr>
<td>CD-1 IP</td>
<td>7</td>
<td>11.3</td>
<td>1.5</td>
<td>0/7</td>
</tr>
<tr>
<td>Control mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BALB/c</td>
<td>0</td>
<td>7.3</td>
<td>3</td>
<td>4/4</td>
</tr>
<tr>
<td>CD-1</td>
<td>0</td>
<td>11.5</td>
<td>1.5</td>
<td>0/4</td>
</tr>
</tbody>
</table>

* Rounded to one decimal place.  ‡ Intrauterine and postpartum.

Table 2. Gross reproductive pathology for pregnant BALB/c and CD-1 mice from which there was moderate and high recovery (TNTC) of *C. jejuni* from the fetoplacental disc.

<table>
<thead>
<tr>
<th>Strain and route</th>
<th>Number of mice with dead pups</th>
<th>Number of mice with dead fetuses</th>
<th>Fetal resorption</th>
<th>Placentitis</th>
<th>Number of pregnant mice with high recovery of <em>C. jejuni</em> IA3902 from the fetoplacental disc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculated mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BALB/c Oral</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>1/10</td>
<td>1</td>
</tr>
<tr>
<td>CD-1 Oral</td>
<td>0/10</td>
<td>1/10</td>
<td>0/10</td>
<td>0/10</td>
<td>2</td>
</tr>
<tr>
<td>BALB/c IP</td>
<td>2/10</td>
<td>2/10</td>
<td>2/10</td>
<td>1/10</td>
<td>4</td>
</tr>
<tr>
<td>CD-1 IP</td>
<td>0/10</td>
<td>1/10</td>
<td>0/10</td>
<td>2/10 ‡</td>
<td>7</td>
</tr>
<tr>
<td>Control mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BALB/c</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0</td>
</tr>
<tr>
<td>CD-1</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0</td>
</tr>
</tbody>
</table>

* Mouse from which there was moderate recovery (50-1000 CFU) of *C. jejuni* IA3902 by microbial culture of the fetoplacental unit.  ‡ One mouse had a bloody vaginal discharge.
Table 3. Sample source and semiquantitative recovery of *C. jejuni* IA3902 cultured from pregnant BALB/c and CD-1 mice at necropsy.

<table>
<thead>
<tr>
<th>Strain and route</th>
<th>Number of samples culture positive*</th>
<th>Low recovery (&lt;50 CFU)§</th>
<th>Moderate recovery (50-1000 CFU)§</th>
<th>High recovery (&gt;1000 CFU)§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Feces</td>
<td>Blood</td>
<td>Uterus</td>
<td>Fetoplacental Disc</td>
</tr>
<tr>
<td>BALB/c Oral</td>
<td>10/1 0</td>
<td>4/10</td>
<td>2/8</td>
<td>1/10</td>
</tr>
<tr>
<td>CD-1 Oral</td>
<td>9/10</td>
<td>3/10</td>
<td>1/5</td>
<td>3/8</td>
</tr>
<tr>
<td>BALB/c IP</td>
<td>10/1 0</td>
<td>5/10</td>
<td>7/7</td>
<td>10/10</td>
</tr>
<tr>
<td>CD-1 IP</td>
<td>9/10</td>
<td>4/10</td>
<td>3/3</td>
<td>10/10</td>
</tr>
<tr>
<td>Control Mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BALB/c</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>CD-1</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
</tbody>
</table>

*Values reported represent number of samples with positive culture results/number of samples available. §Results reported represent number of samples within that category/number of samples with positive culture results.

Table 4. Neutrophil scores of the fetoplacental discs with high recovery (TNTC) of *C. jejuni* IA3902 in pregnant BALB/c and CD-1 mice after oral and intraperitoneal inoculation.

<table>
<thead>
<tr>
<th>Strain and route</th>
<th>Number of samples with high microbial recovery§</th>
<th>Score 1 (&lt;10 neutrophils)</th>
<th>Score 2 (11-25 neutrophils)</th>
<th>Score 3 (26-50 neutrophils)</th>
<th>Score 4 (&gt;50 neutrophils)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BALB/c Oral</td>
<td>1/1</td>
<td>0/1</td>
<td>0/1</td>
<td>1/1</td>
<td>0/1</td>
</tr>
<tr>
<td>CD-1 Oral</td>
<td>2/2</td>
<td>0/2</td>
<td>0/2</td>
<td>1/2</td>
<td>1/2</td>
</tr>
<tr>
<td>BALB/c IP</td>
<td>3/4</td>
<td>0/3</td>
<td>1/3</td>
<td>1/3</td>
<td>1/3</td>
</tr>
<tr>
<td>CD-1 IP</td>
<td>7/7</td>
<td>1/7</td>
<td>4/7</td>
<td>0/7</td>
<td>2/7</td>
</tr>
<tr>
<td>Control Mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BALB/c</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD-1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

§Values reported represent number of samples available for histological assessment/number of samples with high microbial recovery.
Table 5. Lectin histochemistry and special stains of murine reproductive tissue

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Neutral mucin</th>
<th>Acid mucin</th>
<th>Iron$^b$</th>
<th>LTA$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periphery of the placental disc</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Parenchyma of the placental disc</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Yolk sac</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Uterine wall</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
</tbody>
</table>

$^a$ Values reported represent the average of all evaluated placental material

LTA, *Lotus tetragonalobus* lectin agglutinin

$^b$ Scores represent the average of all evaluated tissue from 12 mice: -, low/absent; +, moderate; ++, abundant

$^c$ Multifocal staining

$^d$ Membrane staining

Figures 1-2. Gross reproductive lesions in mice infected with *C. jejuni* IA3902. Figure 1. Two mid-term dead fetuses and placentitis. Figure 2. Two resorbed fetuses (placental discs without macroscopic evidence of subjacent fetuses).
Figure 3-6. Placental discs, mouse. Figure 3. PAS stain with moderate to abundant staining at the periphery of an infected placental disc, staining is within the cytoplasm of trophoblasts (intracellularly) and extracellularly. Figure 4. Perls’ stain (blue colour) multifocally within the parenchyma of the placental disc, mainly within vascular sinusoids. Figure 5. Lectin histochemistry for LTA, with moderate to abundant multifocal, cytoplasmic and membrane staining of cells of the yolk sac and extracellular staining at the periphery of an infected placental disc. Figure 6. Placental disc stained for the membrane outer protein of *C. jejuni* (MOMP). Vascular sinusoids of the placental disc are extensively stained red-brown (large numbers of bacteria).
CHAPTER 3: THE USE OF A SERUM CYTOKINE ASSAY AND IN SITU HYBRIDIZATION TO EVALUATE PRO-INFLAMMATORY AND ANTI-INFLAMMATORY CYTOKINES IN THE UTERUS AND PLACENTA OF CAMPYLOBACTER JEJUNI IA3902 INFECTED MICE

Modified from a paper to be submitted to the Journal of Veterinary Diagnostic Investigation

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Abstract

Objective: To investigate the presence of cytokines in the uterus and fetoplacental unit of mice infected with C. jejuni IA3902

Animals: 48 pregnant BALB/c and CD-1 mice

Procedures: Mice were bred, bled, challenged intraperitoneally and orally, euthanized one week post inoculation, necropsied and blood and tissues collected. Cytokine levels were quantified in the serum from mice with C. jejuni IA3902 (>1000
CFU) cultured from the fetoplacental disc with gross and histological evidence of placentitis. Control mice were culture negative with no evidence of gross or histological pathology. In situ cellular cytokine production and distribution was assessed in infected and control tissues.

**Results:** Interleukin 1 beta (IL1β), interleukin 10 (IL 10), interleukin 17A (IL17A), tumor necrosis factor alpha (TNF α) and interferon gamma (IFN γ) are expressed by specific cells from *C. jejuni* IA3902 infected fetoplacental tissue. There was a statistically significant increase in IL1β, IL6, IL10, IL17A, TNF α and IFN γ in maternal serum post infection.

**Conclusions and Clinical Relevance:** This is the first report of maternal serum cytokine responses in *C. jejuni* IA3902 infected mice combined with in situ analysis of these cytokines. Levels of IL10, an anti-inflammatory cytokine and pro-inflammatory cytokines including IL1 β, IL17A, IFN γ and TNF α are elevated during infection with *C. jejuni* IA3902. More research is needed to determine the exact role of these cytokines in the reproductive pathophysiology of *C. jejuni* and to investigate the possibility of cytokine immunomodulation as a future therapeutic approach in the management of *C. jejuni* abortion.

Abbreviation

MH Mueller-Hinton
Introduction

Campylobacteriosis, currently caused primarily by *C. jejuni*, has been reported as the primary cause of infectious abortion in domestic sheep flocks, yet the pathogenic mechanisms and virulence factors involved in *C. jejuni* infections of the reproductive tract remain poorly understood. To investigate disease pathogenesis, evaluate disease progression and assess therapeutic strategies, it is imperative that the molecular mechanisms of the disease are understood.

Cytokines are small proteins that regulate cells by binding to receptors present on target cells. Receptor binding triggers intracellular signaling, altered gene expression, and the production of other cytokines, surface receptors or feedback inhibition. Cytokines function at both the local and systemic levels and can be classified as chemokines, lymphokines, or interleukins based on target action, cell of secretion or function. It has been proposed that cells in close proximity functioning as a unit interact and respond to stimuli by way of cytokine signals that pass between them. These signals may be amplified, attenuated or provide the stimulus for secretion of other mediators, especially when there is inflammation within the affected organ or tissue.

The analysis of cytokine profiles and their alterations can be very useful to investigate host-pathogen interactions, bacterial persistence and bacterial clearance from infected hosts. Additionally, distinct inflammatory profiles (increased IL1 receptor antagonist and decreased IL10) at the maternal-fetal interface in humans were mirrored by changes in the maternal circulation and may act as biomarkers of placentitis.

Type 1 cytokines e.g. IFN γ are involved with cellular immune responses and are thought to be harmful for pregnancy, for example IFN γ enhances TNF α driven
apoptosis of human trophoblast cells.\textsuperscript{41} In addition, these two cytokines activate prothrombinase, which initiates the blood clotting mechanism and the subsequent production of IL8 which causes endothelial cells and leukocytes to ultimately reduce the blood supply to the developing placenta.\textsuperscript{45}

Type 2 cytokines like IL10, favor antibody responses, inhibit secretion of inflammatory cytokines, stimulate trophoblast outgrowth and invasion \textsuperscript{19} and overall favor the maintenance of gestation.\textsuperscript{31} Placental cytokines like IL6, TNF α and TGF β, have been reported to play roles in trophoblast cell death (either necrotic or aponecrotic) by influencing the activities of caspases and endothelial cell activation.\textsuperscript{12} IL6, IL1 and TNF α are thought to play major roles in early pregnancy and are also elevated in inflammatory states.\textsuperscript{37} TNF α increases with pregnancy and the primary source of its production appears to be the placenta, specifically trophoblast cells. IL1β and TGF β may cause the destruction of intercellular tight junctions between trophoblasts and endothelial cells in placental tissues and facilitate placentitis.\textsuperscript{43}

Cytokines are produced by cells infected with \textit{C. jejuni} when, lipoprotein JlpA, a \textit{C. jejuni} surface protein, binds to host cell heat shock protein, Hsp90, activating nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) via mitogen-activated protein kinases, extracellular signal-regulated kinases (ERK) and p38 mitogen-activated protein kinases (MAPK) intracellularly.\textsuperscript{44,48} This cascade is part of the innate immune response to microbial pathogens which stimulate the transcription of cytokine genes in the \textit{C. jejuni} infected cell.\textsuperscript{11}

\textit{C. jejuni} is thought to also be capable of activating NFκB via receptors and pathways independent of transmembrane Toll-like receptors (TLRs) and intracellular
receptors nucleotide-binding oligomerization proteins (Nods), but these pathways have not yet been fully characterized.\textsuperscript{4} Dendritic cells (antigen presenting cells) infected with \textit{Campylobacter} produce NFκB in addition to IL1β, IL6, IL8, and TNF α which triggered an innate inflammatory response with an initiated Th1-polarized adaptive immune response via IL12, along with increased production of IL10 and IFN γ.\textsuperscript{22, 21}

In an investigation into the cytokine and chemokine profiles of the placenta on the outcome of pregnancy in women exposed to \textit{Plasmodium falciparum (P. falciparum)}, it was found that higher placental plasma IL10 levels were associated with \textit{P. falciparum} infections and that IL10 could be a useful diagnostic marker of this infection during pregnancy.\textsuperscript{13} Sheep infected with \textit{C. jejuni} \textit{SA} with positive identification of bacterial antigens in trophoblasts were found to have increased expression of cytokine genes involved in pro-inflammatory responses and necrosis.\textsuperscript{36}

RNAscope\textsuperscript{®} technology, which uses in situ hybridization methodologies, provides evidence for the cell type, spatial, regional, and possibly temporal specificity of expression of cytokines in the placental disc. RNAscope\textsuperscript{®} in situ hybridization allows for highly sensitive and highly specific detection of cytokine markers within formalin fixed paraffin embedded sections of mice reproductive tissues. This technology amplifies signal detection post probe binding while simultaneously suppressing background staining and eliminating cross hybridization to unintended targets by the use of unique double Z probes that can distinguish RNA sequences with up to 85\% homology.\textsuperscript{47} Based on these unique advantages, this technique was selected to assess cytokine expression in \textit{C. jejuni} infected, murine fetoplacental tissue.
The purpose of this study was to enhance our understanding of the pathogenesis of reproductive disease caused by *C. jejuni* by investigating changes in maternal serum cytokine expression in pregnant mice infected with this abortifacient strain (IA3902) and utilizing in situ hybridization technology to assess cytokine expression following infection of the fetoplacental unit.

**Materials and Methods**

30 BALB/c mice (24 females and 6 males) and 30 CD-1 mice (24 females and 6 males), 6-8 weeks old were sourced from a commercial supplier and acclimated for 5 days. Four female mice were group housed with one male mouse for 14 days. On day 15, females were bled and inoculated either with 0.1 ml intraperitoneally (IP) or 0.5 ml orally (O) 1x10⁹ colony forming units (CFU) /ml of *C. jejuni* IA3902. Blood was collected (0.1-0.2 mls of blood) via the lateral saphenous vein using a 25 G needle. Mice were restrained in 50 ml open ended conical tubes which allowed for the animal’s rear leg to be held through the open end of the tube, hair was shaved using a small electric clipper, petroleum jelly was lightly rubbed onto the skin to help the vein standout, the vein was gently pricked with the needle and blood collected by capillary action into a hematocrit tube. After collection a sterile gauze pad was gently pressed over the puncture site to stop bleeding. Blood was allowed to clot in 1ml Eppendorf tubes, centrifuged at 3000g for 15 mins, serum extracted and re-centrifuged at 10, 000g for 10 mins and stored at -80 °C until tested. Oral inoculation took place after a 12 hour long feed withdrawal on ten female mice from each mouse strain. Mice were lightly anesthetized via gas anesthesia in an anesthetic chamber with 2 % isoflurane and then inoculated orally with 0.5 mls of *C.
jejuni IA3902 at $10^9$ CFU/ml, via gastric lavage using a stainless steel, ball-tipped 24 gauge 1.0 inch animal feeding tube. Ten female mice of each strain were inoculated IP. Each mouse was briefly placed on its back with its head tilted slightly downward and 0.1 ml of C. jejuni IA3902 at $10^9$ CFU/ml injected IP using a 25G needle.

After inoculation female mice were immediately removed and housed in individually identifiable cages for observation twice daily for one week for clinical signs of inactivity, unresponsiveness, ruffled hair coat, diarrhea, vaginal discharge and abortion (fetal loss). All mice were humanely euthanized one week post inoculation. For euthanasia, mice were rendered completely unconscious using 100-200 µl IP Ketamine/Xylazine (80/16 mg/kg). Cardiac blood was collected for serum preparation and storage as described above. This was followed by exsanguination, in accordance with AVMA Guidelines for Euthanasia of Animals, 2013 edition.

Necropsy, microbial culture and histopathology

All mice were subjected to a complete necropsy. At necropsy, all gross lesions were noted and samples collected for microbial culture and histological examination. Cecal swabs were obtained using sterile minitip cotton swabs immediately after euthanasia. Uterus, placenta and fetal tissue were harvested in sterile petri dishes for Campylobacter culture. All samples for microbial culture were refrigerated immediately upon collection and plated on the day of collection. Cecal swabs and blood were directly streaked onto culture media. Uterus and fetoplacental tissue were minced, swabbed and streaked 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). Plates were incubated for 48 hours in anaerobic jars under micro aerobic conditions at 42 °C. *Campylobacter* colonies were counted on each plate to determine the number of CFU in each sample.

Uterus, fetoplacental unit, placenta, maternal liver and gallbladder were examined histologically. Tissues were fixed in 10% neutral buffered formalin for 24 hours. Preserved tissues were paraffin embedded and 5-7 micron diameter sections were mounted on slides and routinely stained with hematoxylin and eosin.

The study was approved by Institutional Animal Care and Use (IACUC), protocol 5-15-8018-M at Iowa State University and fully compliant with the regulations of the Laboratory Animal Resources (LAR) group at whose facility the study was undertaken. Appropriate personal protective equipment was worn in the animal treatment and necropsy rooms.

Serum cytokine testing: The Bio-Plex Pro™ 6 Plex (IL1β, IL6, IL10, IL17A, TNF α and IFN γ) Mouse Cytokine Assay was used to detect cytokines in serum. This assay was able to quantify the levels of six serum cytokines using magnetic beads. The Bio-Plex® 200 reader used a red (635 nm) laser that illuminated the fluorescent dyes within each bead to provide bead classification and identification and a green (532 nm) laser that excited the photoelectrons to generate a reporter signal, which was detected by a photomultiplier tube (PMT). A high-speed digital processor managed the data output and produced median fluorescence intensity (MFI) values as well as concentration in picograms per milliliter (pg/ml). The concentration of analyte bound to each bead was proportional to the MFI of the reporter signal. Prior to serum testing, the Bio-Plex® 200
system was warmed up, calibrated, a wash station prepared and all cytokine standards were reconstituted and diluted according to manufacturer’s instructions. A 96 well plate was used, which after allowing for cytokine standards and duplicate testing of all samples and standards permitted 4 mouse samples to be tested per group (4 per oral CD-1, IP CD-1, oral BALB/c and IP BALB/c) before challenge and these same 4 mice per group had serum tested post challenge (from necropsy) along with 2 control serum samples per strain (2 BALB/c and 2 CD-1) before challenge and 3 control serum samples from these same mice post challenge. Stored sera from mice that had microbiological, gross or histological evidence of \textit{C. jejuni} IA3902 infection were used.

Selected mouse serum samples were removed from -80 °C, thawed and kept refrigerated until tested. Dilutions were prepared just prior to the start of the assay and equilibrated to room temperature before use. Coupled beads were prepared 1X concentration from 20X stock and included 20% excess volume. The Bio-Plex Pro\textsuperscript{TM} 6 Plex (IL1β, IL6, IL10, IL17A, TNF α and IFN γ) Mouse Cytokine Assay was run after all reagents and samples were brought to room temperature. 50 µl of beads were added to wells, wells were then washed twice using 100ul wash buffer. 50 µl standards, blanks, and samples were added to wells and incubated at room temperature on a shaker set to 850 rpm. Wells were washed three times with 100 µl wash buffer, 25 µl of detection antibody was added to the wells and the plate incubated on a shaker at 850 rpm for 30 min. Wells were washed three times with 100 µl wash buffer, 50 µl of streptavidin-PE was added and the plate incubated 850 rpm for 10 mins at room temperature, washed three times with 100 µl of wash buffer and resuspended in 125 µl assay buffer for 30 sec at 850 rpm. The plate was then read on the Bio-Plex 200 system reader.
In order to determine tissue cytokine levels and distribution, the RNAscope® Assay was used. Sections were selected from the 4 infected study groups (oral CD-1, IP CD-1, oral BALB/c and IP BALB/c) along with 2 uninfected control mice that had serum cytokine levels evaluated. Three slides each from oral CD-1, IP CD-1, IP BALB/c and 2 slides from oral BALB/c were used. Each cytokine probe was also tested on control slides from uninfected mice. The C. jejuni IA3902 probe was tested on 4 slides based on histopathological evaluation along with one control (uninfected mouse) and negative and positive assay probes. Slides were prepared by cutting paraffin embedded blocks into 5 microns sections and mounted on Superfrost Plus slides. Slides were then baked for 1 hour, deparaffinized for 20 minutes, and pretreated by applying Pretreat 1 for 10 mins, Pretreat 2 for 30 mins, a hydrophobic barrier was created around the tissues on the slides then Pretreat 3 was applied for 1 hour. The RNAscope® 2.0 HD Detection Kit (Brown) was used. The RNA-specific cytokine probes were: - Probe - Mm-II17a; Probe - Mm-II10; Probe - Mm-II6; Probe - Mm-II1b; Probe - Mm-Ifng and Probe - Mm-Tnfrsf1a. The C. jejuni IA3902 was custom designed based on the sequence, RS: NC_017279 (GB: CP001876). All probes were applied for 2 hours to allow hybridization. Signal was amplified using multiple steps - Amplification reagent (Amp) 1 was applied for 30 mins, Amp 2 for 15 mins, Amp 3 for 30 mins, Amp 4 for 15 mins, Amp 5 for 30 mins and Amp 6 for 15 mins. The amplified signal (target RNA) was detected using Diaminobenzidine (DAB) substrate; slides were counterstained, dehydrated and coverslips were mounted. Tissue sections were examined under an Olympus BX 41 standard bright field light microscope at 20-40 X magnification. The assay allowed for a semi-quantitative scoring based on the number of punctate dots
present within each cell boundary. Assessment of staining was based on the protocol designed by the company and was categorized into five grades (with criteria in brackets):\(-\) 0 (no staining or less than 1 dot per 10 cells), 1 (1 to 2 dots per cell), 2 (4-9 dots per cell, none or very few dot clusters), 3 (10-15 dots per cell and < 10% dots in clusters) and 4 (>15 dots per cell and >10% dots are in clusters) (Figure 1).

**Statistical Analysis**

The analysis was done in SAS\(^1\) with the proc mixed procedure with mice as the random block effect, cytokine levels as the response, and infection, mouse strain and route as the three fixed effects. Results were considered significant at values of \(p \leq 0.05\).

**Results**

Serum was evaluated from 9 mice from which high numbers of *C. jejuni* IA3902 (>1000 CFU) was cultured from the fetoplacental disc and uterus, and from six control mice from which the organism was not recovered. The 9 mice from which *C. jejuni* was isolated had gross evidence of placentitis (edematous and hemorrhagic, yellow or green placental discs), fetal resorption (placental disc without macroscopic evidence of a subjacent fetus, intrauterine fetal death (tan/white mid-sized conceptus) and term dead pups (stillborn pups). In these mice there was histological evidence of increased numbers of neutrophils within the periphery of the placental disc and within the uterine wall. Additionally, the presence of *C. jejuni* IA3902 was confirmed within affected fetoplacental discs with strong positive cytoplasmic in situ RNA hybridization staining in neutrophils and trophoblasts as well as extracellularly within areas of placentitis (Figure 1)
A and B). Control mice were culture negative and had no evidence of gross pathology. Control mice that had given birth, had low to moderate numbers of neutrophils within the uterine wall, an expected postpartum finding (Table 1).

The output done in SAS with the proc mixed procedure with mice as the random block effect, cytokine levels as the response, and infection, mouse strain and route as the three fixed effects indicated that there was a significant difference in the levels of cytokines for mice before and after infection with *C. jejuni* IA3902. Mice with infection tended to have higher cytokine levels. This result held for IL1β with an increase of 216.75 pg/ml and p value 0.0038; IL-10 with an increase of 134 pg/ml and p value 0.0021; IL-17A with an increase of 347.65 pg/ml and p value 0.0201; IFN γ with an increase of 75.84 pg/ml and p value 0.0063) and TNF α with an increase of 1384.69 pg/ml and p value 0.0042. However, there was no significant difference in cytokine levels between the BALB/c and CD-1 strains of mice, and there was no significant difference between the intraperitoneal route and oral route of administration. In addition, there was no significant interaction among the three fixed effects.

IL6 was not present in detectable levels via the Bio-Plex® 200 serum cytokine assay either before or after inoculation in any mouse in the study.

**RNAscope® Results**

In *C. jejuni* IA3902 infected reproductive tracts IL1β originated primarily from the endometrial mucosa and non-degenerate neutrophils within the placenta, especially at the periphery of the placental disc (Figure 1 C). The staining intensity in neutrophils within these areas had a score of 4. IL10 was produced from trophoblasts and
endometrial mucosal cells with a score of 3. IL17A was produced by placental trophoblasts and endometrial mucosal cells with a score of 1. TNF α was produced in trophoblasts (score of 4) (Figure 1 D), endometrial mucosal cells (score of 4), stromal cells of the myometrium (score of 4) and cells of the yolk sac (score of 3). IFN γ was produced from trophoblasts and endometrial mucosal cells with a score of 2. Control slides had a score of 0 (no staining or less than 1 dot per 10 cells) for IL10, IL17A, and IFN γ. Control slides for TNF α had a score of 1 (1 to 2 dots per cell) for trophoblasts, endometrial mucosal cells, stromal cells of the myometrium and cells of the yolk sac. Control slides had a score of 1 for IL1β which originated from neutrophils, located within the vasculature of the placenta and endometrium (Table 2). IL6 did not have significant detectable cellular RNA in either inoculated mice or control mice.

**Discussion**

In this study, high levels of IL1β mRNA were expressed in non-degenerate neutrophils within the placenta, endometrial mucosa and endometrial lumen. IL1β is a central mediator of innate immunity and inflammation and is expressed and/or affected by all cells of the innate immune system. Its main function is to control pro-inflammatory reactions in response to tissue injury by pathogen-associated-molecular patterns (PAMPs, such as bacterial products) or damage- (danger-) associated molecular patterns (DAMPs) released from damaged cells.

IL1β has been detected in placental tissue and is implicated as an endogenous mediator of inflammation and parturition. In chorioamnionitis, IL1β immunoreactivity was noted in trophoblasts, vascular smooth muscle, stromal cells and decidua. IL1β was
found to act as a mediator of endothelial cell function and the placenta contributed to
elevation in serum levels within the maternal circulation. While the placenta was thought
to produce small quantities of IL1β, trophoblasts can be induced to produce significant
quantities of bioactive IL1β in vitro via endotoxin stimulation as evidenced by increased
mRNA expression.

High levels of IL10 mRNA were expressed in trophoblasts with lesser quantities
from the endometrial mucosa. IL10 is an anti-inflammatory cytokine that inhibits the pro-
inflammatory activity of immune cells like Th1, NK cells and macrophages, all of which
are required for optimal pathogen clearance and more than any other cytokine, is an
essential part of the immune regulatory response in almost all infections. IL10 is
proposed to be the key cytokine for the maintenance of pregnancy and inhibits the
secretion of inflammatory cytokines such as TNF α in rats where it was found that
declining IL10 plasma levels were related to embryonic compromise and that it can be
used a predictor of embryo-fetal and placental pathology in diabetic rats.

Small amounts of IL17A mRNA were produced by placental trophoblasts and
endometrial cells. While there are six members in the IL17 cytokine family, biological
function of IL17A and IL17F are the best understood and mediate pro-inflammatory
responses. When pathogens invade mucosal tissues, IL17 induces the production of
antimicrobial proteins such as beta-defensin 2, antimicrobial proteins, lipocalins and
calgranulins, CXC chemokines and granulocyte colony-stimulating factor which results
in protection from bacterial infection. In human recurrent pregnancy loss, high levels of
IL17, IL6 and IL1β have been reported.
IFN γ was produced from trophoblasts and endometrial mucosal cells at moderate levels. This is in agreement with other reports from mouse studies.\textsuperscript{32,50} It is a type II interferon and the coordinator of a wide array of cellular processes because of its ability to regulate the transcription of immunologically relevant genes.\textsuperscript{10} It is the sole type II interferon and its production is controlled by cytokines secreted by antigen presenting cells (APCs), mostly IL12 and IL18.\textsuperscript{38} Negative regulators of IFN γ production include IL4, IL10, transforming growth factor beta and glucocorticoids.\textsuperscript{15} Pathogen products like lipopolysaccharide (LPS) augment local IFN γ production of this pro-inflammatory cytokine, which in turn amplifies the immune response to these agonists.\textsuperscript{40}

IFN γ also has the ability to synergize or antagonize the effects of cytokines, growth factors and pathogen associated molecular patterns (PAMPs) signaling pathways (e.g. TNF α, IL4, IFN alpha/beta, LPS).\textsuperscript{38} It is interesting that elevation in IFN γ has been associated with mid gestation fetal loss in mice,\textsuperscript{28} which is in agreement with our findings in this study. Similarly, an increase in IFN γ production was reported to induce abortion in a mouse model of \textit{B. abortus} infection.\textsuperscript{26}

In this study, high levels of TNF α mRNA as detected by RNAscope® were present in trophoblasts, endometrial mucosa, stromal cells of the myometrium and cells of the yolk sac. While macrophages are believed to be the main source of TNF α, it is also produced by a wide variety of other cells including endothelial cells, fibroblasts and neuronal tissue and its release is triggered by bacterial products and lipopolysaccharides.\textsuperscript{6} TNF α is thought of as a major pro-inflammatory mediator, with strong immunostimulatory activities, is able to induce apoptosis and engage in tissue
regeneration/expansion and destruction and is capable of a wide spectrum of biological activity to which most cells are responsive.\textsuperscript{46}

IL6 is a pro-inflammatory cytokine with roles in Th 2 responses and antibody production.\textsuperscript{9} While IL6 can be found within the reproductive tract,\textsuperscript{33} it did not appear to have a major role in the pathology of abortifacient \textit{C. jejuni} IA3902 in BALB/c and CD-1 mice used in this study. This is in contrast to other work, which suggested roles for IL6 in the immunity and pathogenesis of \textit{Campylobacter}; specifically that it enhanced local enteric and systemic antibodies to \textit{C. jejuni}.\textsuperscript{8} Similar to findings in our study, Abram, 2000, found that \textit{C. jejuni} did not promote the induction of IL6 systemically when C57BL/6 mice were infected by the intraperitoneal route.\textsuperscript{1} The strain of mouse may play a role in cytokine production as IL6 was found to be associated with murine abortion in CBA/J x BALB/c and CBA/J x DBA/2J mating combinations.\textsuperscript{51}

The findings in this study were in agreement with reports by Al-Banna \textit{et al}, 2012, in which there was the production of both pro-inflammatory and anti-inflammatory cytokines in the small and large intestine after infection with \textit{C. jejuni} in mice.\textsuperscript{3} There is also agreement with other studies on human epithelial INT-407 cells found that \textit{C. jejuni} induced high mRNA expression of pro-inflammatory cytokines IL1β, IFN γ and TNF α and anti-inflammatory cytokines IL10.\textsuperscript{2} By analyzing experimental data using structural equation models (SEM) in birds, it was also found that there was primarily a T helper (Th) 17 response (IL17A, IL17F, IL22) in broiler chickens after \textit{C. jejuni} infection that limited the invasion of the bacteria into the caeca.\textsuperscript{34}

Studies on cytokines, more specifically cytokines in gestation can be complicated and many times elucidate results that may conflict with other studies. This is likely based
on the use of different methods of testing and analysis in different animal models and the
fact that there are individual variations coupled with the often dual roles performed by
most cytokines. The cytokine serum assay using the Bio-Plex Pro™ Mouse Cytokine
Assay demonstrated that both pro-inflammatory (IL 1, IL 17, IFN γ and TNF α) as well as
anti-inflammatory cytokines (IL 10) play a role in the reproductive pathology of
*C. jejuni* IA3902 infected BALB/c and CD-1 mice and that maternal serum levels reflect
placental cytokine alterations. The number of fetuses, stage of gestation, dose of
inoculum used and time of infection may influence the type and amount of placental
cytokines produced.

The use of the in situ RNAscope® technology provided visible support that the
source of serum cytokines was the placenta and uterus in infected animals as control mice
did not produce detectable IL10, IL17 and IFN γ and IL1 and TNF α was only produced
at very low levels. Mice with high recovery of *C. jejuni* IA3902 (>1000 CFU) had
cytokine scores above 2 for IL1 β, IL10, IL17 A, IFN γ and TNF α as assessed by the
RNAscope® methodology. IL6 did not have significant detectable cellular mRNA in
either inoculated mice or control mice; neither was a serum cytokine level detectable by
the Bio-Plex® 200 cytokine assay before or after inoculation with *C. jejuni* IA3902.

With the use of RNAscope® technology, there was the additional benefit of being
able to identify the cell types that were involved in producing the placental cytokines and
their spatial distribution within the reproductive tissue. Also, it was possible to semi
quantify or score placental cytokine production. Based on these results, while there
appears to be an anti-inflammatory response as reflected in an IL10 score of 3, as
expected there was an overriding pro-inflammatory response with IL17 and IFN γ scores
of 2 and IL1β and TNF α scores of 4. This supports and emphasizes the role of the reproductive tract and uterus as the main source of circulating cytokines in these mice.

**Conclusion**

This is the first study to report on the maternal serum cytokine responses in *C. jejuni* IA3902 infected BALB/c and CD-1 mice combined with in situ analysis of these cytokines within the murine reproductive tract. This work strongly suggests that (1) IL1β, IL10, IL17A, TNF α and IFN γ are involved in the pathogenic mechanisms of the reproductive tract infection of *C. jejuni* IA3902, (2) that these cytokines can be measured in the maternal serum and in situ production can be demonstrated within fetoplacental and uterine tissue after infection with the organism and (3) that trophoblasts within the placental disc are primary source of these cytokines while endometrial mucosal cells, endometrial stromal cells, cells of the yolk sac and neutrophils also produce significant amounts of cytokines when the pregnant murine uterus is infected with *C. jejuni* IA3902. Levels of IL10, an anti-inflammatory cytokine and pro-inflammatory cytokines including IL1 β, IL17A, IFN γ and TNF α are elevated during infection with *C. jejuni* IA3902. IL6 was not detectable either in maternal serum or within cells of the placenta and thus may not appear to play a significant role in the reproductive pathology of *C. jejuni* IA3902 in BALB/c and CD-1 mice. This study has identified a number of cytokines modulating placental infection with *C. jejuni* IA3902. Further research is needed to determine the role of these cytokines in the pathogenesis of *C. jejuni* abortion and the possibility of cytokine immunomodulation as a future therapeutic approach due to the high prevalence of antibiotic resistance with this pathogen.
Footnotes

a. Charles River, Wilmington, MA.

b. Fisher Scientific, Pittsburgh, PA.

c. BBL CultureSwab, Becton-Dickinson Co, Franklin Lakes, NJ.

d. Preston *Campylobacter* selective supplement, Oxoid Ltd, Cambridge, England

e. *Campylobacter* growth supplement, Oxoid Ltd, Cambridge, England

f, g. BIO RAD, California, USA

h, i. Advanced Cell Diagnostics, Inc. (ACD) USA

j. SAS, version 9.4, SAS Institute Inc., Cary, NC
References


24 Jin W, Dong C: IL-17 cytokines in immunity and inflammation. *Emerg Microbes Infect* 2013:2(9):e60.


Table 1. Summary of infection for BALB/c and CD-1 pregnant mice used for serum cytokine testing following oral and IP inoculation with *C. jejuni* IA3902 based on microbial culture, gross pathology and histopathology.

<table>
<thead>
<tr>
<th>Strain and route of inoculation</th>
<th>Microbial culture positive* (&gt;1000\text{CFU (uterus and/or placenta)})</th>
<th>Gross pathology</th>
<th>Histopathology (neutrophil score)§</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inoculated Mice</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BALB/c Oral</td>
<td>2/4</td>
<td>Placentitis</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD-1 Oral</td>
<td>1/3</td>
<td>Dead fetus</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BALB/c IP</td>
<td>3/4</td>
<td>Dead fetuses</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dead pups</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD-1 IP</td>
<td>4/4</td>
<td>Placentitis</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dead fetuses</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Control Mice</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BALB/c</td>
<td>0/2</td>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>CD-1</td>
<td>0/2</td>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

*values reported represent number of samples with positive culture results/number of samples tested. §Neutrophil count in five 40X fields, scored as follows: ++=<10 neutrophils, ++=11-25 neutrophils, +++=26-50 neutrophils, ++++=>50 neutrophils

Table 2. Summary of semiquantitative RNAscope® scores for cytokines evaluated within placental and uterine cells in inoculated and control mice.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Inoculated mice</th>
<th>Control mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trophoblast</td>
<td>Endometrial mucosal cells</td>
</tr>
<tr>
<td>IFNγ</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>IL17</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>IL10</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>TNFα</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>IL1β</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL6</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

§= Score of 0; ++= Score of 1; +++= Score of 2; ++++= Score of 3; ++++= Score of 4
Figure 1. Photomicrographs of RNAscope sections of murine placental discs infected with *C. jejuni* IA3902, probed and stained for cytokines and *C. jejuni* IA3902. (A) *C. jejuni* staining at the junction of the placental disc and endometrium, there is strong positive red-brown staining within neutrophils and extracellularly. (B) *C. jejuni* Positive staining multifocally within trophoblasts, which are occasionally distended quite markedly. (C) IL1β staining within the cytoplasm of neutrophils, score 4 (>15 dots per cell and >10% dots are in clusters) at the periphery of an infected placental disc. (D) Staining of TNF α within trophoblasts, score 4, in an infected placental disc.
CHAPTER 4: EVALUATION OF THREE DIFFERENT INOCULATION ROUTES FOR THE INDUCTION OF SHEEP ABORTION BY *CAMPYLOBACTER JEJUNI* CLONE SA

Modified from a paper to be submitted to the *American Journal of Veterinary Research*

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Abstract

Objective: To develop a model of ovine abortion and discuss the experimental findings and challenges encountered during this attempt. Two studies were conducted, in which pregnant sheep were inoculated with a highly pathogenic, abortion inducing clone, *Campylobacter jejuni* (SA clone), isolated from a sheep abortion using various routes and doses.

Procedures: Primiparous ewes were administered *C. jejuni* IA3902 (a clone SA isolate) via the oral route, intraperitoneal (IP) route or intravenous (IV) route at approximately 100 days gestation. Animals that aborted or showed significant evidence of impending disease were immediately euthanized and necropsied. Remaining ewes were euthanized at the termination of the study 21 days post inoculation (DPI). Samples
were collected for microbial culture, histologic examination and immunohistochemical analysis.

**Results:** Large doses of *C. jejuni* IA3902 administered orally and IP failed to result in demonstrable fetoplacental infection. Three sheep inoculated IV aborted and three died. *C. jejuni* was recovered in high numbers from placenta and uterus and in moderate numbers from fetal lung and liver of animals inoculated via the IV route. In the placentome, there were histological changes of rounded, swollen trophoblasts, cellular and nuclear debris, vascular congestion and hemorrhage. Immunohistochemistry (IHC) confirmed the presence of the organism within trophoblasts, fetal villi and endometrial glands.

**Conclusions and clinical relevance:** This is the first report of *C. jejuni* within fetal villi and endometrial glands of infected ovine reproductive tissues. Challenges encountered are discussed and suggestions made for future investigations. These include measures for mitigating the effects of endotoxin by washing the culture prior to IV administration, premedicating ewes with non-steroidal anti-inflammatory drugs as well as using confirmed *C. jejuni* clone SA infected placenta from IV inoculated ewes for future oral inoculation studies.

**Abbreviation**

MH  Mueller-Hinton

ABSL 2 Animal Biosafety Level 2

PBS  Phosphate buffered saline
Introduction

*C. jejuni* is a major cause of food-borne bacterial gastroenteritis in humans worldwide and one of the most important causes of abortion, stillbirth and neonatal death in sheep. Historically, there has been significant antigenic and genetic heterogeneity of *Campylobacter* isolates associated with ovine abortion, however within the last decade, a single genetic clone as determined by pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) has become the predominant cause of abortion in sheep in the United States. This clone is universally tetracycline resistant and termed *C. jejuni* clone SA (for Sheep Abortion), of which *C. jejuni* IA3902 represents a clinical isolate obtained from a sheep abortion outbreak in Iowa. *C. jejuni* abortion is regarded as being highly contagious within flocks, with sheep likely getting infected via the oral route following exposure to aborted fetal materials including amnion, placenta and licking the fetus, uterine discharge or feces. Abortion rates and infection of up to 70% of ewes in naïve flocks have been reported.

Infected sheep intermittently shed *Campylobacter* sp. in feces with the highest rates of fecal shedding associated with periods of increased ‘stress’ as a result of lambing, weaning and flock movement. *C. jejuni* clone SA has been identified in raw milk, cattle feces, the feces and bile of healthy sheep as well as in aborted material from sheep, cattle and goats. The combined *Sma*I and *Kpn*I PFGE patterns of clone SA from sheep were indistinguishable from those of 123 (9.03%) human *C. jejuni* isolates (1,361) in the Centers for Disease Control (CDC) database, highlighting its zoonotic potential.

Reports describing the reproductive pathology caused by *C. jejuni* following experimental infection in sheep are limited and findings are sometimes inconsistent.
Hedstrom et al, 1987, demonstrated that *C. jejuni* was abortifacient in sheep after seven IV inoculated ewes aborted within 7 and 12 days post infection (DPI). In another *C. jejuni/sheep* study by Sanad et al, 2014, the same IV dose that Hedstrom used was administered to two sheep and one sheep died 20 hours post inoculation while the other had a normal delivery 6 weeks post inoculation. Additionally, there are disparities between the results of experimental infection compared to descriptions of field outbreaks. For example, there tends to be a low incidence of fetal bronchopneumonia in experimentally inoculated cases whereas this histological lesion is found in the lungs of nearly all fetuses from field cases.

Research in this area is negatively impacted by the high costs associated with sourcing and maintaining sufficient numbers of *C. jejuni* naïve pregnant sheep for lengthy research studies. Despite these obstacles, it is important to develop a reliable pregnant sheep model to further our understanding of important virulence determinants in the host species and to conduct investigations into the development of protective immune mechanisms against the organism in the host species. The ultimate goal would be to develop on farm prevention strategies and vaccination protocols to limit livestock losses as a result of *C. jejuni* associated abortion.

Without the ability to reliably reproduce this disease experimentally, many important aspects of this disease remain a mystery, including the infectious dose of *C. jejuni* needed to induce disease; the incubation period between oral ingestion and abortion and the pathogenesis of abortion, including the precise role that endotoxin may play in abortion induction. Additionally, a reliable pregnant sheep model is necessary to further our understanding of important virulence determinants and to conduct
investigations into protective immune mechanisms against the organism in the host species.

**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 (ISU), Ames, IA 50011, protocol IACUC1-12-7407-OP. The *C. jejuni* IA3902 isolate used in our study is a prototypical, clinical isolate of clone SA recovered from an aborted sheep placenta in 2006 obtained from the Iowa State University Veterinary Diagnostic Laboratory. IA3902 has been shown to be highly virulent in inducing abortion in pregnant guinea pigs. This tetracycline resistant strain is a representative isolate of the predominant clone associated with abortion in sheep as previously determined by PFGE and MLST.

Primiparous crossbred commercial ewes at approximately 100 days of gestation (confirmed via ultrasound) were sourced from a local flock and allowed to acclimate for 3 days upon arrival at the ISU Laboratory Animal Resources (LAR) research facility. Pre-inoculation serology testing indicated that these sheep had low levels of *C. jejuni* specific antibodies via ELISA. A pre-inoculation rectal swab from all ewes was cultured, both by a direct fecal smear and fecal enrichment. A single *Campylobacter* colony was isolated from one sheep from the IP group. This was later identified as *C. sputorum* by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) and confirmed as a non-clone SA isolate by specific PCR. Ewes were housed in individual pens in an ABSL 2 facility and were identified via ear tags and individual
cage identification. All ewes were fed Teklad-Envigo 7060® small ruminant complete 
ration with water available ad lib.

The pilot study used 9 pregnant ewes, divided evenly into 3 groups, one group 
was inoculated orally, another group was inoculated IV and the third group was 
inoculated IP. All groups were inoculated with *C. jejuni* IA3902 cultures that were 
washed in sterile 1 x PBS to remove free endotoxin from the growth media. This was 
done by harvesting the bacterial culture grown on the agar plate in PBS and followed by 
centrifuging the suspension (at 3,000 x g for 15 min) then re-suspending the pellet in PBS 
again to obtain the desired concentration of inoculum. 20mls of 1x10⁹ colony forming 
units (CFU)/ml *C. jejuni* IA3902 was inoculated orally into three ewes with a 20 ml oral 
dosing syringe.

The oral group was fasted for 12 hours prior to inoculation in order to minimize 
rumen fill and to slow gastrointestinal transit time with the aim of improving oral 
infection following oral inoculation. Water was not withheld. IV inoculation was via an 
18G, 2 inch long IV catheter b temporarily placed in the jugular vein. The three ewes in 
the preliminary study received 1 ml of inoculum containing 1x10⁸ CFU/ml. For IP 
inoculation, ewes received 1ml of 1x10⁸ CFU/ml, using an 18 G 1inch sterile needle c and 
ewes were fully conscious and restrained manually. The site of inoculation was localized 
with the use of ultrasound on the right upper quadrant of the flank caudal to the rumen 
and dorsal to the uterus. The inoculation site for both IV and IP administration was 
sheared and aseptically prepared using alternate Chlorhexidine® d (chlorhexidine 
gluconate 2.0%) and Isopropyl alcohol (70%) swabs e performed three times.
The larger study, conducted 4 months later, in May 2016 utilized 15 pregnant ewes divided into 3 groups. One group of 3 ewes was inoculated orally, a group of 6 ewes was inoculated IV and the final group of 6 ewes was inoculated IP, using *C. jejuni* IA3902 cultures that were unwashed (endotoxin not removed). Oral inoculation was via a short orogastric tube placed in the distal esophagus with 50 mls of $2.1 \times 10^{10}$ CFU/ml *C. jejuni* IA3902 inserted directly into the rumen and flushed with an equal volume of culture broth. Six ewes were inoculated IV with 2-5 mls of $1.5 \times 10^9$ CFU/ml. Six ewes were inoculated IP with 5-6 mls of $5 \times 10^9$ CFU/ml. (Table 1).

Following inoculation, animals were monitored three times daily for evidence of depression, loss of appetite, prolonged recumbency, elevated temperatures, and abortion (genital bleeding, expelled fetus). For the oral and IP groups, blood was collected aseptically at 4 and 11 days post inoculation (DPI) and at necropsy for microbial culture. Blood collection was done using a sterile 18G vacutainer needle and 10 ml red top vacutainer tubes to withdraw blood from the jugular vein. Fecal swabs were taken for culture at the same time to assess fecal shedding. Ewes that aborted were immediately euthanized with euthanasia solution (Beuthanasia®, sodium pentobarbital 390mg + sodium phenytoin 50 mg/ml) at 0.22mg/kg, intravenously using an 18 G needle, as per AVMA Guidelines on Euthanasia (AVMA, 2013). The study was concluded 21 days post-inoculation and all remaining animals were euthanized at that time and necropsies performed.
Necropsy and collection of samples

Necropsy involved inspection for gross lesions and sample collection for bacterial culture and histologic examination. Samples collected for culture included maternal heart blood, bile from the gallbladder, a fecal swab, intestinal contents, placenta, fetal stomach contents, and fetal tissue homogenate (lung and liver). Swabs for microbial culture were obtained using sterile minitip cotton swabs\textsuperscript{a} that were placed in liquid Stuart Transport Media\textsuperscript{b}. Maternal bile and fetal stomach contents were collected by use of a sterile tuberculin syringe with a 26 G, 3/8 inch needle and placed in a sterile tube. Samples of uterus, placenta and pooled fetal lung and liver were placed in separate sterile Petri dishes. All tissue and fluid samples were immediately refrigerated and cultured on the day of collection.

For semi-quantitative \textit{Campylobacter} culture approximately 250 microliters of bile, blood or fetal stomach contents were directly streaked onto culture media plates using a sterile cotton swab. Fecal swabs were directly streaked onto agar media. Placenta, uterus as well as pooled fetal and lung tissues were minced with sterile scissors or scalpels, swabbed and streaked onto media. The media was MH agar containing a \textit{Campylobacter} selective supplement\textsuperscript{i} (trimethoprim, rifampicin, polymyxin B and cycloheximide) and a \textit{Campylobacter} growth supplement\textsuperscript{j} (sodium metabisulfite, sodium pyruvate and ferrous sulfate). Incubation took place in sealed jars under micro aerobic conditions at 42 °C for 48 hours. \textit{Campylobacter}-like colonies based on morphology, were counted on each plate to determine the number of CFU in each inoculum.
Samples for histopathologic examination included maternal liver, gallbladder, uterus, placenta, fetal lung and liver. Tissue sections were placed in neutral buffered 10% formalin for 24 hours, transferred to 70% alcohol, trimmed and routinely processed.

Immunohistochemistry

Representative portions of placenta were fixed, processed, embedded and sectioned at 3 microns, mounted on aminoalkylsilane-coated glass slides and baked for 2 hours at 56 °C. Slides were deparaffinized routinely in xylene and rehydrated in graded alcohol solutions and water. Inhibition of endogenous peroxidase was via 2 immersions for 10 mins each in 3% H$_2$O$_2$ in water. Slides were incubated for 15 minutes at 37 °C in 0.1% protease in Tris buffer of pH 7.6, then rinsed 3 times in PBS solution and stained automatically. Nonspecific binding was inhibited by incubation with 10% neutral goat serum at 22 °C for 20 minutes. The primary antibody was directed against the major outer membrane protein (MOMP) of C. jejuni, as described by Zhang and Burrough at a dilution of 1:300 and incubated at 22 °C for 60 minutes and then rinsed in PBS for 5 minutes. A commercially available biotinylated secondary antibody (BioGenex, San Ramon, CA) was used at a dilution of 1:80 and incubated at 22 °C for 15 minutes then rinsed in PBS for 5 minutes. Slides were then incubated with horse radish peroxidase-streptavidin conjugate (Invitrogen, Carlsbad, CA) (1:200 dilution) at 22 °C for 15 minutes and rinsed in PBS for 5 minutes. The final reaction used a commercial chromogen, Nova Red (Vector, Burlingame, CA) for 5 minutes and then slides were rinsed 5 times with Ultra-Pure water. Slides were rinsed and routinely counterstained with Shandon Harris hematoxylin and Scott’s tap water. Slides were dehydrated through graded alcohol and
xylene solutions prior to mounting. Positive controls were sourced from paraffin blocks of ovine placenta that was positive for *C. jejuni* IA3902 by microbial culture. Negative controls were obtained from non-infected (*Campylobacter*-negative) control sheep.

**Results**

All inoculated sheep remained clinically normal for the duration of the initial pilot study. One DPI, all 3 orally inoculated animals were shedding *C. jejuni* clone SA (confirmed by PCR) in the feces, 2/3 at 2 DPI and 3/3 at 7 DPI (Table 2). 1/3 IP inoculated ewe had a positive fecal culture at 7 DPI. No IV inoculated sheep had evidence of intestinal colonization prior to the study termination at 20-21 DPI.

Regardless of the route of inoculation, *Campylobacter* sp were not cultured from the blood, uterus, placenta, fetal lung or fetal stomach content at necropsy. *C. jejuni* was cultured from the feces (2/3 oral, 1/3 IV and 3/3 IP inoculated ewes) and bile (1/3 IV and 1/3 IP inoculated ewes) at necropsy (Table 4) however, none of these isolates were positive for clone SA via PCR.

In the larger follow-up study all sheep inoculated orally and IP remained clinically normal for the duration of the study and *Campylobacter* sp were not cultured from feces, bile, maternal blood, uterus, placenta, fetal lung or fetal stomach content at necropsy (Table 5). Of the 6 pregnant ewes that were inoculated intravenously, 3 animals were euthanized in extremis, two within 12 hours (received 5mls and 2.25mls of 1.5x10⁹ CFU/ml each) and one within 24 hours (dose of 2.25mls) post-inoculation. Two ewes aborted within 24 hours (2 ewes) and one ewe aborted within 48 hours post-inoculation. These animals had received 2, 2.25 and 3mls of 1.5x10⁹ CFU/ml *C. jejuni* IV,
respectively. There was moderate microbial recovery (50-1000 CFU) from the pooled fetal lung and liver and high recovery (>1000 CFU) from the uterus and placenta of one ewe (Table 5). Following abortion, these ewes were promptly euthanized, necropsied and microbiological, histopathological and immunohistochemical analyses performed (Table 6).

Histopathology

The three IV inoculated ewes that aborted all had consistent histopathologic changes in the fetoplacental unit, maternal liver and gallbladder. Within the placentome there were multifocal areas with loss of normal fetomaternal placentome architecture, composed of loosely arranged aggregates of expanded, often rounded trophoblasts with foamy eosinophilic cytoplasm and often pyknotic, karyorrhectic or karyolytic nuclei. These aggregates were surrounded by moderate amounts of eosinophilic cellular debris and small amounts of extravasated erythrocytes (hemorrhage). Blood vessels were markedly congested around these areas (Figure 1A). The submucosa of the uterus was mildly expanded by edema with dilated lymphatics and congested blood vessels. Endometrial glands were multifocally dilated with eosinophilic material or ectatic with large numbers of degenerate and non-degenerate neutrophils (Figure 1B). On the fetal side of the placentome, close to the chorioallantoic membrane, there were large foci of hemorrhage with hemosiderin laden macrophages and trophoblasts, along with accumulations of fibrin, clusters of eosinophilic cellular debris and degenerate trophoblasts with pyknotic, karyorrhectic and karyolytic nuclei accompanied by moderate
edema in the stroma of the fetal villi and congestion of small vessels within the maternal plate with overall loosening of attachment to the body of the placentome.

In the maternal liver, there were small, random multifocal areas of necrosuppurative hepatitis with loss of and necrosis of hepatocytes within these foci and infiltration of moderate numbers of neutrophils (Figure 1C). Within the gallbladder, the mucosal architecture was diffusely disrupted, the apical aspect of which was necrotic. There is a moderate diffuse neutrophilic infiltration within the lamina propria separating and surrounding glands and multifocal lymphoid follicles. Vessels in the lamina propria were congested and there were foci of hemorrhage (Figure 1D).

Immunohistochemistry

In one IV inoculated ewe that aborted and from which high levels of *C. jejuni* were isolated from the placenta and uterus (Table 4), there was positive staining in degenerate trophoblasts for *C. jejuni* as determined by MOMP-specific antibody (Figure 1E). There was also positive staining for the MOMP within endometrial gland content (Figure 1F) and within fetal villi (Figure 1G) of that same animal. There was complete lack of staining for MOMP in other inoculated sheep and in negative control placenta.

**Discussion**

Oral administration of high doses of *C. jejuni* to pregnant sheep did not result in fetoplacental infection, abortion or intestinal colonization. Similarly, high doses administered via the IP route, did not cause clinical signs of ill health, peritonitis, fetoplacental infection or abortion. High IV doses of *C. jejuni* induced abortion and the
organism was recovered in high numbers from the placenta and uterus of one ewe, while moderate numbers were recovered from the lung and liver of the aborted fetus. There were degenerative histological changes in the placentome of this animal with rounded, swollen trophoblasts and cellular and nuclear debris as well as vascular congestion and hemorrhage. Immunohistochemistry (IHC) confirmed the presence of the organism within trophoblasts, fetal villi and endometrial glands.

The oral route has been used to successfully replicate *C. jejuni* IA3902 induced reproductive disease in mice (Lashley, submitted for publication), guinea pigs, and occasionally in sheep, though usually at a lower rate than parenteral routes. The oral route of inoculation replicates the natural route of infection in sheep and represents the preferred model to study disease pathogenesis, organism virulence factors, and assess protective immunity in sheep. In the Pilot study, there was evidence of intestinal colonization from clone SA positive fecal cultures up to 7 DPI but not at 20-21 DPI. This indicates that intestinal colonization by clone SA did not occur or it was only transient. Additionally, the levels of specific serum antibody did not change appreciably by ELISA, further supporting the lack of colonization followed by translocation to the bloodstream by clone SA in the ewes (Table 7). Therefore, despite the instillation of massive numbers of organisms orally (up to 50 mls of 2.1x10^{10} CFU/ml *C. jejuni* IA3902 in the larger study), we were not able to successfully induce fetoplacental infection, abortion or establish intestinal colonization for up to 21 DPI following oral administration of the organism to pregnant sheep. While this may be due to multifactorial reasons, the ability of the organism to adequately colonize and invade the gastrointestinal mucosa may be the most influential factor. The ewes in this study had low titers to *C. jejuni*, which may have
contributed to protection against intestinal colonization by the organism. Secretory IgA is the major immunoglobulin isotype in mucosal secretions and generally responsible for preventing subepithelial translocation of commensal bacteria by preventing their adhesion to epithelial cells or returning bacteria that already reached the basolateral site, without eliciting an inflammatory response. 

IV inoculated sheep in this study had histopathological lesions in the liver and gallbladder similar to lesions identified in guinea pigs and mice orally inoculated with C. jejuni IA3902 (submitted for publication). Liver lesions in all three species consisted of a multifocal necrosuppurative hepatitis speculated to be the result of campylobacteremia. In the larger study, while histopathological changes were noted primarily in the mucosa and submucosa of the gallbladder, the organism was not cultured from bile. Alternatively, it is possible that 48 hours post inoculation was not sufficient time for adequate gallbladder colonization. There was high recovery of the organism (>1000 CFU) from the bile of an IV inoculated ewe in the pilot study cultured 21 DPI. Gallbladder colonization following infection has been reported with both C. fetus and C. jejuni and this feature likely plays a major role in the maintenance of infection within sheep populations. The role and effect of endotoxin in producing related histological lesions are unknown.

The IP route of inoculation has been used to successfully replicate C. jejuni IA3902 induced reproductive disease in both guinea pigs and mice. In both species, IP inoculation resulted in 100% fetoplacental infection (Lashley, submitted for publication), and this challenge route has been described in a model for assessing virulence of Campylobacter spp. Although IP inoculation does not replicate the natural
route of oral exposure by abortifacient *Campylobacter* spp, IP challenge of pregnant ewes was incorporated into this study because of the success of this route in other species and because IP administration would potentially delay access of bacteria and endotoxin into the systemic circulation with the aim of diminishing the rapid systemic impact of endotoxin. Drug absorption following IP administration is reported to be one quarter to one half as fast as that from the IV route. In our studies, even when sheep were administered large doses, up to 6ml of $5 \times 10^9$ CFU/ml IP, they failed to develop clinical signs, peritonitis, fetoplacental infection or abortion.

IV inoculation enables immediate dispersal of the inoculum into the vascular system, allowing rapid exposure to all tissues, including susceptible sites like the fetoplacental unit. IV inoculation of sheep has led to fetoplacental infection in two other studies, albeit in a very limited numbers of animals. Based on the results of our studies, the dose used for IV inoculation appears to be a crucial factor. A low dose failed to result in fetoplacental infection and abortion. High IV doses did result in fetoplacental infection but were met with severe adverse consequences, likely as a result of the endotoxin, leading to the necessity to euthanize animals in extremis within 24 hours of inoculation. The findings in this study are similar to what was found by Sanad *et al.*, 2014 where one ewe died 20 h after IV inoculation with 1.5 mls of $10^9$ CFU of the *C. jejuni* ovine isolate of the abortion associated tetracycline resistant clone SA.

Endotoxins are lipopolysaccharides (LPS) derived from the cell membranes of Gram negative bacteria, like *C. jejuni*, and are mainly released during bacterial growth, multiplication and death. Exposure to high systemic levels can result in endotoxic shock, tissue injury and death of the infected animal. In the initial pilot study *C. jejuni* IA3902
cultures were washed in sterile PBS to remove free endotoxin and 1ml of $1 \times 10^8$ CFU/ml was administered IV. Following inoculation, ewes became slightly depressed and somewhat inappetent for approximately 24 hours, after which time they appeared normal. However, this IV dose failed to result in fetoplacental infection or abortion. Higher IV doses (2-5ml of $1.5 \times 10^9$ CFU/ml) of unwashed cultures did result in fetoplacental infection but were met with severe adverse consequences typical of endotoxic shock, including marked depression, anorexia, elevated heart rate, cool extremities, and petechial hemorrhages on the mucous membranes. These severe clinical signs necessitated the euthanasia of all 6 ewes within 12-48 hours post-challenge. Though the organism was isolated from the fetoplacental unit of three ewes and acute inflammation was identified in the uterus and placenta, the full range of gross and histological lesions typifying field cases of *Campylobacter* abortion were not seen. This is not surprising considering that the ewes aborted within 24 and 48 hours post IV inoculation. Therefore, in this study, it is likely that endotoxic shock was a primary contributing factor in the early reproductive loss in these animals. Future studies will be aimed at mitigating the effects of endotoxin by washing cultures prior to IV administration and premedicating ewes with non-steroidal anti-inflammatory drugs (NSAIDs) in an attempt to ameliorate the clinical effects of high levels of endotoxin in the blood.

Immunohistochemical procedures were based on the use of antibodies for MOMP protein. The MOMP of *C. jejuni*, a porin and adhesin,$^{18}$ has a vital role in producing systemic infection and abortion. IHC confirmed the presence of the organism within trophoblasts which is in agreement with studies in sheep,$^{23,12}$ guinea pigs$^5$ and mice (submitted for publication). As far as we could determine, this is the first report of the
organism within fetal villi and endometrial glands of infected ovine reproductive tissues. As was found in the guinea pig study, immunohistochemical analysis in the sheep placenta was less sensitive than microbial culture as a means of detecting *C. jejuni* in tissues, which is consistent with results in other studies.\(^6,28\)

In a pregnant sheep infected with *C. jejuni*, the organism spreads hematogenously from the gastrointestinal tract to the female reproductive tract resulting in uterine and feto-placental infection.\(^2\) Infected ovine placenta has been reported to have necrotic villi with large colonies of *C. jejuni* within trophoblasts lining chorionic villi, subjacent stroma, vascular endothelium and blood vessel lumen and also within uterine leukocytes.\(^12\) In this study the presence of *C. jejuni* within trophoblasts of the ovine placenta was confirmed via IHC in one animal. Trophoblast infection is likely to be a crucial step in the transfer of infection across the placenta and may occur by direct invasion of bacteria or transcellular migration to and from infected macrophages.\(^1\)

In field cases of *C. jejuni* abortion in sheep, fetal bronchopneumonia has been reported as a consistent finding as well as multifocal hepatic necrosis,\(^12\) however these lesions were not identified in fetal tissue in our studies, possibly due to the short time interval from inoculation to abortion. Since low levels of *C. jejuni* were isolated from pooled fetal lung and liver, it is likely that lesions in these fetal tissues would have developed had the incubation period been a more typical length (8-10 days).

Enzyme linked immunosorbance assay (ELISA) was performed to determine serum titer levels to *C. jejuni* before each study and only sheep that had negligible titre levels were used. Despite this, the possible role of immunity to the pathogen cannot be completely ruled out as a compounding factor. The use of naïve pregnant ewes cannot be
overemphasized for future studies. Despite pre-inoculation fecal testing showing little or no *Campylobacter sp* shedding, the possible role of natural *C. jejuni* intestinal, albeit intermittent colonization and its influence is unknown.

The oral route of inoculation replicates the natural route of infection in sheep and represents the preferred model. Unfortunately, the only inoculation route that resulted in fetoplacental infection in this study was IV. It is possible that organism fitness played a role in the inability to establish intestinal colonization and eventual fetoplacental infection following oral challenge. *C. jejuni* isolated from the intestines of chicks and then re-inoculated into other chicks resulted in cecal colonization to a much greater degree, presumably due to increased organism fitness.\(^\text{13}\) If the fitness of cultured *Campylobacter* is a concern, consistent fetoplacental infection following IV challenge may prove useful as the initial stage of generating infectious material. Infected placenta from abortions following IV inoculation may represent a more productive oral inoculum source.

**Conclusion**

Three separate routes of inoculation were evaluated in pregnant ewes to try and replicate abortion due to *C. jejuni*. At the doses administered, neither oral nor IP administration of the organism induced abortion. IV inoculation caused relatively consistent fetoplacental infection, but the high doses necessary resulted in severe adverse effects in the ewes attributed to endotoxin, and abortions occurred much more rapidly (1-2 days) than is typically reported in field infections. In a ewe that aborted 48 hours post IV inoculation, *C. jejuni* was recovered in high numbers from the placenta and uterus and
in moderate numbers from the lung and liver of the aborted fetus. There were
degenerative histological changes in the placentome of this animal with rounded, swollen
trophoblasts and cellular and nuclear debris we well as vascular congestion and
hemorrhage. Immunohistochemistry (IHC) confirmed the presence of the organism
within trophoblasts, fetal villi and endometrial glands. As far as we could determine, this
is the first report of the organism within fetal villi and endometrial glands of infected
ovine reproductive tissues. Future studies will be aimed at mitigating the effects of
endotoxin by washing the cultures prior to IV administration and premedicating ewes
with non-steroidal anti-inflammatory drugs (NSAIDs). As oral administration of a highly
virulent strain of cultured C. jejuni failed to result in fetoplacental infection, oral
administration of infectious placenta from IV challenged ewes will be considered for
future studies.

Footnotes

a. Envigo, Madison, WI
b, c. Fisher Scientific, Pittsburgh, PA
d, Xttrium Laboratories Inc, Mt. Prospect, IL
e. Science Lab, Houston, TX
f. Merck Animal Health, Madison, NJ
g. BBL CultureSwab, Becton-Dickinson Co, Franklin Lakes, NJ
h. Fisher Scientific, Pittsburgh, PA
i. Preston Campylobacter selective supplement, Oxoid Ltd, Cambridge, England
j. Campylobacter growth supplement, Oxoid Ltd, Cambridge, England
References


15 Kreuder Krull AJ: Investigation of the gallbladder host environment and small RNAs in the pathobiology of Campylobacter jejuni sheep abortion clone IA3902. 2016.


Table 1. Dose and animal distribution for the Pilot study and Larger study

<table>
<thead>
<tr>
<th>Group 1 (Oral challenge)</th>
<th>Dose in January 2016 (CFU/ml)</th>
<th>Dose in May 2016 (CFU/ml)</th>
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<tbody>
<tr>
<td>O1</td>
<td>20mls of 1x10^9</td>
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<tr>
<td>O2</td>
<td>20mls of 1x10^9</td>
<td>50 ml of 2.1x10^10</td>
</tr>
<tr>
<td>O3</td>
<td>20mls of 1x10^9</td>
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<table>
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<td>1ml of 1x10^8</td>
</tr>
<tr>
<td>IV3</td>
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<tr>
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<table>
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</tr>
<tr>
<td>IP6</td>
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Table 2. Pilot Study. Rectal temperatures, fecal cultures and clone SA result by PCR at 0, 1, 2 and 7 DPI

<table>
<thead>
<tr>
<th>ROUTE OF INOCULATION</th>
<th>DAYS POST INOCULATION (DPI)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
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<tr>
<td>Oral</td>
<td>Rectal Temp</td>
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<tr>
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<td>O2</td>
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Negative = -ve, Positive = +ve
Table 3. Pilot Study. Sample source and semiquantitative recovery of *C. jejuni* IA3902 cultured from pregnant ewes at necropsy.

<table>
<thead>
<tr>
<th>Route of inoculation</th>
<th>Volume and concentration of the inoculum (CFU/ml)</th>
<th>Number of samples culture positive&lt;sup&gt;*&lt;/sup&gt;</th>
<th>Low recovery (&lt;50 CFU)&lt;sup&gt;§&lt;/sup&gt;</th>
<th>Moderate recovery (50-1000 CFU)&lt;sup&gt;§&lt;/sup&gt;</th>
<th>High recovery (&gt;1000 CFU)&lt;sup&gt;§&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral</td>
<td>20 mls of 1x10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>2/3 0/3</td>
<td>2/2 0/0</td>
<td>0/2 0/0</td>
<td>0/2 0/0</td>
</tr>
<tr>
<td>IV</td>
<td>1 ml of 1x10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>1/3 1/3</td>
<td>0/1 0/1</td>
<td>1/1 0/1</td>
<td>0/1 1/1</td>
</tr>
<tr>
<td>IP</td>
<td>1 ml of 1x10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>3/3 1/3</td>
<td>3/3 0/1</td>
<td>0/3 1/1</td>
<td>0/3 0/1</td>
</tr>
</tbody>
</table>

<sup>*</sup>Values reported represent number of samples with positive culture results/number of samples tested. <sup>§</sup>Results reported represent number of samples within that category/number of samples with positive culture results.

Table 4. Larger study. Fecal and enriched culture data for oral and IP inoculated ewes at 0, 4 and 11 DPI.

<table>
<thead>
<tr>
<th>Route of Inoculation</th>
<th>0</th>
<th>4</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>23 (not jejuni)</td>
<td>30 (not jejuni)</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>14 (not jejuni)</td>
<td>17 (not jejuni)</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>lawn, white (not Campy)</td>
<td>white not campy (110)</td>
</tr>
<tr>
<td>IP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>lawn, white (not Campy)</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>70 (not jejuni)</td>
<td>4 (not jejuni)</td>
</tr>
<tr>
<td>3</td>
<td>8 (campy, not jejuni)</td>
<td>40 (not jejuni)</td>
<td>9 (not jejuni)</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>lawn, white (not Campy)</td>
<td>2 (not jejuni)</td>
</tr>
<tr>
<td>9</td>
<td>30 (white, not campy)</td>
<td>lawn, white (not Campy)</td>
<td>11 (not jejuni)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>24 (not jejuni)</td>
<td>4 (not jejuni)</td>
</tr>
</tbody>
</table>
Table 5. Larger study. Sample source and semiquantitative recovery of *C. jejuni* IA3902 cultured from pregnant ewes at necropsy.

<table>
<thead>
<tr>
<th>Route of inoculation</th>
<th>Number of samples culture positive*</th>
<th>Low recovery (&lt;50 CFUs)*</th>
<th>Moderate recovery (50-1000 CFUs)*</th>
<th>High recovery (&gt;1000 CFUs)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uterus</td>
<td>Placenta</td>
<td>Pooled fetal lung and liver</td>
<td>Uterus</td>
</tr>
<tr>
<td>Oral</td>
<td>0/3</td>
<td>0/3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IV</td>
<td>2/6</td>
<td>3/6</td>
<td>1/1</td>
<td>1/2</td>
</tr>
<tr>
<td>IP</td>
<td>0/6</td>
<td>0/6</td>
<td>0</td>
<td>0</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. Data in red from one ewe that aborted at 48 hours post inoculation.

Table 6. Summary of clinical findings with microbiological, histopathological and immunohistochemical correlates.

<table>
<thead>
<tr>
<th>Ewe ID (dose of inoculum administered)</th>
<th>Clinical signs post inoculation</th>
<th>Microbial culture results</th>
<th>Histopathology</th>
<th>Immunohistochemistry for MOMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV 2 (2.25mls)</td>
<td>Increased rate and depth of respiration, sternal recumbency, temp within normal range</td>
<td>Uterus +++ Placenta +++ Fetus (lung and liver) ++</td>
<td>Placentitis, endometritis, cholecystitis</td>
<td>Positive identification of <em>C. jejuni</em> in trophoblasts, fetal villi and endometrial glands</td>
</tr>
<tr>
<td>IV 4 (3mls)</td>
<td>Increased rate and depth of respiration, sternal recumbency, temp within normal range, loose stool</td>
<td>Placenta +</td>
<td>Placentitis, endometritis, cholecystitis</td>
<td><em>C. jejuni</em> could not be confirmed</td>
</tr>
<tr>
<td>IV 8 (2mls)</td>
<td>Clinically normal</td>
<td>Placenta +</td>
<td>Placentitis, endometritis, cholecystitis</td>
<td><em>C. jejuni</em> could not be confirmed</td>
</tr>
</tbody>
</table>

* = Low recovery (<50 CFU), ++ = Moderate recovery (50-1000 CFU), +++ = High recovery (>1000 CFU)
Table 7. Pilot Study. ELISA optical density (OD) at 0 and 21 DPI for ewes inoculated by all routes

<table>
<thead>
<tr>
<th>ROUTE OF INOCULATION</th>
<th>DAYS POST INNOCULATION (DPI)</th>
<th>(\text{ELISA OD 1} )</th>
<th>(\text{ELISA OD 2} )</th>
<th>Average OD</th>
<th>(\text{ELISA OD 1} )</th>
<th>(\text{ELISA OD 2} )</th>
<th>Average OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral</td>
<td>0</td>
<td>0.315</td>
<td>0.289</td>
<td>0.302</td>
<td>0.333</td>
<td>0.374</td>
<td>0.355</td>
</tr>
<tr>
<td>Oral</td>
<td>21</td>
<td>0.325</td>
<td>0.322</td>
<td>0.3235</td>
<td>0.323</td>
<td>0.33</td>
<td>0.3265</td>
</tr>
<tr>
<td>O3</td>
<td>0.276</td>
<td>0.246</td>
<td>0.261</td>
<td>0.215</td>
<td>0.215</td>
<td>0.23033333</td>
<td>0.23033333</td>
</tr>
<tr>
<td>IV</td>
<td>0</td>
<td>0.311</td>
<td>0.28</td>
<td>0.2955</td>
<td>0.333</td>
<td>0.357</td>
<td>0.345</td>
</tr>
<tr>
<td>I2</td>
<td>0.225</td>
<td>0.25</td>
<td>0.2375</td>
<td>0.336</td>
<td>0.285</td>
<td>0.3105</td>
<td></td>
</tr>
<tr>
<td>I3</td>
<td>0.329</td>
<td>0.315</td>
<td>0.322</td>
<td>0.286</td>
<td>0.318</td>
<td>0.302</td>
<td></td>
</tr>
<tr>
<td>IP</td>
<td>P1</td>
<td>0.362</td>
<td>0.363</td>
<td>0.3625</td>
<td>0.348</td>
<td>0.327</td>
<td>0.3375</td>
</tr>
<tr>
<td>IP</td>
<td>P2</td>
<td>0.362</td>
<td>0.389</td>
<td>0.3755</td>
<td>0.212</td>
<td>0.224</td>
<td>0.218</td>
</tr>
<tr>
<td>IP</td>
<td>P3</td>
<td>0.294</td>
<td>0.258</td>
<td>0.276</td>
<td>0.29</td>
<td>0.277</td>
<td>0.2835</td>
</tr>
</tbody>
</table>
Figure 1. Photomicrographs of ovine placenta, endometrial glands, liver and gallbladder after inoculation with *C. jejuni* IA3902. (A) H&E of placentome section with fetal villi lined by trophoblasts, loss of architecture and congested blood vessels. (B) H&E – large numbers of neutrophils within endometrial glands (C) H&E of liver – foci of necrosis and neutrophil accumulation (D) H&E of the gallbladder – mucosal architecture is diffusely disrupted, the apical aspect is necrotic, there is moderate diffuse neutrophilic infiltration within the lamina propria separating and surrounding glands and multifocal lymphoid follicles with vascular congestion and hemorrhage. (E) IHC for the membrane outer protein of *C. jejuni* (MOMP), foci of positive stain within endometrial gland content (F) IHC for the membrane outer protein of *C. jejuni* (MOMP), foci of positive stain within endometrial gland content, foci of positive stain within fetal villi.
CHAPTER 5: MUCIN, L-FUCOSE, IRON, TOLL-LIKE RECEPTOR 4 AND CASPASE 3 IN THE OVINE PLACENTA INFECTED WITH C. JEJUNI CLONE SA

Modified from a paper to be submitted to Veterinary Pathology

V. D. Lashley and M. J. Yaeger

Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University, Ames, Iowa.

Abstract

Campylobacter jejuni (C. jejuni) is a foodborne pathogen that causes enteritis in humans and abortion in ruminants. An antibiotic resistant, hypervirulent clone, clone sheep abortion (SA), is the agent responsible for over 90% of Campylobacter abortions in US sheep. This study investigated potential trophic factors for this pathogen within the sheep placenta and explored possible abortifacient mechanisms of action of the organism. Pregnant sheep were inoculated intravenously with C. jejuni IA3902, a representative isolate of clone SA. After abortion, the organism was isolated in high numbers from the placenta and uterus. Immunohistochemistry (IHC) for the major outer membrane protein
(MOMP) of the bacteria established the location of the pathogen in the placenta. Special stains and lectin IHC determined the distribution of known trophic factors for *C. jejuni*, including neutral and acid mucin, iron stores and L-fucose in the uterus and placenta. A commercial assay was used for apoptosis detection. IHC was used to assess caspase 3, Toll-like receptor 4 (TLR4) and transcription of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). *C. jejuni* localized to areas in the ovine fetoplacental unit and uterus where there were significant amounts of neutral and acid mucin, including the fetal villi and endometrial glands and where L-fucose and iron was present, also within endometrial glands. There was abundant, multifocal, cytoplasmic staining of stromal cells and trophoblasts of the chorioallantoic villi for caspase 3 and multifocal, abundant staining of TLR4 within chorioallantoic membranes. There was no difference in NFκB signal from infected or uninfected tissues.

**Introduction**

*C. jejuni* is a Gram negative bacterium that causes significant numbers of sheep abortions with associated economic loss to sheep producers and is a zoonotic gastroenteric human pathogen. The majority of *Campylobacter* abortions in US sheep is currently associated with a specific clone of *C. jejuni*, designated as the SA clone, typified by *C. jejuni* isolate IA3902. *C. jejuni* routinely colonizes the intestines of sheep, is frequently isolated from the ovine gall bladder and has an affinity for the fetoplacental unit in this ruminant species. Studies have indicated that *C. jejuni* IA3902 has strong chemo attraction towards mucin and L-fucose, two agents that also supported
growth of the organism. In that study, iron, mucin and L-fucose were demonstrated to be present within the guinea pig placenta in areas where *C. jejuni* was localized by immunohistochemistry. One goal of this study was to ascertain the distribution and amount of these factors in infected ovine placenta and to ascertain whether the organism also localized in those areas.

Lipopolysaccharide from Gram negative bacteria like *C. jejuni* activate TLR4 mediated NFκB signaling pathways resulting in a proinflammatory response. NFκB is a pleotropic initial-response transcription factor that leads to the expression of genes associated with immunological and inflammatory responses and was found to be activated by the intracellular entry of *C. jejuni* in vitro. TLR4 signaling was the primary agent for the enteritic alterations observed in a murine model of *C. jejuni* infection. However, TLR 4 was not upregulated in guinea pig placenta infected with *Campylobacter*.

Apoptosis is the highly regulated process of cellular death with intrinsic and extrinsic pathways that eventually converge in the generation of executioner caspases, caspase 3 and caspase 7. Caspases are cellular endoproteases that control inflammation and cell death via the production of activated proinflammatory cytokines. Caspase 3 is a member of the interleukin-1 β-converting enzyme family and is thought to be associated with the induction of apoptosis. It is present within the cytoplasm as an inactive proenzyme that is activated during apoptosis. Caspase 3 is an ‘effector’ caspase that initiates the ‘death cascade’ and is a central biomarker of apoptosis. High levels of caspase 3 were detected in mixed viral and bacterial urogenital infections suggesting
increased placental cell death and the triggering of placental dysfunction by this mechanism.\textsuperscript{25}

Another aim was to evaluate factors that contribute to \textit{C. jejuni}'s trophism for ovine fetoplacental tissue by assessing the distribution and amount of known trophic factors for \textit{C. jejuni} in infected ovine placenta and to ascertain whether the organism also localized in these areas.

In order to develop an appreciation for the mechanisms that lead to abortion following fetoplacental \textit{C. jejuni} infection, an apoptosis assay was conducted as well as analysis of an important mediator of apoptosis, specifically caspase 3. TLR4 and NFκB have been reported to be responsible for the majority of intestinal inflammation and the associated enteric pathology caused by \textit{C. jejuni}. The role that TLR4 and NFκB may play in causing reproductive pathology and abortion has not been previously reported.

**Materials and Methods**

\textit{C. jejuni} infected ovine placenta was obtained from a recent live animal study as well as a 2015 field case of abortion obtained from the Veterinary Diagnostic Laboratory, Iowa State University from which \textit{C. jejuni} clone SA was isolated. In the live animal study, six pregnant ewes were inoculated intravenously (IV) with 2-5 mls of $1.5 \times 10^9$ colony forming units (CFU)/ml \textit{C. jejuni} IA3902, 40-45 days prior to lambing. The ewes were first time single and twin pregnant whiteface and black-whiteface crosses, sourced from the Iowa State University Sheep Teaching facility and from which pre-inoculation serology testing showed very low levels of \textit{C. jejuni} specific antibodies via Enzyme
Linked Immunosorbent Assay (ELISA). *Campylobacter* was not isolated from a pre-inoculation rectal swab from any of the IV inoculated ewes either by culture of a direct fecal smear or fecal enrichment. Ewes were housed in individual pens in an ABSL 2 facility and were identified via ear tags and individual cage identification. Ewes were fed Teklad-Envigo 7060 small ruminant complete ration with water available ad lib. A group of 6 ewes was inoculated IV via an 18G, 2 inch long IV catheter placed in the jugular vein. The inoculation site was sheared and aseptically prepared using alternate Chlorhexidine® (chlorhexidine gluconate 2.0%) and Isopropyl alcohol (70%) swabs performed three times. The ewes received 2.0, 2.5 (3), 3.0 and 5.0 mls of 1.5x10⁹ CFU/ml *C. jejuni* IA3902.

Ewes were monitored three times per day for signs of ill health including depression, loss of appetite, prolonged recumbency, elevated temperatures, and abortion (genital bleeding, expelled fetus). Ewes that aborted were immediately euthanized with euthanasia solution (Beuthanasia®, sodium pentobarbital 390mg + sodium phenytoin 50 mg/ml) at 0.22mg/kg, intravenously using an 18 G needle, as per AVMA Guidelines on Euthanasia (AVMA, 2013).

Inspection for gross lesions and sample collection for bacterial culture and histologic examination were undertaken at necropsy. Samples collected for culture included maternal heart blood, bile from the gallbladder, fecal swab, intestinal contents, placenta, fetal stomach content, and fetal tissue homogenate (lung and liver). Swabs for microbial culture were sterile minitip cotton swabs with transport media. Fetal bile and stomach contents were collected by use of a sterile tuberculin syringe with a 26 G, 3/8 inch needle. Samples of uterus, placenta and pooled fetal lung and liver were placed in
separate sterile Petri dishes. Samples were immediately refrigerated after collection and bacterial culture was done on the day of collection,

For *Campylobacter* culture and semiquantitative enumeration of *C. jejuni* from necropsy samples approximately 250 microliters of bile, blood or fetal stomach contents were directly streaked onto culture media plates using a sterile cotton swab. Fecal swabs were directly streaked onto media. Placenta and uterus as well as pooled fetal and lung tissues were minced with sterile scissors or scalpels, swabbed and streaked onto media. The media was MH agar containing a *Campylobacter* selective supplement (trimethoprim, rifampicin, polymyxin B and cycloheximide) and a *Campylobacter* growth supplement (sodium metabisulfite, sodium pyruvate and ferrous sulfate). Incubation took place in anaerobic jars under micro aerobic conditions at 42 °C for 48 hours. *Campylobacter*-like colonies were counted on each plate to determine the number of CFU in each sample.

Samples harvested for histopathology included uterus, liver and gallbladder from each ewe, placenta, fetal lung and liver. Tissues for histopathology were placed in neutral buffered 10% formalin for 24 hours then transferred to 70% alcohol.

Special stains

Perls’ iron stain, Alcian blue pH 2.5 for acid mucins and the Periodic acid-schiff (PAS) reaction with and without diastase pretreatment for neutral mucins were performed on sections of ovine placenta confirmed positive by microbial culture and immunohistochemistry for *C. jejuni*. Serial sections were cut to 5 µm, stained and
assessed to determine the presence or absence of stained material with characteristics consistent with iron, acid mucin and neutral mucin within the placentome, chorioallantoic membrane, endometrium and endometrial glands. Staining was subjectively classified as low/absent, moderate or abundant, intracellular, extracellular, multifocal or diffuse.

Lectin Histochemistry

This technique follows the protocol described by Burrough et al., 2012. Three micron (3 µm) serial sections were set on aminoalkylsilane-coated glass slides, baked at 56 °C for 2 hours and 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% hydrogen peroxide (H₂O₂) in water. Antigen was unmasked by treating sections with Tris-EDTA (pH 9.0) in a stream bath for 20 minutes, cooled to room temperature, and rinsed 3 times in phosphate buffered saline (PBS) prior to placement in an automated cell staining system (BioGenex, US). The lectin used was the commercially available biotinylated *Lotus tetragonolobus* lectin agglutinin I (LTA; Vector, US) at a dilution of 1:100, 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) and chromogen (NovaRED, Vector) as per the manufacturer’s instructions; 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. Negative controls were prepared from serial sections with the lectin omitted and
replaced with dilution buffer. Lectin binding was subjectively quantified in the placentome, chorioallantoic membranes, endometrium and endometrial glands as low/absent, moderate or abundant and whether cytoplasmic or membranous.

Immunohistochemistry

Immunohistochemical analysis was performed for TLR4, NFκB and Caspase 3 on ovine placenta sections for which there was positive microbial recovery and positive MOMP antigen detection. These criteria were also applied to a recently confirmed *C. jejuni* positive field case that was obtained from the Veterinary Diagnostic Laboratory of Iowa State University and included here as a positive control. Healthy, confirmed uninfected ovine placenta was sourced as a negative control. Placenta collected at necropsy and embedded were sectioned at 3 microns, mounted on aminoalkylsilane-coated glass slides and baked for 30 minutes for at least 57 °C (2 hours at 56 °C for MOMP). Slides were deparaffinized routinely in xylene and rehydrated in graded alcohol solutions and water. Inhibition of endogenous peroxidase was via 2 immersions for 10 mins each in 3% H$_2$O$_2$ in water followed by 3 rinses in Ultra-Pure water. Slides were incubated for specific times at temperatures and in various antigen retrieval buffers (Table 1), then rinsed 3 times in PBS solution and stained automatically (BioGenex, San Ramon, CA). Nonspecific binding was inhibited by incubation with 10% neutral goat serum at 22°C for 20 minutes. The primary antibody used to detect *C. jejuni* was directed against the MOMP of the organism as described by Zhang$^{33}$ and Burrough$^7$ and used at dilution of 1:300. Antibodies directed against TLR4, NFκB and caspase 3 (Abcam,
Cambridge, MA) were each at dilutions of 1:100, and incubated at 22°C for specific times (Table 1), followed by 2 PBS rinses, a 5 minute PBS bath and then 2 PBS rinses. A commercially available biotinylated secondary antibody (BioGenex) was used at a dilution of 1:80 and incubated at 22°C for 15 minutes followed by 2 PBS rinses, a 5 minute PBS bath and then 2 PBS rinses. Slides were then incubated with horse radish peroxidase-streptavidin conjugate (Invitrogen, Carlsbad, CA) (1:200 dilution) at 22°C for 15 minutes followed by 2 PBS rinses, a 5 minute PBS bath and then 2 PBS rinses. The final reaction used a commercial chromogen, Nova Red (Vector, Burlingame, CA) for 5 minutes and then rinsed 5 times with Ultra-Pure water. Slides were routinely counterstained with Shandon Harris hematoxylin and Scott’s tap water. Slides were dehydrated through graded alcohol and xylene solutions prior to mounting. Positive controls were sourced from paraffin blocks of ovine placenta that was positive for *C. jejuni* IA3902 by microbial culture. Negative controls were obtained from uninfected control sheep. Internal controls were also included.

Apoptosis detection

The cascade of biochemical and molecular events that result in apoptosis involves endonucleases that cleave DNA in fragments with free 3’-OH groups at the ends which can be labeled and detected. In situ hybridization can be used to recognize and enable an understanding of the spatial distribution of apoptotic nuclei via detection of these labeled DNA fragments in paraffin-embedded ovine placental tissues. The Abcam *In situ* Apoptosis Detection Kit® was used according to manufacturer’s instructions via
completion of multiple steps in a standard IHC based assay. Slides were cut to 10 um and rehydrated. Reagents were thawed 30 mins prior to use, tissue was permeabilized using Proteinase K, endogenous peroxide was inactivated using 3% H$_2$O$_2$ for specified times. Tissues were labelled with TdT Enzyme, blocked with proprietary blocking buffer, incubated with conjugate and incubated with assay prepared DAB solution, counterstained with methyl green counterstain; dehydrated, cover-slipped and evaluated using an Olympus light microscope.

In this apoptosis assay, terminal deoxynucleotidyl transferase (TdT) binds to exposed 3’-OH ends of DNA fragments and catalyzes the binding of biotin-labeled deoxynucleotides which are detected using a streptavidin-horseradish peroxidase (HRP) conjugate. Diaminobenzidine (DAB) reacts with the HRP-labeled tissue section to produce an insoluble brown substrate at the location of the fragmented DNA. Methyl green was used to aid morphological evaluation of normal and apoptotic cells.  

The IHC method used to detect caspase 3 in this study was a very specific and sensitive method to identify apoptotic cells in paraffin-embedded tissue. This is because Caspase 3 is normally present intracellularly as a procaspase and once cleaved through the activation of the apoptotic cascade mediates further cleavage of itself and other downstream caspases and therefore its detection is a unique and sensitive indicator of apoptosis.  

Results

One sheep that was inoculated with 2.25mls *C. jejuni* IA3902 IV and aborted 48 hours post inoculation, had high microbial recovery (>1000 CFU) of *C. jejuni* from the
uterus and placenta and moderate microbial recovery (50-1000 CFU) from fetal lung and liver. There was positive red-brown staining for the MOMP \textit{C. jejuni} within trophoblasts of the placentome, within the lumen of endometrial glands (Figure 1) and within fetal chorionic villi (Figure 2) in this animal.

PAS stains were utilized to detect neutral mucins. There was moderate diffuse PAS positive staining of placentome basement membranes and trophoblasts of the chorioallantoic membranes had abundant, multifocal cytoplasmic staining. Within the endometrium, there were moderate amounts of staining for neutral mucins at the apical membrane and occasional low cytoplasmic staining. There was abundant staining for neutral mucins multifocally within endometrial glands (Figure 3).

Alcian blue stains were utilized to detect acid mucins. Diffusely, the stroma of the endometrium, fetal villi of the placentome (Figure 4) and chorioallantoic membranes had abundant positive staining for neutral mucins. Multifocally at the apical aspect of cell membranes of the endometrium and endometrial glands, there was abundant alcian blue positive staining for acid mucin.

There was low iron staining multifocally within the placentome and chorioallantoic membranes. These were located within trophoblast cytoplasm or as occasional extracellular deposits. There was also rare extracellular staining for iron within luminal content of endometrial glands in small amounts (Table 2).

\textit{Lotus tetragonolobus} (LTA) lectin-peroxidase conjugates are specific for fucose-containing glycoconjugates were used to detect L-fucose in tissue sections. Multifocally, there were small amounts of lectin staining at the apical cytoplasmic membrane of the
endometrial glands and endometrium (Figure 5). There were occasional foci of staining within luminal contents of endometrial glands (Figure 5).

There was abundant cytoplasmic staining in trophoblasts for NFκB in the positive control (field case), in the uninfected tissue (negative control) and in the IV inoculated sheep. Staining for TLR 4 was not detected in uninfected tissue or in the experimentally inoculated sheep but there was abundant multifocal staining in infected chorioallantoic villi from the field abortion case, (Figures 6 and 7).

There were small amounts of apoptotic nuclei randomly distributed throughout sections of placenta in no significantly quantifiable amount in the field case of C. jejuni abortion, the negative control and the experimentally inoculated ewe. There was a complete lack of caspase 3 signal detection from placental sections from inoculated sheep and from the negative control. In the field case, multifocally, there was abundant cytoplasmic staining for caspase 3 within stromal cells and trophoblasts of the chorioallantoic villi (Figure 8).

**Discussion**

In culture, mucins promote the growth of C. jejuni and in infected guinea pigs the organism localizes to regions of the placenta containing both neutral and acid mucin. Special stains and immunohistochemistry were used in order to determine potential factors that may contribute to this fetoplacental trophism in sheep. In this study the organism consistently localized to regions in the infected ovine placenta and uterus with strong staining for both neutral and acid mucin, that is, C. jejuni was found in the fetal
villi of the ovine placentome and within endometrial glands where there were significant amounts of mucin.

L-fucose is reported to be both growth promoting and chemoattractive for *C. jejuni*.\(^8\) Lectin histochemistry demonstrated that there was L-fucose present at the apical cytoplasmic membrane of the endometrial glands and endometrium and occasional foci within lumen contents of endometrial glands. This correlates well with the immunohistochemical detection of the organism within ovine endometrial glands in this study. Virulent strains of *C. jejuni*, of which IA3902 is an isolate, possess a genomic island (cj0480c-cj0490) that is upregulated in the presence of mucin and L-fucose, where fucose metabolism is independent of ATP or GTP activation\(^27\) and this may help to explain the localization of the organism within the uterus.

In murine studies,\(^19\) it was also found that *C. jejuni* is trophic for mucin and L-fucose. Overall, these findings suggest that after bacteremia, localization of the organism may be initially to the endometrium and endometrial glands, then to the fetoplacental unit. This is in agreement with histological findings in which there were large numbers of neutrophils within endometrial glands (endometritis) and few to no neutrophils within the body of the placentome 48 hours post intravenous inoculation of *C. jejuni* IA3902 (submitted for publication). Interestingly, endometritis was also reported in guinea pigs 48 hours post-challenge with IA3902\(^7\) and was a feature of *C. jejuni* induced abortion in sheep after IV inoculation.\(^14\)

Previously, it was shown that some strains of *C. jejuni* readily obtained iron from external sources like hemin and hemoglobin by a common pathway and possibly from
compounds other than heme.\textsuperscript{22} It was also thought that iron was necessary for \textit{Campylobacter} infection as there was decreased growth of the organism in iron deficient environments.\textsuperscript{9} In the current study, there was low iron staining multifocally within the ovine placentome, chorioallantoic membranes and in the endometrial glands where \textit{C. jejuni} was also localized by IHC. This is in agreement with findings from the guinea pig abortion model, where there was a low to moderate amount of iron intracellularly and extracellularly in the guinea pig placenta.\textsuperscript{8} While \textit{C. jejuni} IA3902 was found to be trophic for iron in the murine placenta which had moderate iron deposits within the parenchyma of the placental disc, the organism was also located at the periphery of the placental discs where iron deposits were low or absent (submitted for publication).

Interestingly, it was demonstrated that there was down-regulation of iron uptake systems in IA3902.\textsuperscript{31} Therefore it is possible that the hypervirulent strain may have adapted to multiply even in the apparent low iron environment of the ovine placenta.

The lack of significant signal for apoptosis and complete lack of signal in the caspase 3 IHC may suggest that placental apoptosis was not a significant feature of abortion 2 days post inoculation. In the field abortion case, multifocally, there was abundant cytoplasmic staining for caspase 3 within stromal cells and trophoblasts of the chorioallantoic villi. This is in agreement with another study that reported the upregulation of apoptosis genes with \textit{C. jejuni} abortion in sheep.\textsuperscript{24} Of note is that the sheep in that study aborted 2 weeks post oral inoculation and the effect of the delay in onset of abortion on apoptotic processes is unknown. \textit{C. jejuni} was reported to cause rapid oncotic rather than apoptotic death in enterocytes which was not prevented by caspase inhibition and which also suggests oncosis via a caspase independent
mechanism. This feature of *C. jejuni* is distinctly different from other enteric bacterial pathogens like *Salmonella* spp. that induce cell death via activation of caspases yet is in agreement with findings of *C. jejuni* caspase independent macrophage cell death. Additionally, *C. jejuni* has also been reported to activate cellular inflammasomes upon cellular invasion without cytotoxicity or cell death.

In different murine infection models TLR4 dependent signaling of *C. jejuni* lipopolysaccharide has been determined to be a key factor in immunopathology associated with the organism. *C. jejuni* endotoxin bound to its carrier protein engages the TLR4 along with the cytoplasmic membrane receptor CD14 and an accessory protein MD4. Once this occurs, through the activation of the transcription factor, NFκB and a signaling complex with downstream signaling through mitogen-activated protein (MAP) kinase and phosphatidylinositol-3 (PI3) kinase pathways, there is ultimately the transcription of proinflammatory cytokine genes and cellular release of cytokines like IL1 and TNF.

Three sheep from this study aborted between 12 and 48 hours post IV inoculation and this was hypothesized to be due to the effects of endotoxin because of the short time interval between inoculation and abortion and the presence of clinical signs in the ewes consistent with endotoxemia. Yet there was no signal detected in response to TLR4 antibody in the placentome from these sheep or the uninfected control, but there was abundant positive signal from the field case. This may suggest that while TLR4 endotoxin activation may not play a significant role in rapidly-induced experimental sheep abortions, such as occurred in our study, it likely plays a role in nature as there was
abundant staining for both *C. jejuni* and TLR4 in the chorioallantoic membranes of the field abortion case.

There was no discernible difference in NFκB signal from field case, uninfected control or inoculated sheep. One possible explanation for these results is that placental activation of TLR4 and production of NFκB was not the cause of the abortions seen in these experimentally infected sheep. It has also been reported that *C. jejuni* could activate NFκB independently of either TLR2 or TLR4.² From previous work from our lab, it was found that murine placental cytokine production played a role in the reproductive pathology of *C. jejuni* (submitted for publication). It is therefore also possible that different pathways are involved in *C. jejuni* endotoxin mediated abortion in sheep that are not primarily dependent on placental activation of NFκB production. Another possibility was that these abortions were more likely influenced by endotoxin mediated prostaglandin production, though Sanad *et al* reported down-regulation of the gene encoding prostaglandin E receptor 4 in a sheep that aborted compared to control sheep.²⁴

Very little is known about the pathogenic mechanisms of *C. jejuni* mediated abortion or the spatial localization of the organism and its trophic factors within infected ovine placenta. We confirmed the presence of the organism in uterus and fetoplacental tissue via high microbial recovery and IHC for the MOMP. Within the ovine placenta, *C. jejuni* IA3902 is trophic for L-fucose as indicated by multifocal areas of lectin staining at the apical cytoplasmic membrane of the endometrial glands and endometrium and occasional foci of staining within lumen content of endometrial glands. Another trophic factor within the sheep placenta was mucin, both neutral mucin and acid mucin, primarily
located within endometrial glands and fetal villi of the placentome, two locations with intense immunohistochemical staining for the organism. *C. jejuni* IA3902 was present within endometrial glands where low iron deposits were also rarely identified. NFκB did not appear to play a discernable role in ovine abortion caused by *C. jejuni* IA3902 in our studies.

Two days post inoculation significant amounts of apoptosis were not detectable in the ovine placenta after infection with *C. jejuni* IA3902 using a commercial assay and this was supported by absence of caspase 3 activation, a central executioner involved in both the intrinsic and extrinsic apoptotic pathways. In the *C. jejuni* confirmed field abortion case, multifocally there was abundant cytoplasmic staining of stromal cells and trophoblasts of the chorioallantoic villi for caspase 3 and multifocal, abundant staining of TLR4 within trophoblasts of the chorioallantoic membranes. Therefore the roles of caspase 3 and TLR4 in the pathogenesis of ovine *Campylobacter* abortion warrant further investigation.

**Acknowledgements**

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References


3 BDBiosciences: Caspase-3 Activation: An Indicator of Apoptosis in Image-Based Assays; 2012.


Table 1. Final optimized conditions for incubation times and Antigen Retrieval specifications for Immunohistochemistry and Histochemistry procedures

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Incubation</th>
<th>Antigen Retrieval Buffer</th>
<th>Heat induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOMP</td>
<td>1 hr</td>
<td>Protease (0.1% Tris pH7.4)</td>
<td>20 min at RT</td>
</tr>
<tr>
<td>Lectin</td>
<td>30 min</td>
<td>Tris at pH 9</td>
<td>20 min steam</td>
</tr>
<tr>
<td>NFκB</td>
<td>2 hr</td>
<td>Citrate buffer at pH 6</td>
<td>20 min steam</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>1 hr</td>
<td>Citrate buffer at pH 6</td>
<td>5 min microwave</td>
</tr>
<tr>
<td>TLR4</td>
<td>1 hr</td>
<td>Citrate buffer at pH 6</td>
<td>20 min steam</td>
</tr>
</tbody>
</table>

Table 2. Lectin histochemistry and special stains of ovine reproductive tissue

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Neutral mucin</th>
<th>Acid mucin</th>
<th>Iron&lt;sup&gt;b&lt;/sup&gt;</th>
<th>LTA&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placentome</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Chorioallantoic</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>membranes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometrium</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Endometrial glands</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values reported represent the average of all evaluated placental material
<sup>*</sup> - = negative; + = low; ++ = moderate; +++ = abundant
LTA, *Lotus tetragonolobus* lectin agglutinin
<sup>b</sup> Multifocal staining
<sup>c</sup> Apical membrane staining
Figures 1-8. Photomicrographs of ovine placenta and endometrial glands after infection with *C. jejuni* IA3902. (1 and 2) Placentome after IHC for the membrane outer protein of *C. jejuni* (MOMP), 1 – foci of positive stain within endometrial gland content and 2 – foci of positive stain within fetal villi. (3) Abundant PAS stain for neutral mucin within endometrial gland content. (4) Alcian blue stain for acid mucin with abundant staining of fetal villi. (5) Lectin histochemistry for LTA, with moderate to abundant multifocal, apical membrane staining of endometrial cells of the yolk sac and extracellular staining of endometrial gland content. (6) TLR4 IHC with abundant multifocal TLR4 staining within trophoblasts of the chorioallantoic fetal membranes (7) Higher magnification of TLR4 staining within trophoblasts x40 mag. (8) Caspase 3 IHC with cytoplasmic staining of stromal cells and trophoblasts of the chorioallantoic villi.
Conclusions and Recommendations for Future Research

A tetracycline resistant *C. jejuni* clone, *C. jejuni* clone SA has replaced *C. fetus* as the predominant *Campylobacter* species causing sheep abortion in the United States.\(^1\) Specific amino acid substitutions in loop 4 of the MOMP encoded by *porA* are responsible for the hypervirulence of this clone.\(^3\) The pathogenesis of placental infection caused by this agent and the mechanism of abortion induction are unknown. A primary objective of the previously described studies (chapters 2-5) was to attempt to elucidate important aspects of the pathogenesis of *C. jejuni* clone SA on the pregnant uterus.

Towards this end, several components of this research attempted to characterize aspects of the cellular and molecular pathology of *C. jejuni* clone SA in the fetoplacental unit and to improve animal models used to study the pathogenesis of this disease.

Pregnant mice can be used as a model to study the reproductive pathology of *C. jejuni* IA3902 using the endpoint of positive microbial culture of the organism from the fetoplacental unit.

In chapter 2, the pregnant murine model of *C. jejuni* IA3902 infection was investigated and it was demonstrated that the organism is trophic for neutral mucin, iron and L-fucose within the murine placental. These findings as well as the histological changes are similar to what has been demonstrated for infected guinea pig placentas. *C. jejuni* IA3902 has affinity for the murine reproductive tract, specifically the fetoplacental unit, where it is capable of producing a necrotizing placentitis with positive
microbial recovery after both IP and oral challenge in BALB/c and CD-1 pregnant mice. Gross reproductive pathology following inoculation included necrosuppurative placentitis, fetal resorption, intrauterine fetal death, stillborn pups and multifocal hepatitis. With further modifications of the murine model, it is likely that abortion, a feature of the ideal laboratory model would be induced. Experimental design changes to improve the model might include higher oral doses, challenge earlier in pregnancy and the use of genetically modified mice.

Pro-inflammatory and anti-inflammatory cytokines were produced from the C. jejuni infected murine placenta and these could be measured in the maternal serum and semi quantitated within the uterus and placenta of Campylobacter jejuni IA3902 infected mice by the use of in situ hybridization.

Interleukin 1 beta (IL1β), interleukin 10 (IL 10), interleukin 17A (IL17A), tumor necrosis factor alpha (TNF α) and interferon gamma (IFN γ) are expressed by specific cells from C. jejuni clone SA infected fetoplacental tissue. There was a statistically significant increase for each of these cytokines post infection with C. jejuni IA3902. The findings of chapter 3 demonstrate that: (1) Cytokines play a role in modulating the placental infection and reproductive pathology of C. jejuni IA3902 infected pregnant BALB/c and CD-1 mice (2) Cytokines can be measured in the maternal serum and that levels are increased after fetoplacental and uterine infection with the organism and (3) Trophoblasts within the placental disc are the main source of these cytokines. Interleukin 6 (IL6) did not appear to have a significant role in the uterine or placental pathology with C. jejuni IA3902 in either BALB/c or CD1 mice.
The cytokine studies identified cells producing specific pro-inflammatory and anti-inflammatory cytokines after the murine placenta is infected with \textit{C. jejuni} IA3902. This work demonstrated that cytokines could be semi quantitated using in situ hybridization and quantitated via maternal cytokine serum testing. Further work is needed to determine the exact role of each of these cytokines in modulating the course of infection with \textit{C. jejuni} and in the pathogenesis of fetal death and abortion caused by the pathogen. The use of cytokine agonists and antagonists in the therapeutic management of a wide variety of inflammatory conditions suggests consideration for the possible use of these agents to study the potential roles of these cytokines in the pathogenesis of abortion, with the eventual goal of evaluating the potential for pharmacologic management of infectious abortion.

\textbf{Fetoplacental infection in sheep was established following IV inoculation, though abortion within 48 hours does not mimic what is observed with naturally occurring disease.}

In Chapter 4, we report on our attempts to establish a sheep model for \textit{C. jejuni} clone SA abortion and investigated the effects on pregnant sheep after oral, intraperitoneal and intravenous inoculation of the pathogen. Large doses of \textit{C. jejuni} IA3902 administered orally and IP failed to result in demonstrable fetoplacental infection. Three sheep that were inoculated intravenously (IV) aborted and three died. In one of these sheep, \textit{C. jejuni} was recovered in high numbers from the placenta and uterus and in moderate numbers from the fetal lung and liver. In the placentome of this animal, there were degenerative histological changes with rounded, swollen trophoblasts, cellular
and nuclear debris as well as vascular congestion and hemorrhage. Immunohistochemistry (IHC) confirmed the presence of the organism within trophoblasts, fetal villi and endometrial glands. This is the first report of the organism within fetal villi and endometrial glands of infected ovine reproductive tissues.

Further analyses conducted on confirmed infected sheep placenta were reported in Chapter 5. Our ovine studies found that *C. jejuni* was localized to areas in the ovine placentome where there were significant amounts of neutral and acid mucin, including the fetal villi and endometrial glands and where L-fucose was present, also within the endometrial glands. *C. jejuni* IA3902 was found within endometrial glands where low iron deposits were also rarely identified. Significant amounts of apoptosis were not detectable and this was supported by absence of caspase 3 signal identification. In the *C. jejuni* confirmed field abortion case multifocally there was abundant cytoplasmic staining of stromal cells and trophoblasts of the chorioallantoic villi for caspase 3 and multifocal, abundant staining of TLR4 within chorioallantoic membranes. Therefore the roles of caspase 3 and TLR4 in the pathogenesis of ovine *Campylobacter* abortion therefore warrant further investigation.

**Sheep placenta infected with *C. jejuni* IA3902 demonstrate increased caspase 3 activity by stromal cells and trophoblasts of the chorioallantoic membrane compared with normal uninfected pregnant chorioallantoic ovine membranes.**

While this feature was not observed in experimentally inoculated sheep, this finding suggests that apoptosis is part of the mechanism of placental injury induced by *C. jejuni* clone SA and requires further examination. Caspase 1 and caspase 11 are involved
in NLRP3 inflammasome activation after Gram-negative bacteria LPS bind to TLR4 and the activation of these caspases in the pathophysiologic mechanisms of *C. jejuni* clone SA abortion should be analyzed.

*C. jejuni* IA3902 infected sheep placenta had increased expression of TLR4 from trophoblasts of the chorioallantoic membranes.

This is the first report of TLR4 up-regulation and warrants further investigation using larger numbers of infected sheep placenta. NFκB, a main downstream mediator that is activated upon binding of TLR4 did not demonstrate increased expression compared to uninfected placenta using light microscopy. These studies should be repeated using other techniques and testing for other downstream targets.

**References**


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