Immunogenic and protective properties of recombinant proteins from a highly pathogenic Campylobacter jejuni clone associated with sheep abortion

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Immunogenic and protective properties of recombinant proteins from a highly pathogenic *Campylobacter jejuni* clone associated with sheep abortion

by

Fei Wang

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

MASTER OF SCIENCE

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# TABLE OF CONTENTS

ABSTRACT .......................................................................................................................... iii

CHAPTER 1. GENERAL INTRODUCTION ........................................................................ 1

  THESIS ORGANIZATION ................................................................................................ 3

  LITERATURE REVIEW ..................................................................................................... 4

   Campylobacter: microbiology and genetics .................................................................. 4
   Human campylobacteriosis ......................................................................................... 6
   Ovine campylobacteriosis ......................................................................................... 8
   Virulence factors of C. jejuni .................................................................................. 12
   Treatment ................................................................................................................. 21
   Prevention and Vaccines ......................................................................................... 22

  REFERENCES .................................................................................................................. 24

CHAPTER 2. IMMUNOGENIC AND PROTECTIVE PROPERTIES OF RECOMBINANT
     PROTEINS FROM A HIGHLY PATHOGENIC CAMPYLOBACTER JEJUNI CLONE
     ASSOCIATED WITH SHEEP ABORTION ................................................................. 43

   INTRODUCTION ........................................................................................................ 44
   MATERIALS AND METHODS ................................................................................... 49
   RESULTS .................................................................................................................... 59
   DISCUSSION .............................................................................................................. 64
   CONCLUSION ........................................................................................................... 69
   ACKNOWLEDGEMENTS .......................................................................................... 70
   REFERENCES ............................................................................................................. 70

CHAPTER 3. GENERAL CONCLUSION ...................................................................... 86

ACKNOWLEDGMENTS .................................................................................................... 88
ABSTRACT

*Campylobacter jejuni* clone SA has recently emerged as the prevalent cause of *Campylobacter*-associated sheep abortion in the United States. To develop effective vaccines against *C. jejuni* clone SA in sheep, it is necessary to identify the antigens that elicit protective immune responses. Recently, by using immunoproteomic approaches we successfully identified a number of clone SA proteins that were consistently immunoreactive with multiple convalescent sheep and guinea pig sera. In this study, as a first step towards developing an efficacious subunit vaccine against sheep abortion, we began to further characterize these proteins. Accordingly, 7 clone SA proteins were selected, which included HtrA, CgpA, CJSA_0852, Peb4, FabG, MetK and FlgL. Recombinant proteins for each of these antigens were produced in an *E. coli* expression system, and their reactivity with a panel of convalescent sera obtained from *C. jejuni*-infected ewes and guinea pigs were tested using immunoblotting. The results showed that CgpA, MetK, FabG had the strongest antigenicity, while HtrA, FlgL and Peb4 were less antigenic, and CJSA_0852 had little reactivity with the sera tested. CgpA, HtrA and FlgL were chosen to further evaluate the induction of protective immunity against bacterial challenge in the mouse model of systemic infection and bacteremia. Immunization of mice with recombinant CgpA, HtrA and FlgL induced high level of specific antibodies, but only CgpA-immunized mice showed a significant decrease in the level of bacteremia compared with the control mice. Analysis of different cellular fractions demonstrated that CgpA is a periplasmic protein. These results indicate that CgpA may be a potential subunit vaccine candidate against sheep abortion caused by *C. jejuni*. 
CHAPTER 1. GENERAL INTRODUCTION

Introduction

*C. jejuni* has become one of the most predominant etiologic agents of acute gastroenteritis in humans all over the world (1), which leads most research of *Campylobacter* to focus on *C. jejuni*. Patients who are susceptible to *Campylobacter* infection are different between developing and developed countries. *Campylobacter* infection is also one of the most predominant causes of ovine abortion worldwide (2). Infection is mostly associated with oral exposure to infected products of parturition (3). Historically, *C. fetus* subsp. *fetus* was regarded as the majority of the *Campylobacter* spp. associated with ovine abortion, nevertheless, recent studies pointed that *C. jejuni* is increasingly related with ovine abortions, instead of *C. fetus* (2-7). Although the highly variable genetics and strains of *Campylobacter* associated with sheep abortion worldwide, in the United States there is a predominant *C. jejuni* clone causing the majority of *Campylobacter*-associated ovine abortions (named clone SA for sheep abortion) (2). This is a surprising finding and demonstrates that clone SA has developed means to adapt to the agricultural practices in sheep production and/or owns novel virulence traits, which have facilitated its dominance as a successful pathogen in the United States. Furthermore, *C. jejuni* clone SA poses an important threat to public health due to zoonotic transmission of this emergent clone to humans mainly via raw milk, which has been recently demonstrated by molecular and epidemiological evidence (8). Moreover, it is possible that chemoprophylaxis
might have in part enhanced the emergence of this tetracycline-resistant clone because tetracyclines are commonly used in the United States for the prevention and treatment of campylobacteriosis in sheep (2, 9).

*C. jejuni* is an enteric pathogen and is transmitted mainly via the fecal-oral route. To cause an infection, first, *C. jejuni* must be able to tolerate the acidity in the stomach and colonize the intestines (10-12). Colonization of the gut mucosa is determined by the corkscrew morphology, flagellar motility, and its ability of adhesion to the mucosal layer (11, 12). Once colonized, the organism may trigger inflammation and epithelial cell damage with fluid secretion (13). As with other bacterial pathogens, almost all *Campylobacter* virulence factors are located on the bacterial cell surface or are secreted (14).

It is widely believed that a vaccine must be able to stimulate intestinal immunity to be effective against an enteric bacterial pathogen (15-18). Three different strategies have been considered for the development of a reliable vaccine against *C. jejuni*. The first strategy is live attenuated *Campylobacter* vaccine, which is considered as an attractive approach (19). Nevertheless, a study using a viable non-colonizing strain of *C. jejuni* failed to elicit protective immunity in chicks (20). Furthermore, the association of the neuropathy and autoimmune disease Guillain–Barre syndrome (GBS) with *C. jejuni* infections impedes the development of whole cell vaccines (21). The second one is killed whole cell vaccine, which can offer several advantages as potential vaccines for mucosal immunization. Due to their microparticle nature, whole dead cells should enhance the interactions between their surface and mucosal lymphoid tissues (22). A recent
study has demonstrated that a limited protection was conferred by *C. jejuni* killed cells after oral vaccination in ferrets (23). Using specific proteins/antigens of *Campylobacter* as subunit protein vaccines is another promising strategy for vaccination. Two *Campylobacter* antigens, PEB1 and flagellin (24, 25), have been evaluated as vaccine candidates. However, *Campylobacter* flagellins have different levels of glycosylation and high antigenic diversity, which makes the development of a flagellin-based vaccine problematic (26, 27). A recent study also demonstrates that significant levels of anti-PEB serum IgG did not protect against *C. jejuni* after oral challenge in mice (28). In this study, we selected 7 of these proteins to evaluate their potential as subunit vaccine candidates. The selection is based on the predicted functions, cellular localization, consistent sero-reactivity, and sequence conservation among *C. jejuni*. This list included 3 periplasmic proteins (HtrA, CgpA, CJSA_0852), one outer membrane protein (Peb4), two cytoplasmic proteins (FabG and MetK), and one extracellular membrane protein (FlgL). Our results suggest that CgpA may be a promising subunit vaccine candidate against sheep abortion caused by *C. jejuni*.

**Thesis Organization**

This thesis consists of three chapters. The first chapter is a literature review that includes a general overview of *Campylobacter* (mainly *C. jejuni*) and *Campylobacter* abortion, virulence factors of *Campylobacter* and Current vaccine development. The second chapter talks about Immunogenic and protective properties of recombinant proteins from a highly pathogenic *Campylobacter jejuni*
clone associated with sheep abortion. The third chapter is a general conclusion. The references cited throughout the text are located immediately after each chapter.

**Literature review**

**Campylobacter: microbiology and genetics**

*Campylobacter*, a genus of small and gram-negative microorganisms that have curve or spiral shaped morphology, has attracted broad attention and research efforts over the last decades. Most *Campylobacter* species are motile bacterial organisms with one or two unsheathed polar flagella attached to their polar ends (29, 30). *Campylobacter jejuni* is inhabitant in a microaerophilic environment for optimal growth at 5% O$_2$, 10% CO$_2$ and 85% N$_2$ between 37 °C to 42 °C (31, 32). *Campylobacter* spp. are relatively fastidious organisms, which do not usually make use of carbohydrates, but utilize amino acids as the main carbon source (33).

The first reports of the organism originated from an investigation on epizootic abortion of cattle initiated at the request of the British Board of Agriculture and Fisheries in 1913 (34, 35). In 1927, Smith and Orcutt discovered a microorganism isolated from feces of the cattle, which were infected with diarrhea, and named this bacterium as *Vibrio jejuni* (36). Later, another bacterium was isolated from feces of diarrheal pig, which was named *Vibrio coli* by Doyle in 1944 (33, 36, 37). The disease in humans was first reported from outbreak of enteritis in 1938 due to unpasteurized milk (38). Fecal cultures were negative, but fecal smears and blood cultures were observed to contain *V. jejuni*-like organisms (35, 38).
The genus *Campylobacter* was initially known as *V. fetus* and *V. bubulus* in 1963 (39). Separation from *Vibrio* was later proposed due to the low G+C content, non-fermentative metabolism and microaerophilic growth nature of *Campylobacter* (40). Currently, the genus *Campylobacter* consists of more than 25 heterogeneous species, which are *C. coli*, *C. jejuni*, *C. lari*, *C. fetus*, *C. upsaliensis*, *C. helveticus*, *C. hyointestinalis*, *C. concisus*, *C. mucosalis*, *C. curvus*, *C. rectus*, *C. showae*, *C. gracilis* and *C. sputorum and other more*. It is possible, but not always reliable to differentiate between *Campylobacter* species by traditional biochemical methods and growth characteristics, especially for the biochemical tests (39). For instance, based on the growth temperature, *Campylobacter* species have been characterized as non-thermophilic, such a *C. fetus*, and thermophilic such as *C. jejuni* and *C. coli* (41, 42). Molecular methods are commonly used to differentiate *Campylobacter* species and subspecies today. Some examples include restriction length fragment polymorphism (RFLP), pulse field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), and amplification/sequencing of 16S/23S rRNA genes or species specific genes by PCR (8, 43-46).

*C. jejuni* is the main pathogenic specie implicated in human infections, which leads most research of *Campylobacter* to focus on *C. jejuni*. Currently an increasing number of *C. jejuni* strains are being whole-genome sequenced, which include RM1221, 81-176, 81116, IA3902, ICDCCJ07001, M1, S3, NCTC 11168, 260.94, CG8421, CG8486, BH-01-0142, HB93-13, CF93-6, 84-25 and doylei. A rather small circular chromosome, which is only 1.6 to 1.8 megabases, exists in *C. jejuni* with low G+C content (47), which is especially low in the
lipooligosaccharide (LOS) and extracellular polysaccharide (EP) regions (48). *C. jejuni* can take up DNA directly from the environment and produce more genetic diversity, including the development of antibiotic resistance (12). The genome of *C. jejuni* contains a high rate of variable sequences particularly in the genes of modification or biosynthesis in surface structures (49). Multiple genome sequences of *C. jejuni* strains with different phenotypic traits have demonstrated the epidemiology and pathobiology of this organism through the use of comparative genomics.

**Human campylobacteriosis**

*C. jejuni* has become one of the most predominant etiologic agents of acute gastroenteritis in humans all over the world (1). Patients who are susceptible to *Campylobacter* infection are different between developing and developed countries. Campylobacteriosis occurs in all ages, especially for children who are less than 4 years old and adults who are 15 to 39 years old in developed countries (50). In contrast, developing countries witness a peak in clinical cases during childhood and then a sharp decline in cases with age (29). Additionally, in developed countries, *C. jejuni* infection is more serious than in developing countries. The prevalence of asymptomatic infections are approximately 15% in developing countries (29). This appears to be mimicked in developed countries where occupationally exposed individuals, such as poultry abattoir workers,
experience clinical disease in the initial stage of employment, but rarely develop clinical disease as employment continues (29, 50, 51).

Campylobacteriosis typically has an incubation period of 1-2 days (52). Symptoms include fever, nausea, bloody and watery diarrhea, and abdominal pain with fever sometimes starting 1-2 days before diarrhea (52). It takes approximately 2-5 days to develop to acute diarrhea after infected with *Campylobacter*. Generally, the symptoms can persist nearly two weeks. Only about 15% of infected persons vomit, though most patients feel nauseous (11). Immunocompromised individuals, particularly those with humoral immune deficiencies like AIDS tend to have more severe disease with bacteremia (29). One third of patients may have influenza virus-like clinical symptoms like headache, fever, myalgia and dizziness before they suffer from diarrhea. This phenomenon indicates that systemic immune response influences the effect of local infection (29, 53). Furthermore, the variable phenotypic expressions of the disease also associate with diversity of bacterial virulence ability and immune response from different hosts (54, 55).

Post-infectious sequelae, like Guillain Barre syndrome (GBS) and reactive arthritis (ReA), may appear after acute phase for some individuals with self-limiting of *Campylobacter* infections (29, 56, 57). GBS is a disease defined as “a progressive motor weakness of more than one limb with low or absent reflexes” by Asbury and Cornblath (57, 58). Reactive arthritis is a sterile inflammation of the joints occurring within 4 weeks of a bacterial intestinal or urinary infection (56). Both GBS and ReA are believed to result from autoimmune disease secondary to immune recognition of *C. jejuni* like molecules, noted as molecular mimicry (56,
Previous studies indicate that the primary reason of GBS caused by *Campylobacter* is molecular mimicry, which happens between structures on the LOS (lipooligosaccharides) of some *Campylobacter* strains and peripheral nerve glycolipids or myelin proteins (1, 59).

**Ovine campylobacteriosis**

One of the most vital factors related with the profitability of small ruminant operations is reproductive performance (60). Moreover, such epizootics of abortion of small ruminants might cause severe economic losses (61). *Campylobacter* infection is one of the most predominant causes of ovine abortion worldwide (2). Infection is mostly associated with oral exposure to infected products of parturition (3). However, it is quite common that water supplies are also contaminated by *Campylobacter* of feces origin (62) and the time of fecal excreting of the organism has been observed to increase with the rate of abortion in sheep flocks (3). Historically, *C. fetus* subsp. *fetus* was regarded as the majority of the *Campylobacter* spp. associated with ovine abortion, nevertheless, recent studies pointed that *C. jejuni* is increasingly related with ovine abortions, instead of *C. fetus* (2-7). Kirkbride et al. first reported this shift in the late of 1980s (6), and the proportion of *C. jejuni* isolates in ovine abortion has increased substantially in recent years (2, 5). Although genetically diverse strains of *Campylobacter* are associated with sheep abortion worldwide, in the United States there is a predominant *C. jejuni* clone causing the majority of *Campylobacter*-associated
ovine abortions (named clone SA for sheep abortion) (2). This is a surprising finding and demonstrates that clone SA has developed means to adapt to the agricultural practices in sheep production and/or owns novel virulence traits, which have facilitated its dominance as a successful pathogen in the United States. Furthermore, *C. jejuni* clone SA poses an important threat to public health due to zoonotic transmission of this emergent clone to humans mainly via raw milk, which has been recently demonstrated by molecular and epidemiological evidence (8). Moreover, it is possible that chemoprophylaxis might have in part enhanced the emergence of this tetracycline-resistant clone because tetracyclines are commonly used in the United States for the prevention and treatment of campylobacteriosis in sheep (2, 9).

The pathogenesis of ovine campylobacteriosis is shown to entail ingestion of contaminated material after oral exposure (63) with mucosal invasion and placental localization subsequent to intestinal colonization (64). *Campylobacter* can be carried in the gall bladder and intestines of healthy sheep with no risks of clinical diseases (2, 65-67). Previous studies have reported that gall bladder colonization by *C. fetus* follows oral inoculation (68), intravenous inoculation (69, 70), natural abortion (68), and in ewes intravenously inoculated, gall bladder colonization was always related with concurrent intestinal colonization (70), thus supporting the potential role of gall bladder colonization in overall disease epidemiology and in the development of carrier ewes (69). Infection in sheep is predominantly intestinal (71), and involvement of the uterus is regarded by some to be an accidental result of bacteremia in non-immune pregnant ewes. However, systemic infections can be
caused by highly virulent *C. jejuni* strains which can invade into circulation (72). One significant step in the pathogenesis of *Campylobacter*-associated abortion is accessing to the blood stream and systemic spread followed by invasion of the intestinal epithelium. For *C. fetus* subsp. *fetus*, systemic infection is depended on the expression of surface layer (S-layer) proteins (73), which suppress complement 3b (C3b) binding (74) and therefore inhibit complement mediated killing mechanisms. Nevertheless, these proteins are not expressed by *C. jejuni*. Pathogenic isolates of *C. jejuni* are thought to have a microtubule dependent invasion system (75), and generally invasive (76), whereby bacteria travel along microtubules to the perinuclear area (77) after taken into an endosomal vacuole and then replicate within intestinal cells (77). These internalized bacteria move within vacuoles (78) to the basolateral membrane for exocytosis (75). The presence of functional flagella is a critical factor in cellular uptake of *C. jejuni* (79) and efficient colonization (80, 81), whereas flagellar paralysis allows adherence instead of invasion of eukaryotic cells (82). Many strains of *C. jejuni*, as well as *C. fetus* and *C. coli*, generate cytolethal-distending toxin (CDT) (83), a nuclease that induces double strand breaks in DNA of both non-proliferating and proliferating host cells, thereby resulting in cell death or cell cycle arrest (84). These dying or dead cells in the mucosa may cause considerable disruption of the epithelial barrier to allow bacteria invade into the lamina propria. In sum, these findings provide an overall understanding of the pathogenesis of septic abortion associated with *Campylobacter* spp.; nevertheless, the mechanisms of the observed variation in
virulence between *Campylobacter* strains and species and the requisite steps in this process remain further explored.

For pregnant ewes that are susceptible to infection, initial exposure may cause intestinal invasion, followed with placentitis, fetal infection and abortion, which commonly occurs in the last third period of pregnancy (3, 72, 85, 86). During the initial stage of infection, there is no signs of clinical disease in the ewes; however, ewes may die due to uterine sepsis and septicemia occasionally if the *fetus* dies and remains in utero (2, 3). Abortion generally takes place 7 to 25 days post-exposure (61) and initially, only a small quantity of ewes abort, followed by a rapid increase in abortion (i.e., abortion storms) 2 to 3 weeks later (87). If the start of placental infection is delayed until the very late stages of pregnancy, lambs may be born live, but are typically too weak to survive.

Thickened uterine walls with edema, swollen caruncles and placentas with mottled swollen cotyledons are typical lesions in aborted ewes (88). Aborted *fetuses* can be autolyzed mildly to severely and have serosanguinous fluid in the thorax and abdomen, and focal liver necrosis (2, 3, 88, 89). Generally, lesions are focused on the placentomes whereas the intercotyledonary spaces are less severely infected (71). Fetal lesions are not always the same and the characteristics include nonspecific edema, suppurative bronchopneumonia and random necrotizing hepatitis with a targetoid gross appearance (71). Histologically, the aborted placentas have the signs of acute suppurative placentitis with necrosis of cotyledons and congestion; trophoblasts can be distended with intracytoplasmic organisms that can be stained with Giemsa stain (88, 89).
Virulence factors of C. jejuni

C. jejuni is an enteric pathogen and is transmitted mainly via the fecal-oral route. To cause an infection, first, C. jejuni must be able to tolerate the acidity in the stomach and colonize the intestines (10-12). Colonization of the gut mucosa is determined by the corkscrew morphology, flagellar motility, and its ability of adhesion to the mucosal layer (11, 12). Once colonized, the organism may trigger inflammation and epithelial cell damage with fluid secretion (13). As with other bacterial pathogens, almost all Campylobacter virulence factors are located on the bacterial cell surface or are secreted (14). Below is the description on some of the best characterized virulence factors involved in Campylobacter pathogenesis.

a) Flagella

In the period of hosts’ infection, C. jejuni lives primarily within the thick mucus layer lining the intestinal epithelium (90-92). C. jejuni flagella and its corresponding motility are of utmost importance in the pathogenic process, including host cell invasion and protein secretion, and host colonization; non-motile C. jejuni strains are attenuated for colonization of human or animal hosts (93-95). Moreover, Wassenaar and Blaser (52) concluded that aflagellated C. jejuni mutants displayed a dramatic decline of internalization by host cells, indicating the significant role flagella plays in invasion. Two different viscosity dependent modes of motility exists in C. jejuni in cecal mucosal scrapings from
infected mice (92). At low viscosity, *C. jejuni* swims via rather straight vertical movements without direction changes (96, 97). While at high viscosity, a darting motility characterized by oscillation along a relatively short and straight path is observed (97, 98). Regarding the structure, the flagellar filament is comprised of flagellin subunits encoded by FlaA (major) and FlaB (minor) (99). Whereas FlaA is essential for flagellation and motility, the requirement of FlaB varies among *Campylobacter* species (100-103). A two-component system, including the sensor FlgS and the response regulator FlgR, is crucial in the control of the transcriptional expression of *Campylobacter* flagellum (104). The flagellar hook is primarily composed of the protein FlgE and hook-filament junction proteins FlgK and FlgL. The *C. jejuni* FlgL mutant is not capable of exporting and delivering the *Campylobacter* invasion antigens, Cia proteins (105). Apart from agglutination, attachment and biofilm formation, bacterial flagella also enable chemotaxis that plays a vital role in both the commensal and pathogenic lifestyle of *C. jejuni* (12). Initially, *C. jejuni* was reported to be attracted to amino acids, carbohydrates and organic acids (106). Several of these responses, however, were subsequently found to occur in non-chemotactic mutants and thus represent false-positive responses (107). Several compounds have withstood additional chemotaxis assays, however, fucose, pyruvate, aspartate, fumarate, and formate can be attracted for *C. jejuni* (106, 108-111). To sum up, these findings demonstrate that flagella and associated components are indispensable factors for the initial interaction of *C. jejuni* with the host, causing colonization of the intestine and subsequent pathology to host.
b) Cell wall structures

Several virulence factors are located in/on the cell wall of *C. jejuni*. There are three layers in the outer-membrane of Gram-negative bacteria, an outer lipoprotein layer, a middle lipopolysaccharide layer and an inner mucopeptide layer. The main component of bacterial surface is polysaccharides, which are crucial in the interactions among pathogens, hosts and the environment (112, 113). During the 1990s, Aspinall and co-workers discovered that *Campylobacter* (*C. jejuni*, *C. coli*, and *C. lari*) exposed polysaccharides (PSs) that were considered to be O-chain PS regions of cell wall lipopolysaccharides (LPS) (114-119). However, subsequent genomic analysis demonstrated that the observed PSs of *C. jejuni* were actually capsular polysaccharide (CPS) (120). *Campylobacter* species, like other Gram negative mucosal pathogens, express a CPS, but unlike other enteric pathogens it possesses a lipooligosaccharide (LOS) instead of a full length LPS (121). Polysaccharides of *C. jejuni* mainly include CPS, LOS, N-linked protein glycans and O-linked flagellar sugars. The LOS and CPS generated from distinct strains of *C. jejuni* are diverse in structure and highly variable. A large quantities of genes in these hypervariable regions are also subject to phase variation, leading to phenotypic and genetic diversity among *C. jejuni* strains, which may play a crucial role in immune evasion during *C. jejuni* infection (122).

The LOS structure is composed of a lipid A anchor and an oligosaccharide which has an unchanged inner core and a variable outer core, therefore it is denoted as LOS, for it does not contain the O-antigen, which exists in lipopolysaccharides (123-125). To date, the relationship between *C. jejuni* and the debilitating
neuropathy GBS has been thoroughly characterized (126, 127), in which the LOS structures on some C. jejuni strains resemble the human gangliosides on peripheral nerves. The capsular polysaccharide (CPS), which is the outermost structure on the bacterial cell, may play a vital role in the interaction among the pathogen, host and environments (127). The CPS is a key virulence factor due to its importance in epithelial cell adherence, serum resistance and invasion for C. jejuni (124, 128). CPS-based vaccine strategies have been very successful in reducing the overall disease incidence of several encapsulated bacteria including Neisseria meningitidis, Streptococcus pneumoniae, and Haemophilus influenza (129, 130) and are currently in the testing phase for controlling C. jejuni infection. Previous studies have shown that N-linked glycosylated proteins might play a key role in adherence, invasion and colonization in C. jejuni (93, 131-136). The first sequenced C. jejuni (strain NCTC11168) genome in 2000 confirmed that a genetic locus termed pgl encodes the oligosaccharyltransferase (OST) PglB, which is involved in general protein glycosylation (137, 138). Functional recombinant expression of the general glycosylation locus in Escherichia coli (139), together with the relaxed specificity of PglB, enable the production of novel glycoprotein combinations with potential applications in vaccine development (140). The most successful human vaccines so far are usually glycoconjugate-derived where the combination of a protein coupled to a glycan elicits both T-cell-dependent and T-cell-independent responses, evoking a protective and lasting immunity as mentioned above.
c) Cytolethal distending toxin

Cytolethal distending toxin (CDT), the only *Campylobacter* toxin which is verified to date, is thought to be a key virulence factor in this pathogen (141). It is produced by multiple *Campylobacter* spp. such as *C. lari, C. jejuni, C. coli, C. fetus,* and *C. upsaliensis* (104, 142). Previously, CDT was considered as periplasmic protein of *C. jejuni*, implicated in adherence, invasion of and cytotoxicity towards host cells (143). A recent study demonstrated that outer membrane vesicles (OMVs) function as the vehicle for CDT to release to the outer surface of *C. jejuni* (144). OMVs are common to a wide spectrum of Gram-negative bacteria and are generated during the normal metabolism and cell growth or under stress (145, 146). Once the OMVs are free from the bacterium, they appear as small membrane vessels including periplasmic constituents and outer membrane components. OMVs may serve as delivery vehicles for bacterial virulence factors without typical signal sequences (147, 148) and are potentially rich in antigens that are the initial targets for innate and adaptive immune recognition (149), and thus may generate protective immunity against bacterial challenge when used as an immunogen (150). CDT holotoxin is comprised of three units which are encoded by the genes *cdtA, cdtB* and *cdtC* (151). It seems that CdtA and CdtC interact with CdtB to produce a tripartite CDT holotoxin, which is essential to deliver CdtB, the enzymatically active subunit (152). Subsequently, the CdtB active subunit, which shows DNaseI-like activity, breaks double strand in the host, causing DNA damage (153). In sum, precise role of CDT in *Campylobacter*
pathogenesis is not clear; CDT might play a critical role in the invasiveness and modulation of the immune response instead of inducing diarrhea directly (154).

d) Invasion and adhesion

One of the most important aspects of virulence in *Campylobacter* is interaction with intestinal cells (155). *Campylobacter* adheres to intestinal cell lining at first and then may become internalized within the cells. The organism can get access to sub-mucosal tissue leading to tissue damage and inflammation, followed by gastroenteritis. This is thought to be the virulence mechanism of *C. jejuni* moving across epithelial cell barrier. Previous studies have confirmed certain putative adhesions and binding factors of *C. jejuni*, such as the surface exposed lipoprotein JlpA (156), periplasmic binding protein Peb1 (157), outer membrane fibronectin-binding protein CadF (158), and the auto-transporter protein CapA. The surface-exposed lipoprotein JlpA is essential to bind the host (156). Peb1 is the periplasmic protein binding component of an amino acid ABC transporter in *C. jejuni* (157), can bind glutamate and aspartate, and if they are severed as the major carbon source, Peb1 mutants cannot survive (159). CadF, an outer membrane protein (OMP), is expressed in all *C. coli* and *C. jejuni* strains. CadF is indispensable for maximal binding and invasion by *C. jejuni* by binding to the cell matrix protein, fibronectin (104). The CadF mutants are dramatically decreased in ability in chick colonization compared with the wild type (160, 161). CapA is another auto-transporter lipoprotein which adheres to epithelial cells in human and chicken (162). A number of studies with variable animal models have shown that
early mucosal damage can be caused by the invasion of C. jejuni in the colonic epithelial cells. Therefore, the invasion ability of C. jejuni has been confirmed as an important pathogenicity-associated factor (163-166). Nevertheless, distinct strains of C. jejuni show different ability to invade (167). C. jejuni strains invade gut intestinal cells by a microtubule- and actin filament-dependent mechanism (168-172).

e) Stress response

Compared to other food-borne bacterial pathogens, C. jejuni has a limited ability to grow in the environment, which partially is owing to its complex nutritional requirements and lack of corresponding adaptive responses (104). Campylobacter lives on intermediates of the tricarboxylic acid (TCA) cycle as a source of energy and cannot readily metabolize carbohydrates due to the lack of glycolytic enzyme phosphofructokinase. Therefore, it seems that C. jejuni mainly depends on scavenging amino acids and keto acids from the host or from intestinal microbial flora. Genome analyses concluded that C. jejuni does not possess the global stress response regulator RpoS, which is prerequisite for the survival of Gram-negative bacteria upon exposure to various types of environmental stresses (120).

The ability to acquire iron in the animal host is important to pathogenic bacteria in establishing infection (173). Less availability of free iron in mammalian fluids is a main problem for C. jejuni in establishing infection. The forms for most of the iron in the cells are lactoferrin, transferrin, iron binding glycoproteins, heme
or ferritin (174). Iron sulphur proteins participate in anaerobic respiration, energy metabolism, electron transport and amino acid metabolism. *Campylobacter* is exposed to the reactive oxygen species in human immune response, which is important when it is internalized by the phagocytes. Heme compounds like hemin, ferric iron, ferrous iron and hemoglobin can support the growth of *C. jejuni*. Additionally, *C. jejuni* has many systems for siderophore-mediated uptake of iron, like enterochelin transport system, siderophore systems encoded by gene cj0718 and cfr A. This iron uptake system is controlled by a global regulator, Fur, in *C. jejuni* (174).

Most of *Campylobacter* species are sensitive towards high levels of oxygen and are microaerophilic, including the important species (such as *C. jejuni*) directly associated with food-borne infection. Superoxide dismutase can catalyze the dissimilation of superoxides into hydrogen peroxide and oxygen and remove superoxide. Further peroxidase or catalase removes the produced hydrogen peroxide. *Campylobacter* converts hydrogen peroxide to water and oxygen under the help of heme cofactor and catalase (175). *C. jejuni* and *C. coli* express a single catalase designated Per R which is the regulator and Kat A is iron repressed (175). Many species of bacteria utilize their osmo-regulatory mechanisms to cope with osmotic stress, but in *Campylobacter* such system has not been identified yet (120).

*Campylobacter* is able to produce cold shock proteins. At temperature as low as 4ºC, *Campylobacter* can still respire, generate ATP, but is unable to replicate. However, *C. jejuni* exhibits chemotaxis even at this low temperature, indicating that it may migrate to places which has more favorable conditions and avoid certain
environmental stress conditions (176). Overall, these strategies may explain the ubiquitous distribution of *C. jejuni* and its prominent role as an enteric bacterial pathogen.

**Epidemiology**

*C. jejuni* normally lives in gastrointestinal tracts of copious food producing animals as a commensal organism. The most prone hosts for *Campylobacter* are the avian species due to their higher body temperature (36, 177). Therefore, *C. jejuni* has a widespread presence in turkeys, chickens, and wild birds such as seagulls and geese (178) (179). Carriage of *Campylobacter* by wild birds results in contamination of waterways, and as *Campylobacter* can survive in water for weeks, open waters may serve as a source of infection for poultry and domestic animals (54). The majority of *Campylobacter* infections in humans are associated with unprotected handling of raw chicken or consumption of undercooked chicken (180-182). *Campylobacter* also commonly exists in many types of food animals other than chickens, such as turkeys, cattle, pigs, dairy cows, lamb or duck (183). Another significant reservoir for a number of *Campylobacter* spp. is the digestive tract of healthy cattle, in which prevalence ranges at 0-80% (184), while the prevalence in sheep is about 20-50% (185). Moreover, high carriage rate of *Campylobacter* in pigs has also been reported (186), but the role of pork in human infections does not appear to be a major concern. Besides contaminated poultry meat, contact with farm animals and pets, and consumption of raw cow milk and untreated water are significant risk factors for human *Campylobacteriosis*. 
A recent study (2) by Sahin et al. concluded that a highly pathogenic *C. jejuni* clone (clone SA) has become the predominant cause of ovine abortions associated with *Campylobacter* in the United States, indicating that this *C. jejuni* clone is well adapted to ruminants. Furthermore, clone SA was found in healthy sheep and cattle, and in cows and goats with abortion, suggesting that the clone SA acts as both commensal and a pathogen in ruminants.

**Treatment**

*C. jejuni* infections are often self-limiting and there is usually no rationale for antimicrobial treatment, but under certain circumstances, medication can alleviate severe symptoms. Antibiotic therapy is often prescribed to patients who are immunocompromised or exhibit severe clinical symptoms including bloody diarrhea, high fever, pregnancy, and prolonged infection (187). *C. jejuni* is known to be intrinsically resistant to several antibiotics, such as vancomycin, rifampin, trimethoprim, amoxicillin, ampicillin, metronidazole, and cephalosporin (1). Fluoroquinolones, macrolides, tetracyclines are commonly reserved for the treatment of *C. jejuni* infections. Fluoroquinolones are often used for the empirical treatment of enteric infections, because they can alleviate the infection by multiple pathogens like *Salmonella* and *Campylobacter* (188). Nevertheless, high levels of resistance to fluoroquinolones, erythromycin and tetracycline in *C. coli* and *C. jejuni* strains may weaken the efficiency of the antibiotic treatment (189-192).
Antimicrobial susceptibility of *C. jejuni* isolated from food animal production is commonly monitored. A recent study by Sahin *et. al.* suggests oxytetracycline is ineffective for *Campylobacter*-induced abortion in sheep (2). To date, erythromycin is considered as the choice for *Campylobacter* infections because it has relatively low resistance in *Campylobacter* species. Furthermore, erythromycin is easy and safe to be administered to immunosuppressed or immune-immature patients, such as children and pregnant women (188). Its advantages also include low toxicity, low cost, and narrow activity (1).

**Prevention and Vaccines**

Since foodstuffs (e.g., poultry meat) is the primary source of *Campylobacter* infection in humans, prevention should take a farm-to-fork approach to reduce the infection level among poultry houses, minimize contamination of carcasses in processing plants, and ultimately educate consumers for proper handling and cooking poultry products. It is necessary to install hygienic barriers between the external and internal environments of poultry houses, and implement strict hygienic routines such as washing and sanitizing hands, changing boots and coveralls before entering into the poultry houses. However, such control measures are often ignored on farms (36). Limiting the bacterial load on poultry farms is useful for prevention of *C. jejuni* infections. Additionally, a number of studies have been performed to reduce carcass contamination in slaughter houses (193, 194). However, currently
there are no practical and effective strategies that can be taken in effect to reduce *Campylobacter* colonization in live birds.

It is widely believed that a vaccine must be able to stimulate intestinal immunity to be effective against an enteric bacterial pathogen (15-18). For this logistical reason, the oral route of immunization has been identified as the preferred approach in the Vaccine Initiative among children (195). It is simpler to administer than parenteral immunization. Furthermore, it takes advantage of the huge amounts of lymphoid tissue in the oropharynx and intestine. Three different strategies have been considered for the development of a reliable vaccine against *C. jejuni*. The first strategy is live attenuated *Campylobacter* vaccine, which is considered as an attractive approach (19). Nevertheless, a study using a viable non-colonizing strain of *C. jejuni* failed to elicit protective immunity in chicks (20). Furthermore, the association of the neuropathy and autoimmune disease Guillain–Barre syndrome (GBS) with *C. jejuni* infections impedes the development of whole cell vaccines (21). The second one is killed whole cell vaccine, which can offer several advantages as potential vaccines for mucosal immunization. Due to their microparticle nature, whole dead cells should enhance the interactions between their surface and mucosal lymphoid tissues (22). A recent study has demonstrated that a limited protection was conferred by *C. jejuni* killed cells after oral vaccination in ferrets (23). Using specific proteins/antigens of *Campylobacter* as subunit protein vaccines is another promising strategy for vaccination. Two *Campylobacter* antigens, PEB1 and flagellin (24, 25), have been evaluated as vaccine candidates. However, *Campylobacter* flagellins have different levels of
glycosylation and high antigenic diversity, which makes the development of a flagellin-based vaccine problematic (26, 27). A recent study also demonstrates that significant levels of anti-PEB serum IgG did not protect against *C. jejuni* after oral challenge in mice (28).

The above cited vaccine studies and epidemiologic information suggest that immune protection against disease caused by *C. jejuni* is possible. But high amount of antigenic variation among different *Campylobacter* strains causing sheep abortion may result in vaccine failure, and result in inadequate amount of cross-protection produced by the vaccine strain(s) against the field isolates. These observations indicate the need for developing a more effective subunit-based vaccine incorporating the immunogens of predominant *Campylobacter* strains associated with sheep abortion in the U.S.

**References**


CHAPTER 2. IMMUNOGENIC AND PROTECTIVE PROPERTIES OF RECOMBINANT PROTEINS FROM A HIGHLY PATHOGENIC CAMPYLOBACTER JEJUNI CLONE ASSOCIATED WITH SHEEP ABORTION

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Abstract

Campylobacter jejuni clone SA has recently emerged as the prevalent cause of Campylobacter-associated sheep abortion in the United States. To develop effective vaccines against C. jejuni clone SA in sheep, it is necessary to identify the antigens that elicit protective immune responses. Recently, by using immunoproteomic approaches we successfully identified a number of clone SA proteins that were consistently immunoreactive with multiple convalescent sheep and guinea pig sera. In this study, as a first step towards developing an efficacious subunit vaccine against sheep abortion, we began to further characterize these proteins. Accordingly, 7 clone SA proteins were selected, which included HtrA, CgpA, CJSA_0852, Peb4, FabG, MetK and FlgL. Recombinant proteins for each of these antigens were produced in an E. coli expression system, and their reactivity with a panel of convalescent sera obtained from C. jejuni-infected ewes and guinea pigs were tested using immunoblotting. The results showed that CgpA,
MetK, FabG had the strongest antigenicity, while HtrA, FlgL and Peb4 were less antigenic, and CJSAX_0852 had only little reactivity with the sera tested. CgpA, HtrA and FlgL were chosen to further evaluate the induction of protective immunity against bacterial challenge in the mouse model of systemic infection and bacteremia. Immunization of mice with recombinant CgpA, HtrA and FlgL induced high level of specific antibodies, but only CgpA-immunized mice showed a significant decrease in the level of bacteremia compared with the control mice. Analysis of different cellular fractions demonstrated that CgpA is a periplasmic protein. These results indicate that CgpA may be a potential subunit vaccine candidate against sheep abortion caused by *C. jejuni*.

**Introduction**

*Campylobacter* spp. is one of the most predominant causes of ovine abortion in the United States and worldwide, contributing to an overall abortion rate of 5 to 50% (average, 23.2%) in affected flocks (1). A national study from the USDA/APHIS/Veterinary Services in cooperation with the American Sheep Industry Combination to investigate the etiology of sheep abortion indicated that *Campylobacter* species ranked first among infectious causes of abortion in the United States, with 8.8% of the sheep producers reporting the cause as *Campylobacter* spp. (2). *Campylobacter* species can also be carried in the gall bladder and intestines of healthy sheep without causing clinical diseases (3-5). In addition, *Campylobacter* is a main cause of bacterial foodborne gastroenteritis in
humans worldwide. A latest estimate by the CDC points out that *Campylobacter* is not only among the most popular causes of domestically acquired food-borne illnesses in humans (over 800,000 cases per year), but also among the main causes of hospitalization (over 8,000 annually) in the United States (6).

Ovine abortions caused by *Campylobacter* were first reported in Britain (McFadyean and Stockman 1913) and have since been reported in most sheep-rearing countries (7). Historically, *Campylobacter fetus* subsp. *fetus* has been regarded as the main *Campylobacter* species associated with ovine abortion. However, the proportion of *C. jejuni* isolates from ovine abortion cases has increased steadily during the last two decades and *C. jejuni* has now replaced *C. fetus* as the predominant *Campylobacter* species causing sheep abortion in the United States (8-11). The most recent examination of the species distribution, genotypes and antimicrobial susceptibilities of abortion-associated *Campylobacter* isolates obtained from multiple lambing seasons on different farms in Iowa, Idaho, South Dakota and California revealed that 89% of the isolates were identified as *C. jejuni* and the remaining 11% were classified as *C. fetus* (11). More strikingly, the majority (93%) of the *C. jejuni* isolates associated with sheep abortion belonged to a single genetic clone (named sheep abortion, SA clone). Furthermore, zoonotic transmission of this emergent clone to humans has been demonstrated by molecular and epidemiological evidence, posing an important threat to public health (12). These findings indicate that clone SA has evolved to possess novel virulence traits and agricultural practices in sheep production in the U.S. may have favored the dominance of this hypervirulent clone.
A previous immunoproteomics study in our laboratory was conducted to characterize the immunogenic antigens from the whole membrane fraction of *C. jejuni* clone SA associated with sheep abortion (13). Twenty-six antigens were identified to be strongly recognized by convalescent sheep sera, including 8 cytoplasmic proteins, 2 cytoplasmic membrane proteins, 11 putative periplasmic proteins, 3 outer membrane proteins and 2 extracellular proteins (13). Since the majority of bacterial virulence factors are commonly located on the bacterial cell surface or are secreted (14), immune response to membrane-related virulence factors is potentially protective against infection. Among all 8 cytoplasmic proteins and 2 cytoplasmic membrane proteins, only two cytoplasmic proteins (MetK and FabG) that lack a typical signal peptide but predicted to be secreted by SecretomeP V2.0 (13) may be considered as potential vaccine candidates against *C. jejuni*. For the 3 outer membrane proteins, the major outer membrane protein MOMP is involved in solute transport across the bacterial cell wall and adhesion to the intestinal mucosa (15, 16). Recombinant MOMP was shown to provide more than 42% protective efficacy against intestinal colonization in the mouse model (17). VirB10, located in the outer membrane, is not widely distributed among the *C. jejuni* isolated from sheep abortion and thus not suitable as a vaccine candidate for *C. jejuni*-induced sheep abortion. Outer membrane peptide PEB4 is an antigenic virulence factor implicated in host cell adhesion, invasion and colonization in *Campylobacter* (18), and its immunogenicity has also been reported in human sera (19). The 2 extracellular membrane proteins FlgE and FlgL are both flagellar hook-associated proteins. The high sequence homology of proteins or antigens among *C.
jejuni strains may promote the development of broadly protective vaccines against C. jejuni infections in sheep. However, FlgE has relatively low identity with its homologs in other strains and a previous study indicated that monoclonal antibodies raised against FlgE bound exclusively to this antigen (20). For the 11 periplasmic proteins, most of them have been previously shown to be immunogenic and somewhat protective against intestinal colonization in various animal models. For example, LivK could protect against intestinal colonization in a mouse model (21), whereas ZnuA, CJSA_0392 and CJSA_0679 were not protective in the mouse immunization studies (21). CjaA was highly immunogenic and shown to be protective in the colonization of chickens when given as an oral Salmonella-vectored vaccine (22) although the same effect was not repeatable in the follow-up studies.

Vaccines for prevention ovine abortion caused by Campylobacter have been used around the world and have been reported to be somewhat effective (23-25). Nevertheless, previous experimental studies (24, 26) have indicated that protection against either homologous or heterologous Campylobacter challenge in ovine is not always obvious following vaccination with commercial vaccines. Moreover, Campylobacter associated abortion cases have been also documented in sheep flocks given commercial monovalent and bivalent vaccines (27, 28). Therefore, improved vaccines are needed to fully protect sheep against abortion caused by diverse Campylobacter strains and serotypes (29). Currently, there are two commercial Campylobacter vaccines available for controlling sheep abortion in the U.S. Both vaccines are killed whole-cell bacterins and contain both C. fetus subsp.
fetus and C. jejuni strains. Despite their common use on farms, Campylobacter still remains a major cause of ovine abortion [12]. Antigenic variation among different Campylobacter strains may result in vaccine failure due to inadequate amount of cross-protection produced by the vaccine strain(s) against the field isolates. Thus, in addition to whole-cell based bacterin preparations, newer vaccine formulation (such as subunit vaccines) incorporating conserved proteins/antigens of C. jejuni are needed to elicit a robust immune response against multiple Campylobacter genotypes/serotypes associated with sheep abortion. This notion is further supported by our group’s recent observation where whole-cell based vaccines including the commercial ones and in-house made bacterin provided only limited protection against systemic infection/abortion in a pilot study with guinea pigs (29). Although a vaccine incorporating three serotypes of C. fetus subsp. fetus plus a single strain of C. jejuni has been overall successful in New Zealand (CampyVax4), the vaccine may not work in the U.S. because C. jejuni clone SA has emerged as the predominant cause of sheep abortion in the U.S. (9).

Considering the significance of C. jejuni clone SA in causing sheep abortion and foodborne illnesses, protective vaccines are needed to control its transmission and spread. Recently, in an attempt to identify antigens of clone SA that may be useful for vaccine development, we identified a number of recombinant proteins of C. jejuni strain IA3902 (a clone SA isolate) that were consistently immunoreactive with multiple convalescent sheep and guinea pig sera. In this study, we selected 7 of these proteins to evaluate their potential as subunit vaccine candidates. The selection is based on the predicted functions, cellular localization, consistent sero-
reactivity, and sequence conservation among \textit{C. jejuni}. This list included 3 periplasmic proteins (HtrA, CgpA, CJSA\_0852), one outer membrane protein (Peb4), two cytoplasmic proteins (FabG and MetK), and one extracellular membrane protein (FlgL). Our results suggest that CgpA may be a promising subunit vaccine candidate against sheep abortion caused by \textit{C. jejuni}.

**Materials and methods**

**Bacterial strains and growth conditions**

\textit{C. jejuni} strain IA3902 is a clinical isolate of clone SA from an ovine abortion case described previously (9), and is the wild-type strain used in this study (Table 1). It was grown on Mueller-Hinton (MH) agar under microaerobic (5\% oxygen, 10\% carbon dioxide and 85\% nitrogen) conditions at 42°C for up to 48 h. As needed for specific experiments, the culture medium was supplemented with \textit{Campylobacter} selective (polymyxin B, rifampicin, trimethoprim, and cycloheximide) and growth (sodium pyruvate, sodium metabisulfite, and ferrous sulfate) supplements (Oxoid, Cambridge, England).

**Sheep sera and guinea pig sera**

The sheep sera used in this study were collected in 2008 and 2011 from two farms where \textit{C. jejuni} SA clone was epidemic. Serum samples WI, WI96, and G190 were all from aborted ewes of the same flock and were collected in 2008.
Serum samples 9010, 9417, 9069, 9070, 9078, 9092 and 9097 were all from aborted ewes in the same flock and were collected in 2011, but serum 9200 was from a non-pregnant ewe in this flock. Each of the serum samples was from a different ewe that was infected with a clone SA isolate. The lamb serum sample came from a colostrum-deprived neonatal lamb of healthy sheep flock in 2008, which had no reported cases of Campylobacter-associated abortion. Whether this flock was colonized (in the intestine) by C. jejuni was unknown. Sera A9, A6, B7, D1, D11, D2, C6, G4, D5, D10 were all from aborted guinea pigs due to C. jejuni clone SA.

**Expression and purification of recombinant proteins**

The entire coding sequence without the signal peptide for each protein was amplified from C. jejuni IA3902 genomic DNA by PCR. CgpA, HtrA, Peb4, FlgL, MetK, FabG and CJS_A0852 coding sequences (not including the N-terminal lipoprotein signal peptide sequence) were amplified by PCR using primers specific for each gene (Table 2). The amplified products containing specific restriction enzyme sites at the 5’-ends and 3’-ends were purified and digested with appropriate restriction enzymes and separately ligated into pQE-30 vectors (Qiagen, Valencia, CA, USA), resulting in the addition of a 6 × His N-terminal leader sequence to each coding sequence. The fidelity of the recombinant plasmid was confirmed by sequencing. Plasmids encoding the respective fusion genes were transformed into *Escherichia coli* JM109. Protein induction was then carried out by adding 1 mM isopropyl-D-thiogalactoside (IPTG) in shaking bacterial cultures for 3 hours at
37°C. Induced cells were harvested and washed with cold PBS and centrifuged at 8,000 × g for 15 min. The cell pellet was resuspended in Ni-NTA lysis buffer (300 mM NaCl, 50 mM sodium phosphate buffer, pH 8.0) at 50 g/mL wet weight. Bacteria were lysed by sonication and the lysate was centrifuged at 12,000 × g for 30 min. Supernatants were then loaded onto a Ni-NTA Superflow Columns (Qiagen, Valencia, CA, USA) and the His-tagged proteins were eluted using elution buffer containing various imidazole concentrations.

**Insertional mutation of cgpA**

An isogenic cgpA mutant was constructed by insertional mutagenesis in C. jejuni IA3902. The kanamycin resistance (KanR; ~1200 bp) cassette was amplified from pMW10 (30) with Kan-F and Kan-R primers (Table 2). An 800-bp fragment (CgpA1) comprising the first 19 bp of cgpA gene and its upstream flanking region was amplified from gDNA of IA3902 via PCR using primers CgpA1F and CgpA1R where the reverse primer (44 bp long) was also added with 25 bp that was complementary to the 5’-end of the KanR cassette. Another 800-bp fragment (CgpA2) containing the last 19 bp of cgpA gene and its downstream flanking region was amplified via PCR using the primer set CgpA2F and CgpA2R where the forward primer (43 bp long) was added with additional 24 bp complementary to the 3’-end of the KanR cassette. CgpA1 fragment and KanR gene were first fused together and amplified via PCR using primers CgpA1F and Kan-R (Table 2) to obtain CgpA1-KanR. Then, CgpA1-KanR and CgpA2 fragment were merged and amplified in a PCR with CgpA1F and CgpA2R to generate CgpA-Kan. The
CgpA-Kan construct (~2.8 kb) was introduced into *C. jejuni* IA3902 by natural transformation. Transformants were selected on MH agar containing kanamycin (50 µg/ml). Disruption of *cgpA* in IA3902 by deletion of the most of the ORF and insertion of the KanR gene with the correct orientation was confirmed by PCR; the *cgpA* mutant strain was named ΔCgpA.

**Preparation of cell fractions**

The cell fractions of cytoplasm, periplasm, inner membrane, and outer membrane were obtained using the methods described previously (31), with slight modifications. In total, 500 ml of an overnight culture of *C. jejuni* strain IA3902 were harvested by centrifugation at 5000 x g for 30 min, and the cell pellet was resuspended in 20 ml of the ST buffer (20% w/v sucrose, 30 mM Tris-HCl, pH 8.0) at room temperature. EDTA was added to the cell suspension to a final concentration of 1 mM and the suspension was incubated for 10 min at room temperature with shaking. The cells were then centrifuged at 8000 x g for 10 min at room temperature to collect the cell pellet. The supernatant was discarded. The pellet was resuspended and stirred in ice-cold 10 mM Tris-HCl (pH 7.5) at 4 ºC for 10 min followed by centrifugation at 15,000 x g at 4 ºC for 15 min and it was kept as the periplasmic fraction. The pellet was again resuspended in 5 ml of ice-cold 10 mM Tris- HCl (pH 7.5), followed by sonication (Virsonic, an SP Industries Company, Warminster, PA) by three bursts of 30 s at 6 mm amplitude and centrifugation at 13,000 x g at 4 ºC for 15 min. The pellet was discarded and the supernatant was ultracentrifuged at 100,000 x g at 4 ºC for 1 hour. The supernatant
was then transferred to a new tube and again centrifuged at 100,000 x g at 4°C for 1 hour, corresponding to the cytoplasmic fraction. The pellet collected was the total membrane fraction. The isolation of inner and outer membrane was performed using the method of Carlone et al. (32). The pellet of membrane fraction was washed three times with ice-cold 10 mM Tris-HCl (pH 7.5) and resuspended in 0.2 ml of 10 mM HEPES buffer (pH 7.4). An equal volume of sarkosol buffer (2% sodium N-lauroylsarkosine; SIGMA) was added and incubated at room temperature for 60 min, during which the solution was mixed occasionally. The inner membrane proteins were solubilized at this step. The mixture was centrifuged at 15,600 x g for 30 min at 4°C. The supernatant was kept as the soluble inner membrane fraction. The pellet was washed with 0.5 ml of 10 mM HEPES buffer followed by resuspension in 200 ml of 10 mM HEPES buffer. This fraction was the outer membrane fraction. The fractions were analyzed by SDS-PAGE and immunoblotting.

**SDS-PAGE and Western blotting**

Recombinant proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 4% stacking gel and 10% separating gel (18 cm × 16 cm in size; using low florescent glass plates). Protein samples were boiled in sample buffer at 100°C for 3 min prior to electrophoresis. The SDS-gels were run at 15°C with parameters as 80V for 30 min, then 200V for 45 min and stopped until the dye front running out of the gels. For Western blotting, the proteins were transferred to LF-PVDF membrane (Millipore, Billerica,
MA), and then blocked with the blocking buffer (5% skim milk with 0.1% Tween-20 in PBS) for 1 h at room temperature. The membrane was incubated with primary antibodies (sheep serum and guinea pig serum; 1:100 dilution in the blocking buffer) at 4 °C overnight. After three washings with washing buffer (0.1% Tween-20 in PBS), the blot was incubated with rabbit anti-sheep or goat anti-guinea pig conjugated with horseradish peroxidase (Kirkegaard & Perry Laboratories, KPL, Gaithersburg, Maryland) (diluted in 1:1000 in the blocking buffer) for 1 hour at room temperature. After three washings with the washing buffer, the membrane was developed with the 4CN Horseradish Peroxidase Substrate system (KPL). The images were captured by a CCD imager (AlphaImager® 2200, ProteinSimple, Santa Clara, CA).

ELISA

An enzyme-linked immunosorbent assay (ELISA) was used to determine the level of *C. jejuni*-specific IgG antibodies in mice. For the bacterin group (positive control) and the alum group (sham control), microtiter plates (Nunc-Immune Plate; Nunc, Roskild, Denmark) were first coated with 100 µl of whole membrane proteins (ca. 60 ng/well) of *C. jejuni* IA 3902 in coating buffer (sodium carbonate [pH 9.6]) overnight at 25°C. For recombinant protein vaccine (CgpA, HtrA and FlgL) groups, the microtiter plates were first coated with 100 µl recombinant CgpA, HtrA or FlgL recombinant protein, respectively, (CgpA ca. 960 ng/well; HtrA ca. 120 ng/well; FlgL ca.960 ng/well). Then, plates were incubated with a blocking buffer (PBS containing 2% milk, 2% bovine serum albumin, and 0.1%
Tween 20) at 37°C for 1 h. Serum samples were diluted in the blocking buffer to 1:100, and then 100 µl of each dilution was added to individual wells. Duplicate wells were used for each sample. After incubation at 25°C for 2 h, the plates were washed three times with wash buffer (PBS containing 0.1% Tween 20). Goat anti-mouse IgG conjugated to peroxidase (Kirkegaard & Perry) was diluted to 1:1,000 in blocking buffer and added to the wells (100 µl/well). After 2 h of incubation at 25°C, the plates were washed three times with the wash buffer, and then 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS)-peroxidase substrate (Kirkegaard & Perry) was added. Optical density (OD) values of individual wells were measured using an ELISA reader at 405 nm. Serum samples from *Campylobacter*-colonized sheep (9200 sheep serum) were used as positive control in each plate.

**Vaccines**

Appropriate amount of purified recombinant proteins of CgpA, HtrA and FlgL were dissolved in sterile PBS and mixed with Alhydrogel 2% (alum) (InvivoGen, San Diego, California) in 2:1 ratio to give a final concentration of 20 µg/100 µl. The formulations were rotated at room temperature for 30 min prior to use. A bacterin of *C. jejuni* IA3902, which was demonstrated to provide protection against homologous challenge in a previous study (33) was included as positive control. For preparation of the bacterin, a frozen stock of *C. jejuni* IA3902 was grown microaerobically on Mueller-Hinton agar for 48 hours at 42°C, and then sub-passaged on Mueller-Hinton agar for an additional 24 hours at 42°C under the same
atmospheric conditions. Colonies of *C. jejuni* were harvested in PBS solution directly from plates by washing and then pelleted by centrifugation (15 minutes at 7,000 X g). The pellet was washed and resuspended in 10 mL of PBS solution containing 0.3% formalin. This suspension was centrifuged again (30 minutes at 7,000 X g), the supernatant was discarded, and the washing process repeated 2 times. The final pellet was weighed and resuspended in sterile PBS solution to achieve a final concentration of 5 mg of *C. jejuni* cells/mL. This final concentration was selected on the basis of a previous report comparing antigen dose and various adjuvant types in which this concentration provided 93% protection against homologous challenge (33). A few drops of the diluted cells were streaked on Mueller- Hinton agar or added to enrichment broth, both of which were placed in a microaerophilic environment to evaluate efficacy of formalin killing. The bacterin and Alhydrogel 2% were mixed in a ratio of 2:1 and the volume was adjusted to 100 µl /mouse. Alhydrogel 2% mixed with sterile PBS in 1:2 ratio was included as negative control (sham vaccine).

**Mouse immunization and challenge experiments**

A key step in *Campylobacter*-induced abortion is the development of bacteremia. Previously we have developed a mouse model to evaluate bacteremia induced by *C. jejuni* clone SA (34). This model was used in this study to assess the immunogenicity of the selected recombinant proteins. Wild-type HA-ICR (CD-1) female mice at 8-10 weeks of age were purchased from Charles River Laboratories
(Wilmington, MA) and maintained by Laboratory Animal Resources at the College of Veterinary Medicine at Iowa State University. All animal experiments were approved by the Iowa State University Institutional Animal Care Committee. The mice were housed in polycarbonate microisolator cages with bedding (4-5 mice/cage) and provided with water and a commercial feed ad libitum. The animals were acclimated for a minimum of three days before being used for experiments. Mice were injected subcutaneously twice at 2 weeks interval with 100 µl of each of the recombinant protein vaccine and control formulations described above (16 mice/group). Blood samples (~0.1-0.2 ml) were taken from saphenous vein using a 25G needle 2 weeks after the second immunization. Serum was obtained from each blood sample and used in ELISA to determine the antibody levels specific for each vaccine.

Seventeen days after the second immunization, the mice were challenged with C. jejuni IA3902. This strain used for inoculation was recovered from a freezer stock (-80°C, 20% glycerol), plated on selective medium and incubated for 48 h as described above. Bacterial cultures obtained from these plates were sub-passaged under the same conditions for 18 h. Fresh cultures were harvested and suspended in MH broth, diluted to the desired cell density based on optical density (OD$_{600}$ =1), and subsequently confirmed by viable counts. Ten mice from each immunized group were challenged and each mouse received 100 µl bacterial culture (approximately 1 x 10$^8$CFU/mouse) via gastric gavage using a curved, ball-tipped, 18-gauge, 2-inch needle, under light sedation with 2% isoflurane as previously
described (35). Additionally, 6 mice for each vaccine group were not inoculated, and were used solely for serum collection from the heart blood.

### Quantitation of systemic infection

Mice were euthanized at 8 h post-inoculation via intraperitoneal (IP) injection of a ketamine/xylazine mixture followed by exsanguination. Fecal swabs were taken from the non-inoculated mice at necropsy to confirm all mice were *Campylobacter*-free and no cross contamination occurred. Samples harvested for *Campylobacter* culture included cardiac blood and liver. Cardiac blood was collected by use of a sterile tuberculin syringe with a 22-gauge needle and placed in blood collection tubes (0.5 mL Greiner Vacuette MiniCollect K3 EDTA, Fisher Scientific). Within a couple of hours of sample collection, 250 µL of undiluted blood and appropriate serial dilutions thereof were plated on selective medium and incubated for 48 h under microaerobic conditions. Liver tissues were placed in a separate sterile plastic bag (Whirl-Pak bags, Nasco, Fort Atkinson, WI), weighed, homogenized in MH broth, serially diluted, plated on selective medium and incubated for 48 h. *C. jejuni* recovery was expressed as $\log_{10}$ CFU/ml blood or g of liver.

### Statistical analysis

A Fisher exact test for binomial variables was used when comparing *Campylobacter* culture results in experimental and control vaccine groups of mice.
A 1-way ANOVA was used to detect differences in ELISA results, and a Tukey’s adjustment for multiple comparisons was made. A $p$ value of $\leq 0.05$ was used in ELISA result to determine significant differences in antibody levels whereas $p \leq 0.1$ was used to evaluate significant differences in bacteremia in challenge experiments.

**Results**

**Production of recombinant antigens**

Seven proteins including CgpA, CJSA_0852, FabG, MetK, HtrA, FlgL, Peb4 of *C. jejuni* IA3902 were cloned and expressed in *E. coli* as recombinant proteins. These antigens were chosen for further evaluation since they all had strong and consistent reactivity with convalescent sera as determined in a previous immunoproteomics study (13). The recombinant proteins were successfully prepared and purified from *E. coli* (Figure 1). All of 7 recombinant proteins were soluble in water and showed the expected sizes (Table 3) on SDS-PAGE. However, HtrA had two bands, in which the top one matched the excepted size, which is 51kDa. Previous study demonstrated that mature HtrA was partially degraded both *in vivo* and *in vitro* due to self-cleavage following Cys$^{69}$ and Gln$^{82}$ of the mature protein (36, 37). The cleaved protein was also called short-HtrA (37). Then the 7 recombinant proteins were purified and blotted by a convalescent sheep serum. The results showed that all the recombinant proteins were reactive to the serum (Figure 1). Both bands of HtrA can be recognized by the convalescent sheep serum.
The purified recombinant proteins were further blotted with 11 convalescent sheep sera from aborted ewes due to natural infection with clone SA and 10 convalescent sera from guinea pigs aborted due to experimental infection with *C. jejuni* IA3902. The results showed that, except for CJS_A0852, all other six recombinant antigens were reactive with all of the tested sera (Figure 2). However, the strength of reactivity varied markedly with different antigen-serum combinations (Figure 2). In general, the convalescent sheep sera reacted more strongly with all recombinant proteins than the sera from the aborted guinea pigs (Figure 2). This was not surprising since sheep sera were collected from naturally infected and aborted ewes with longer duration of infection (~months) whereas the guinea pig sera were obtained from experimentally infected and aborted animals with much shorter duration of infection (~ a few days to 3 weeks). Immunogenicity of each protein is considered as one of the important factors for selecting promising vaccine candidates. The stronger the reactivity of the antigen with convalescent sera from sheep and guinea pig is, the more likely the antigen may elicit an effective immune response in vaccinated animals. Consequently, we selected 3 of these proteins (CgpA, HtrA, and FlgL) as potential subunit vaccine candidates on the basis of predicted functions (novel or previously suggested as a good vaccine candidate or a virulence factor), cellular localization (surface associated or secreted), consistent sera-reactivity with convalescent sera, and sequence conservation among *C. jejuni* (Table 3). These three antigens were further characterized in detail and evaluated for the induction of protective immunity.
against oral bacterial challenge in a mouse model of systemic infection and bacteremia.

**Immunogenicity of the recombinant subunit vaccines in mice**

No apparent side effects were noted in mice following subcutaneous injections of any of the vaccine formulations including the recombinant subunit (rCgpA, rHtrA and rFlgL) and the control (bacterin and sham) vaccines, indicating their safety. Levels of specific IgG antibodies in the systemic circulation for each vaccine was determined 2 weeks after the second immunization using ELISA plates coated with the corresponding antigen. The results showed the production of high-level specific antibodies in animals immunized with each of the subunit vaccines or the bacterin, compared with the sham-vaccine (alum only), which only induced very weak antibody response (Figure 3A). The sera from animals immunized with each vaccine also strongly reacted with whole membrane proteins of *C. jejuni* IA3902 (Figure 3B), which demonstrates that these subunit vaccines induced specific antibody that recognize not only recombinant antigens, but also native proteins expressed in *Campylobacter*. Specific antibody can react with their specific recombinant protein stronger than whole membrane protein, which can be reflected by the lower OD value of recombinant protein in Figure 3B. This finding showed that CgpA, HtrA and FlgL are all highly immunogenic in mice when given S.C. in combination with alum, suggesting their feasibility as subunit vaccine candidates against *Campylobacter*-induced abortion.
**Efficacy of recombinant subunit vaccines in mice**

To assess the potential of the recombinant subunit vaccines in protecting mice from bacteremia, the mice (n=10 per group) were orally challenged with *C. jejuni* IA3902 17 days after the second immunization with the corresponding vaccine. At 8 h after the inoculation, cardiac blood and liver tissues were collected and cultured for *C. jejuni*. The number of mice positive with bacteremia and/or liver infection was used to measure the protective ability of each vaccine (Figure 4). Compared with the control (sham vaccine) group in which 50% of blood samples yielded *Campylobacter* growth (with the mean $\log_{10} \text{CFU/ml}$ being 0.97), only 10% of mice showed evidence of bacteremia in rCgpA vaccine group (mean $\log_{10} \text{CFU/ml}$: 0.24) ($p \leq 0.1$). In the rHtrA vaccine group, 40% of mice were bacteremic with mean of bacteremia rate at 0.85 $\log_{10} \text{CFU/ml}$ ($p>0.1$). In the group of mice immunized with the bacterin, none of the animals developed detectable levels of bacteremia ($p \leq 0.1$)(Figure 4). Values of $p \leq 0.1$ were considered significant. Since the liver plays a potential role in clearing *C. jejuni* from the bloodstream, homogenized liver tissues were also cultured. In general, quantitative cultures of the livers of the orally infected mice yielded much less *Campylobacter* compared with those in the bloodstream for all groups irrespective of the vaccine they received (Figure 4). Only 10% of mice showed evidence of liver infection in rCgpA vaccine group while both the rHtrA vaccine and sham groups had 20% of mice being *Campylobacter* culture positive in the liver. None of the mice were positive for *Campylobacter* culture in the liver of bacterin group (Figure 4).
positivity in the liver of different vaccine vs. control groups were not statistically significant. In the second experiment, the protective capacity of rFlgL subunit vaccine was assessed. Although 80% of the vaccinated mice did not have *Campylobacter* recovered in the blood/liver, the validity of this data was not assured due to the extremely low-level bacteremia (10%) in the sham control group (data not shown). Together, the results from the immunization/challenge experiments indicate the high immunogenicity of the all three recombinant proteins, and further suggest that subunit vaccine formulations incorporating CgpA could be a potential candidate for protection against *C. jejuni*-induced abortion in sheep.

**CgpA is a periplasmic protein**

In order to determine the cellular localization of CgpA, *C. jejuni* strain IA3902 was fractionated to isolate proteins from the periplasm, cytosol, inner membrane and outer membrane. The fractions were examined by immunoblotting with anti-rCgpA antibodies produced in mice (Figure 5). CgpA was detected as multiple bands predominantly in the periplasmic fraction (Figure 5A). This result is consistent with the fact that CgpA in *C. jejuni* is modified by differential levels of glycosylation with neutral sugar residues (38). Interestingly, the CgpA bands showed larger sizes when the whole-cell extract of *C. jejuni* IA3902 was blotted with anti-rCgpA antibody (Figure 5B, lanes 3 and 4). On the other hand, the recombinant CgpA produced in *E. coli* was not glycosylated and was shown as a single band (Figure 5B, lane 1), which was smaller in size than the multiple forms of the native protein detected in the periplasmic fraction and the whole-cell extract. This finding further
indicated that glycosylation affects the migration patterns of glycosylated proteins on SDS-PAGE. As control, an isogenic CgpA mutant strain was blotted with the anti-rCgpA antibody, which showed no apparent reaction (Figure 5B, lane 2). Additionally, we used CmeB, CmeR and MOMP as controls for various fractions and they were predominantly detected in the inner membrane, cytoplasmic and outer membrane fractions, respectively (Figure 5A), consistent with the previous findings that CmeB is an inner membrane protein (39), CmeR is a cytoplasmic protein (40), and MOMP is an outer membrane protein (41). These results confirm the predicted periplasmic location of CgpA in \textit{C. jejuni}.

**Discussion**

\textit{Campylobacter jejuni} clone SA is the predominant cause of sheep abortion in the United States and incurs a significant economic loss to sheep producers. In order to identify the proteins of clone SA that may be useful for vaccine development, we recently employed an immunoproteomics approach to identify the immunoreactive antigens of \textit{C. jejuni} 1A3902 (a clone SA strain). The results suggested 7 such proteins on the basis of their consistent reactivity with multiple convalescent sheep and guinea pig sera. This list included 3 periplasmic proteins HtrA, CgpA, CJSA_0852, one outer membrane protein, Peb4, two cytoplasmic proteins, FabG and MetK, and one extracellular membrane protein FlgL. In this study, we selected 3 of these proteins (CgpA, HtrA, and FlgL) based on the predicted functions, cellular localization, consistent sero-reactivity, and sequence
conservation among *C. jejuni*, and further evaluated their potential for protection against bacteremia in mice. Our results suggest that CgpA may be a promising subunit vaccine candidate against sheep abortion caused by *C. jejuni*.

As a major foodborne pathogen, *Campylobacter* is widely distributed in food producing animals (42). Despite the recent advancements in understanding its ecology and its increasing global importance in animal health as well as public health, research on vaccines against *Campylobacter* is still in its infancy. Several strategies have been evaluated for developing vaccines against *C. jejuni* infections or disease. Using genetics to develop live attenuated *Campylobacter* vaccines (43) was proposed to be a promising approach. However, a study using a viable non-colonizing strain of *C. jejuni* failed to provoke protective immunity in chicks (44). Furthermore, the association of the neuropathy and autoimmune disease Guillain–Barre syndrome with *C. jejuni* infections makes the development of whole-cell human vaccines problematic (45). Killed whole-cell vaccines, which are naturally occurring microparticles, have several advantages as potential vaccines when formulated with appropriate adjuvants for mucosal immunization. They can enhance the interaction between their mucosal lymphoid tissue and surface (46). A recent study demonstrated that oral vaccination with killed *C. jejuni* cells can only provide a limited protection of ferrets (47). Cross protection against major serotypes of *Campylobacter* is probable but it proves to be difficult due to genetic and antigenic variations. Using subunit antigens or native proteins of *Campylobacter* purified and isolated from the bacterial surface as vaccines is another possible method for vaccination. Two *Campylobacter* antigens, a protein
called PEB1 and flagellin (48, 49) have been advised as *Campylobacter* vaccine candidates. However, *Campylobacter* flagellins have diverse antigenicity and the fact that immunogenic flagellin proteins need to be glycosylated, which may impede the development of a flagellin-based vaccine (50, 51). Additionally, a recent study demonstrated that significant levels of anti-PEB serum IgG did not protect vaccinated mice against *C. jejuni* after oral challenge (52).

Almost all bacterial virulence factors are located on the cell surface of bacteria or are secreted (14). Antibodies to membrane-related virulence factors could neutralize the virulence antigens and cease the pathogenic process. Periplasmic proteins possess a short N-terminal motif, which is known to be the reason of their transport to the periplasm, but no signal sequences can be identified to lead their transport to the bacterial surface. However, it is possible that periplasmic proteins could still be exposed to surface after being transported to the periplasm (13). For example, cytolethal distending toxin (CDT) can be transported from periplasm to bacteria surface by outer membrane vesicles (OMVs) (53). Thus, common immunogenic nature of the clone SA periplasmic proteins identified in this study is not surprising since these proteins can be exposed on the bacterial cell surface (or secreted) and readily recognized by the host immune system. Therefore, depending on their biological functions, specific antibodies produced against periplasmic antigens could interfere with pathogenesis of bacterial infections, indicating the feasibility of development of effective subunit vaccines formulated with such proteins.
CgpA, a 220 aa-long and N-linked glycosylated periplasmic protein in *C. jejuni*, is commonly recognized by convalescent sera from infected animals (13). Previous studies have evidenced that *C. jejuni* has an N-linked protein glycosylation pathway that is indispensable for efficient cell invasion (54). N-linked glycosylated proteins might be involved in adherence, invasion and colonization in *C. jejuni* (55-61). Linton et al. also presented evidence indicating CgpA as a N-linked GalNAc-containing glycoprotein (62). In this study, a subunit vaccine incorporating rCgpA and alum adjuvant was shown to be highly immunogenic following two S.C. injections in mice, as measured by high levels of IgG in systemic circulation. Moreover, the same vaccine was found to confer a significant level of protection against bacteremia and liver infection in mice orally challenged with the homologous strain. Thus CgpA is a potential vaccine candidate for *C. jejuni* abortion in sheep. Future studies on the protective role of CgpA-based subunit vaccines using pregnant animal disease models (e.g., guinea pigs and sheep) are warranted. Interestingly, CgpA showed multiple bands in both whole cell extract and periplasmic fraction (Figure 5B). However, the bands in the whole-cell extract of *C. jejuni* IA3902 were larger than those in the periplasmic fraction. The size discrepancy is probably due to the chemical and physical treatment used in the purification of different fractions, which may have caused breakage of glycosylated chains and resulted in smaller size bands in the periplasmic fraction.

The periplasmic protein HtrA has both protease and chaperone activity and is required for efficient binding to epithelial cells in *C. jejuni* (63). Another study showed that HtrA could be secreted during infection with an unknown mechanism
and HtrA’s protease activity played a key role in rapid crossing of the intestinal epithelial barrier (64). Although a previous study demonstrated that HtrA mutants of Salmonella and Yersinia strains protected mice from subsequent challenge with virulent strains (65), a rHtrA-based subunit vaccine did not show apparent protection against bacteremia in mice in our study. FlgL is a flagellar hook-associated protein and a novel immunogen of C. jejuni identified recently by proteomics (13). Bacterial outer membrane proteins, due to the direct interaction with the host cells and being potential pathogenicity factors, are good vaccine candidates. Although the rFlgL-based subunit vaccine was found to elicit a high-level antibody response in immunized mice, the protective role of this vaccine could not be measured in this study due to unexpected issues with the animal model.

Guinea pigs have been represented as a well-established model to study Campylobacter-induced abortion by previous studies (66-68), because the induced disease is similar to that seen in sheep and they are susceptible to Campylobacter. Although the model is available for researching Campylobacter-induced abortion, it is time consuming, cumbersome, and expensive. Therefore, using pregnant guinea pigs is not always feasible for large-scale studies involving multiple experiments. Previously, we developed a tractable and inexpensive mouse model to study bacteremia/systemic infection induced by C. jejuni (34). Since the process of bacteremia/systemic infection is a key prerequisite in the pathogenesis of Campylobacter induced sheep abortion, we reasoned that our mouse model would also be a suitable screening tool for evaluating a large number of subunit proteins.
for in-vivo immunogenicity and vaccine potential. In this study using the mouse model, we significantly narrowed down the number of antigens to be evaluated as promising vaccine candidates in more relevant (but highly expensive and complex) animal models using pregnant guinea pigs and/or sheep. Unexpectedly, our second challenge experiment with rFlgL vaccinated mice failed because of extremely low-level bacteremia even in the sham control group. Although the mice were from the same commercial source and were confirmed Campylobacter-free, they might have developed resistance to Campylobacter for unknown reasons. Additional experiments are needed to assess the protection of rFlgL.

**Conclusion**

In conclusion, our finding showed that CgpA, HtrA and FlgL are all highly immunogenic in mice when given S.C. in combination with alum, suggesting their feasibility as subunit vaccine candidates against Campylobacter-induced abortion. Furthermore, the results suggest that subunit vaccine formulations incorporating CgpA could be a potential candidate for protection against *C. jejuni*-induced abortion in sheep. Future studies are needed to evaluate the functions of these recombinant proteins in protection against ovine abortion caused by *C. jejuni* using more relevant disease models including pregnant guinea pigs and sheep.
Acknowledgements

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References


Table 1. Bacterial strains and plasmids used in this study.

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<tr>
<th>Strain or Plasmid</th>
<th>Description a</th>
<th>Reference</th>
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<tr>
<td><strong>C. jejuni</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA3902</td>
<td>Wild type</td>
<td>(9)</td>
</tr>
<tr>
<td>ΔCgpA mutant</td>
<td>IA3902 derivative CgpA:: kan'</td>
<td>This study</td>
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<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM 109</td>
<td>endA1 recA gyrA96 thi hsdR17(rk2, mk+) relA1 supE44D(lac proAB) [FreaD36proABlacIqZDM15]</td>
<td>Promega</td>
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<td><strong>Plasmid</strong></td>
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<tr>
<td>pMW10</td>
<td><em>E. coli-C. jejuni</em> shuttle vector with promoter less <em>E. coli</em> lac Z gene, kan'</td>
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a kan' confers kanamycin resistance
### Table 2. Primer sequences used in this study.

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<td>pQE30_R</td>
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<td>CgpA_R</td>
<td>CCCAAGCTTTCGAGATTTATATATC(HindIII)</td>
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<tr>
<td>MetK_F</td>
<td>CGGGGTACCTATCTATTTCCAGAAGTCGTAA(KpnI)</td>
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<td>Peb4_F</td>
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<td>Peb4_R</td>
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<td>FlgL_F</td>
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<tr>
<td>Kan_R</td>
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$^a$The underlined sequences in the primers indicate the restriction sites for specific restriction enzymes, which are shown in parenthesis.
Table 3. Characteristics of *C. jejuni* recombinant proteins evaluated for subunit vaccine potential in this study.

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<th>CJS_A_0852</th>
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<th>FabG</th>
<th>MetK</th>
<th>Peb4</th>
<th>FlgL</th>
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<td>+++</td>
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<tr>
<td><strong>Predicted Function</strong></td>
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<td>Major antigenic peptide</td>
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<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
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<tr>
<td><strong>Protein MW(KDa)</strong></td>
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<td>37.74</td>
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<td>Cytoplasm</td>
<td>Cytoplasm</td>
<td>Periplasm</td>
<td>Cell surface</td>
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*Based on reactivity with sheep and guinea pig sera of infected/aborted animals.
†Based on sequence similarity among *C. jejuni* isolates from sheep abortions.
Figure 1. Expression and purification of 7 recombinant antigens from *C. jejuni* IA3902 with convalescent sheep sera. (A) SDS-PAGE image of the purified recombinant proteins. The identity of each protein is labeled underneath the panels. (B) Immunoblotting image of recombinant antigens with sheep serum 9010. HtrA shows two specific bands due to its autocleavage during maturing.
Figure 2. Immunoblot analysis of the 7 recombinant proteins of C. jejuni IA3902 with convalescent sera. For each recombinant protein, the results are shown for 11 sheep sera (SP) and 10 guinea pig sera (GP). For sheep sera, the serum numbers refer to animal IDs 9010, 9069, 9070, 9078, 9092, 9097, 9417, 9200, WI, WI96, and G190, respectively. For guinea pig sera, the lane numbers correspond to animal IDs A9, A6, B7, D1, D11, D2, C6, G4, D5 and D10, respectively. HtrA shows two bands, which are indicated by arrows.
Figure 3. Immunogenicity of alum-adjuvanted recombinant proteins (CgpA, HtrA, and FlgL) and bacterin of *C. jejuni* IA3902 in mice. Systemic IgG ELISA levels specific for each recombinant proteins were measured 2-weeks after the 2nd immunization with the respective antigen given s.c. at two weeks intervals. (A) In CgpA, HtrA, FlgL and Bacterin groups, the sera (black bars) were tested with their corresponding antigens coated onto plates in comparison with the sera from the sham control group (grey bars). (B) Sera from each group were tested with whole membrane protein as the coating antigen on plates. *p < 0.05 indicates significant differences.
Figure 4. Protective effect of recombinant subunit vaccines against C. jejuni IA3902 in mice. CD-1 mice vaccinated with the corresponding recombinant protein as described in Figure 2 or bacterin were orally challenged with IA3902 (~10^8 CFU/mouse) and were necropsied at 8 hour post-inoculation. Levels of bacteremia (Figure 3A) and liver infection (Figure 3B) were determined as described in materials and methods. Each bar represents percentage of infected mice in each vaccine group. Statistically significant differences are shown at *p < 0.1. **P > 0.1 in liver infection shows no significant differences.
Figure 5. Identification of CgpA as a periplasmic protein in C. jejuni IA3902. (A) The periplasmic (lane 1), cytosol (lane 2), inner membrane (lane 3), and outer membrane fractions (lane 4) were separated by SDS-PAGE and probed with anti-rCgpA (panel A-I), anti-rCmeR (panel A-II), anti-rCmeB (panel A-III), and anti-rMOMP (panel A-IV) antibodies, respectively. (B) Recombinant CgpA (lane 1), whole cell lysate of CgpA mutant (lane 2), whole cell lysate of wild type IA3902 (lane 3), and the periplasmic fraction (lane 4) of IA3902 were separated by SDS-PAGE and probed with anti-rCgpA.
CHAPTER 3. GENERAL CONCLUSION

*Campylobacter jejuni* is one of the most predominant causes of ovine abortion in the United States and worldwide. Our work recently demonstrated the majority (93%) of the *C. jejuni* isolates associated with sheep abortion belonged to a single genetic clone (named sheep abortion, SA clone). Considering the significance of *C. jejuni* clone SA in causing sheep abortion and foodborne illnesses, protective vaccines are needed to control its transmission and spread. In an attempt to identify antigens of clone SA that may be useful for vaccine development, a previous immunoproteomics study in our laboratory was conducted to characterize the immunogenic antigens from the whole membrane fraction of *C. jejuni* clone SA associated with sheep abortion. Twenty-six antigens were identified to be strongly recognized by convalescent sheep sera.

In this study, we produced recombinant proteins of *C. jejuni* strain IA3902 (a clone SA isolate) that were consistently immunoreactive with multiple convalescent sheep and guinea pig sera. We selected 7 of these proteins to evaluate their potential as subunit vaccine candidates. The selection is based on the predicted functions, cellular localization, consistent sero-reactivity, and sequence conservation among *C. jejuni*. This list included 3 periplasmic proteins (HtrA, CgpA, CJSA_0852), one outer membrane protein (Peb4), two cytoplasmic proteins (FabG and MetK), and one extracellular membrane protein (FlgL). Recombinant proteins for each of these antigens were produced in an *E. coli* expression system,
and their reactivity with a panel of convalescent sera obtained from *C. jejuni*-infected ewes and guinea pigs were tested using immunoblotting. The results showed that CgpA, MetK, FabG had the strongest antigenicity, while HtrA, FlgL and Peb4 were less antigenic, and CJSJSA_0852 had only little reactivity with the sera tested. CgpA, HtrA and FlgL were chosen to further evaluate the induction of protective immunity against bacterial challenge in the mouse model of systemic infection and bacteremia. Immunization of mice with recombinant CgpA, HtrA and FlgL induced high level of specific antibodies, but only CgpA-immunized mice showed a significant decrease in the level of bacteremia compared with the control mice. Analysis of different cellular fractions demonstrated that CgpA is a periplasmic protein.

Together, our finding showed that CgpA, HtrA and FlgL are all highly immunogenic in mice when given S.C. in combination with alum, suggesting their feasibility as subunit vaccine candidates against *Campylobacter*-induced abortion. Furthermore, the results suggest that subunit vaccine formulations incorporating CgpA could be a potential candidate for protection against *C. jejuni*-induced abortion in sheep. Future studies are needed to evaluate the functions of these recombinant proteins in protection against ovine abortion caused by *C. jejuni* using more relevant disease models including pregnant guinea pigs and sheep.
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