Molecular Epidemiological and Pathogenesis Studies on *Campylobacter* Species in Cattle and Sheep

**DISSERTATION**

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By

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Abstract

Campylobacteriosis is recognized as a leading bacterial cause of human gastroenteritis in both developed and developing countries. Though it has been known for a long time that poultry is the main source of *Campylobacter* infection, recent studies have suggested that cattle and sheep are potential emerging sources for human infections. Cattle can contribute to human infections through several routes of transmission such as direct contact, environmental contamination, and ingestion of contaminated food and milk. *C. jejuni* and *C. coli* usually colonize cattle without showing clear symptoms. Some studies have reported that 80% of livestock herds and 40–60% of individual animals may shed *Campylobacter*. However, the direct and indirect contributions of cattle and sheep in spreading of human infections are still under investigation. In addition, the sources of *Campylobacter* and its routes of transmission at dairy farms have not been clearly established. Several studies have also indicated that wild birds may play a potential role in the epidemiology of human campylobacteriosis by transmitting *Campylobacter* from animals to humans. In our study we hypothesized that dairy cattle and feedlot beef cattle act as potential sources of human campylobacteriosis. Further, the prevalence, and genotypic relatedness of *C. jejuni* and *C. Coli* isolated from dairy and beef cattle are different depending on the region where they originate. We also hypothesized that wild birds’ reservoirs may spread *Campylobacter* species to dairy farm environments which
can be transmitted to dairy cattle and humans. Thus, both wild birds and dairy cattle play an important role in human infections. For testing our hypothesis, in the first study, we determined the prevalence, genotypic and phenotypic properties of these pathogens. Further, characterization of the public health relevance of the cattle-associated *Campylobacter* by determining their invasion and intracellular potential to human intestinal cells (INT407), as well as their antimicrobial resistance profiles. Fecal samples were collected from beef and cull-cows at slaughter plants from four different representative geographical locations in the US. to determine the rate of *Campylobacter* infection. Our results showed that the prevalence of *Campylobacter* varied regionally and was significantly (*P*<0.05) higher in fecal samples collected from the South as compared to North, East and Midwest. Pulsed Field Gel Electrophoresis (PFGE) analysis showed that *C. jejuni* and *C. coli* isolates were genotypically diverse and certain genotypes were shared across two or more of the geographic locations. Further, MLST analysis demonstrated that the cattle associated *C. jejuni* strains harbored sequence types that were commonly shared in human cases and also showed varying invasion and intracellular survival capacity to human intestinal cells. Furthermore, many cattle associated *Campylobacter* isolates showed resistance to several antimicrobials including ciprofloxacin, erythromycin, and gentamicin. Conclusively, our results highlight the importance of cattle as an important reservoir of *Campylobacter* spp clinically important to human.

In the second study, we investigated the occurrence of the invasion associated marker (*iam*) in *C. jejuni* isolated from cattle in order to determine the contribution of this marker (**iam**) in *C. jejuni* isolated from cattle in order to determine the contribution of this
reservoir to human infections with invasive *Campylobacter*. Additionally, we assessed *iam*’s contribution to the colonization of multiple hosts by characterizing the potential of *iam*-containing cattle isolates for chicken colonization and human intestinal cell invasion. Simultaneous RAPD typing and *iam*-specific PCR analysis of cattle and human-associated *C. jejuni* isolates showed that the prevalence of *iam* in cattle *C. jejuni* is relatively lower as compared to isolates occurring in humans and chickens. In addition, *iam* was polymorphic and certain alleles occurred in cattle isolates that were capable of colonizing and invading chickens and human intestinal cells, respectively. However, the *iam* did not appear to contribute to the invasion, intracellular survival, and antimicrobial resistance potential of cattle-associated *C. jejuni*. We propose that the screening of the *iam* as a virulence determinant for epidemiological purposes in *C. jejuni* must be carefully considered.

In the third study, we determined the prevalence, genotypic, and phenotypic properties of *Campylobacter* that were isolated from paired fecal samples of dairy cattle and starlings (*Sturnus vulgaris*) in Northeastern Ohio. Our results showed that the prevalence of *C. jejuni* in birds was significantly (*P*<0.01) higher than that in dairy cattle. Further, PFGE analysis showed that *C. jejuni* were mostly genetically diverse and host restricted. However, there were several shared genotypes between dairy and starlings isolates. Furthermore, clonal complexes (CC) ST-45, and ST-21 were frequently shared between dairy and starlings by MLST analysis, which were also commonly found in humans. However, unique starlings’ isolates associated STs such as CC ST-177 and ST-682 as well as CC ST-42 that was restricted to dairy isolates were detected. Further, two
new STs were detected in *C. jejuni* isolated from dairy cattle. Interestingly, cattle and starlings *Campylobacter* showed high resistance to multiple antimicrobials including ciprofloxacin, erythromycin, and gentamicin. In conclusion, our results highlight starlings as potential reservoirs for *C. jejuni* and may play important role in the epidemiology of clinically important *C. jejuni* in dairy cattle population.

In the last study, we conducted a pilot study to understand the pathogenesis of *C. jejuni* induced abortion in sheep. *Campylobacter jejuni* has been recently shown to be increasingly associated with sheep abortion; however, it is still not known which circumstances and factors contribute to emergence of virulent *C. jejuni* strains that can cause abortion in sheep. We determined the genotypic and phenotypic properties of ovine and bovine abortion associated *Campylobacter* isolates which were acquired from the diagnostic laboratories of the Ohio Department of Agriculture (ODA). Further analyses included invasion and intracellular survival potentials in human INT407 cells, along with in-vivo experiment in pregnant ewes. PFGE analysis showed that the fingerprints of ovine and bovine abortion *C. jejuni* were identical. Furthermore, by MLST analysis, ovine and bovine abortion isolates were classified as (Sequence Type) ST-8 which belonged to Clonal Complex (CC) ST-21 which is also commonly found in humans. Additionally, the ovine and bovine abortion associated *C. jejuni* strains showed varying invasion and intracellular survival capacity to human intestinal INT407 cells; however, one isolate showed a significantly higher invasion potential compared to other strains (*P*<0.01) which matched that of *C. jejuni* 81-1176, a hyper-invasive strain. Furthermore, the ovine and bovine abortion associated *Campylobacter* isolates showed resistance to
several antimicrobials including ciprofloxacin, erythromycin, and gentamicin. Abortion-like symptoms and pathological lesions including suppurative necrotizing placentitis, suppurative and necrotic endometritis, along with occasional lymphadenitis and hepatitis were detected in pregnant ewes after inoculation with the abortion-associated \textit{C. jejuni} strains. Taken together, our results showed that these \textit{C. jejuni} isolates are capable of causing abortion in sheep, which indicated that the virulence characters of abortion inducing \textit{C. jejuni} can be better studied in a natural host, sheep. Since this clone of \textit{C. jejuni} share genotypic similarities with clones that exist in human population, it may have zoonotic potential.

Our study fills the knowledge gap of prevalence, distribution, genotypic diversity and molecular epidemiology of \textit{C. jejuni} and \textit{C. coli} strains from cattle (beef and dairy) as well as from European Starlings that exists particularly in the US. The results from our study would enhance preharvest efforts to establish effective control and preventive measures, which interm limite the public health impact of these pathogens. Further, the pathogenesis studies of ovine and bovine abortion-associated \textit{C. jejuni} in pregnant ewes would provide a better understanding of the pathogenic mechanisms used by these pathogens and may identify novel targets to develop control strategies.
This work is dedicated to my loving parents and family
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CHAPTER 1

Introduction and Literature Review

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Food Animal Health Research Program, Ohio Agricultural Research and Development Center, Department of Veterinary Preventive Medicine, The Ohio State University, Wooster, OH 44691.
1.1 *Campylobacter* as a major food borne pathogen

The majority of human campylobacteriosis is caused by *C. jejuni*, a Gram-negative, microaerophilic bacterium commonly found in water and gastrointestinal tract of avian species and mammalian animals. *C. jejuni* and *C. coli* are small (approximately 1.5 – 6.0 µm by 0.2 – 0.5 µm), uni or bi-polar flagellate, non-spore forming, curved rods (Inglis *et al.*, 2007), (Koneman *et al.*, 1997). It is a member of the Campylobacteraceae family, originally named and placed in the Vibrio genus due to their spiral or “seagull” shape (Doyle, 1944), (Jones *et al.*, 1931). *Campylobacter* are easily identifiable by microscopic examination. A spiral shaped microorganism was first characterized by Escherich in 1884 from human stool (Vandamme, 2000). Later, the same spiral shaped bacteria was identified by McFadyean and Stockman from sheep abortion cases (McFadyean and Stockman, 1913). However, *Campylobacter* was first characterized and described as a Vibrio-like bacteria associated with dysentery in cattle and calves by Smith *et al* (Smith and Orcutt, 1927) and Jones *et al* (Jones *et al*. 1931). This organism was named *Vibrio jejuni* and shown to be the etiology of “Winter dysentery” in cattle (Jones *et al*. 1931). The first documented outbreak of campylobacteriosis occurred in 1938 which was a milk borne outbreak and ‘bovine-Vibrio’ was reported as the causative agent (Levy, 1946; Vandamme, 2000). The second most common *Campylobacter* species, *C. coli*, was first called *Vibrio coli* and was identified by Doyle *et al* in 1944 from pigs with dysentery. This was also suggested to be another possible agent of the 1938 outbreak (Doyle, 1944). *C. jejuni* and *C. coli* have fastidious growth requirements (Koneman *et al*., 1997). They are considered to be the most important *Campylobacter*
species causing human infections (Frost et al., 1999). Nearly 90% of human campylobacteriosis are associated with \textit{C. jejuni}, while less than 5% of the cases are attributed to \textit{C. coli} infections (Frost et al., 1999).

\textit{Campylobacter} is recognized as a leading bacterial cause of human gastroenteritis in both developed and developing countries (Diker and Istanbulluoglu, 1986). \textit{Campylobacter} was reported as the second most common bacterial cause of gastroenteritis with over one million cases of campylobacteriosis occurring annually in the United States (Scallan et al., 2011). \textit{Campylobacter} infections are most likely to occur as sporadic cases rather than as outbreaks and more commonly reported in summer seasons than winter months (Olson et al., 2008). The occurrence of campylobacter infections is commonly reported in developed countries more than in developing countries, with higher incidence in children less than a year old (Olson et al., 2008).

The genus \textit{Campylobacter} contains species that are known for their adaptation capabilities, readily acquiring genetic material necessary for survival and persistence (Meinersmann et al., 2002). \textit{Campylobacter} spp. can asymptotically colonize major food-animals such as poultry and cattle, subsequently contaminating foods of animal origin including meats and dairy products, which are the potential sources of human campylobacteriosis (Allos, 2001; Miller and Mandrell, 2005). Additionally, direct occupational transmission of campylobacter infections among farmers, veterinarians, or workers in animal and poultry processing plants through direct contact with infected animals or animal products has been reported (Butzler, 2004). Further, the contact with \textit{Campylobacter} infected pets or exposure to environment contaminated with infected
fecal matter from wildlife or birds in addition to direct individual contact has been considered a risk factor for human campylobacteriosis (Butzler, 2004). Vertical transmission from infected dam to fetus, either through ascending route from infected urogenital tract or descending infection through trans-placental route, was also suggested (Butzler, 2004; Mcdonald and Gruslin, 2001).

_Campylobacter_ species can cause mild to severe diarrhea, with loose, watery stools often followed by bloody diarrhea (Tracz et al., 2005). The true incidence of campylobacteriosis may be higher than the figures indicated by previous studies. It was previously suggested that for every reported case there are 38 unreported cases (Mead et al., 1999). _Campylobacter_ infections are also associated with secondary complications. Neuropathies such as Guillian-Barre syndrome (GBS) is the most serious complication, which has been estimated to occur in 1 of 1000 cases of campylobacteriosis (Godschalk et al., 2006). Further, it has been reported that myocarditis might be associated with _Campylobacter_ infections, which is fatal in the majority of cases (Cox et al., 2001; Cunningham and Lee, 2003). _Campylobacter_ are commensal bacteria, and can be found in the oral cavity and intestines of humans (Young et al., 2007). However, it was estimated that human _campylobacter_ infections may cause more than 100 cases of deaths annually in the USA (Mead et al., 1999).

### 1.2 Molecular epidemiological studies of _Campylobacter_

_C. jejuni_ epidemiology in food animal populations, which are the main source of human infection for public health, is still limited. Several factors, such as low
discriminatory power, inapplicability of sharing results between laboratories, and inadequate reproducibility of strain characterization methods, have an impact in limiting the available data (Kwan et al., 2008). Serotyping, as a phenotypic tool has been widely used (Wassenaar and Newell, 2000). However, the serotyping of *Campylobacter* was found inefficient since; i) it requires intensive labor, ii) considerable time is required in the production of antisera, and iii) significant number of strains are non-typeable by this tool (Patton and Wachsmuth, 1992). Therefore, alternative subtyping schemes were clearly needed (Wassenaar and Newell, 2000). It was suggested that DNA-based genotyping of *Campylobacter* strains are most desired for efficient epidemiological studies and outbreak investigation (Owen et al., 1995). Several molecular typing techniques were developed with a major goal of making these tools universally available. Some of these techniques, like ribotyping, pulsed-field gel electrophoresis (PFGE), and flagellin typing (*fla* typing) using restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) analysis, Random amplified polymorphic DNA (RAPD) (Newell and On, 1988) in addition to the multilocus sequence typing (MLST) (Dingle et al., 2001) are being commonly used for typing of *Campylobacter* species. Several of these techniques have major disadvantages; for example, AFLP requires sophistication and skills, RAPD lacks reproducibility and RFLP has low discriminatory power (Wassenaar and Newell, 2000). Therefore, regardless of the cost and requirement for skilled labor, the combination of PFGE, as a highly discriminatory typing tool, (Nielsen, 2002) and MLST, as a robust and accurate molecular characterization method (Dingle et al., 2005), was found to be very useful in
studying the molecular epidemiology of *Campylobacter* (Fakhri et al., 2005; Sahin et al., 2008).

Pulsed-field gel electrophoresis (PFGE) was developed in 1983 by Schwartz et al. to overcome the inability to monitor and separate large DNA fragments (<30 kb) by regular agarose gel electrophoresis (Upcroft and Upcroft, 1993; Chu and Gunderson, 1991). Larger size DNA fragments cannot migrate in a straight path and need to be stretched and squeezed to be able to move through the pores of gel matrix (Eby, 1990; Townsend and Dawkins 1993). Often the DNA needs to move in a snake like movement through the gel until it reaches the suitable pore size (Townsend and Dawkins, 1993) to be resolved. PFGE resolved this problem by forcing the previously restricted DNA fragments to travel in a straight line by pulsing the electrical current from different directions around the gel at calculated time intervals (Chu and Gunderson, 1991; Townsend and Dawkins, 1993). PFGE has been shown by many studies to be an accurate and more effective tool in molecular epidemiology of *Campylobacter* (Fitzgerald et al., 2001; Kuusi et al., 2005; Sails et al., 2003). However, PFGE is a labor intensive tool of genotyping the whole genome of bacterial isolates (Fiett et al., 2004). The main drawback of PFGE is that it requires well trained and skilled individuals to get accurate and reproducible results and also the inter-laboratory PFGE gels were often hard to compare due to use of different processing schemes by laboratories which consequently resulted in poor epidemiological investigations for the shared pathogenic bacteria between different countries in early 1990s (Swaminathan et al., 2001). However with
advances in technology, now PFGE is widely accepted epidemiological tool for typing *Campylobacter.*

The other most commonly used genotyping technique nowadays is the Multilocus sequence typing (MLST). It is a robust and accurate molecular characterization method. MLST has been demonstrated to be an invaluable typing tool for better understanding of molecular epidemiology and population structure of *C. jejuni* (Dingle et al., 2005). MLST was first suggested by Maiden et al 1998. It employs the relatively conserved sequences of several genes which have housekeeping functions and the variations are more likely to occur under neutral selectivity. This typing tool assumes that the housekeeping genes are under less influence of a selective pressure (Maiden et al., 1998). Multiple unlinked housekeeping genes were chosen for the subtyping scheme with the expectation that if recombination occurred at one locus then the increased number of gene loci would lessen the likelihood of incorrect assumptions about bacterial relationships. MLST overcame the common drawbacks associated with phenotyping techniques, providing robust, accurate, reproducible and importantly portable evidence of bacterial relationships (Maiden et al., 1998). Thus, MLST has been used widely to determine the diversity and prevalence of *C. jejuni* genotypes from various sources including cattle to help better understand *C. jejuni* epidemiology (Kwan et al., 2008, add more references). However, PFGE is still generally recognized as the gold standard for tracking outbreaks of pathogenic bacteria, because of its high discriminatory power (Nielsen, 2002). Because the discriminatory ability of MLST is less than the PFGE,
MLST is not recommended as a tool to be used singularly for subtyping in an outbreak investigation, rather to describe bacterial populations structure (Fakhr et al., 2005).

1.3 Epidemiology of *Campylobacter* in cattle and its contribution to human infection

*Campylobacter* usually colonizes the gastrointestinal tract of a wide range of animals and birds. *Campylobacter* has been isolated from poultry such as chickens (Bull et al., 2006) and ducks (Savill et al., 2003), ruminants such as cattle (Colles et al., 2003), (Wesley et al., 2000) and sheep (Colles et al., 2003), (Stanley and Jones, 2003), and also found in pigs (Jensen et al., 2006). *Campylobacter* has been also isolated from a broad variety of mammalian and bird reservoirs (Kwan et al., 2008b). The most important risk for human campylobacteriosis is known to be the handling and consumption of raw or undercooked poultry meat and cross contamination with uncooked meat products (Tauxe, 1992). However, there is now a rising concern, based on molecular epidemiological studies, that the potential role of non-poultry sources for human clinical infections has been underestimated. The relative direct and indirect contributions of cattle and sheep to human infections are still unknown (Frost, 2001). Epidemiological studies, either case-controlled or prevalence studies on meat, suggested that red meat is an emerging and potential risk factor for humans infection (Grau, 1991). Gross microbial contamination of the carcass with gut contents may occur during evisceration but it is thought that most contamination occurs during removal of the hide or from cross-contamination from personnel or slaughter instruments (Gannon, 1999). Furthermore, cattle-associated *Campylobacter* also pose an additional indirect public health risk (Piddock et al., 2000;
Vandeplas et al., 2008). Interestingly, up to 80% of cattle herds and 40–60% of the individual animals shed *Campylobacter* (Besser et al., 2005; Milnes et al., 2007; Wesley et al., 2000). In a recent study using Multilocus Sequence Typing (MLST), it was shown that cases of human *Campylobacter* infections in Finland could be attributed equally to cattle and poultry (de Haan et al., 2010). Similarly, another study in Lancashire, England, reported that cattle were the source of human infections in 35% of examined cases (Wilson et al., 2008).

Furthermore, the consumption of unpasteurized milk is also most likely a potential cause of *Campylobacter* sporadic infections and outbreaks in the United States (Allos, 2001; Skirrow, 1990). Direct and indirect fecal contamination of milk as well as asymptomatic *Campylobacter* mastitis, which might shed a high number of bacteria to milk, are the most common sources of *Campylobacter* contamination (Orr et al., 1995). Among 57 reported food-borne outbreaks caused by *C. jejuni* in the USA in the period from late seventies to mid-eighties, twenty six outbreaks were associated with consumption of raw milk (Tauxe, 1992). Not only in the US, but also in England, *Campylobacter* were responsible for 26% of milk-borne outbreaks that occurred during 1992 to 2000 (Gillespie et al., 2003).

Literature from different countries on the prevalence of *campylobacter* infection in adult cattle in the last two decades estimated that 0.8% to 46.7% of cattle shed *Campylobacter* in feces (Rosef et al., 1983; Cabrita et al., 1992; Giacoboni et al., 1993; Nielsen 2002; Hoar et al., 2001; Wesley et al., 2000). Although, the estimated bacterial carriage varies significantly between herds, thermophilic *Campylobacters* are readily
isolated from the intestinal tract of healthy ruminants. Variable factors, such as herd size and type, season, age of animal, sample site, sample frequency and isolation method, geography, diet and husbandry policies, have been suggested to be responsible for discrepancies in prevalence of *Campylobacter* in cattle reported in the literature. For example, *C. jejuni* incidence in feedlots cattle was reported to be higher than that in grazing cattle (Garcia *et al.*, 1985). An enrichment step has often been found to increase the recovery of *Campylobacter* from ruminant samples. This may be because the average number of *Campylobacter* in adult bovine and ovine intestinal samples is lower than in broiler fecal samples (Stanley and Jones 2003). In 2008, Ragimbeau *et al* reported that cattle could be an underestimated reservoir of human *C. jejuni* cases (Ragimbeau *et al.*, 2008).

The genotypic diversity of *Campylobacter* might serve as an indicator for the value of a reservoir in providing a suitable niche for the persistence and evolution of these microorganisms (Wilson *et al.*, 2010). High genotypic diversity has been reported in *Campylobacter* isolated from cattle, sheep, and pigs (Oporto *et al.*, 2007). Molecular typing using PFGE analysis of cattle-associated *Campylobacter* isolates obtained from different locations across the USA showed that cattle can be considered a potential reservoir for genetically diverse *Campylobacter* and many of these *Campylobacter* have public health relevance (Sanad *et al.*, 2011). Molecular typing of cattle-associated *Campylobacter* using MLST showed that CC ST-21, CC ST-45, CC ST-42 and ST-61 were predominant in cattle samples from many countries, including the UK, Canada, New Zealand, Finland, and USA (Dingle *et al.*, 2002; Kwan *et al.*, 2008a; Levesque *et
These clonal complexes harbor 60% of *C. jejuni* associated with human infections (Dingle *et al.*, 2001; Grove-White *et al.*, 2010).

### 1.4 The role of wild birds in dissemination of *Campylobacter*

Free-living birds were suggested to be potential reservoirs that can play an important role in maintenance and dissemination of *C. jejuni* in the environment through their great motivation and carriage rate for this pathogen. Although it was reported in several articles on the occurrence of *Campylobacter* spp. in wild birds (Luechtfeld *et al*., 1980; Kapperud and Rosef, 1983; Matsusaki *et al*., 1986), the epidemiology of *C. jejuni* in wild birds populations and the link between the transmission of *C. jejuni* from wild birds to food producing animals are limited. For example; the prevalence of *C. jejuni* isolated from different locations and bird species ranged from 2 to 35% in Japan (Ito *et al*., 1988) and 45.5% in Northeast Portugal (Cabrita *et al*., 1992). Fecal bacteriological isolation of *Campylobacter* spp. from wild birds in Helsinki, Finland was 34% (Pitkala *et al*., 1992). The sources of infection and routes of transmission of *Campylobacter* on dairy farms have not been clearly established. However, some studies have indicated that wild birds, flies, and rodents may play a role in the epidemiology of human infections by transmitting *Campylobacters* from livestock to humans through food (Jones, 2001; Rosef *et al*., 1983; Cabrita *et al*., 1992; Waldenstrom *et al*., 2005).

The importance of wild birds as sources of *C. jejuni* for dairy cows was described (Cabrita *et al*., 1992). Wild birds are usually found near animal facilities and around
grazing areas which present an opportunity for environmental contamination in dairy farms by wild bird’s fecal droppings. Further, *Campylobacter* can be transmitted from wild animal reservoirs to cattle through contamination of stored concentrates and animal feed. These wild bird reservoirs may also serve to spread *Campylobacter* spp. to the environment which can be transmitted to other animals and humans (Cabrita *et al.*, 1992; Jones 2001). It was suggested that wild birds play a potential role in contamination of milking cups at dairy establishments (Southern *et al.*, 1990). In 2005, Waldenström, *et al* (Waldenström *et al.*, 2005) determined the antimicrobial susceptibility of 137 *C. jejuni* strains isolated from free living birds and they suggested that resistant genotypes can persist in the farm environment and can be later spread to any environmental reservoirs such as wild birds which increase the risk of transmission of infection from one farm to another (Waldenström *et al.*, 2005).

Although the huge public health efforts and control measures are being taken to control the *Campylobacter* contamination in poultry populations, foodborne *Campylobacter* infections still persist which indicates that there are multiple pathways for the infection (French *et al.*, 2009). Thus, it is necessary to identify these pathways to further improve the control measures to prevent *Campylobacter* contamination. These pathways include cattle and sheep meat, occupational exposure, direct contact and environmental contamination with wildlife and livestock fecal material (French *et al.*, 2009). Specifically, European Starlings (*Sturnus vulgaris*) have the tendency to gather by large numbers at livestock feeding operations in the United States (Linz *et al.*, 2007). They were also suggested to be a potential source of *Campylobacter* for human and
livestock infection because of their high shedding rates of *Campylobacter* (Colles *et al*., 2008; Nielsen *et al*., 2004) as well as their ability to disseminate infections between farms (LeJeune *et al*., 2008).

Prevalence data for *Campylobacter* in wild birds in the US are very limited. Several studies in the US were conducted to determine the occurrence of *Campylobacter* in wild birds (Lu *et al*., 2011; Keller *et al*., 2011). *C. jejuni* was detected in six avian families with the highest prevalence of 25% in Californian Gulls (Keller *et al*., 2011). Further, the prevalence of *Campylobacter* in wild birds as well as other phenotypic characteristics such as antibiotic resistance has been investigated outside the USA (Waldenström *et al*., 2002; Waldenström *et al*., 2005; French *et al*., 2009). *Campylobacter* prevalence in wild birds was 21.6% in Sweden (Waldenström *et al*., 2002), 30.6% in New Zealand (French *et al*., 2009) and the percentage ranged between 2 to 50% in some of the wild bird species including starlings in Northern England (Hughes *et al*., 2009). *C. jejuni* represented the majority of the retrieved isolates from starlings’ samples (Luechtefeld *et al*., 1980; Kapperud and Rosef, 1983; Waldenström *et al*., 2002; Broman *et al*., 2004; Keller *et al*., 2011).

Several phenotypic and genotypic properties strongly supported the potential role of wild birds as a reservoir in the transmission of *campylobacter* infection and their public health impact (Broman *et al*., 2004). Phenotypically, for example; the antimicrobial resistance prevalence of bird isolates for several antimicrobials including ciprofloxacin and nalidixic acid was (0.7 to 3.6%) (Waldenström *et al*., 2002). Horizontal transfer of resistance features among species of *Campylobacter* has been suggested to
play an important role in the emergence of antimicrobial resistance (Velazquez et al., 1995). In addition, transfer of resistant *Campylobacter* between different host species is possible, for example; transfer of fluoroquinolone-resistant *Campylobacter* from poultry to humans (Federal Register, 2000). In Northwest England, genotypic studies using multilocus sequence typing (MLST) indicated that the most commonly recovered genotypes from dairy cattle, wildlife, and environmental sources were frequently associated with human disease (Kwan et al., 2008).

1.5 Antibiotic resistance (Phenotypic) study of *Campylobacter*

The initial intention of using antimicrobial drugs in humans and veterinary medicine, more than fifty years ago, was to get health benefits for both humans and animals. However, microbial resistance to some important classes of antimicrobials was discovered which can have a serious public health impact (FDA, 2010). Thus, the misuse of antimicrobials would increase the persistence of these emerging resistant pathogens which subsequently can be transmitted to humans and cause a serious incurable infections (FDA, 2010). However, a debate is still going on the use and misuse of antibacterials as feed additives for livestocks and sometimes their indiscriminate prescription for therapeutic use in humans (Aarestrup and Wegener, 1999, Hecker et al., 2003). From the veterinary aspect, balanced farm economics and the maintenance of animal health are of particular interest rather than the concern on the acquired microbial resistance to antimicrobials and its affiliated risks (Phillips et al., 2004). *Campylobacter* is considered an important foodborne zoonotic disease. Thus, the highly resistant strains of this
pathogen, which can acquire resistance in response to antimicrobial selection pressures in food animal production, could cause severe economic losses for humans especially when the resistance to antimicrobials that are used as drugs of choice for treating human infections is acquired (Aarestrup and Wegener, 1999).

Resistance to antimicrobials has become a serious problem worldwide, and the numbers of *Campylobacter* resistant to multiple antibiotics continue to increase. Resistant *Campylobacter* strains can subsequently emerge because of the administration of antimicrobials to treat bacterial infections in animals, and may possibly contaminate human food (Piddock et al., 2008). The ability of livestock-associated *Campylobacter* to resist antibiotics possesses a significant problem and emphasizes the need for further research (Luangtongkum et al., 2009; Sanad et al., 2011). Several recent studies indicated that livestock-associated *Campylobacter* are becoming increasingly resistant to multiple antimicrobials of high importance for human treatment (Inglis et al., 2006; Châtre et al., 2010; Sanad et al., 2011), and is an increasingly important emerging threat to public health (Inglis et al., 2006; Sanad et al., 2011). Additionally, there is a clear increase in the antimicrobial resistance among pathogens isolated from dairy cattle and the use of antimicrobials in dairy cows and other livestock does contribute to increased antimicrobial resistance (Oliver et al., 2011). In a study conducted by Sato et al., 2004, there was no significant difference in the prevalence of *Campylobacter* spp. in organic and conventional farms (Sato et al., 2004). Moreover, resistance of human-associated *Campylobacter* isolates to fluoroquinolones (FQ) is proportionally increasing with the wide spread use of the same drug in animals and poultry in the United States (Price et al.,
2005; Oliver et al., 2011). Of particular interest, Campylobacter strains isolated from food animals has been exhibiting a remarkably increased resistance to fluoroquinolone and macrolides, which are the drugs of choice for treatment of human campylobacteriosis when clinical treatment is required (Luangtongkum et al., 2009).

1.6 Campylobacter jejuni induced abortion in sheep:

Campylobacter spp. can asymptptomatically colonize the intestinal tract of several species of food animals. Campylobacter spp. can colonize the intestines and gall bladder of healthy sheep without causing clinical diseases (Acik and Cetinkaya, 2006; Milnes et al., 2008; Smith, 2002). However, Campylobacter infection in sheep can cause abortions stillbirths, and weak lambs which lead to severe economic losses. Campylobacter fetus ssp. fetus, C. jejuni and C. coli are mainly the causative agents of abortions (Diker et al., 1988; Varga et al., 1990). Although it was known historically that C. fetus subsp. fetus is the main cause of ovine abortion, it appears recently that C. jejuni is increasingly associated with ovine abortion cases all over the world including the USA (Delong et al., 1996; Kirkbride, 1993; Skirrow 1994; Sahin et al., 2008). Campylobacter induced abortion rates usually ranges between 20% and may reach up to 90% in some cases (Spronk, 2000). Bacteremia, placentitis, uterine and fetal infection, and consequently abortion, which usually occurs in the third trimester of pregnancy, are common symptoms associated with ovine campylobacteriosis (Skirrow, 1994; Hedstrom et al., 1987). Further, occasionally retention of dead fetus is observed which may lead to death of the pregnant ewes due to septicemia and uterine sepsis. However, no clinical signs can
be seen in these ewes at the beginning of infection (Skirrow, 1994). The Veterinary Diagnostic Laboratory (VDL) report for 1993 indicated that 184 (10.3%) out of 1,784 of ovine abortions cases in South Dakota were caused by *Campylobacter* species (Kirkbride, 1993). In another study, (Delong et al., 1996), *Campylobacter* were identified from abortion cases in 14 different sheep flocks in three different States in the USA during the same lambing season. The majority (14/15) of *Campylobacter* isolated from these cases were identified as *C. jejuni* and only one as *C. fetus* subsp. *fetus*. It is still not clear the reasons for the emergence of this unique clone with altered virulence that can cause abortions (Sahin et al., 2008). Therefore, understanding the virulence properties of the abortion associated *C. jejuni* and pathogenesis of ovine abortion is important for the development of control strategies.

The genotypic properties of *Campylobacter* isolates from ovine abortions in the United States are still limited (Delong et al., 1996). In a recent study conducted on *C. jejuni* isolates retrieved from sheep abortions during a single lambing season in different States in the USA showed diverse genetic profiles among those isolates (Sahin et al., 2008). Molecular characterization of ovine abortion-associated *Campylobacter* isolates should provide a better understanding of the epidemiology and ecology of this important pathogen in sheep populations (Sahin et al., 2008). The genotyping analysis using PFGE for 33 *C. jejuni* isolates that were obtained from ovine abortion cases, on various farms at different times, revealed identical fingerprints for the majority (32/33) of the isolates. This finding suggests that a single genetically stable clone of *C. jejuni* is responsible for most of the abortions and is widely distributed in the US (Sahin et al., 2008). Further,
MLST analysis for a subset of those isolates classified all ovine abortion-associated isolates as ST-8 which belonged to Clonal Complex (CC) ST-21. Interestingly, ST-8 was also recorded previously in different hosts including humans based on the PubMLST database (Sahin et al., 2008).

In an effort to understand the pathogenesis of ovine abortion, Hedstrom et al. inoculated pregnant ewes in their third trimester with abortion associated *C. jejuni* (Hedstrom et al., 1987). Abortion occurred in all of the inoculated ewes 7 to 12 days post-inoculation. Specifically, gross lesions of placentitis occurred in the majority of the *C. jejuni* inoculated ewes along with uterine purulent endometritis and enlarged caruncles. Overall, it was suggested that *C. jejuni* is an important ovine abortifacient organism (Hedstrom et al. 1987). Since *C. jejuni* abortions were not common, no further studies have been designed. However, the recent emergence of *C. jejuni* as a leading cause of ovine abortion increased the need to better understand the virulence factors and pathogenesis of abortion-associated *C. jejuni*. In a recent study Burrough et al. (Burrough et al., 2009) investegated the pathogenesis of ovine inducing abortion *C. jejuni* in a guinea pigs model. Pregnant guinea pigs were inoculated via oral or intrapretonial (IP) routes with abortion associated *C. jejuni*. All IP inoculated animals as well as 6 out of 10 of the orally inoculated animals aborted and *C. jejuni* was retrieved from fetoplacental and intestinal tissues. This study indicated that ovine abortion-associated *C. jejuni* is highly abortifacient strain and can colonize the intestines, and cause systemic infection, leading to abortion (Burrough et al., 2009).
Campylobacter infection is usually acquired through ingestion of contaminated food, or water, as well as through the contact with infected animal reservoirs. The organism also can be transmitted between individuals by close contact. Moreover, the fetuses may get infected either by descending route through hematogenous transplacental transmission or via ascending infection from infected genital tract of the dam which might also occur during the normal vaginal delivery (Mcdonald and Gruslin, 2001; Butzler, 2004). Nevertheless, the zoonotic potential of the C. jejuni clone from sheep abortion described in Sahin et al. study is still unknown (Sahin et al., 2008). Studies looking at the role of several possible virulence mechanisms such as enterotoxins, motility, adherence, and invasion potentials in the pathogenesis of C. jejuni induced ovine abortion are needed to characterize the virulence traits of this clone (Skirrow, 1994; Bourke et al., 1998). Further, comparative functional genomics studies between abortifacient and non- abortifacient C.jejuni would provide critical information that would help better understand the virulence factors. Comparative genomic analysis between abortifacient C. jejuni and non- abortifacient C.jejuni (NCTC11168) strains revealed that the abortion associated strain possessed 58 unique genes and 2605 non-synonymous SNPs (Wu et al., 2011). Although these unique genes were highly conserved among abortion associated strains, most of these genes could not be detected in the abortion-associated C. jejuni isolates from UK using the array-based Comparative Genomic Hybridization (CGH) analysis (Wu et al., 2011). Comparative transcriptomic analysis of genes, revealed differential expression of genes that are likely associated with microbial virulence like flagellar assembly, motility, and energy metabolism, while genes
associated with transport functions were down-regulated in abortion inducing strain in comparison to NCTC11168 strain. Further, proteomic analysis detected 18 proteins that were differentially expressed between abortion associated strain and NCTC11168. In general, multiple proteins involved in iron utilization were down regulated in the sheep abortion strain (Wu et al., 2011). These findings suggested that the virulence of the abortion-associated C. jejuni is related to multiple genomic changes (Wu et al., 2011).

However, studies looking at the change in the host tissues following infection with abortion-associated C. jejuni are needed to better understand the host-pathogen interaction. These studies would be valuable to develop effective control strategies. The zoonotic potential of the abortion associated C. jejuni is not explored. Based on the initial finding that clones sharing similar genotypes exist in human population suggest a potential risk. The observation that a preterm labor in a farm working women at 22 weeks of gestation who had a history of C. jejuni induced diarrhea, abdominal pain, and fever one week prior to hospitalization suggest a possible role for C. jejuni in inducing abortions in humans as well (Mcdonald and Gruslin, 2001). Therefore, future studies should be directed to understand the zoonotic potential of the abortion associated C. jejuni to humans.

1.7 Research statement

Campylobacter is a leading cause of bacterial enteric illness in the United States. Campylobacteriosis is estimated to affect over one million people in the United States every year. Campylobacter species can cause mild to severe diarrhea, with loose watery
stools often followed by bloody diarrhea. *C. jejuni, C. coli,* and *C. lari* account for more than 99% of the human infections with *C. jejuni* accounting for 90% of these infections. Thermophilic species such as *C. jejuni* are occasionally invasive and the infections can manifest as meningitis, pneumonia, abortion, and neuropathic complications. *Campylobacter* infection can lead to polyneuropathies such as Guillain-Barre syndrome (GBS). Thermophilic *Campylobacters* are small, non-spore forming, Gram-negative, motile bacteria. Campylobacteriosis is a self-limiting disease often requiring no treatment; however, antibiotic treatment is necessary in some cases of *Campylobacter* infections.

Chickens and pigs have been considered for a long time the main sources of campylobacteriosis in humans. However, it was suggested in recent studies that cattle are a potential emerging source of human infections. Cattle can contribute to human infections through direct contact, environmental contamination, and ingestion of contaminated food and milk. *C. jejuni* and *C. coli* usually colonize cattle without showing clear symptoms. It was reported in some studies that 40–60% of individual cattle and 80% of herds shed *Campylobacter*. The colonization of *Campylobacter* in dairy and beef cattle is not only significant as a potential source for contamination of milk at the farm and the carcass at processing plans, but also the contamination of water sources from the waste of abattoir and animal slurries to the environment. In addition, many studies have reported that the presence of farm animals, such as cattle and sheep, on broiler farms increases the risk of infection in broiler flocks.
The direct or indirect contributions of cattle and sheep to sporadic human infections are still under recognized. Gross microbial contamination of the carcass with gut contents most probably occurs during evisceration but it has also been thought that the contamination can occur during the process of hide removal or through cross-contamination of carcass from hide via workers hands and instruments. The sources of campylobacteriosis and its routes of transmission at dairy farms have not been clearly established. However, studies have indicated that wild birds may play a potential role in the epidemiology of human campylobacteriosis by transmitting *Campylobacter* from livestocks to humans. Thus, more epidemiological and genotyping studies on *Campylobacter* isolates from cattle and identifying the shared sequence types among bird, cattle and human isolates, would be useful in developing ways to control and prevent human infection and improve the safety of our food supply.

It has been well known that *Campylobacter fetus* infection (vibrios) is one of the major causes of ovine abortions worldwide. However, recently, *Campylobacter jejuni* has been shown to be increasingly associated with sheep abortion. *C. jejuni* was isolated from aborted sheep in multiple lambing seasons on different farms in Iowa, Idaho, South Dakota, and California. Further, it was found that *C. jejuni* has dominated and replaced *C. fetus* as the *Campylobacter* species causing sheep abortion in the US. A single tetracycline-resistant *C. jejuni* clone has emerged as the major cause of *Campylobacter*-associated sheep abortion in the US. Recently, the Animal Disease Diagnostic Lab at the Ohio Department of Agriculture (ODA) isolated two *C. jejuni* isolates from aborted sheep fetuses from ewes in their third trimester. However, the virulence characters of the
abortion associated \( C. \textit{jejuni} \) and the pathogenesis of ovine abortion are poorly understood. Further, whether these ovine abortions associated \( C. \textit{jejuni} \) have any zoonotic potential for human health is unknown.

**Specific objectives of this work:**

1) To determine the genotypic and phenotypic properties of cattle-associated \textit{campylobacter} and their implications to public health in the USA.

2) To investigate the occurrence of the invasion associated marker (\textit{iam}) in \textit{Campylobacter jejuni} isolated from cattle.

3) To understand the molecular epidemiology of \textit{Campylobacter} isolates from dairy cattle farms and wild birds in Ohio.

4) To conduct pathogenesis studies on ovine and bovine abortion-associated \textit{Campylobacter jejuni} isolates.
### References

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CHAPTER 2

Genotypic and Phenotypic Properties of Cattle-Associated
Campylobacter and their Implications to Public Health in the USA

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2.1 Abstract

Since cattle are a major source of food and the cattle industry engages people from farms to processing plants and meat markets, it is conceivable that beef-products contaminated with *Campylobacter* spp. would pose a significant public health concern. To better understand the epidemiology of cattle-associated *Campylobacter* spp. in the USA, we characterized the prevalence, genotypic and phenotypic properties of these pathogens. *Campylobacter* were detected in 181 (19.2%) out of 944 fecal samples. Specifically, 71 *C. jejuni*, 132 *C. coli*, and 10 other *Campylobacter* spp. were identified. The prevalence of *Campylobacter* varied regionally and was significantly (*P*<0.05) higher in fecal samples collected from the South (32.8%) as compared to those from the North (14.8%), Midwest (15.83%), and East (12%). Pulsed Field Gel Electrophoresis (PFGE) analysis showed that *C. jejuni* and *C. coli* isolates were genotypically diverse and certain genotypes were shared across two or more of the geographic locations. In addition, 13 new *C. jejuni* and two *C. coli* sequence types (STs) were detected by Multi Locus Sequence Typing (MLST). *C. jejuni* associated with clinically human health important sequence type, ST-61 which was not previously reported in the USA, was identified in the present study. Most frequently observed clonal complexes (CC) were CC ST-21, CC ST-42, and CC ST-61, which are also common in humans. Further, the cattle associated *C. jejuni* strains showed varying invasion and intracellular survival capacity; however, *C. coli* strains showed a lower invasion and intracellular survival potential compared to *C. jejuni* strains. Furthermore, many cattle associated *Campylobacter* isolates showed resistance to several antimicrobials including
ciprofloxacin, erythromycin, and gentamicin. Taken together, our results highlight the importance of cattle as a potential reservoir for clinically important *Campylobacter*.

### 2.2 Introduction

Campylobacteriosis is estimated to affect over one million individuals in the United States annually, with symptoms ranging from mild diarrhea to more serious neuropathies (Scallan *et al*., 2011). Of major concern are *C. jejuni*, a species responsible for the majority of human campylobacteriosis, and *C. coli*, which has been exhibiting increased resistance to antimicrobials (Allos, 2001; Saenz *et al*., 2000). Both *C. jejuni* and *C. coli* can readily and asymptotically colonize major food-animals such as poultry and cattle, subsequently contaminating foods of animal origin including meats and dairy products (Miller and Mandrell, 2005). Therefore, both species highlight the potential public health impact of *Campylobacter* contamination of food animals.

Although it is known that chickens constitute a major reservoir for *Campylobacter* spp., the occurrence of these pathogens in other food animals such as cattle and its potential impact on human health remain largely uncharacterized. The latter can be partially attributed to the sporadic nature of *Campylobacter* infections and difficulties in isolating these fastidious pathogens. However, since cattle are a major source of food and the multi-faceted cattle industry engages people from farms to processing plants and meat markets, it is conceivable that both live cattle and contaminated cattle products could contribute significantly to *Campylobacter* infections in humans. Furthermore, cattle-associated *Campylobacter* also pose an additional indirect public health risk (Piddock *et al*., 2000; Vandeplas *et al*., 2008). For example,
contamination of surface and ground water may occur with waste run-off from cattle farming and processing operations. Thus, it is important to further investigate the epidemiology of *Campylobacter* in the cattle population in order to assess associated risks to public health.

Recent studies have shown that the contributions of non-poultry associated *Campylobacter* to human infections were considerable and warrant investigation (Ragimbeau *et al*., 2008; Wilson *et al*., 2008). For example, evidence collected using Multilocus Sequence Typing (MLST) showed that cases of human *Campylobacter* infections in Finland could be attributed equally to cattle and poultry (de Haan *et al*., 2010). Moreover, another study reported that cattle were the source of human infections in 35% of the cases examined in Lancashire, England (Wilson *et al*., 2008). This is not surprising since up to 80% of cattle herds and 40–60% of the individual animals shed *Campylobacter* (Besser *et al*., 2005; Milnes *et al*., 2007; Wesley *et al*., 2000). Consequently, the role of cattle as reservoirs for these pathogens might be important for understanding the epidemiology of *Campylobacter* infections. However, particularly in the US, the prevalence of *Campylobacter* in cattle, their characteristics and relationship to isolates from humans have not been extensively described in the peer reviewed literature.

Host cell invasion and intracellular survival and resistance to antibiotics are important characteristics that affect *Campylobacter* infections in humans. However, the potential role of these properties in facilitating human infections with *Campylobacter* isolated from cattle is not clear. Although epidemiological studies deploy typing analysis to glean information about the relationships between cattle-associated *Campylobacter*
and clinically-important human isolates, the capability of cattle-associated *Campylobacter* to invade and persist in the human host might require further assessment, especially when previously unknown sequence types are identified. This also applies to antimicrobial resistance properties, which have been posing a serious concern in *Campylobacter* collected from animal hosts along with the possibility of the transmission of these isolates to humans through the food chain. Therefore, epidemiological studies concerning cattle-associated *Campylobacter* would gain from attempts to amend molecular typing analysis with *in vitro* invasion studies using human intestinal cell lines and phenotypic assays for determining antibiotic resistance.

Since molecular typing data of cattle-associated *Campylobacter* in the United States are limited and little is known about their impact on human health, it is important to adopt a multiphasic approach to characterize cattle-associated *Campylobacter* by using a combination of molecular typing and *in vitro* assays. Therefore, in this study, we determined the genotypes of *C. jejuni* and *C. coli* isolated from cattle slaughtered for meat purpose in different geographical locations in the U.S. and investigated their antimicrobial susceptibility profiles as well as virulence associated phenotypes such as their potential for invasion and persistence in human intestinal epithelial cells.

### 2.3 Materials and Methods

**Distribution of sampling sites and collection and processing of fecal samples from beef cattle**
A total of 944 fresh fecal samples (10 g each) were collected during the summer and early fall of 2008 from cattle presented to slaughter which included both feed-lot (n=482) as well as mature cows and bulls (n= 462) culled from milking and breeding herds (Table 2.1). The samples were collected from colon on the conveyer belt during the normal harvest process from slaughtered cattle. To account for potential spatial heterogeneity between different beef processing plants, sampling efforts included seven plants that were distributed across four major geographical locations [North (N), East (E), Midwest (M), and South (S)] in the U.S. The time interval of sample collection per plant spanned two consecutive days and was divided into four periods during which at least four different lots of cattle were sampled in order to capture the genetic diversity of cattle-associated *Campylobacter*. To further limit sampling bias, the number of samples collected per plant was calculated in terms of the total number of animals expected for slaughter on each day, and a statistically calculated sample size to detect at least a single sequence type of *Campylobacter* should it be present in each group of animals at a prevalence of greater than 2.5% with a confidence of 95%. Samples were stored on ice immediately after collection and shipped overnight to the laboratory for *Campylobacter* isolation.

**Isolation and identification of *Campylobacter* species from fecal samples**

To isolate *Campylobacter* spp., 1 g of each fecal sample was enriched in Preston broth for 48 h at 42 °C under microaerobic conditions (5% O₂, 10% CO₂, and 85% N₂) (Krause *et al*., 2006). From the enrichments, an inoculum (100 µl) was spread onto
modified Cefoperazone Charcoal Deoxycholate Agar (mCCDA) plates, which were then incubated for an additional 48 h at 42° C under microaerobic conditions (Engberg et al., 2000). Three to five colonies suspected as Campylobacter were selected from each plate and sub-cultured onto Muller-Hinton agar plates, after which DNA was extracted from each isolate using the Genomic DNA Purification Kit (Epicenter, Madison, WI) as described by the manufacturer. The DNA was then quantified using the NanoDrop 1000 Spectrophotometer V3.7.1 (Fisher Scientific, Pittsburgh, PA) and subjected to species-specific PCR analysis to confirm the identity of the isolates as described elsewhere (Denis et al., 2008). PCR analysis targeted a 16S rRNA gene fragment (F) 5'-ATCTAATGGCTTAACCATTAAAC-3' and (R) 5'-GGACGGTAACTAGTTTAGTATT-3', mapA, (F) 5'-CTATTTTATTTTGTAGTGCTTG-3' and (R) 5'-GCTTTATTTGCCATTGTTTTATTA-3' and ceuE, (F) 5'ATTTGAAAATTGCTCCAACATG-3' and (R) 5'-TGATTTTATTATTGTAGCAGCG-3' which indicated the specific detection of Campylobacter spp., C. jejuni, and C. coli, respectively (Denis et al., 1999; Linton et al., 1997). All PCR products were resolved on a 1.5% agarose gel containing 0.5 µg/ml of ethidium bromide. The size of the PCR products was determined using a 1Kb DNA ladder and detection was confirmed by comparison to PCR products generated from C. jejuni 81-176 (wild-type strain) and C. coli (ATCC 33559), which were used as positive controls in all PCR analysis. Negative controls (reactions with no DNA templates) were included in all PCR analyses to ensure specific product amplification.
Pulsed Field Gel Electrophoresis (PFGE) of *Campylobacter* isolates

To determine their genotypic relatedness, the *Campylobacter* isolated from fecal samples were analyzed using PFGE analysis as described in Ribot et al. (Ribot et al., 2001). Briefly, *C. jejuni* and *C. coli* isolates were harvested from MH agar plates and suspended to an OD$_{610}$ of 1.4 in 1X PBS. To prepare agarose plugs, OD adjusted suspensions were gently mixed with 1% SeaKem Gold agarose (SKG, Fisher scientific, Pittsburgh, PA) that was pre-melted in TE (10 mM Tris, 1 mM EDTA, pH 8.0). The plugs were then incubated with shaking (200 rpm) in lysis buffer [50 mM Tris, 50 mM EDTA (pH 8.0), 1% sarcosine, 0.1 mg ml$^{-1}$ of proteinase K] for 1 h at 55° C. After lysis the plugs were washed four times and suspended in 5 ml of fresh TE. The plugs were then sliced and digested overnight with SmaI at room temperature. The digested slices were loaded onto a 1% SKG agarose gel and DNA fragments were separated by electrophoresis for 20 h using the CHEF Mapper system (Bio-Rad, Hercules, CA) followed by post staining with ethidium bromide. The resulting PFGE patterns were documented and analyzed using the BioNumerics 5.1 software (Applied Maths Inc, Austin, TX). Similarity and clustering analysis of the PFGE patterns were performed using the Dice Coefficient and the unweighted pair-group method with arithmetic averages (UPGMA) with optimization of 1% and position tolerance of 1.5% (Ragimbeau et al., 2008), respectively. The PFGE analysis was also performed on *C. jejuni* 81-176 and *C. coli* (ATCC 33559), which were used as controls for facilitating gel to gel comparison. Furthermore, Lambda Ladder PFG Marker (50-1,000 kb, New England
BioLabs, Ipswich, MA) was used as a molecular marker. A cut-off similarity value of 75%, was used to determine the sub-clusters of macro restriction profiles (MRPs).

**Determining the antimicrobial-resistance properties of the *Campylobacter* isolates**

Minimal inhibitory concentrations (MIC) for *C. jejuni* and *C. coli* isolates were determined using commercially available 96-well plates containing antimicrobials (Sensititre Campy plates, TREK Diagnostic Systems Inc., Cleveland, OH, USA). These Sensititre Campy plates were used as described by the manufacturer and include *Campylobacter*-relevant antimicrobials such as azithromycin (AZI) (MIC for a resistant *Campylobacter* isolate: ≥8 µg ml⁻¹); ciprofloxacin (CIP) (MIC: ≥4 µg ml⁻¹); clindamycin (CLI) (MIC: ≥8 µg ml⁻¹); erythromycin (ERY) (MIC: ≥32 µg ml⁻¹); gentamicin (GEN) (MIC: ≥8 µg ml⁻¹); nalidixic acid (NAL) (MIC: ≥64 µg ml⁻¹); telithromycin (TEL) (MIC: ≥8 µg ml⁻¹); florfenicol (FEN) (MIC: ≥8 µg ml⁻¹); and tetracycline (TET) (MIC: ≥16 µg ml⁻¹). *Campylobacter* isolates were grown to mid-log phase and then suspended in Mueller-Hinton broth to achieve an OD₆₀₀ of 0.05. For each isolate, one hundred microliter of the suspension were transferred to each well in the Sensititre Campy plates, including a control well that did not contain any antibiotics, while *C. jejuni* 81-176 and *C. coli* (ATCC 33559) were used for quality control. The plates were then incubated under microaerobic conditions at 42°C for 24 h after which the minimum inhibitory concentration (MIC) was measured. The MIC for each antibiotic was defined as the absence of bacterial growth in the well with the lowest concentration of the antibiotic and
susceptibility was interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2006).

**Multilocus sequence typing (MLST) of Campylobacter isolates**

To investigate the clonality of the *Campylobacter* isolates and to assess similarity to strains associated with human infections, MLST analysis was performed on a total of 112 isolates (62 *C. jejuni* and 50 *C. coli*). Nine *C. jejuni* isolates were not typed because either they yielded incomplete profiles or last culturability upon storage. However, since the *C. coli* isolates showed limited genetic diversity using MLST, the tested number of isolates (n=50) was considered adequate to meet the objectives of this study. These isolates were selected to be representative of different PFGE clusters. MLST analysis was conducted as described by Dingle *et al.* (Dingle *et al.*, 2001). Briefly, loci from seven housekeeping genes (*aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt*, and *uncA*) were amplified using gene specific primers by PCR and anticipated sizes of the amplicons were confirmed by agarose gel electrophoresis. The *Campylobacter* MLST oligonucleotides (Dingle *et al.*, 2001) were obtained from Integrated DNA Technologies (Coralville, IA). PCR products were purified (QIA quick 96 PCR purification kit, QIAGEN, Valencia, CA), sequenced in both directions as described previously (Thakur and Gebreyes, 2005), forward and reverse sequences were then aligned using ClustalW (www.ebi.ac.uk/clustalw). Allelic profiles were determined by performing BLAST analysis using the single-locus query function, while sequence types (STs) were assigned using the allelic profile query function available in the MLST *Campylobacter* database.
STs were then traced to their respective clonal complexes using BURST at http://pubmlst.org/.

**Invasion and Intracellular survival potential of the *Campylobacter* isolates in INT407 cells**

Representative *C. jejuni* (n = 19) and *C. coli* (n = 12) that belonged to clonal complexes associated with human infections as well unassigned clonal complexes including the newly identified STs were further tested for their virulence associated phenotypes by assessing their ability to invade and survive within human intestinal epithelial cells. The invasion studies were conducted as described in Konkel *et al.* (Konkel and Cieplak, 1992) and Prasad *et al.* (Prasad *et al.*, 1996). Briefly, $10^5$ cells ml$^{-1}$ of INT407 (human embryonic intestine, ATCC CCL 6) were seeded into each well of a 24-well tissue culture plates after suspension in Eagle’s Minimum Essential Medium (MEM, Fisher scientific, Pittsburgh, PA) supplemented with 10% fetal bovine serum (FBS, Fisher scientific, Pittsburgh, PA). The plates were then incubated at 37 °C in a humidified incubator with 5% CO$_2$ until semi-confluent mono-layers were obtained. In preparation for infection with *Campylobacter*, the INT407 mono-layers were washed three times and covered in MEM supplemented with 1% FBS. Similarly, *Campylobacter* cultures were washed three times and suspended in MEM supplemented with 1% FBS to obtain $10^7$ bacteria ml$^{-1}$. One ml of bacterial suspension was added to each well containing the INT407 cell monolayer, achieving a 1:100 multiplicity of infection (MOI) then incubated for 3 h. After 3 h of incubation with bacteria, cells were treated with
gentamicin (150 µg/ml) and incubated for additional 2 h. The infected mono-layers were then washed three times with MEM, lysed using 0.1% Triton X-100 (Fisher scientific, Pittsburgh, PA), serially diluted (10-fold) in MEM and 100 µl of each dilution were spread on MH agar plates. The agar plates were then incubated for 48 h at 42° C under microaerobic conditions, after which colony forming units (CFU) were counted to determine the number of *Campylobacter* that invaded the monolayers. Each isolate was tested in duplicate per assay, and the experiment was repeated three times on separate occasions. *C. jejuni* 81-176 (highly invasive) (Bacon *et al.*, 2000), *C. jejuni* NCTC11168 (poorly invasive) (Bacon *et al.*, 2000) and *C. coli* (ATCC 33559) were used as controls in all invasion assays.

To assess intracellular survival (Konkel and Cieplak, 1992), *Campylobacter* cultures and the INT407 cells were processed as described above. However, after treatment with gentamicin, MEM containing 3% FBS and bacteriostatic concentration of gentamicin (10 µg ml\(^{-1}\)) were added to each well to allow strict quantitation of intracellular bacteria. After further incubation at 37 °C for 24 h, the monolayers were washed three times, lysed and serially diluted in MEM as described earlier and plated on MH agar to determine CFU of *Campylobacter* surviving within the intestinal cells. In addition, we also cultured the supernatant of gentamicin treated monolayers to ensure the quality of the gentamicin protection assay. The experiment was repeated three times on separate occasions. *C. jejuni* 81-176, *C. jejuni* NCTC11168, and *C. coli* (ATCC 33559) were used as controls in all intracellular survival assays.
Statistical analysis

A Chi square test was used to evaluate data collected from the Campylobacter prevalence analysis. The data derived from cell culture assays were analyzed using one-way ANOVA followed by Tukey's Multiple Comparison Test. A $P$ value of $<0.05$ was considered statistically significant for all experiments. Measurements expressed as mean $\pm$ SE (standard error) were averages of at least three replicas.

2.4 Results

Occurrence and distribution of Campylobacter spp. in feces sampled from cattle

The occurrence and distribution of Campylobacter in cattle feces was investigated in order to determine the role of cattle as a potential reservoir for these bacteria. Campylobacter were detected in 181 (19.2%) out of 944 fecal samples, while a total of 213 isolates were retrieved from the Campylobacter-positive fecal samples. The $C.\ jejuni$ and $C.\ coli$ constituted the majority of the isolates and were detected in 71 (7.5%) and 132 (14%) of the samples, respectively (Table 2.1). There was no significant difference ($P<0.12$) in the number of $C.\ jejuni$ isolated from fecal samples obtained from; either feed lot (6.2%) or the cull cattle (8.9%), while $C.\ coli$ was more frequently isolated from cull cattle (17%) as compared to feed lot cattle (11%) ($P<0.01$). Furthermore, the occurrence of Campylobacter in cattle feces varied according to geographic location of the sampling sites. Specifically, Campylobacter species were retrieved from 32.8% of the fecal samples collected from the South, which was significantly higher ($P<0.05$) than those from the North (14.8%), Midwest (15.83%), and East (12%) (Table 2.1).
C. jejuni and C. coli strains were genotypically diverse across different geographic regions

PFGE analysis was performed on C. jejuni and C. coli isolated from feces in order to determine the genetic diversity of these bacteria and the relationship between the isolates retrieved from different geographic locations. PFGE analysis was successfully performed on 67 out of 71 C. jejuni, while 4 isolates could not be typed using the aforementioned method. Regardless, analysis of the PFGE profiles of the C. jejuni suggested that the isolates possessed diverse genotypes, especially when comparing profiles of isolates belonging to different geographic locations (Fig. 2.1A). Furthermore, there were 7 main clusters (Fig. 2.1A) and by using a cut-off similarity value of 75%, profiles of the C. jejuni were classified to 15 sub-clusters. With the exception of 4 clusters that included isolates that were collected from 2 to 3 different geographic locations, C. jejuni profiles were mostly found to form geographically homogenous groupings. For example, one cluster was composed of three isolates collected from the East, the Midwest, and the South, respectively, while others included 4 isolates, 3 of which were isolated from the Midwest and one from the South (see arrows in Fig. 2.1A). Overall, it was interesting to observe that profiles of the isolates from the North did not group with those collected from the East and the South.

Similarly, PFGE analysis was successfully performed on 115 out of 132 C. coli isolates, while the remaining isolates could not be typed using the aforementioned method (Fig. 2.1B). Unlike the C. jejuni, PFGE profiles of certain C. coli isolated from all four geographic locations were found to share a 100% similarity. Specifically, analysis
grouped the *C. coli* isolates into 4 main clusters and by using a cut-off similarity value of 75%, 21 sub-clusters were identified, 2 of which included identical profiles (100% similar) for isolates from different geographic locations (See arrows Fig. 2.1B). For example, a cluster was composed of 5 isolates (4 from the North and 1 from the East) that displayed identical profiles, while another cluster similarly contained identical profiles of 4 isolates (3 from the North and 1 from the South). However, other clusters contained profiles of *C. coli* isolates from different geographic locations that were not identical, yet exhibited high similarity (between 79 to 95%) than that of the cut-off (75%). Similar to PFGE analysis of *C. jejuni*, many *C. coli* isolated from the same geographic location showed profiles that tend to cluster together.

**Antimicrobial susceptibility of *C. jejuni* and *C. coli* isolates**

To better assess the potential public health impact of the *Campylobacter* spp. associated with cattle, the isolates were assayed for their potential to resist antibiotics that are of both clinical and veterinary importance. Antimicrobial susceptibility was assessed on 66 (the 5 remaining isolates lost cultivability after storage) *C. jejuni* isolates using commercially available Sensititre Campy plates. The *C. jejuni* isolates were resistant to different antimicrobials including, ciprofloxacin (MIC: 4-64 μg ml\(^{-1}\)), erythromycin (MIC: 32 μg ml\(^{-1}\)), tetracycline (MIC: 16-64 μg ml\(^{-1}\)), and clindamycin (MIC: 8-16 μg ml\(^{-1}\)) (Fig. 2A). However, resistance to tetracycline was observed for the majority of the tested *C. jejuni* (72.7%), while resistance to nalidixic acid was observed for only 27.3% of the isolates (Fig. 2.2). Furthermore, 10 (15.1%) tested *C. jejuni* were resistant only to
tetracycline, while 34 (51.5 %) isolates were resistant to 3 or more antimicrobials, including ciprofloxacin, tetracycline, nalidixic acid, erythromycin, and clindamycin. Overall, a higher percentage of *C. jejuni* isolates from the South region showed decreased resistance to most of the antimicrobials (See asterisk Table 2.2). The MRPs and antimicrobial resistance profiles of the *C. jejuni* isolates analyzed by MLST are summarized in (Table 2.5).

Since, *C. coli* isolates belonging to different PFGE clusters were highly similar, we tested representative isolates from each cluster for a total of 63 isolates. Although the range of MIC to disparate antibiotics was at instances different from those observed for *C. jejuni*, the *C. coli* isolates also exhibited resistance to the several of the antimicrobials tested (Fig. 2.2). For example, *C. coli* isolates were resistant to ciprofloxacin (MIC: 4-64 µg ml⁻¹), erythromycin (MIC: 32-64 µg ml⁻¹), tetracycline (MIC: 16-64 µg ml⁻¹), and clindamycin (MIC: 16 µg ml⁻¹) (Table. 2.3). Furthermore, resistance to telethromycin was observed for the majority of the tested *C. coli* (66.7%), while resistance to flourofenicol was observed for only 25.4% of the isolates (Fig. 2.2). Of the 63 tested *C. coli*, 3 (4.8%), 9 (14.3%) and 48 (76.2%) isolates were resistant to only one (e.g. nalidixic acid), two (e.g. ciprofloxacin and nalidixic acid), and three or more antimicrobials, respectively. Furthermore, a higher percentage of *C. coli* isolates from the South showed increased resistance to all the antimicrobials (Table 2.2).

**MLST analysis identifies STs belonging to human clonal complexes**
To determine the genotypic properties of *Campylobacter* spp. isolated from cattle as well as their relationship to those of human origin, MLST analysis was performed on selected *C. jejuni* and *C. coli* isolates. A total of 112 isolates, 62 *C. jejuni* and 50 *C. coli* that represented the main PFGE clusters and different geographic locations, were selected for MLST analysis. Thirteen new *C. jejuni* STs were identified and designated as STs 4922 to 4932, 5447, and 5448 (Table 2.3). These new STs were recognized following query with the PubMLST database. Furthermore, three new alleles, glyA; 416 (Cj-M-46), pgm; 537 (Cj-M-63) and tkt; 436 (Cj-M-64) were detected for 3 STs out of 13 new *C. jejuni* STs. Both pgm and tkt alleles had only a single nucleotide polymorphism, while glyA had a two nucleotide polymorphism.

In general *C. jejuni* isolates showed high genetic diversity by MLST analysis (Table 2.3). A total of 25 different STs were identified. Twenty isolates with 8 STs belonged to Clonal Complex (CC) ST-21, while 3 of these isolates were assigned to new STs. Additional isolates (n= 19) were grouped into 3 STs that belonged to CC ST-42, while five, three, and 11 isolates were identified as ST-459 which belonged to CC ST-42, ST-590, and ST-1013, respectively. Finally, one isolate belonged to CC ST-45, one belonged to CC ST-48, and 9 belonged to undefined CC (Table 2.3). Interestingly, we also detected in our study three isolates belonging to clonal complex ST-61, and two of them were identified as ST-61, which is clinically important and has not been reported previously in cattle in the USA according to the PubMLST database. The most frequently observed clonal complexes (CC) were CC ST-21, CC ST-42, and CC ST-61, which are
also common in humans. Six isolates in our collection had incomplete allelic profiles and could not be assigned to any ST.

In contrast to *C. jejuni* isolates, *C. coli* isolates showed very limited diversity (Table 2.3). Of the 50 *C. coli* isolates analyzed (Table 2.3), a total of 8 STs were identified with 1 isolate belonging to new ST-4933 and ST-5446. Interestingly, 40 (80%) isolates were identified as ST-1068. Twenty three of those 40 isolates were isolated from the South as well as 8 from the North, 7 from the Midwest and 2 from the East. Additionally, seven different STs were identified from the remaining 10 strains that were isolated from all four regions. Two of these isolates were identified as ST-902 (from Midwest), while the remaining two isolates (from the North and the South, respectively) were identified as ST-2501. Additional isolates (n=2) were identified as ST-1110 (East), while three more isolates were identified as ST-3866, ST-5372, and ST-4933, respectively. Additionally, one isolate was identified as a new ST-5446 (Midwest). All 50 *C. coli* isolates belonged to CC ST-828 (Table 2.3).

**Invasion and intracellular survival potential of cattle associated *C. jejuni* and *C. coli***

To investigate the virulence-associated potential of the cattle-associated *C. jejuni* and *C. coli*, we tested selected isolates for their ability to invade and survive in human INT407 intestinal epithelial cells. *C. jejuni* and *C. coli* isolates that belonged to all the detected CCs were selected for these assays; in addition isolates that exhibited disparate antibiotic resistance profiles and/or originated from different geographic locations were also included in this analysis. All tested *C. jejuni* isolates (n=19) invaded INT407 (Fig.
2.3A); however, the invasion potential varied between isolates, ranging from an average of 2.75x10^1 to 8.0x10^4 CFU ml^-1 (Fig. 3A). The invasion capability of 17 of the 19 isolates tested was significantly lower than that of the highly invasive strain *C. jejuni* 81-176 (*P<0.01*); however, 11 of these isolates invaded the INT407 cells with higher numbers as compared to the poorly invasive *C. jejuni* NCTC11168 (Fig. 2.3A). Furthermore, two *C. jejuni* isolates Cj-M-13 and Cj-N-33 exhibited high invasion potential that matched that of *C. jejuni* 81-176 (Figure 2.3A). Interestingly, all tested *C. jejuni* isolates were capable of intracellular survival, albeit the potential varied between isolates, ranging from an average of 8.0x10^1 to 1.3x10^4 CFU ml^-1(Fig. 2.3B). Additionally, the same aforementioned two isolates showed a significantly higher (*P<0.01*) potential for intracellular survival as compared to that of *C. jejuni* 81-176.

On the other hand, only nine *C. coli* isolates could invade the INT407 cells with average numbers ranging between 2.75x10^1 and 1.34x10^4 CFU ml^-1 (Fig. 2.4A). There was a significant difference in the invasion capabilities between the *C.coli* isolates (*P<0.05*). Furthermore, only seven isolates were capable of intracellular survival (Fig. 2.4B). Three isolates that did not invade were not tested for intracellular survival. In general *C. coli* isolates were less invasive and displayed reduced intracellular survival compared to *C. jejuni*.

Our data also show that invasive *Campylobacter* possessed a variable antimicrobial phenotype that ranged from complete susceptibility to resistance to 7 different antimicrobials (Table 2.4). However, it was notable that *C. jejuni* isolates with
high invasion potential (Cj-M-13 and Cj-N-33) were also resistant to multiple antimicrobials (see asterisks Table 2.4; Fig. 2.3A).

2.5 Discussion

We investigated the occurrence of two important Campylobacter spp. (C. jejuni and C. coli) in cattle that are slaughtered for meat production across 4 major geographic locations in the USA. This is in contrast to previous studies of cattle-associated Campylobacter in the USA, which have been mostly geographically confined and/or limited to investigating the prevalence of certain species as well as other phenotypic characteristics such as antibiotic resistance (Wesley et al., 2000; Englen et al., 2007; Hoar et al., 2001). Therefore, our approach was unique as it included analysis of genotypic diversity and antimicrobial resistance properties as well as attempts to understand the potential role of cattle-associated Campylobacter in virulence related phenotypes that might impact human health. Significantly, our findings highlight the importance of cattle as a reservoir for genotypically diverse, antimicrobial resistant, and potentially virulent Campylobacter in the USA.

Although the association of Campylobacter spp. with cattle in the USA has been investigated previously, available literature reported differing prevalence numbers for these pathogens. For example, Hoar et al. (Hoar et al., 2001) isolated Campylobacter from 5% of the fecal samples collected from cattle, while Sato et al (Sato et al., 2004) and Gharst et al (Gharst et al., 2006) showed that these bacteria occurred in 27.9% and 23.4% of their samples, respectively. These differences in the prevalence of cattle-associated Campylobacter can be attributed to several factors, including methods for
isolation, sample size and type (e.g. dairy versus feedlot), seasonal variations, and geographical location (Allos, 2001). However, the latter factor, geographical location, was a prominent difference in the aforementioned prevalence studies, which were conducted in California, Wisconsin and Southeastern region of the USA, respectively (Hoar et al., 2001; Sato et al., 2004; Gharst et al., 2006). Since the prevalence of cattle-associated *Campylobacter* in the USA seemed to vary according to the geographic location, it was necessary in this study to investigate this assumption as well as the overall occurrence of these bacteria in cattle. Our results showed that the occurrence of *Campylobacter* in Southern USA (prevalence of 32.8%) was significantly (*P*<0.05) higher than those observed in Northern (14.8%), Midwestern (15.83%), and Eastern (12%) locations. The precise reasons that contribute to the observed differences in geographic distribution of the *Campylobacter* are not clear. Nevertheless, previous studies have suggested that environmental conditions such as temperature, humidity, and sunlight might contribute to increases in human infections with *Campylobacter* (Patrick et al., 2004). Subsequently, the climate might be a driver of the relatively higher prevalence of cattle-associated *Campylobacter* in the Southern USA, which is generally warmer as compared to the other locations. Interestingly, Stanley et al. (Stanley et al., 1998) suggested that indirect-temperature dependent factors such as migratory animals might impact the occurrence of *Campylobacter* in dairy cattle. Additionally, *C. coli* have been previously reported to predominantly occur in samples collected from broiler flocks during warmer months (Denis et al., 2008). Strikingly higher prevalence of *C. coli* (83 isolates) in our samples from the South (Table 2.1) further support a role for climate in
affecting the geographic prevalence of *Campylobacter*. Regardless, the overall prevalence of cattle-associated *Campylobacter* in this study was 19.2%, falling between the percentages (0.8 to 46.7%) reported for cattle from other countries (Bae et al., 2005). However, it is important to note that samples in our study were collected only over a two-day period from a limited number of locations in each region. Furthermore, to emphasize a public health relevance of cattle as potential reservoir for *Campylobacter* (discussed below), our sampling efforts focused on the seasons that normally witness an increase in *Campylobacter* incidences (Wilson et al., 2008). As such our prevalence data don’t account for possible seasonal and temporal variations and, from this study alone, the extent to which differences between geographic locations contributed to *Campylobacter* prevalence in cattle cannot be conclusively ascertained. Nevertheless, our prevalence data provide evidence that like in other countries cattle in the USA might constitute a considerable reservoir for these pathogens, promoting their potential persistence in cattle-derived products and environment, which might pose a risk for human consumers and cattle-handlers.

The genus *Campylobacter* contains species that are known for their adaptation capabilities, readily acquiring genetic material necessary for survival and persistence (Meinersmann et al., 2002). High genotypic diversity has been reported in *Campylobacter* isolated from humans, chickens, sheep, pigs, and cattle (Levesque et al., 2008; Oporto et al., 2006; Oporto et al., 2007; Wilson et al., 2010). This is important since the genotypic diversity of these bacteria might serve as an indicator for the value of a reservoir in providing a suitable niche for the persistence of these microorganisms and
their evolution (Wilson et al., 2010). As elegantly demonstrated by Wilson et al. (Wilson et al., 2010), the broiler gastrointestinal tract constitutes such a niche, allowing multiplication of C. jejuni and enhancing its genetic diversity. Subsequently, it was important to investigate the genetic diversity of Campylobacter in cattle in order to assess the potential attributes of this reservoir. Our PFGE analysis of the cattle-associated Campylobacter revealed that all C. jejuni and C. coli isolates were generally grouped into 7 and 4 major clusters, respectively (Fig 2.1). Remarkably, some C. jejuni isolates possessed genotypes that were 100% similar on 3 different occasions (see stars in Fig. 1A). Similarly, several C. coli exhibited 100% similar genotypes and were encountered on seven occasions (see asterisks in Fig 2.1B). Taken together, these observations confirm a high genetic diversity within the cattle-associated C. jejuni and C. coli isolated in the USA, which also corroborates the findings of studies conducted on cattle in other countries, including Spain (Oporto et al., 2007), the United Kingdom (Grove-White et al., 2010), Denmark (Litrup et al., 2007), Finland (de Haan et al., 2010), and Turkey (Açik and Çetinkaya, 2006). Additionally, our PFGE analysis revealed clustering of some C. jejuni isolates (more than 75% similarity) that originated from different geographic locations. Likewise, some clusters included C. coli isolates with identical genotypes originating from different geographic locations (see arrows Fig 2.1). This indicated that isolates with identical genotypes can occur in different herds, which highlights the possibility for certain strains to be transmitted among disparate herds/individuals. However, the latter requires further investigation to elucidate potential routes and mechanisms of transmission. Our PFGE analysis shows that cattle can be considered a
potential reservoir for genetically diverse *Campylobacter* that is worth investigating in order to determine its relevance to public health in the USA.

An outstanding feature in livestock associated *Campylobacter* is its ability to resist antibiotics (reviewed in Luangtongkum *et al.* (Luangtongkum *et al.*, 2009), an increasingly important emerging threat to public health (Inglis *et al.*, 2006). Furthermore, previous studies have demonstrated that cattle-associated *Campylobacter* in the USA can potentially resist front-line therapeutic drugs for treating human infections. For example, 31.8% and 44% of *C. coli* collected from calves and feedlot cattle were resistant to erythromycin and ciprofloxacin, respectively (Bae *et al.*, 2005). Additionally, 47.7% and 49.1% of *C. jejuni* isolated from dairy and feedlot cattle, respectively, were resistant to tetracycline (Englen *et al.*, 2007, Englen *et al.*, 2005). In agreement with these studies, our antibiotic resistance analysis showed that a high percentage of the cattle-associated strains were resistant to erythromycin, ciprofloxacin, tetracycline and others (Fig 2.2 and Table 2.2). Remarkably, 23.7% and 12.1% of the cattle-associated *C. jejuni* that were analyzed in our study exhibited resistance to ciprofloxacin and erythromycin, respectively (Fig 2.2). This was an important and marked difference when compared to previous studies in the US that reported a relatively lower frequency of resistance to ciprofloxacin (1.8% to 5%) and erythromycin (0.4% to 2.9%) in *C. jejuni* isolated from different cattle operations (Englen *et al.*, 2007; Bae *et al.*, 2005; Englen *et al.*, 2005). Our data further support previous findings and predictions that livestock-associated *Campylobacter* are becoming increasingly resistant to important antibiotics (Inglis *et al.*, 2006; Châtre *et al.*, 2010). These observations highlight the need for rigorous
surveillance of antibiotics used in cattle operations in order to facilitate interventions and curb further emergence of antibiotic resistant *Campylobacter*.

Understanding the contributions of cattle to human infections with *Campylobacter* relies heavily on: i) assessing the relationship between the cattle-associated *Campylobacter* and those isolated from- or previously implicated in human infections and ii) directly examining the potential of these isolates to invade and persist in the human intestine. However, these two criteria were rarely investigated together in previous research that focused on cattle-associated *Campylobacter* and are, to our knowledge, absent in similar studies conducted in the USA. To meet the aforementioned criteria, we typed a pool of the cattle-associated *Campylobacter* using MLST and further analyzed a subset of isolates for their properties in an in vitro surrogate for the human host (i.e. INT407 intestinal cell line). Since interpretation of the MLST typing depends on pre-existing online data bases (PubMLST) to assign sequence types (STs) to the isolates, it is possible to compare the cattle-associated *Campylobacter* from this study to others from different sources (e.g. humans) and locations, including other countries. Subsequently, our MLST analysis confirmed that cattle-associated *C. jejuni* (n = 62) were diverse and belonged to disparate STs (n= 25; Table 2.3), while only 8 STs were assigned to *C. coli* (n = 50) (Table 2.3). Furthermore, we identified 12 *C. jejuni* that belonged to unassigned clonal complexes (n = 10), which along with newly discovered alleles (n = 3) and STs (n = 13) (Table 2.3) emphasize the genetic diversity of this bacterium in cattle, possibly indicating that certain *C. jejuni* might be highly associated with this potential source of infection. The latter is supported by reports showing that CC ST-21 and ST-61
were predominant in cattle samples from other countries, including the UK, Canada, New Zealand, and Finland (de Haan et al., 2010; Levesque et al., 2008; Dingle et al., 2002; Kwan et al., 2008). The detection of CC ST-61 and CC ST-21 (Table 2.3) in this study was of particular interest, since these complexes constitute two of six CCs that harbor 60% of C. jejuni associated with human disease (Dingle et al., 2001; Grove-White et al., 2010). Although all tested cattle-associated C. jejuni were capable of invading the human INT407 cells with variable efficiency, two isolate (Cj-M-13) and (Cj-N-33) that belong to CC ST-21 not only exhibited an invasive potential that was comparable to the hyper-invasive strain C. jejuni 81-176 (Hu and Kopecko, 1999) but also showed a higher capability to survive in the intracellular milieu (Fig 2.3). Furthermore, these two isolates were resistant to 7 out of 9 tested antimicrobials, including ciprofloxacin, erythromycin, and gentamicin (Table 2.4). Along the same lines, 11 out of 19 C. jejuni with varying invasive capacities were able to either match or outperform the intracellular survival potential of C. jejuni 81-176 (Fig 2.3). No specific association was observed with either invasion or intracellular survival of the tested C. jejuni isolates and their MRPs, antimicrobial resistance properties or their geographical distributions. Since many of the tested C. jejuni (13 out of 19) were moderate to highly invasive (Table 2.4) and given this bacterium’s aforementioned capability for natural transformation, the occurrence of invasive strains in a reservoir that is witnessing a rise in antibiotic-resistant Campylobacter constitutes a cause for caution and increased surveillance. Since invasion and intracellular survival of pathogens facilitates evading the immune system of the host.
and allows for persistent and recurrent infection (Day et al., 2000), it can be argued that the cattle-associated 
*C. jejuni* would pose a plausible risk to human health.

Furthermore, MLST data and invasion studies also showed relatively lower clinical relevance of selected cattle-associated *C. coli* isolates, which all belonged to CC ST-828, a complex that include strains which are mainly isolated from agricultural and environmental sources and some from clinical cases (Sheppard et al., 2010). Although the invasion and intracellular survival capacities of *C. coli* were overall lower than those of *C. jejuni*, one cattle associated *C. coli* (CC-S-10) invaded the cells with approximately $10^4$ CFU per ml, while also surviving inside the cells in relatively high numbers (Fig 2.4). Taken together, our data confirm the observations of Sheppard et al. (Sheppard et al., 2010) that ruminants also are among the most likely sources of human infections with *C. coli*.

We conclude that cattle in the USA constitute a suitable niche for the persistence of *Campylobacter* and a potentially important source for human infections with these pathogens. The potential of cattle-associated *Campylobacter* to resist antibiotics, invade and persist in human cells, and their genetic classification into clinically important clonal complexes warrant highlighting the contribution of cattle to the epidemiology of these pathogens in the USA.

2.6 Acknowledgement

We thank Dr. M. Koohmararie for assistance in logistics of samples collections. This research was supported by funds appropriated to Dr. Rajashekar from National
Cattlemen Beef Association, and by the Ohio Agricultural Research and Development Center, The Ohio State University and a scholarship to Yasser Sanad from the Egyptian Ministry of Higher Education.

2.7 References


**Table 2.1:** *Campylobacter* species isolated from cattle fecal samples that were collected from four major geographical locations in the USA.

<table>
<thead>
<tr>
<th>Regions</th>
<th>Number of samples</th>
<th><em>Campylobacter</em> prevalence/ region (%)</th>
<th>Number of <em>C. jejuni</em></th>
<th>Number of <em>C. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>North</td>
<td>210</td>
<td>31/210 (14.8%)</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td>Midwest</td>
<td>240</td>
<td>38/240 (15.83%)</td>
<td>36</td>
<td>12</td>
</tr>
<tr>
<td>South</td>
<td>244</td>
<td>80/244 (32.8%)</td>
<td>8</td>
<td>83</td>
</tr>
<tr>
<td>East</td>
<td>250</td>
<td>32/250 (12%)</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td>944</td>
<td>181/944 (19.2%)*</td>
<td>71</td>
<td>132</td>
</tr>
</tbody>
</table>

*A total of 213 *Campylobacter* isolates occurred in 181 fecal samples including 10 *Campylobacter* spp. other than *C. jejuni* and *C. coli*. 

64
Table 2.2: Antimicrobials resistance of *C. jejuni* and *C. coli* isolated from four different locations.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>AZI</th>
<th>CIP</th>
<th>ERY</th>
<th>GEN</th>
<th>TET</th>
<th>FGN</th>
<th>NAL</th>
<th>TEL</th>
<th>CLI</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>5(7.6%)</td>
<td>6(9.1%)</td>
<td>1(1.5%)</td>
<td>3(4.5%)</td>
<td>9(13.6%)</td>
<td>3(4.5%)</td>
<td>8(12.1%)</td>
<td>7(10.6%)</td>
<td>4(6.1%)</td>
</tr>
<tr>
<td></td>
<td>8-32a</td>
<td>4-64</td>
<td>32</td>
<td>8-16</td>
<td>16-64</td>
<td>8-16</td>
<td>64</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>S*</td>
<td>0(1.5%)</td>
<td>2(3.0%)</td>
<td>16-64</td>
<td>0(0.0%)</td>
<td>2(3.0%)</td>
<td>0</td>
<td>64</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M</td>
<td>6(9.1%)</td>
<td>4(6.1%)</td>
<td>4(6.1%)</td>
<td>26(39.4%)</td>
<td>14(21.6%)</td>
<td>4(6.1%)</td>
<td>9(13.6%)</td>
<td>11(16.7%)</td>
<td>8-16</td>
</tr>
<tr>
<td></td>
<td>8-16</td>
<td>16-32</td>
<td>32</td>
<td>8-16</td>
<td>16-64</td>
<td>8-16</td>
<td>64</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>7(10.6%)</td>
<td>4(6.1%)</td>
<td>3(4.5%)</td>
<td>6(9.1%)</td>
<td>11(16.7%)</td>
<td>4(6.1%)</td>
<td>4(6.1%)</td>
<td>6(9.1%)</td>
<td>8-16</td>
</tr>
<tr>
<td></td>
<td>8-32</td>
<td>16-32</td>
<td>32</td>
<td>8-16</td>
<td>16-64</td>
<td>8-16</td>
<td>64</td>
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<th>Isolates</th>
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<th>CIP</th>
<th>ERY</th>
<th>GEN</th>
<th>TET</th>
<th>FGN</th>
<th>NAL</th>
<th>TEL</th>
<th>CLI</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>0(0.0%)</td>
<td>2(3.2%)</td>
<td>1(1.6%)</td>
<td>1(1.6%)</td>
<td>0</td>
<td>6(9.5%)</td>
<td>4(6.3%)</td>
<td>2(3.2%)</td>
<td>8-16</td>
</tr>
<tr>
<td></td>
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<td>8-16</td>
<td>16</td>
<td>4-16</td>
<td>4-16</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>S*</td>
<td>10(15.9%)</td>
<td>24(38.1%)</td>
<td>13(20.6%)</td>
<td>20(31.7%)</td>
<td>10(15.9%)</td>
<td>23(36.5%)</td>
<td>26(41.3%)</td>
<td>16(25.4%)</td>
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</tr>
<tr>
<td>M</td>
<td>3(4.8%)</td>
<td>2(3.2%)</td>
<td>4(6.3%)</td>
<td>3(4.8%)</td>
<td>0</td>
<td>2(3.2%)</td>
<td>5(7.9%)</td>
<td>3(4.8%)</td>
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</tr>
<tr>
<td></td>
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<td>32-64</td>
<td>8-16</td>
<td>16-64</td>
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<td>64</td>
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<td>E</td>
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<td>3(4.8%)</td>
<td>8(12.7%)</td>
<td>8(12.7%)</td>
<td>6(9.5%)</td>
<td>6(9.5%)</td>
<td>7(11.1%)</td>
<td>10(15.9%)</td>
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<td>16-64</td>
<td>8-16</td>
<td>64</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

*Decreased resistance to most of antimicrobials.

a MIC range of antimicrobials
Table 2.3: Distribution of multilocus sequence types among cattle *C. jejuni* and *C. coli*.

<table>
<thead>
<tr>
<th>ST*</th>
<th><em>C. jejuni</em> Isolates</th>
<th>Isolates/Region</th>
<th>ST- CC</th>
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<tr>
<td></td>
<td></td>
<td>N   E   M   S</td>
<td></td>
</tr>
<tr>
<td>590</td>
<td></td>
<td>3   0   1   2   0</td>
<td>ST-42</td>
</tr>
<tr>
<td>1013</td>
<td></td>
<td>11  2   0   7   2</td>
<td></td>
</tr>
<tr>
<td>459</td>
<td></td>
<td>5   0   0   5   0</td>
<td></td>
</tr>
<tr>
<td>797</td>
<td></td>
<td>7   0   1   5   1</td>
<td></td>
</tr>
<tr>
<td>4924</td>
<td></td>
<td>5   0   0   3   2</td>
<td></td>
</tr>
<tr>
<td>2876</td>
<td></td>
<td>2   0   0   2   0</td>
<td></td>
</tr>
<tr>
<td>4026</td>
<td></td>
<td>2   1   0   1   0</td>
<td></td>
</tr>
<tr>
<td>4930</td>
<td></td>
<td>1   0   1   0   0</td>
<td>ST-21</td>
</tr>
<tr>
<td>4922</td>
<td></td>
<td>1   0   0   1   0</td>
<td></td>
</tr>
<tr>
<td>4923</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>239</td>
<td></td>
<td>1   0   0   1   0</td>
<td></td>
</tr>
<tr>
<td>61</td>
<td></td>
<td>2   0   2   0   0</td>
<td>ST-61</td>
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<tr>
<td>500</td>
<td></td>
<td>1   0   0   1   0</td>
<td></td>
</tr>
<tr>
<td>3091</td>
<td></td>
<td>1   1   0   0   0</td>
<td>ST-45</td>
</tr>
<tr>
<td>3084</td>
<td></td>
<td>1   0   0   0   1</td>
<td>ST-48</td>
</tr>
<tr>
<td>4929</td>
<td></td>
<td>2   0   2   0   0</td>
<td></td>
</tr>
<tr>
<td>4925</td>
<td></td>
<td>1   0   0   1   0</td>
<td></td>
</tr>
<tr>
<td>4926</td>
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<td></td>
</tr>
<tr>
<td>4927</td>
<td></td>
<td>1   1   0   0   0</td>
<td></td>
</tr>
<tr>
<td>4928</td>
<td></td>
<td>1   0   1   0   0</td>
<td></td>
</tr>
<tr>
<td>4931</td>
<td></td>
<td>1   0   0   1   0</td>
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<tr>
<td>4932</td>
<td></td>
<td>1   0   0   0   1</td>
<td></td>
</tr>
<tr>
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<td></td>
<td>1   0   1   0   0</td>
<td></td>
</tr>
<tr>
<td>5447</td>
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<td></td>
</tr>
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<td>5448</td>
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<table>
<thead>
<tr>
<th>ST</th>
<th><em>C. coli</em> Isolates</th>
<th>Isolates/Region</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N   E   M   S</td>
</tr>
<tr>
<td>1068</td>
<td>40</td>
<td>8   2   7   23</td>
</tr>
<tr>
<td>902</td>
<td>2</td>
<td>0   0   2   0</td>
</tr>
<tr>
<td>2501</td>
<td>2</td>
<td>1   0   0   1</td>
</tr>
<tr>
<td>1110</td>
<td>2</td>
<td>0   2   0   0</td>
</tr>
<tr>
<td>3866</td>
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<td>0   0   0   1</td>
</tr>
<tr>
<td>5372</td>
<td>1</td>
<td>0   0   0   1</td>
</tr>
<tr>
<td>4933</td>
<td>1</td>
<td>0   0   0   1</td>
</tr>
<tr>
<td>5446</td>
<td>1</td>
<td>0   0   1   0</td>
</tr>
</tbody>
</table>

*Complete allelic profiles for six *C. jejuni* isolates could not be ascertained.
Table 2.4: The properties of *C. jejuni* and *C. coli* isolates that were tested for invasion and intracellular survival in INT407 cell line.

<table>
<thead>
<tr>
<th><em>C. jejuni</em> Isolates</th>
<th>Invasiona</th>
<th>Survival</th>
<th>Antimicrobial Resistance Profile</th>
<th>ST</th>
<th>CC ST</th>
<th>MRP cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cj-M-6</td>
<td>Moderate</td>
<td>High</td>
<td>Susceptible</td>
<td>797</td>
<td>CC ST21</td>
<td>II</td>
</tr>
<tr>
<td>Cj-M-11</td>
<td>Moderate</td>
<td>High</td>
<td>GEN, TET, NAL</td>
<td>4922</td>
<td>CC ST21</td>
<td>II</td>
</tr>
<tr>
<td>Cj-M-52</td>
<td>Moderate</td>
<td>High</td>
<td>CIP, TET, TEL, CLI</td>
<td>4924</td>
<td>CC ST21</td>
<td>V</td>
</tr>
<tr>
<td>Cj-M-13a</td>
<td>High</td>
<td>High</td>
<td>AZI, CIP, ERY, GEN, FFN, TEL, CLI</td>
<td>2876</td>
<td>CC ST21</td>
<td>II</td>
</tr>
<tr>
<td>Cj-N-33a</td>
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<td>High</td>
<td>AZI, CIP, GEN, TET, NAL, TEL, CLI</td>
<td>4026</td>
<td>CC ST21</td>
<td>II</td>
</tr>
<tr>
<td>Cj-M-48</td>
<td>Moderate</td>
<td>High</td>
<td>ERY, CLI</td>
<td>4923</td>
<td>CC ST21</td>
<td>V</td>
</tr>
<tr>
<td>Cj-M-41</td>
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<td>Poor</td>
<td>TET, FFN, NAL</td>
<td>239</td>
<td>CC ST21</td>
<td>IV</td>
</tr>
<tr>
<td>Cj-E-29</td>
<td>Poor</td>
<td>Moderate</td>
<td>CIP, TET, TEL</td>
<td>4930</td>
<td>CC ST21</td>
<td>II</td>
</tr>
<tr>
<td>Cj-M-60</td>
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<td>Poor</td>
<td>TET</td>
<td>459</td>
<td>CC ST42</td>
<td>VII</td>
</tr>
<tr>
<td>Cj-S-14</td>
<td>Moderate</td>
<td>High</td>
<td>Susceptible</td>
<td>4924</td>
<td>CC ST21</td>
<td>II</td>
</tr>
<tr>
<td>Cj-E-58</td>
<td>Poor</td>
<td>Moderate</td>
<td>AZI, GEN, TET, NAL, TEL</td>
<td>4929</td>
<td>UA</td>
<td>VI</td>
</tr>
<tr>
<td>Cj-M-63</td>
<td>Moderate-High</td>
<td>High</td>
<td>TET</td>
<td>4925</td>
<td>UA</td>
<td>VII</td>
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<td>High</td>
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<td>I</td>
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<td>Cj-M-64</td>
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<td>High</td>
<td>AZI, CIP, ERY, GEN, TET, CLI</td>
<td>4926</td>
<td>UA</td>
<td>VII</td>
</tr>
<tr>
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<td>AZI, ERY, FFN, TEL, CLI</td>
<td>4927</td>
<td>UA</td>
<td>II</td>
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<tr>
<td>Cj-E-57</td>
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<td>Poor</td>
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<td>VI</td>
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<td>Cj-E-42</td>
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<td>CIP, GEN, TET, NAL, CLI</td>
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<td>V</td>
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<td>Cj-E-45</td>
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<td>Poor</td>
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<td>CC ST61</td>
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<table>
<thead>
<tr>
<th><em>C. coli</em> Isolates</th>
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<th>Survival</th>
<th>Antimicrobial Resistance Profile</th>
<th>ST</th>
<th>CC ST</th>
<th>MRP cluster</th>
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<td>Poor</td>
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<td>NAL</td>
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*aClassification index:
High= higher or comparable to *C. jejuni* 81-176.
Moderate-High: Significantly lower than *C. jejuni* 81-176 but significantly higher than moderate.
Moderate: Significantly higher than *C. jejuni* NCTC11168.
Poor: significantly lower or comparable to *C. jejuni* NCTC11168.

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Table 2.5: Antimicrobial resistance profiles, Sequence Types, and MRP clusters of *C. jejuni* isolates.

<table>
<thead>
<tr>
<th>Isolates*</th>
<th>Antimicrobial Resistance Profile</th>
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* Highly susceptible isolates were not presented. Four isolates belonged to CC ST21, one belonged to CC ST45, one belonged to CC ST48, and two belonged to unassigned CC.
Figure 2.1. Dendrogram showing the Pulsed-field gel electrophoresis patterns for
SmaI restricted cattle-associated *C. jejuni* (A) and *C. coli* (B). Similarity analysis was
performed using the Dice coefficient, and clustering was performed by the unweighted
pair-group method with arithmetic averages UPGMA (optimization, 1% and position
tolerance, 1.5%). Clustering cut-off was 75% similarity, which was represented by dashed
line. Numbers on bootstraps represent Cophenetic correlations. N=North, S=South,
M=Midwest, and E=East. Cj= *C. jejuni*, CC= *C. coli*. 
Figure 2.1: Dendrogram showing the Pulsed-field gel electrophoresis patterns for SmaI restricted cattle-associated *C. jejuni* (A) and *C. coli* (B).
Figure 2.2: Antimicrobial resistance of *Campylobacter* isolated from cattle. *C. jejuni* and *C. coli* isolates obtained from four different locations across the US. Percentage of isolates resistant to different antimicrobials used in this study is shown. *C. jejuni* 81-176 and *C. coli* ATCC 33559 strains were used for quality control.
Figure 2.3. Invasion and intracellular survival of cattle *C. jejuni* isolates in INT407 cells. **A.** CFU ml$^{-1}$ representing the number of the internalized bacteria which could be retrieved after treatment of cells with gentamicin. **B.** Intracellular survival of *C. jejuni* isolates in INT407 cells. CFU ml$^{-1}$ representing the numbers of internalized bacteria retrieved after 24 h of incubation following the gentamicin treatment. The INT407 were infected with 1:100 MOI of *C. jejuni* strains. *C. jejuni* 81-176 and NCTC11168 were used as controls. The detection limit of the assay is represented by the dashed line. Each bar represents the mean ± SE of three independent experiments done in duplicates for each sample (*P*<0.01).
Figure 2.3: Invasion and intracellular survival of cattle C. jejuni isolates in INT407 cells.
Figure 2.4. Invasion and intracellular survival of cattle *C. coli* isolates in INT407 cells. A. CFU ml\(^{-1}\) representing the number of the internalized bacteria which could be retrieved after treatment of cells with gentamicin. B. Intracellular survival of *C. coli* isolates in INT407 cells. CFU ml\(^{-1}\) representing the numbers of internalized bacteria retrieved after 24 h of incubation following the gentamicin treatment. The INT407 cells were infected with 1:100 MOI of *C. coli* strains. *C. coli* (ATCC 33559) and *C. jejuni* NCTC11168 were used as controls. The detection limit of the assay is represented by the dashed line. Each bar represents the mean ± SE of three independent experiments done in duplicates for each sample (*P*<0.01).
Figure 2.4: Invasion and intracellular survival of cattle *C. coli* isolates in INT407 cells.
CHAPTER 3

Occurrence of the Invasion Associated Marker (iam) in Campylobacter jejuni Isolated from Cattle

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3.1 Abstract

The invasion associated marker (iam) has been detected in the majority of invasive Campylobacter jejuni retrieved from humans. No data exist regarding the occurrence of this marker in C. jejuni isolated from non-poultry food-animals such as cattle, an increasingly important source of human infections. Furthermore, the detection of iam in C. jejuni isolated from two important hosts, humans and chickens, suggested a role for this marker in C. jejuni’s colonization of multiple hosts. However, little is known about the genetics associated with C. jejuni’s capability for colonizing physiologically disparate hosts. Therefore, we investigated the occurrence of the iam in C. jejuni isolated from cattle in order to determine the contribution of this reservoir to human infections with invasive Campylobacter. Additionally, we assessed iam’s contribution to the colonization of multiple hosts by characterizing the potential of iam-containing cattle isolates for chicken colonization and human cell invasion. Simultaneous RAPD typing and iam-specific PCR analysis of 129 C. jejuni isolated from 1171 cattle fecal samples showed that 8 (6.2%) of the isolates were iam-positive, while 7 (54%) of human-associated isolates were iam-positive. The iam sequences were mostly heterogeneous and occurred in diverse genetic backgrounds. However, all isolates were motile and possessed important genes (cadF, ciaB, cdtB) associated with adhesion and virulence. Furthermore, certain iam-containing isolates invaded and survived in INT-407 cells in high numbers and successfully colonized live chickens and displayed a capacity to resist important antibiotics. However, there was no clear association between the occurrence, sequence type, and expression levels of the iam and the aforementioned phenotypes. In conclusion,
we show that the prevalence of *iam* in cattle *C. jejuni* is relatively lower as compared to isolates occurring in humans and chickens. In addition, *iam* was polymorphic and certain alleles occur in cattle isolates that were capable of colonizing and invading chickens and human intestinal cells, respectively. However, the *iam* did not appear to contribute to the invasion, intracellular survival, and antibiotic resistance potential of cattle-associated *C. jejuni*. We propose that the inclusion of the *iam* as a virulence determinant for *C. jejuni* needs to be carefully considered.

### 3.2 Introduction

*Campylobacter jejuni* is an important foodborne pathogen that can cause a variety of infections in humans (Vandamme, 1991). Additionally, *C. jejuni* colonizes important food-animals such as chicken and cattle, which together constitute an important source for human infections with this pathogen (Allos, 2001). Although *C. jejuni* can occur in multiple hosts, it is more readily transmissible within species (McCarthy et al., 2007). Once established in a new host, *C. jejuni* has a remarkable capacity for acquiring genetic material that facilitates adaptation to the host environment (McCarthy et al., 2007). Efficient host colonization can be essential in pathogenesis mechanisms, including host cell invasion and associated sequelae (Konkel et al., 2001). Furthermore, numerous studies, using live chicken models and in vitro human cell lines, have suggested multiple genetic determinates that are important in *C. jejuni*’s host colonization (Konkel et al., 2001; Hendrixson and DiRita, 2004). However, little is known about genetic factors that might be important for *C. jejuni*’s adaptation to multiple hosts, which is important since
the broad host range of *C. jejuni* complicates on-farm control measures aiming for decreasing its transmission to humans.

An important factor in *C. jejuni*’s host colonization is its capability to attach to- and/or invade epithelial cells in the host’s gastrointestinal tract (Ketley, 1997). Yet, different strains of *C. jejuni* display varying capacities for cellular adherence and invasion, which could be attributed to the presence, absence and/or acquisition of certain genetic determinants that contribute to the pathobiology of this bacterium (Fearnley *et al*., 2008; Hofreuter *et al*., 2008). Although invasion and host adaptation would be influenced by the interaction of multiple genetic factors, several individual components, including outer-membrane proteins and secreted antigens can impact *C. jejuni*’s adherence to and invasion of enterocytes (Fearnley *et al*., 2008; Konkel *et al*., 1997; Konkel *et al*., 1999b). Of particular interest is the invasion associated marker (*iam*) that was significantly associated with invasive *Campylobacter* (Carvalho *et al*., 2001). This marker was discovered using random amplified polymorphic DNA (RAPD) analysis that identified a diagnostic DNA band (1.6 Kb) containing a genetic element (designated later as *iam*).

Although specific PCR analysis showed that the *iam* was observed in 63% of the invasive isolates retrieved from diarrheic children, the authors reported that it was not detected in every potentially invasive isolate and occurs in a low percentage of the non-invasive ones. Consequently, it was concluded that mutations/ allele variations might have impacted both the detection of the marker and its role in mediating the invasion (Carvalho *et al*., 2001). However, these assumptions were not tested further and the role of *iam* in *C. jejuni*’s invasion and adaptation to hosts, whether humans or animals, has not
been fully investigated. The limited data available suggest that the occurrence of the *iam* in *C. jejuni* might be both dependent on the characteristics of the human population understudy and associated sources of infection (Al-Mahmeed *et al.*, 2006; Rozynek *et al.*, 2005). The latter is important since the majority of *Campylobacter* isolated from chicken carcasses, an established source for *Campylobacter* infections, also possessed the *iam* (Rozynek *et al.*, 2005, Korsak *et al.*, 2004), which suggests that the *iam* might play a role in the transmission of *Campylobacter* and/or its adaptation to different host(s). Since no data are available concerning the occurrence of *iam* in *C. jejuni* isolated from other hosts, the aforementioned conclusion regarding the *iam* association with multiple-host colonization might be spurious. Consequently, it was important to investigate the occurrence of *iam* in *C. jejuni* from other important sources such as cattle (de Haan *et al.*, 2010; Wilson *et al.* 2008; Grove-White *et al.*, 2010) and test their potential for colonization of humans and chickens, respectively. If *iam* is associated with *Campylobacter*’s potential for colonization of multiple hosts, this would facilitate understanding the interactive impact of major animal sources such as chicken and cattle in the transmission of *Campylobacter*. Therefore, we investigated the occurrence of the *iam* in *C. jejuni* isolated from cattle and determined the association of this element and its alleles with the pathogen’s invasion potential of a human intestinal cell line and colonization of 1-day old chickens.

3.3 Materials and Methods

**Isolation of Campylobacter jejuni from cattle and human samples**
Fecal samples (n = 1171) were collected from cattle at slaughter plants located in 4 geographic locations (North, Mid-West, East, South) across the U.S. as well as from dairy cattle in Northeastern Ohio. To isolate *C. jejuni*, 1 g of each fecal sample was enriched in Preston broth for 48 h at 42 °C under microaerobic conditions (5% O₂, 10% CO₂, and 85% N₂). From the enrichments that showed growth, an inoculum (100 µl) was spread onto modified Cefoperazone Charcoal Deoxycholate Agar (mCCDA) plates, which were then incubated for an additional 48 h at 42° C under microaerobic conditions (Engberg *et al*., 2000). Colonies exhibiting *Campylobacter* phenotype (flat and grey with metallic sheen) were selected from the plates and subjected to species-specific PCR analysis to confirm their identity (Linton *et al*., 1997; Denis *et al*., 1999). The size of the PCR products was determined using a 1Kb DNA ladder and detection was confirmed by comparison to a PCR product generated from a *C. jejuni* 81-176 (wild-type strain), which was used as a positive control in all PCR analysis. Negative controls (reactions with no DNA templates) were included in all PCR analysis to ensure specific product amplification.

Additional *C. jejuni* isolates from human hosts were acquired from a medical center (The Ohio State University). These isolates represented different sporadic human infections and their identity was further confirmed using the aforementioned PCR analysis.

**Detection of *iam* using RAPD typing and PCR**

To determine if the *C. jejuni* originating from cattle and human samples carried the *iam* locus (a diagnostic 1.6 Kb band), all isolates were subjected to DNA
fingerprinting using RAPD analysis as described in Carvalho et al. (Carvalho et al., 2001). RAPD-PCR products were then analyzed using 1.4% agarose gels, containing 0.5 µg/ml of ethidium bromide. RAPD fingerprints were documented and dendrograms were constructed using BioNumerics 5.1 software (Applied Maths, Inc, USA). C. jejuni isolates that carried the iam locus as identified by the RAPD fingerprinting were tested to further confirm the presence of the iam locus. This was achieved using PCR analysis to detect a 518-bp DNA fragment inside the 1.6 kb band that was earlier identified as the iam locus by RAPD fingerprinting as described in Carvalho et al. (Carvalho et al., 2001).

**Detection of virulence-associated genes using PCR**

C. jejuni isolates that were identified as iam-positive using both RAPD and iam-specific PCR analysis, hereafter referred to as the iam-positive C. jejuni, were screened for carrying genes that are important in the pathobiology of this pathogen. Specifically, PCR analysis was performed for the detection of the cadF (Campylobacter adherence factor), ciaB (Campylobacter invasion antigens), and cdtB (cytolethal distending toxin) genes as described elsewhere (konkel et al., 1999b; Datta et al., 2003; Bang et al., 2003). Genomic DNA from C. jejuni strain 81-176 was used as a positive control, while negative controls contained the PCR reagent mix with no DNA templates.

**Motility assay**

To establish that iam-positive C. jejuni were putatively capable of host invasion and colonization, it was important to establish that the isolates were not defective in
motility. For this purpose, the motility of the _iam_-positive _C. jejuni_ was tested using semi-solid (0.4%) Mueller-Hinton agar plates as described by Fields and Thompson (Fields and Thompson, 2008). The diameter of the zone of motility was measured and compared to that of _C. jejuni_ 81-176 (positive control). The motility assays were repeated twice for each isolate, which were also tested in duplicates per each assay.

**In vitro cell invasion and intracellular survival assay using human epithelial cell lines (INT-407)**

The human intestinal cell invasion assays were performed using _iam_-negative and _iam_-positive _C. jejuni_ isolated from both cattle and humans. For this purpose, $10^5$ cells ml$^{-1}$ of INT407 (human embryonic intestine, ATCC CCL 6) were seeded into each well of a 24-well tissue culture plates after suspension in Minimum Essential Medium Eagle (MEM, Fisher scientific, USA) supplemented with 10% fetal bovine serum (FBS, Fisher scientific, PA, USA). The plates were then incubated at 37 °C in a humidified incubator with 5% CO$_2$ until semi-confluent mono-layers were obtained (Monteville _et al._, 2003; Konkel _et al._, 1999a). In preparation for infection with _C. jejuni_, the INT407 mono-layers were washed three times and covered in MEM supplemented with 1% FBS. Similarly, the _C. jejuni_ cultures were washed three times and suspended in MEM supplemented with 1% FBS to obtain $10^7$ bacteria ml$^{-1}$. Respectively, 1 ml of each suspension was then added to each well containing the INT407 cell monolayer, achieving a 1:100 multiplicity of infection (MOI). After 3 h of incubation, gentamicin (150 µg ml$^{-1}$) was added to the wells to inhibit the bacteria that did not invade the cells. The infected
mono-layers were washed with 1X PBS, lysed using 0.01% Triton X-100 (Fisher scientific, PA, USA) and serially diluted (10-fold) in 1X PBS. One hundred µl of each dilution were spread on MH agar plates. The agar plates were then incubated for 48 h at 42° C under microaerobic conditions, after which colony forming units (CFU) were counted. Each isolate was tested in duplicate per assay, while the experiment was repeated twice on separate occasions. C. jejuni 81-176 and NCTC11168 were used as controls in all invasion assays.

For the intracellular survival assays (Konkel et al., 1992), Campylobacter cultures and the INT407 cells were processed as described above. However, after treatment with gentamicin, MEM containing 3% FBS and gentamicin (10 µg ml⁻¹) were added to each well. After further incubation at 37 ºC 24 h, the monolayers were washed three times with MEM containing 1% FBS and then lysed and processed as described above. The number of viable intracellular bacteria was determined by counting the CFU that grew on Muller-Hinton plates.

**Phylogenetic analysis of the iam alleles**

To determine if the iam sequences were heterogeneous and examine relationship between the iam occurring in cattle C. jejuni isolates and those from human samples, the iam fragments were sequenced and subjected to a phylogenetic analysis. Briefly, the products resulting from the iam-specific PCR analysis (described above) of the cattle and human isolates were purified using the QIAquick PCR purification kit (Qiagen, CA, USA) and commercially sequenced (Molecular and Cellular Imaging Center, OARDC, 84
The identity of the sequences was confirmed by conducting BLAST analysis. The sequences were then exported to MEGA4 software (Tamura et al., 2007) aligned and analyzed. The phylogenetic tree was drawn using the Neighbor-Joining method to determine the evolutionary relationship among the sequences. *iam* sequences that were analyzed in this work were deposited in GenBank [HM533957-HM533968, JF927289-JF927291 and HQ317917].

**Typing of *iam*- positive *C. jejuni* using pulsed field gel electrophoresis (PFGE)**

To determine the relatedness and diversity of their genomic backgrounds, *iam*-positive *C. jejuni* isolated from cattle and human samples, respectively, were analyzed using PFGE analysis as described by Ribot et al. (Ribot et al., 2001). The resulting PFGE patterns were documented and analyzed using the BioNumerics 5.1 software (Applied Maths Inc, TX, USA). Similarity and clustering analysis of the PFGE patterns were performed using the Dice Coefficient and the unweighted pair-group method with arithmetic averages, UPGMA (optimization of 1% and position tolerance of 1.5%), respectively.

**Expression analysis for the *iam* using quantitative real-time PCR (q-RT PCR)**

Quantitative real-time PCR was used to investigate an association between the expression of *iam* and the phenotypes of the cattle-associated *C. jejuni*. q-RT PCR primers (Table 3.1) targeting different fragments of the *iam* were designed using Beacon Designer 7.0 software (Premier Biosoft International, Palo Alto, CA). An RNeasy Mini
kit (Qiagen) was used to extract RNA from  *iam*-positive  *C. jejuni* isolated from cattle. Subsequently, cDNA from each sample was synthesized following a manufacturer's protocol (SuperScript III First-Strand Synthesis SuperMix, Invitrogen) and used for q-RT PCR (SensiMixPlus SYBR RT-PCR kit, Quantance) in a Mastercycler ep realplex² thermal cycler (Eppendorf). The relative expression of *iam* in the control ( *C. jejuni* 81-176 and NCTC11168) and *iam*-containing strains was normalized with the 16S rRNA gene. The q-RT PCR analysis was repeated three times with two replicates for each sample per assay and threshold cycle (C₇) values for each sample were then averaged to represent the expression levels of the *iam*.

**In vivo chicken colonization assay**

*iaw*-positive  *C. jejuni* were selected for in vivo chicken colonization assays based on *iam*-sequence type and the *iam* expression profile. Subsequently, Bov-6 was selected to represent Bov-9, Bov-10, and 11 (see Fig 3.4A), while Bov-9 was excluded because it did not express the *iam* product. For this purpose, one day-old chicken (specific pathogen free) were divided into groups, each containing seven birds. Before the experimental infection, the chickens were confirmed to be *Campylobacter* free by randomly testing cloacal samples collected from each group. Groups were divided to simultaneously test *iam*-positive isolates versus *iam*-negative ones and isolates from human hosts versus those from cattle. Subsequently,  *C. jejuni* isolates were suspended in Muller-Hinton broth to achieve an OD₆₀₀ of 0.04 and individual chickens in each group were inoculated orally with 200 µl of the suspensions (~ 2 x 10⁵ CFU of *Campylobacter*),
respectively. Seven days post-inoculation, the chickens were euthanized and the caeca were aseptically collected, weighed, and homogenized in 1X PBS (pH 7.4). The caecal extracts were serially diluted (10-fold) and 100 µl from each dilution were spread onto Muller-Hinton agar plates supplemented with SR117E (Oxoid, KS, USA), a Campylobacter selective supplement. The plates were then incubated at 42 °C under microaerobic conditions for 48 h and the number of colony forming units (CFU) per gram of caecal contents was calculated to determine the colonization capability of the tested C. jejuni isolates. The chickens were cared for according to the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC).

**Antimicrobial resistance assay**

To further determine a possible clinical relevance of the iam-positive C. jejuni isolates, their susceptibility to important antimicrobials was determined. For this purpose, commercially available 96-well plates containing antimicrobials relevant to Campylobacter (Sensititre Campy plates, TREK Diagnostic Systems Inc., OH, USA) were used. Briefly, the iam-positive C. jejuni isolates were suspended in Mueller-Hinton broth to achieve an OD$_{600}$ of 0.05. For each isolate, One hundred µl of the suspension were then transferred to each well in the Sensititre Campy plates, including a control well that did not contain any antibiotics. The plates were incubated under microaerobic conditions at 42°C for 24 h after which the minimum inhibitory concentration (MIC) was measured. The MIC for each antimicrobial was defined as the absence of bacterial growth in the well with the lowest concentration of the antimicrobial and susceptibility was
interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2006). Antibiotic resistance assays were repeated twice for each isolate, which were also tested in duplicates per each assay.

**Statistical analysis**

Data were presented as means ± standard error (SE) and assessed using analysis of variance (ANOVA), followed by Tukey’s significance test. A *P* value of <0.05 was used to indicate if differences were statistically significant.

### 3.4 Results and Discussion

A total of 129 *C. jejuni* were isolated from fecal samples (*n* = 1171) collected from cattle at slaughter plants located in 4 geographic locations ((North, South, Midwest, East) as well as from some Ohio dairy establishments in the U.S. Additionally, 13 *C. jejuni* isolates were acquired from different sporadic human infections. The identity of the *C. jejuni* isolates was confirmed using PCR (Figure 1A). RAPD analysis was performed as described by Carvalho *et al.* (Carvalho *et al*., 2001) and showed that 24% of the cattle isolates possessed the 1.6 Kb DNA band that potentially harbors *iam* as compared to 54% of the human isolates (Table 3.2). Furthermore, *iam*-specific PCR (Carvalho *et al*., 2001) showed that 9% of the cattle isolates were *iam*-positive as compared to 54% of the human isolates (Table 3.2). The discrepancy between the RAPD and the PCR results for the cattle isolates was not surprising since, as suggested by Carvalho *et al.* (Carvalho *et al*., 2001), the PCR primers might not necessarily detect *iam*
mutated fragments/alleles. However, randomly selected isolates that did not show the 1.6 Kb band after RAPD analysis were also observed to be *iam*-negative using *iam*-specific PCR analysis. Additionally, mutations might spuriously give rise to a 1.6 Kb band that can be mistaken for the *iam* locus. Therefore, in order to limit *iam* false-positives that might be detected using either method, we selected isolates that possessed both the 1.6 Kb fragment and the *iam*-PCR product, referred to as *iam*-positive strains, for further analysis.

*i-ami*-positive cattle isolates and 2 *iam*-positive human isolates (H-5, H-7) were assessed for their capability to invade and survive in human intestinal cells (INT-407) (Hendrixson and DiRita, 2004; Hu and Kopecko, 1999). Two *iam*-negative cattle isolate (Bov-1, Bov-5) and 2 *iam*-negative human isolates (H-1, H-4) were used for comparison. Although all the *iam*-positive strains were motile on semi-solid Mueller-Hinton agar (Fig. 1C), our results show that 5 (Bov-2, 3, 6, 7, and 10) of the *iam*-positive cattle isolates invaded INT-407 with numbers higher than those of *C. jejuni* NCTC-11168, while only two (Bov-7 and Bov-10) were also more invasive as compared to the *C. jejuni* 81-176, a highly invasive strain (Fig 3.2A). Furthermore, 4 of the *iam*-positive cattle isolates (Bov-2, 3, 6 and 7) exhibited either equivalent or higher capacity for intracellular survival in INT-407 as compared to *C. jejuni* 81-176 (Fig. 2B). In contrast, independent of the occurrence of the *iam*, all tested human isolates exhibited significantly decreased capacity for invasion and intracellular survival in the INT-407 cell line as compared to *C. jejuni* 81-176 (Fig. 3.2A and 3.2B). Consequently, the occurrence of the *iam* in the cattle and human isolates did not seem to confer any clear advantage in terms of invasiveness and
the intracellular survival potential in the human cell line. However, since the *iam* was previously detected in 16% of non-invasive *Campylobacter* (Carvalho et al., 2001), Carvalho *et al.* (Carvalho et al., 2001) speculated that the non-invasive strains might carry a mutated variant of this marker and that different *iam* alleles might result in discrepancies in the invasion potential of disparate *C. jejuni* strains. This is plausible since allelic variations in other targets were also suggested to impact the virulence of different strains (Fearnley *et al.*, 2008; Poly *et al.*, 2007).

Genomic analysis of the *iam* sequence deposited by Carvalho *et al.* (Carvalho *et al.*, 2001) showed that the *iam* encodes an ABC transporter (Table 3.2), which is important since potential virulence factors, including surface structures such as transporters, are predicted to harbor much of the genetic diversity that characterize disparate *C. jejuni* strains (McCarthy *et al.*, 2007). Interestingly, the *iam* was only 84% and 83% similar to sequences harbored in *C. jejuni* 11168 and 81-176, respectively (Table 3.3) and 86% similar to sequences [Genbank:HQ317917] that occurred in a newly sequenced cattle isolate; *C. jejuni* JL11 (Zeng *et al.*, 2010), which highlights the polymorphism of this marker.

To examine if the *iam* retrieved in our study exhibited sequence polymorphism, we sequenced the *iam* fragment in the strains retrieved from both cattle and human samples. Subsequent phylogenetic analysis showed that only 4 cattle isolates (Bov-6, 10, 11, and 12) shared the same *iam* sequence type, while the other *iam* sequences from cattle and humans were heterogeneous, clustering in groups that mostly contained *iam*-isolates from both hosts (Fig 3.4A). Interestingly, the cattle isolates with identical *iam* sequences
(Bov-6, 10, 11 and 12), did not exhibit similar invasion and intracellular survival properties in INT-407 cells (Fig 3.2A and 3.2B), which suggested that there were no clear associations between an *iam* sequence type and the aforementioned phenotypes. Furthermore, the *iam* did not also appear to affect the invasion and intracellular survival potential in the tested human strains, as both *iam*-positive and *iam*-negative isolates showed similar properties, respectively.

The virulence traits of *C. jejuni* might likely be affected by the interaction of several genetic elements (Konkel *et al.*, 1997; Konkel *et al.*, 1999b; Poly *et al.*, 2007). Hence, the role of the *iam* in the pathobiology of *C. jejuni*, if any, would likely depend on other factors (e.g. flagella, adhesins), which in turn might need to occur in specific allelic sequences to mediate their impact. It was interesting to note that the pulsed field gel electrophoresis analysis showed that the genotypes of the *iam*-positive strains were mostly not highly similar (< 80%) (Fig 3.3B). This indicated that the *iam* is occurring in diverse genetic backgrounds that, along with the *iam* sequence heterogeneity, might impact the role of this locus in the pathobiology of *C. jejuni*. Subsequently, it was important to investigate whether the *iam*-containing isolates harbored genes that are commonly associated with *C. jejuni* adherence and virulence in order to ensure that our observations can be attributed to *iam* and not other possible genetic defects. Subsequently, PCR analysis showed that the *iam*-containing isolates carried the *cadF*, *ciaB*, and *cdtA* genes (Table 3.3) that are important for *C. jejuni* pathogenesis (Konkel *et al.*, 1997; Konkel *et al.*, 1999b; Al-Mahmeed *et al.*, 2006). The PCR detection of these
genes was satisfactory for the aforementioned purpose since it appeared that many of the tested isolates were not defective in invasion of INT-407 cells.

The possibility that the *iam* carriage might not be necessarily associated with the expression of its products might explain the lack of an apparent relationship between *iam* and invasiveness. Therefore, the expression of the *iam* was assayed for the cattle isolates using q-RT PCR, which showed that the expression levels of the *iam* varied between the strains (Fig 3.4). Bov-3 and Bov-6 with low *iam* expression levels and Bov-9 with no detectable expression were still capable of invade and survive in INT-407 cells (Fig 3.2A and 3.2B). Although isolates (Bov-7 and Bov-10) with *iam* expression similar to that of *C. jejuni* 81-176 exhibited high invasion and intracellular survival potential, isolates with relatively the highest *iam* expression (Bov-11 and Bov-12) did not possess the highest capacities for the aforementioned phenotypes. Consequently, the expression of *iam* did not also seem to confer any clear advantage in terms of invasion and intracellular survival.

We attempted to examine if there was a potential role for the *iam* in other important phenotypes. Therefore, we examined the potential of *iam*-containing cattle *C. jejuni* to colonize chickens and resist antibiotics. Out of 5 *iam*-containing cattle isolates tested, only one (Bov-2) colonized the chickens with numbers that were significantly higher than those of the *iam*-negative strains (Fig 3.5). Bov-7 that relatively highly expressed the *iam* was not detected in the chickens (Fig 3.5). Selected human isolates colonized the chickens in similar numbers, regardless from the *iam*. Furthermore, although many of the *iam*-containing isolates, from human and cattle, exhibited high
resistance to multiple antibiotics, these properties were also shared by \textit{iam}-negative isolates (Table 3.5). Therefore, the occurrence and expression of \textit{iam} apparently did not contribute to the chicken colonization and antibiotic resistance potential of \textit{C. jejuni}. Carvalho \textit{et al.} (Carvalho \textit{et al.}, 2001) only correlated the occurrence of the \textit{iam} in a certain number of invasive \textit{C. jejuni} using typing techniques, while acknowledging the existence of the marker in a relatively smaller percentage of non-invasive strains. Our analysis was more rigorous and included attempts to associate the \textit{iam} with several important phenotypes. However, we report that the \textit{iam} does not contribute to invasion and survival in human intestinal cells, chicken colonization, or antibiotic resistance. Subsequently, we propose that the use of the \textit{iam} as a virulence determinant in epidemiological studies might need careful consideration.

3.5 Acknowledgments

This work was supported by the National Cattlemen’s Beef Association grant (RF60015457). Research at Dr. Rajashekara’s laboratory is also supported by the Ohio Agricultural Research and Development Center (OARDC), The Ohio State University, and the USDA grant 2007-03109, and a scholarship to Yasser Sanad from the Egyptian Ministry of Higher Education.

3.6 References


Table 3.1: List of primers used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Properties</th>
<th>Product size</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA-F</td>
<td>5'−ATCTAATGGCTTAACCATTAAAC-3'</td>
<td><em>Campylobacter</em> specific</td>
<td>850 bp</td>
<td>[21]</td>
</tr>
<tr>
<td>16S rRNA-R</td>
<td>5'−GGACGGTAACCTAGTATTTAGATT-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>mapA</em>-F</td>
<td>5'−CTATTTATTTGCTTGCTTG-3'</td>
<td><em>C. jejuni</em> specific</td>
<td>589 bp</td>
<td>[7]</td>
</tr>
<tr>
<td><em>mapA</em>-R</td>
<td>5'−GCTTTAATTTGCCATTGTTTATT-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>cadE</em>-F</td>
<td>5'−TTGGAAGGTAATTAGATATG-3'</td>
<td><em>cadE</em> detection</td>
<td>400 bp</td>
<td>[19]</td>
</tr>
<tr>
<td><em>cadE</em>-R</td>
<td>5'−CTAATACCTAAAGTTGGAAC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>cdnB</em>-F</td>
<td>5'−GTTAAAATCCCCTGCTATCAACCA-3'</td>
<td><em>cdnB</em> detection</td>
<td>495 bp</td>
<td>[3]</td>
</tr>
<tr>
<td><em>cdnB</em>-R</td>
<td>5'−GTTGGCAGTGTGGAATTGGAAGC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>ciaB</em>-F</td>
<td>5'−TTTTTATCAGTCTCTTA-3'</td>
<td><em>ciaB</em> detection</td>
<td>986 bp</td>
<td>[6]</td>
</tr>
<tr>
<td><em>ciaB</em>-R</td>
<td>5'−TTTCGATATCATTAGC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer 1290</td>
<td>5'−GTGGATGGCA-3'</td>
<td>RAPD-typing</td>
<td>Variable</td>
<td>[4]</td>
</tr>
<tr>
<td>1.6-F</td>
<td>5'−GCCCAAATATTATCACCC-3'</td>
<td>Ism-specific</td>
<td>518 bp</td>
<td>[4]</td>
</tr>
<tr>
<td>1.6-R</td>
<td>5'−TTCACGACTACTATGCG-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>iam1</em>-F</td>
<td>5'−AACATTACGGGAAGAT-3'</td>
<td><em>iam</em>-specific</td>
<td>160 bp</td>
<td>This study</td>
</tr>
<tr>
<td><em>iam1</em>-R</td>
<td>5'−GTATATTCTTTAAGAGGGTAG-3'</td>
<td>(qRT-PCR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>iam2</em>-F</td>
<td>5'−AACATTACGGGAAGAT-3'</td>
<td><em>iam</em>-specific</td>
<td>160 bp</td>
<td>This study</td>
</tr>
<tr>
<td><em>iam2</em>-R</td>
<td>5'−TCATTAAACCAGCATT-3'</td>
<td>(qRT-PCR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>iam</em>-81176-F</td>
<td>5'−AAGATAGCATACACAAAGACT-3'</td>
<td><em>iam</em>-specific (qRT-PCR)</td>
<td>160 bp</td>
<td>This study</td>
</tr>
<tr>
<td><em>iam</em>-81176-R</td>
<td>5'−ATTCAAGCTACTATAAGG-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2: Percentage of *iam*-positive *C. jejuni* from cattle and human hosts as determined using RAPD and *iam*-specific PCR.

<table>
<thead>
<tr>
<th>C. jejuni source (Total number of isolates)</th>
<th><em>iam</em>-positive (RAPD)</th>
<th><em>iam</em>-positive (PCR)</th>
<th>Percentage <em>iam</em>-positive (RAPD)</th>
<th>Percentage <em>iam</em>-positive (PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle (129)</td>
<td>14</td>
<td>8</td>
<td>10.8%</td>
<td>6.8%</td>
</tr>
<tr>
<td>Human (13)</td>
<td>7</td>
<td>7</td>
<td>54%</td>
<td>54%</td>
</tr>
</tbody>
</table>
Table 3.3: Analysis of the *iam* marker using the BLAST algorithm.

The sequence reported by Carvalho et al. (4) was used for query. Cds stands for coding sequence. *JL11* is a cattle isolate that was sequenced in Dr. Lin’s laboratory; its sequence and complete annotations have not been submitted to Genbank, yet (29). However, the *iam* homolog in JL11 was deposited in Genbank [Genbank: HQ317917]. NA: Not available.

<table>
<thead>
<tr>
<th>Description</th>
<th>Accession</th>
<th>Locus Tag</th>
<th>Query coverage (%)</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. jejuni</em> hypothetical integral membrane protein (<em>iamB</em>) gene (partial cds); and ABC transporter (<em>iamA</em>) gene (complete cds)</td>
<td>AF023133.1</td>
<td>AF023133</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td><em>C. jejuni</em> subsp. <em>jejuni</em> IA3902</td>
<td>CP001876.1</td>
<td>CJS_A1559</td>
<td>100%</td>
<td>84%</td>
</tr>
<tr>
<td><em>C. jejuni</em> subsp. <em>jejuni</em> NCTC 11168</td>
<td>AL111168.1</td>
<td>Cj1647</td>
<td>100%</td>
<td>84%</td>
</tr>
<tr>
<td><em>C. jejuni</em> RM1221</td>
<td>CP000025.1</td>
<td>CJE1819</td>
<td>100%</td>
<td>84%</td>
</tr>
<tr>
<td><em>C. jejuni</em> subsp. <em>jejuni</em> 81116</td>
<td>CP000814.1</td>
<td>C8J_1549</td>
<td>100%</td>
<td>83%</td>
</tr>
<tr>
<td><em>C. jejuni</em> subsp. <em>jejuni</em> 81-176</td>
<td>CP000538.1</td>
<td>C8J1176_1638</td>
<td>100%</td>
<td>83%</td>
</tr>
<tr>
<td><em>C. jejuni</em> subsp. <em>jejuni</em> JL110034*</td>
<td>NA</td>
<td>NA</td>
<td>100%</td>
<td>86%</td>
</tr>
<tr>
<td><em>C. jejuni</em> subsp. <em>doylei</em> 269.97</td>
<td>CP000768.1</td>
<td>JJD26997_2007</td>
<td>100%</td>
<td>85%</td>
</tr>
<tr>
<td><em>C. lari</em> RM2100</td>
<td>CP000932.1</td>
<td>Cla_0118</td>
<td>94%</td>
<td>81%</td>
</tr>
</tbody>
</table>
Table 3.4: Detection of virulence-associated genes in *iam*-containing *C. jejuni* using PCR.

<table>
<thead>
<tr>
<th>Strain</th>
<th>cdtB</th>
<th>ciaB</th>
<th>cadF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bov-2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bov-3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bov-6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bov-7</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bov-9</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bov-10</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bov-11</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bov-12</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hum-3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hum-5</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hum-6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hum-7</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hum-9</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hum-12</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hum-13</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 3.5: Antibiotic resistance profile of *iam*-containing *C. jejuni* isolated from cattle and humans, respectively.

Isolates underlined are *iam*-negative. Values that indicate resistance to the antibiotic according to the CLSI guidelines (5) are shaded in grey. H-13 grew poorly and could not be tested for antibiotic resistance using our assays. AZI: azithromycin; CIP: ciprofloxacin; ERY: erythromycin; GEN: gentamicin; TET: tetracycline; FFN: florfenicol; NAL: nalidixic acid; TEL: telithromycin; CLI: clindamycin. *C. jejuni* 81-176 and NCTC-11168 were used for quality control purposes.

<table>
<thead>
<tr>
<th>Human Isolates</th>
<th>Inhibitory Concentrations (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>H-1</td>
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Figure 3.1: C. jejuni specific and iam specific PCR confirmation. A. Examples of 16S rRNA gene PCR products (Campylobacter-specific; upper panel) and mapA PCR products (C. jejuni-specific; lower panel). Lanes 1-7 represent C. jejuni isolated from cattle feces. Lane 8 represents C. jejuni 81-176 (positive control). B. Detection of iam-positive C. jejuni from bovine (Bov) and human- (H) samples using PCR analysis as described by Carvalho et al. (4). M: 1Kb DNA ladder marker. -ve: negative control. C. Examples showing the motility exhibited by selected iam-positive strains as compared to C. jejuni 81-176.
Figure 3.1: *C. jejuni* specific and *tam* specific PCR confirmation.
Figure 3.2: Invasion and intracellular survival of cattle and human *C. jejuni* isolates in INT407 cells. A. *C. jejuni* invasion potential of human INT-407 cells. B. Intracellular survival of *C. jejuni* isolates in INT-407 cells. Bov-1, Bov-5, H-1, and H-4 are *iam*-negative *C. jejuni* used for comparison with *iam*-containing isolates. *C. jejuni* 81-16 and NCTC-11168 are wildtype strains used as controls. Data were log transformed and presented as mean ± SE (standard error).
Figure 3.2: Invasion and intracellular survival of cattle and human *C. jejuni* isolates in INT407 cells.
Figure 3.3: The evolutionary and phylogenetic analyses of cattle and human *C. jejuni* isolates. **A.** The evolutionary relationship between *iam* sequences detected in *C. jejuni* isolated from human and cattle samples. The tree was drawn to scale using the Neighbor-Joining method. The percentage of replicate trees in which the *iam* sequences clustered together in the bootstrap test (10000 replicates) is shown next to the branches. The evolutionary distances are in the units of the number of base substitutions per site. **B.** Dendrogram showing the pulsed-field gel electrophoresis profile for the *iam*-positive *C. jejuni*. H-9 and H-13 could not be typed using PFGE. Numbers on the nodes represent the Cophenetic correlations.
Figure 3.3: The evolutionary and phylogenetic analyses of cattle and human *C. jejuni* isolates.
Figure 3.4: Expression of *iam* as determined using qRT-PCR analysis.

Bov-1 is an *iam*-negative strain. Wild type strains (*C. jejuni* 81-176 and NCTC-11168) that harbor the *iam* were also included for comparison with *iam*-containing cattle isolates.
Figure 3.5: Chicken colonization with *C. jejuni* isolated from cattle and human hosts.

Results were represented as means of CFU g$^{-1}$ of caecum retrieved from at least six chicks per isolate tested. Bov-1, Bov-5, H-1, and H-4 are *iam*-negative *C. jejuni* used for comparison with *iam*-containing isolates. *C. jejuni* 81-176 and NCTC-11168 were used as controls. Data were log transformed and presented as mean ± SE (standard error).
CHAPTER 4

Molecular Epidemiological Studies on *Campylobacter* Isolated from Dairy Cattle and European Starlings in Ohio, USA

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4.1 Abstract

Dairy cattle serve as a potential source for Campylobacter infection in human. Outbreaks associated with consumption of either Campylobacter contaminated raw milk or contaminated milk after treatment, were previously recorded in the US. However, sources of Campylobacter infection and its routes of transmission in dairy farms have not been clearly established. Studies outside the US have indicated that wild birds (starlings) may play a potential role in the epidemiology of Campylobacter in dairy cattle; however, this information still remains very limited in the US. Here, we determined the prevalence, genotypic, and phenotypic properties of Campylobacter that were isolated from paired fecal samples of dairy cattle and starlings in Northeastern Ohio. Campylobacter were detected in 83 (36.6%) and 57 (50.4%) out of 227 dairy and 113 starlings fecal samples, respectively. Specifically, 79 C. jejuni, 5 C. coli, and 2 other Campylobacter spp. were isolated from dairy feces, while all the retrieved isolates from starlings’ samples were C. jejuni. Our results showed that the prevalence of C. jejuni in dairy cattle was significantly (P<0.05) different among different dairy establishments in Northeastern Ohio. Moreover, the prevalence of C. jejuni in birds was significantly (P<0.01) higher than that in dairy cattle. Further, PFGE analysis showed that C. jejuni were mostly genetically diverse and host restricted. However, there were several shared genotypes between dairy and starling isolates. Furthermore, many shared clonal complexes (CC) between dairy and starlings were observed by MLST analysis. Both in cattle and starlings, the most frequently represented CCs were CC ST-45, and CC ST-21 which are also commonly found in humans. As previously reported, CC ST-177 and CC ST-682
were restricted to the bird isolates, while CC ST-42 was restricted to dairy isolates. Further, two new STs were detected in *C. jejuni* isolated from dairy. Interestingly, cattle and starlings *Campylobacter* showed high resistance to multiple antimicrobials including ciprofloxacin, erythromycin, and gentamicin. In conclusion, our results highlight starlings as potential reservoirs for *C. jejuni* and may play important role in the epidemiology of clinically important *C. jejuni* in dairy population.

### 4.2 Introduction

Campylobacteriosis is estimated to affect over one million individuals in the United States annually, with symptoms ranging from mild diarrhea to more serious neuropathies (Scallan et al., 2011). *C. jejuni* and *C. coli*, are species of major concern and responsible for the majority of human campylobacteriosis (Allos, 2001). Both *Campylobacter* spp. can asymptptomatically colonize major food-animals such as poultry and cattle, subsequently contaminating foods of animal origin including meats and dairy products (Miller and Mandrell, 2005). However, little is known about the transmission and epidemiology of cattle-associated *Campylobacter* in the USA. Recent studies showed that cattle play a potential role in the transmission of human infections (Ragimbeau et al., 2008; Wilson et al., 2008). Cattle can contribute to human infections through several routes of transmission such as direct contact, environmental contamination, and ingestion of contaminated food and milk. Moreover, consumption of unpasteurized or inadequately pasteurized milk has long been identified as a source of campylobacteriosis (Stanley and Jones, 2003). Twenty six of the 57 food-borne outbreaks caused by *C. jejuni* in the period
from 1978 to 1986 were associated with raw milk consumption (Tauxe, 1992). However, limited number of Campylobacter sources has been identified in dairy and further investigations are needed to better understand the role of reservoirs and their relative contribution to human infection (Ragimbeau et al., 2008). Several studies have indicated that wild birds may play a potential role in the epidemiology of human infection by transmitting Campylobacter from livestocks to humans (Jones, 2001; Rosef et al., 1983; Cabrita et al., 1992; Waldenström et al., 2005). There are several alternative routes for transmission of Campylobacter infection to humans which includes cattle byproducts, occupational exposure, direct contact, and exposure to environment contaminated with wild life and livestock fecal material (French et al., 2009). Campylobacter genotypes could spread from farms into the environment by several ways, and further spread to any environmental reservoirs such as wild birds, may facilitate their transmission from one farm to another (Waldenström et al., 2005). In a study at Northwest England using multi locus sequence typing (MLST), most commonly recovered genotypes from dairy cattle, wildlife, and environmental sources were frequently associated with human disease (Kwan et al., 2008b). Further, wild European Starlings (Sturnus vulgaris) were suggested to be a potential source of human and livestock infection because of their high shedding rates of Campylobacter (Colles et al., 2008; Nielsen et al., 2004) as well as their capability to disseminate infections between farms (LeJeune et al., 2008). Furthermore, European Starlings have the tendency to invade and gather in thousands at dairy farms and animal feeding operations (Linz et al., 2007). Consequently, the role of European Starlings and dairy cattle as reservoirs for these pathogens might be important for
understanding the epidemiology of *Campylobacter* infections. Particularly in the US, the prevalence of *Campylobacter* in dairy cattle and wild birds (starlings), their genotypic characteristics and relationship to isolates from humans’ origin have not been extensively described in the peer reviewed literature. Thus, more epidemiological and genotyping studies on *Campylobacter* isolates from cattle and identifying the shared sequence types among birds, cattle and humans isolates is important to further understand the epidemiology of *Campylobacter* in the dairy populations in order to assess the associated risks to public health. Further, these studies would be useful in developing ways to control and prevent human infection and improve the safety of our food supply.

Here, we hypothesized that starlings may play a potential role as a source of *Campylobacter* infection and/or transmission to dairy cattle through contaminated droppings. To address our hypothesis, a cross-sectional study was conducted to determine the prevalence, genotypic relatedness between *Campylobacter* isolated from dairy cattle and European Starlings captured on those farms in Northeastern Ohio.

### 4.3 Materials and Methods

**Distribution of sampling sites, collection and processing of fecal samples from dairy cattle and starlings**

A total of 227 fresh dairy fecal samples as well as 113 starlings’ fecal samples were collected during the summer and early fall of 2009 from 11 dairy farms in northern Ohio, USA (Table 4.1). The sampling was done during the summer months and early fall; this marks the period of peak bovine shedding of *Campylobacter* and when starlings start
congregating in large flocks on farms. Ten g of dairy feces were collected as well as starlings were captured during each visit using mist nets or decoy cages (during some visits, capture of starlings was not possible) (Table 4.1). All birds were euthanized by cervical dislocation and carcasses were placed on ice for transport to the laboratory. All work was conducted with approval from The Ohio State University Institutional Animal Care and Use Committee and necessary government permits.

**Isolation and identification of Campylobacter spp. from fecal samples**

To isolate *Campylobacter* spp., 1 g of each fecal sample was enriched in Preston broth for 48 h at 42 °C under microaerobic conditions (5% O₂, 10% CO₂, and 85% N₂) (Krause *et al*., 2006). From the enrichments, an inoculum (100 µl) was spread onto modified Cefoperazone Charcoal Deoxycholate Agar (mCCDA) plates, which were then incubated for an additional 48 h at 42°C under microaerobic conditions (Engberg *et al*., 2000). Three to five colonies suspected as *Campylobacter* were selected from each plate and sub-cultured onto Muller-Hinton agar plates, after which DNA was isolated from each culture using the Genomic DNA Purification Kit (Epicenter, USA) as described by the manufacturer. The DNA was then quantified using the NanoDrop 1000 Spectrophotometer V3.7.1 (Thermo Fisher Scientific, USA) and subjected to genus and species-specific PCR analysis to confirm their identity as described elsewhere (Denis *et al*., 2008). PCR analysis targeted a 16S rRNA gene fragment, *mapA*, and *ceuE*, which indicated the specific detection of *Campylobacter* spp., *C. jejuni*, and *C. coli*, respectively (Denis *et al*., 1999, Linton *et al*., 1997). All PCR products were resolved on a 1.5%
agarose gel containing 0.5 µg/ml of ethidium bromide. The size of the PCR products was
determined using a 1Kb DNA ladder and detection was confirmed by comparison to PCR
products generated from *C. jejuni* 81-176 (wild-type strain) and *C. coli* (ATCC 33559),
which were used as positive controls in all PCR analysis. Negative controls (reactions
with no DNA templates) were included in all PCR analyses to ensure specific product
amplification.

**Pulsed Field Gel Electrophoresis (PFGE) of *Campylobacter* isolates**

To determine their genotypic relatedness, the *Campylobacter* isolated from fecal
samples were analyzed using PFGE analysis as described in Ribot et al. (Ribot et al.,
2001). Briefly, *C. jejuni* isolates were harvested from MH agar plates and suspended to
an OD₆₅₀ of 1.4 in 1X PBS. To prepare agarose plugs, OD adjusted suspensions were
gently mixed with 1% SeaKem Gold agarose (SKG, Fisher scientific, PA, USA) that was
pre-melted in TE (10 mM Tris, 1 mM EDTA, pH 8.0). The plugs were then incubated
with shaking (200 rpm) in lysis buffer [50 mM Tris, 50 mM EDTA (pH 8.0), 1%
sarcosine, 0.1 mg ml⁻¹ of proteinase K] for 1 h at 55°C. After lysis the plugs were
washed four times and suspended in 5 ml of fresh TE. The plugs were then sliced and
digested overnight with *SmaI* at room temperature. The digested slices were loaded onto
a 1% SKG agarose gel and DNA fragments were separated by electrophoresis for 20 h
using the CHEF Mapper system (Bio-Rad, CA, USA) followed by post staining with
ethidium bromide. The resulting PFGE patterns were documented and analyzed using the
BioNumerics 5.1 software (Applied Maths Inc, TX, USA). Similarity and clustering
analysis of the PFGE patterns were performed using the Dice Coefficient and the unweighted pair-group method with arithmetic averages (UPGMA) with optimization of 1% and position tolerance of 1.5% (Ribot et al., 2001). The PFGE analysis was also performed on C. jejuni 81-176 which was used as control for facilitating gel to gel comparison. Further, Lambda Ladder PFG Marker (50-1,000 kb, BioLabs, New England) was used as a molecular marker.

**Determining the antimicrobial-resistance properties of the *Campylobacter* isolates**

Minimal inhibitory concentration (MIC) of *C. jejuni* and *C. coli* isolates were determined using commercially available 96-well plates containing antimicrobials (Sensititre Campy plates, TREK Diagnostic Systems Inc., OH, USA). These Sensititre Campy plates were used following the protocol described by the manufacturer and include *Campylobacter*-relevant antimicrobials such as azithromycin (AZI) (MIC: ≥8 µg ml⁻¹); ciprofloxacin (CIP) (MIC: ≥4 µg ml⁻¹); clindamycin (CLI) (MIC: ≥8 µg ml⁻¹); erythromycin (ERY) (MIC: ≥32 µg ml⁻¹); gentamicin (GEN) (MIC: ≥8 µg ml⁻¹); nalidixic acid (NAL) (MIC: ≥64 µg ml⁻¹); telithromycin (TEL) (MIC: ≥8 µg ml⁻¹); florfenicol (FEN) (MIC: ≥8 µg ml⁻¹); and tetracycline (TET) (MIC: ≥16 µg ml⁻¹). *Campylobacter* isolates were grown to mid-log phase and then suspended in Mueller-Hinton broth to achieve an OD₆₀₀ of 0.05. For each isolate, one hundred microliter of the suspension were transferred to each well in the Sensititre Campy plates, including a control well that did not contain any antibiotics, while *C. jejuni* 81-176 was used for quality control. The plates were then incubated under microaerobic conditions at 42°C for 24 h after which the
minimum inhibitory concentration (MIC) was measured. The MIC for each antibiotic was defined as the absence of bacterial growth in the well with the lowest concentration of the antibiotic and susceptibility was interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2006).

**Multilocus sequence typing (MLST) of Campylobacter isolates**

To further ascertain the genotypic relationship of the *Campylobacter* isolates and to assess similarity to strains associated with human infections, MLST analysis was performed on a total of 72 *C. jejuni* isolates. The isolates were selected to be representative of different PFGE clusters. MLST analysis was conducted as described by (Dingle *et al*., 2001). Briefly, loci from seven housekeeping genes (*aspA, glnA, gltA, glyA, pgm, tkt,* and *uncA*) were amplified using gene specific primers by PCR and anticipated sizes of the amplicons were confirmed by agarose gel electrophoresis. Oligonucleotides (Dingle *et al*., 2001) were obtained from Integrated DNA Technologies (USA). The PCR products were purified (ExoSAP-IT for PCR Product Clean-Up-Affymetrix, Inc., Cleveland, OH, USA), sequenced in both directions as described previously (Dingle *et al*., 2001), forward and reverse sequences were then aligned using ClustalW (www.ebi.ac.uk/clustalw). Allelic profiles were determined by performing BLAST analysis using the single-locus query function, while sequence types (STs) were assigned using the allelic profile query function available in the MLST *Campylobacter* database (http://pubmlst.org/campylobacter/). STs were then traced to their respective clonal complexes using BURST at http://pubmlst.org/.
The adjusted sample size for this study was calculated statistically by choosing a hypothetical prevalence in wild birds of 5%, Thus, initial estimates for simple random sampling with study power of 0.7, \( \alpha=0.05 \), and error margin 0.1. For adjusted clustering we used (STATA, ver. 9.0) and interclass correlation coefficient=0.2. A Chi square test was used to evaluate data collected from the Campylobacter prevalence analysis. \( P \) values of <0.05 were the criteria chosen for statistical significance.

4.4 Results

Occurrence and distribution of Campylobacter spp. in feces sampled from dairy cattle and starlings

The occurrence and distribution of Campylobacter in dairy cattle and starlings feces was investigated in order to determine the role of starlings as a potential reservoir for these bacteria on dairy farms as well as to determine the potential public health significance of dairy cattle associated Campylobacter isolates. Campylobacter were detected in 81 (36.6\%) out of 227 dairy fecal samples and 57 (50.4\%) out of 113 starlings fecal samples. A total of 86 Campylobacter isolates occurred in 83 dairy fecal samples. The C. jejuni represented the majority of the isolates which were detected in 79 (91.9\%) and 5 (5.8\%) were C. coli, while 2 (2.3\%) Campylobacter isolates were other than C.jejuni and C.coli (Table 4.1). A total of 57 C.jejuni isolates were isolated from starlings, while no C. coli were isolated from starlings’ feces. Our results showed that the prevalence of C. jejuni in dairy cattle was significantly \( (P<0.05) \) different among
different dairy establishments in Northeastern Ohio. Furthermore, the prevalence of *C. jejuni* in starlings was significantly (*P* < 0.01) higher than that in dairy cattle.

*C. jejuni* strains were genotypically diverse among different spp.

PFGE analysis was performed on *C. jejuni* isolated from dairy cattle and starlings feces in order to determine the genotypic diversity of these bacteria and the relationship between the isolates retrieved from both species. PFGE analysis was successfully performed on 130 out of 136 dairy and starlings-associated *C. jejuni* isolates, while 6 isolates could not be typed using the aforementioned method. Analysis of the PFGE profiles of the *C. jejuni* suggested that the isolates possessed diverse genotypes, especially when comparing profiles of isolates from different farms (Fig 4.1). Furthermore, there were 4 main clusters identified with cluster I mainly consisting of cattle associated *C. jejuni* (Fig 4.1). All clusters included profiles of *C. jejuni* isolates associated with both dairy cattle and starlings with different percentages of similarities. However, *C. jejuni* isolates from dairy and starlings with 100% similarity were observed in 11 sub clusters (see arrows in Fig. 1). Only 3 of those 100% similarity sub clusters possessed shared macro restriction profiles (mrp) for *C. jejuni* isolates from both species from the same location (farm) (see stars in Fig 4.1). Further, the other 8 sub clusters constituted cattle and bird associated *C. jejuni* isolates from different farms including one with 5 isolates from 4 different farms (see asterisk Fig 4.1).
Antimicrobial susceptibility of dairy and starlings associated C. jejuni isolates

Due to the increasing resistance capacity of livestock-associated Campylobacter to clinically important antimicrobials as well as to better assess the potential public health impact of the Campylobacter spp. associated with cattle and starlings, the isolates were assayed for their potential to resist antibiotics that are of both clinical and veterinary importance. A total 117 C.jejuni isolates (72 dairy cattle associated and 45 starlings associated isolates) were tested for antimicrobial susceptibility using commercially available Sensititre Campy plates. Isolates tested including a subset of isolates representing different PFGE clusters. Those isolates that were un-typeable by PFGE were not included for analysis. The dairy associated C. jejuni isolates were resistant to different antimicrobials including, ciprofloxacin (MIC: 4-8 µg ml⁻¹), erythromycin (MIC: 32-64 µg ml⁻¹), gentamicin (MIC: 8-32 µg ml⁻¹), tetracycline (MIC: 16-64 µg ml⁻¹), and clindamycin (MIC: 8-16 µg ml⁻¹) (Fig 4.2). However, resistance to tetracycline was observed for the majority of the dairy cattle associated C. jejuni (68.0%) (Fig.2A). Furthermore, 28 (38.9%) dairy-associated C. jejuni isolates were resistant only to tetracycline, 3 (4.16%) isolates were resistant to only two antimicrobials, 7 (9.7%) isolates were resistant to only 3 antimicrobials, while 18 (25%) isolates were resistant to 4 or more antimicrobials including ciprofloxacin, tetracycline, nalidixic acid, erythromycin, and clindamycin (Fig 4.2). However, 15 (20.8%) isolates showed high susceptibility to all the tested antimicrobials.

On the other hand, a total 45 bird-associated C. jejuni isolates were tested for antimicrobials susceptibility (Fig 4.2). Although the resistance rate to most of the tested
antimicrobials for cattle and bird associated \textit{C.jejuni} were different, the later also exhibited resistance to several antimicrobials including ciprofloxacin, gentamicin, nalidixic acid, tetracycline, and clindamycin. Furthermore, resistance to tetracycline was observed for the majority of bird \textit{C.jejuni} isolates (42.2%), while only one (2.2%) isolate showed resistance to erythromycin (Fig 4.2). Of the 45 bird associated isolates tested, 8 (17.8%) were resistant to only one antimicrobial (e.g. tetracycline), 5 (11.1%) were resistant to two antimicrobials and 7 isolates (15.5%), were resistant to only three antimicrobials, while 5 (11.1%) isolates were resistant to four or more antimicrobials. However, nineteen isolates (42.2%) were susceptible to all antimicrobials (Fig 4.2 and Fig 4.3).

**MLST analysis identifies STs belonging to clonal complexes of public health significance**

To further determine the genotypic relationship of \textit{C. jejuni} isolated from dairy cattle and starlings to those of human origin, MLST analysis was performed on selected \textit{C. jejuni} isolates and Sequence Types (STs) were identified by interrogation of the PubMLST data base (http://pubmlst.org/campylobacter/). A total of 75 isolates (44 dairy and 31 starlings associated) that represented the main PFGE clusters and different locations (farms), were selected for MLST analysis. Two new \textit{C. jejuni} STs were identified and designated as STs 5463 and 5464 (Fig 4.3).

In both species, a total of 15 different STs (8 dairy associated and 5 starlings associated) were identified, and two STs (ST-45 and ST-8) were shared between both
species (Fig 4.3). Specifically, 34 (23 dairy associated and 11 starlings associated) isolates representing 4 STs belonged to Clonal Complex (CC) ST-45. Additional 9 isolates (7 dairy associated and 2 starlings associated) were grouped into ST-8 that belonged to CC ST-21 (Fig 3). However, eleven dairy cattle-associated _C. jejuni_ isolates representing 4 STs including the two newly identified STs (ST-459, ST-42, ST-5463, and ST-5464), belonged to CC ST-42. In addition, 3 isolates were identified as ST-2197, one as ST-399, one as ST-782 which all belonged to CC ST-45. One isolate was identified as ST-2038 which belonged to CC ST-21. On the other hand, ten starlings-associated isolates were identified as ST-177, two as ST-1506 and one as ST-685 which all belonged to CC ST-177. Further, four isolates were identified as ST-1020 and one as ST-1021 belonged to CC ST-682 (Fig 4.3). The complete profile for 2 isolates could not be obtained.

4.5 Discussion

We investigated the occurrence of _Campylobacter_ spp. (_C. jejuni_ and _C. coli_) in dairy cattle and European starlings in dairy establishments in Northeastern Ohio, USA, which represent a leading section in Ohio with many dairy farms concentrated in Wayne, Holmes, Ashland, Crawford, and Huron counties. Previous studies in the USA are restricted only to dairy cattle-associated _Campylobacter_ or wild birds (starlings)-associated _Campylobacter_ and have been limited to investigating the prevalence of certain and phenotypic characteristics such as antibiotic resistance (Englen _et al._, 2007, Hoar _et al._, 2001; Wesley _et al._, 2000; Waldenström _et al._, 2002; Waldenström _et al._,
Furthermore, particularly in the USA, the role of wild birds in the epidemiology of *Campylobacter* in dairy establishments as well as the prevalence of *Campylobacter* in dairy cattle and wild birds (starlings), their genotypic characteristics and relationship to isolates from humans’ origin have not been extensively described in the peer reviewed literature. Thus, it was necessary to investigate this assumption as well as to determine the overall occurrence of these bacteria in dairy cattle and European Starlings. Therefore, our approach was comprehensive as it included determining *Campylobacter* prevalence, analysis of genotypic relatedness, antimicrobial resistance properties as well as public health relevance of dairy cattle and European Starlings-associated *Campylobacter*. Significantly, our findings highlight the importance of dairy cattle and starlings as reservoirs for genotypically diverse and antimicrobial resistant *Campylobacter* in the USA.

Although the association of *Campylobacter* spp. with dairy cattle in the USA has been investigated previously, available literature reported variable prevalence numbers for these pathogens. For example, Harvey *et al* (Harvey *et al*., 2004) isolated *Campylobacter* from 0 to 10% of the fecal samples collected from lactating dairy cows from various regions of the US, while Englen *et al* (Englen *et al*., 2006), and Wesley *et al* (Wesley *et al*., 2000) stated that these bacteria occurred in 51.2% and 37.7% of dairy feces, respectively. On the other hand, the prevalence data for *Campylobacter* in wild birds in the US are very limited. In a study conducted by Keller *et al*. (Keller *et al*., 2011) *C.jejuni* was isolated from six avian families with the highest prevalence of 25%. Our results showed that the occurrence of *Campylobacter* in some dairy establishments in
Northeastern Ohio, USA, from dairy was 36.6% (Table 4.1). Our data falling between the percentages (0 to 51.2%) previously reported for dairy from the US (Harvey et al., 2004; Wesley et al., 2000; Sato et al., 2004; Bae et al., 2005; Dodson and LeJeune, 2005; Englen et al., 2007) is in concordance with those reported outside the USA (35.9%) and UK (Kwan et al., 2008a). On the other hand, the occurrence of Campylobacter spp. in starlings samples in our study was higher (50.4%) than most of that previous reports from other countries such as Sweden; 21.6% (Waldenström et al., 2002), and New Zealand; 30.6% (French et al., 2009). However, it was in concordance with the reported percentage range 2 to 50% in some of wild bird species including starlings (Hughes et al., 2009). As previously reported, C. jejuni represented the majority of the retrieved isolates from dairy samples (Wesley et al., 2000; Bae et al., 2005) as well as starlings’ samples (Luechtefeld et al., 1980; Kapperud and Rosef, 1983; Waldenstrom et al., 2002; Broman et al., 2004; Keller et al., 2011). Our results showed that the prevalence of C. jejuni in dairy cattle was significantly different (P<0.05) among different dairy establishments in Northeastern Ohio. The reasons for the observed discrepancies in Campylobacter prevalence are not clear. As such our prevalence data don’t account for possible seasonal and temporal variations. Our sampling efforts focused on the late summer and early fall season that normally witness an increase in Campylobacter incidence (Wilson et al., 2008). Nevertheless, our prevalence data provide evidence that dairy cattle and European starlings in the USA possibly constitute considerable reservoirs for these pathogens, promoting their potential persistence in dairy cattle-derived products and environment, which might pose a risk for human consumers of cattle byproducts.
Genotyping properties of dairy and wild birds’ isolates in the USA have not been extensively investigated. Subsequently, it was important to investigate the genetic diversity of *Campylobacter* in dairy cattle in order to assess the potential attributes of this reservoir as well as to investigate the distribution of *C. jejuni* genotypes among wild birds (European Starlings). Furthermore, the bird isolates were also compared with dairy cattle isolates collected during the same time period and from the same location (farm), to investigate the possible impact of wild birds on dairy *C. jejuni* infections and their potential role in transmission of infection. Our PFGE analysis of the dairy cattle and starlings-associated *C. jejuni* revealed that all isolates were generally grouped into 4 major clusters (Fig 4.1). Remarkably, some *C. jejuni* isolates possessed genotypes that were 100% similar on 29 different occasions (Fig 4.1), eleven out of which included shared isolates from both species (see arrows in Fig 4.1). These observations supports the findings of studies conducted on cattle in other countries, including Spain (Oporto *et al.*, 2007), the United Kingdom (Grove-White *et al.*, 2010), Denmark (Litrup *et al.*, 2007), Finland (de Haan *et al.*, 2010), Turkey (Açık and Çetinkaya, 2006), and also in the USA (Sanad *et al.*, 2011) as well as in wild birds (Broman *et al*. 2004). Additionally, our PFGE analysis revealed clustering of some *C. jejuni* isolates from both dairy and starlings that originated from same locations in 3 occasions as well as isolates from both species originated from different farms (see stars in Fig 4.1). These results support the notion that European starlings are highly mobile (Johnson and Glahn, 1992) and can serve as a vector for transmission of pathogens between farms (LeJeune *et al.*, 2008).
Campylobacter resistance to antibiotics is an increasingly important emerging threat to public health (Inglis et al., 2006; Englen et al., 2005). Furthermore, previous studies have demonstrated that cattle-associated Campylobacter in the USA can potentially resist antimicrobial drugs of clinical importance to human health. For example, 47.4%, 4% and 2.5% of C. jejuni collected from dairy cows in the US were resistant to tetracycline, nalidixic acid, and ciprofloxacin, respectively (Englen et al., 2007). Additionally, 47.7% and 49.1% of C. jejuni isolated from dairy and feedlot cattle, respectively, were resistant to tetracycline (Englen et al., 2005; Englen et al., 2007). In agreement with these studies, our antibiotic resistance analysis showed that a high percentage of the dairy cattle-associated strains were resistant to erythromycin, ciprofloxacin, gentamicin, tetracycline and other antimicrobials (Fig 4.2 and Fig 4.3). Specifically, 20.8% and 6.9% of the cattle-associated C. jejuni that were analyzed in our study exhibited resistance to ciprofloxacin and erythromycin, respectively (Fig 4.2). This was an important and marked difference when compared to previous studies in the US that reported a relatively lower frequency of resistance to ciprofloxacin (1.8% to 5%) and erythromycin (0.4% to 2.9%) in C. jejuni isolated from different cattle operations (Bae et al., 2005; Englen et al., 2005; Englen et al., 2007). It has been suggested that there is a clear increase in the antimicrobial resistance among pathogens and this is attributed to the use of antimicrobials in the dairy cows and other livestock (Oliver et al., 2011). Our data further support predictions that livestock-associated Campylobacter are becoming increasingly resistant to important antibiotics (Châtre et al., 2010; Inglis et al., 2006). Similarly, starling associated C. jejuni isolates also displayed higher resistance than those
previously reported outside the USA from wild birds. For example; the resistance prevalence of bird isolates for several antimicrobials including ciprofloxacin and nalidixic acid was (0.7 to 3.6%) (Waldenström et al., 2002). Horizontal transfer of resistance features among species of *Campylobacter* as well as transmission of resistant *Campylobacter* between different species provides potential mechanisms for emerging antimicrobial resistance (Velazquez et al., 1995; Federal Register, 2000). These observations highlight the need for strict surveillance of antibiotics used in dairy cattle operations in order to facilitate interventions and curb further emergence of antibiotic resistant *Campylobacter*.

Understanding the contributions of dairy cattle associated *Campylobacter* to public health mainly depends on assessing the relationship between the dairy cattle *Campylobacter* with those previously isolated from- or implicated in human infections. To determine the aforementioned criteria, we typed a pool of the dairy cattle-associated *Campylobacter* and starlings-associated isolates using MLST. Since interpretation of the MLST typing depends on pre-existing online data base (PubMLST) to identify sequence types (STs) of isolates, it is possible to compare the dairy cattle and starlings *Campylobacter* from this study to others from different sources (e.g. humans) and locations, including other countries. MLST analysis confirmed that dairy cattle and starlings *C. jejuni* [n = 75 (44 dairy, and 31 starlings isolates)] were diverse and belonged to 8 and 5 disparate STs, respectively, in addition to two shared STs (ST-45 and ST-8) between both species (Fig 4.3) emphasizing the genetic diversity of this bacterium in both species. Further, our results indicated that certain *C. jejuni* may be highly host associated.
(CC ST-42; cattle, CC ST-177 and CC ST-682; starlings) (Waldenstrom et al. 2002; Colegrave and Buckling, 2005). CC ST-21, CC ST-45, CC ST-42 and ST-61 were predominant in cattle samples from other countries, including the UK, Canada, New Zealand, and Finland (de Haan et al., 2010; Dingle et al., 2002; Kwan et al., 2008a; Levesque et al., 2008; Kwan et al., 2008b). The detection of CC ST-45, CC ST-21 and CC ST-42 (Fig 4.3) in this study was of particular interest, since these complexes constitute three of six CCs that harbor 60% of C. jejuni associated with human disease (Dingle et al., 2001; Grove-White et al., 2010). The clear absence of unique starlings-associated strains of C. jejuni in dairy population suggests that the i) infection is most likely transmitting from dairy to birds (Hughes et al., 2009) and the starlings are involved in transmission of infection between dairy operations and ii) some C. jejuni strains are host adapted and cannot survive (transmit) well in desperate host reservoirs.

Taken together, our studies indicate European starlings may play a potential role in transmission of Campylobacter infection in dairy cattle populations as well as dissemination of clinically important C. jejuni clonal complexes. Further, the potential of dairy cattle and starlings Campylobacter to resist multiple antimicrobials including those antibiotics of treatment choice for human cases, reassert the contribution of starlings to the epidemiology of these pathogens to dairy populations and consequently to human through consumption of contaminated dairy and dairy byproducts in the USA. Thus, better understanding of molecular epidemiology of Campylobacter would enhance the efforts to establish effective control and preventive measures, and limit the public health impact of this pathogen from dairy and associated reservoirs.
4.6 Acknowledgement

Research at Dr. Rajashekara’s laboratory is also supported by the Ohio Agricultural Research and Development Center (OARDC), The Ohio State University, and the USDA grant 2007-03109. This research was partially supported by funds appropriated to Dr. LeJeune; NRI-2006-01227. Yasser sanad’s PhD scholarship is sponsored by the Egyptian Ministry of Higher Education.

4.7 References


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Table 4.1: *Campylobacter* species isolated from dairy cattle and starlings fecal samples from dairy establishments in Northeastern Ohio.

<table>
<thead>
<tr>
<th>Farm</th>
<th>Cattle samples</th>
<th>Positives Dairy (%)</th>
<th>Dairy Isolates</th>
<th>Bird samples</th>
<th>Positives Birds (%)</th>
<th>Birds Isolates</th>
<th>Total Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C. jejuni</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>10</td>
<td>5 (50.0)</td>
<td>2</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>5</td>
</tr>
<tr>
<td>B</td>
<td>25</td>
<td>6 (24.0)</td>
<td>2</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>7</td>
</tr>
<tr>
<td>C-M</td>
<td>17</td>
<td>6 (17.6)</td>
<td>7</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>3</td>
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<tr>
<td>C-R</td>
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<td>1</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>D</td>
<td>25</td>
<td>7 (28.0)</td>
<td>8</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>8</td>
</tr>
<tr>
<td>E</td>
<td>20</td>
<td>8 (53.3)</td>
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<td>12</td>
<td>7 (58.3)</td>
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<td>13</td>
<td>12</td>
<td>6 (50.0)</td>
<td>6</td>
<td>19</td>
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<td>22</td>
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<td>18</td>
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<td>15</td>
</tr>
<tr>
<td>J</td>
<td>25</td>
<td>15 (75.0)</td>
<td>12</td>
<td>26</td>
<td>24 (92.3)</td>
<td>24</td>
<td>39</td>
</tr>
<tr>
<td>TOTAL</td>
<td>227</td>
<td>83 (36.6)*</td>
<td>79</td>
<td>113</td>
<td>57 (50.4)</td>
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<td>143</td>
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</tbody>
</table>

* A total of 86 *Campylobacter* isolates occurred in 83 dairy fecal samples including two *Campylobacter* spp. other than C. jejuni and C. coli.
Figure 4.1: Dendrogram showing the Pulsed-field gel electrophoresis patterns for *Sma*I restricted dairy cattle and starlings *C. jejuni*. Similarity analysis was performed using the Dice coefficient, and clustering was performed by the unweighted pair-group method with arithmetic averages UPGMA (optimization, 1% and position tolerance, 1.5%). Numbers on bootstraps represent Cophenetic correlations. A-J= Farms, D= dairy, Br= birds.
Figure 4.1: Dendrogram showing the Pulsed-field gel electrophoresis patterns for SmaI restricted dairy cattle and starlings C. jejuni.
Figure 4.2: Antimicrobial resistance of *Campylobacter jejuni* isolated from dairy cattle (n=72) and starlings (n=45). Percentage of isolates resistance to different antimicrobials used in this study is shown. All resistant values are according to CLSI. *C. jejuni* 81-176 strain was used as Quality Control.
Figure 4.3: Phylogenetic tree for the sequence types detected in dairy cattle and starlings associated *C. jejuni* isolates. The tree is drawn using UPGMA method in MEGA5. The percentage values of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches. The tree is drawn to scale, with branch lengths (above the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Antimicrobial resistance profiles, Sequence Types, and MRP clusters of dairy and starlings *C.jejuni* isolates were combined to the tree. A-J= Visited farms, D= dairy, Br= birds.
Figure 4.3: Phylogenetic tree for the sequence types detected in dairy cattle and starlings associated C. jejuni isolates.
CHAPTER 5

Preliminary Studies on Ovine and Bovine Abortion Associated with

*Campylobacter jejuni*


Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, Ohio 44691.
5.1 Abstract

*Campylobacter jejuni* is commonly found as a commensal in the intestinal tract of many food producing animals such as poultry, cattle, sheep etc.; however, recently it has been shown to be increasingly associated with abortion in sheep and cattle in the USA. Pathogenesis studies of *C. jejuni* induced abortion in sheep and cattle are limited. Here, we determined the genotypic and phenotypic properties of ovine and bovine abortion associated with *Campylobacter* isolates. These isolates were acquired from the diagnostic laboratories of the Ohio Department of Agriculture (ODA) and were genotyped using Pulsed Field Gel Electrophoresis (PFGE) and multilocus sequence typing (MLST). Further analyses included antibiotic susceptibility, invasion and intracellular survival potential in human INT407 cells, as well as *in-vivo* experimental infection in pregnant ewes. PFGE analysis showed that the fingerprints of ovine and bovine abortion *C. jejuni* were identical and were classified as Sequence Type 8 (ST-8) by MLST. The ST-8 belongs to Clonal Complex (CC) ST-21 which is also commonly found in humans. Additionally, the ovine and bovine abortion associated *C. jejuni* strains had varying invasion and intracellular survival capacity in human intestinal INT407 cells; however, ovine abortion isolate (Ovine abortion-II) showed significantly higher invasion potential compared to other strains (*P*<0.01) which matched that of *C. jejuni* 81-1176, a hyper-invasive strain. Furthermore, the ovine and bovine abortion associated *Campylobacter* isolates, though resistant to tetracycline, showed different susceptibility patterns compared to previously described sheep abortion associated isolates in the US. Experimental infection of pregnant ewes resulted in abortion like symptoms, such as
uterine prolapse, and partial protroton of placenta, and lesions such as suppurative necrotizing placentitis, suppurative and necrotic endometritis, along with occasional lymphadenitis and hepatitis. Taken together, our results strongly suggest the possible public health importance and provide model to study the pathogenesis of sheep abortion.

5.2 Introduction

Campylobacter jejuni is a leading cause of bacterial gastroenteritis in humans resulting in serious economic losses. C. jejuni is commonly found in the intestinal tract of many food producing animals such as poultry, cattle, sheep etc. In these species, the bacteria are present without causing any symptoms of disease and however serve as a major source for human infections leading to gastroenteritis. Although it has been known historically that C.fetus subsp fetus (vibriois) is the main cause of ovine Campylobacter abortion, it appears recently that C.jejuni is increasingly implicated in ovine abortion cases all over the world including the US (Delong et al., 1996; Kirkbride, 1993; Skirrow 1994, Sahin et al., 2008), Campylobacter induced abortion rates usually ranged between 20% to 80% and may reach 90% in some outbreaks (Spronk, 2000). C. jejuni was isolated from aborted sheep in multiple lambing seasons on different farms in Iowa, Idaho, South Dakota, and California (Sahin et al., 2008). Bacteremia, placentitis, uterine and fetal infection, and consequently abortion, which usually occurs in the third trimester of pregnancy in ewes, are common symptoms associated with ovine campylobacteriosis (Skirrow, 1994; Hedstrom et al., 1987). A single tetracycline-resistant C. jejuni clone has emerged as the major cause of Campylobacter-associated sheep abortion in the US (Sahin et al., 2008). Further, occasionally infection can result in retention of dead fetus in uterus.
which may cause death of pregnant ewes due to septicemia and uterine sepsis; however, no clinical signs can be seen at the beginning of infection (Skirrow, 1994). It is still unknown what conditions are required for a harmless infection to turn into an acute abortion. Further, it is currently unknown whether these abortion inducing *C. jejuni* strains have any zoonotic potential. However, PFGE analysis of ovine abortion isolates has revealed a striking similarity with human gastroenteritis associated *C. jejuni* isolates (Zhang, 2011). This strongly suggests significance of these abortion isolates to human health. Therefore there is a critical need for understanding the pathogenesis of *C. jejuni* induced abortion. In a recent study on the pathogenesis of *C. jejuni* induced abortion it was shown that the abortion can be effectively induced in experimentally infected pregnant guinea pigs following intraperitoneal or oral inoculation and the bacteria can be recovered from fetoplacental tissues (Burrough et al., 2009), These findings have indicated that ovine abortion-associated *C. jejuni* is highly abortifacient and could colonize the intestines, cause systemic infection, and induce abortion. Though this study provided a valuable animal model to understand the pathogenesis of abortion, further *in vivo* studies in a natural host (sheep) is needed to better characterize their virulence.

In order to gain more insights into the pathogenesis of ovine *C. jejuni* induced abortion, we conducted; 1) Genotypic and phenotypic characterization of ovine and bovine *C. jejuni* abortion isolates, 2) *In vitro* invasion and intracellular survival studies on human intestinal epithelial cells INT407, and 3) *In vivo* pathogenesis of ovine abortion in pregnant ewes by challenging them with *C. jejuni* ovine and bovine abortion isolates. Our results suggested that abortions inducing *C. jejuni* isolates are very clonal similar to
previously described tetracyclin resistant abortion associated *C. jejuni* and have a potential to cause abortion in sheep. Presence of similar clones in human population warrants further investigation into their implications on public health.

### 5.3 Materials and Methods

#### History of abortion associated *C. jejuni* isolates

Four *Campylobacter* isolates were obtained from the Ohio Department of Agriculture (ODA). Specifically, two *C. jejuni* isolates (Ovine abortion-I and Ovine abortion-II) were retrieved from aborted Suffolk sheep fetuses from ewes in their third trimester of pregnancy in 2008, and one *C. jejuni* isolate (Bovine abortion) was isolated from aborted cow as well as one *C. coli* isolate (Ovine feces) was isolated from sheep feces in 2009.

#### Identification of *Campylobacter* isolates

The DNA was extracted from each isolate using the Genomic DNA Purification Kit (Epicenter, Madison, WI) as described by the manufacturer. The DNA was then quantified using the NanoDrop 1000 Spectrophotometer V3.7.1 (Fisher Scientific, Pittsburgh, PA) and subjected to species-specific PCR analysis to confirm the identity of the isolates as described elsewhere (Denis *et al.*, 2008; Sanad *et al.* 2011). PCR analysis targeted a 16S rRNA genus specific fragment, and *mapA*, and *ceuE*, the species specific for *C. jejuni* and *C. coli*, respectively. All PCR products were resolved on a 1.5% agarose gel containing 0.5 µg/ml of ethidium bromide. The size of the PCR products was
determined using a 1Kb DNA ladder and detection was confirmed by comparison to PCR products generated from *C. jejuni* 81-176 (wild-type strain) and *C. coli* (ATCC 33559), which were used as positive controls in all PCR analysis. Negative controls (reactions with no DNA templates) were included in all PCR analyses to ensure specific product amplification.

**Pulsed Field Gel Electrophoresis (PFGE)**

To determine their genotypic relatedness, abortion-associated *Campylobacter* isolates were analyzed using PFGE as described previously (Ribot *et al.*, 2001). Briefly, *C. jejuni* and *C. coli* isolates were harvested from MH agar plates and suspended to an OD\textsubscript{610} of 1.4 in 1X PBS. To prepare agarose plugs, OD adjusted suspensions were gently mixed with 1% SeaKem Gold agarose (SKG, Fisher scientific, Pittsburgh, PA) that was pre-melted in TE (10 mM Tris, 1 mM EDTA, pH 8.0). The plugs were then incubated with shaking (200 rpm) in lysis buffer [50 mM Tris, 50 mM EDTA (pH 8.0), 1% sarcosine, 0.1 mg ml\textsuperscript{-1} of proteinase K] for 1 h at 55° C. After lysis the plugs were washed four times and suspended in 5 ml of fresh TE. The plugs were then sliced and digested overnight with SmaI at room temperature. The digested slices were loaded onto a 1% SKG agarose gel and DNA fragments were separated by electrophoresis for 20 h using the CHEF Mapper system (Bio-Rad, Hercules, CA) followed by post staining with ethidium bromide. The resulting PFGE patterns were documented and analyzed using the BioNumerics 5.1 software (Applied Maths Inc, Austin, TX). Similarity and clustering analysis of the PFGE patterns were performed using the Dice Coefficient and the
unweighted pair-group method with arithmetic averages (UPGMA) with optimization of 1% and position tolerance of 1.5% (Ragimbeau et al., 2008), respectively. The PFGE analysis was also performed on *C. jejuni* 81-176 and *C. coli* (ATCC 33559), which were used as controls for facilitating gel to gel comparison. Furthermore, Lambda Ladder PFG Marker (50-1,000 kb, New England BioLabs, Ipswich, MA) was used as a molecular marker.

**Antimicrobial-resistance properties of the Campylobacter isolates**

Minimal inhibitory concentrations (MIC) for all isolates were determined using commercially available 96-well plates containing various antimicrobials (Sensititre Campy plates, TREK Diagnostic Systems Inc., Cleveland, OH, USA). These Sensititre Campy plates were used as described by the manufacturer and included; azithromycin (AZI) (MIC for a resistant *Campylobacter* isolate: ≥8 µg ml-1); ciprofloxacin (CIP) (MIC: ≥4 µg ml-1); clindamycin (CLI) (MIC: ≥8 µg ml-1); erythromycin (ERY) (MIC: ≥32 µg ml-1); gentamicin (GEN) (MIC: ≥8 µg ml-1); nalidixic acid (NAL) (MIC: ≥64 µg ml-1); telithromycin (TEL) (MIC: ≥8 µg ml-1); florfenicol (FEN) (MIC: ≥8 µg ml-1); and tetracycline (TET) (MIC: ≥16 µg ml-1). *Campylobacter* isolates were grown to mid-log phase and then suspended in Mueller-Hinton broth to achieve an OD600 of 0.05. For each isolate, one hundred microliter of the suspension were transferred to each well in the Sensititre Campy plates, including a control well that did not contain any antibiotics, while *C. jejuni* 81-176 and *C. coli* (ATCC 33559) were used for quality control. The plates were then incubated under microaerobic conditions at 42ºC for 24 h after which the
minimum inhibitory concentration (MIC) was measured. The MIC for each antimicrobial was defined as the absence of bacterial growth in the well with the lowest concentration of the antimicrobial and susceptibility was interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2006).

**Multilocus sequence typing (MLST) of Campylobacter isolates**

To investigate the clonality of the *Campylobacter* isolates and to assess similarity to strains associated with human infections, MLST analysis was performed on all isolates. MLST analysis was conducted as described by Dingle *et al.* (Dingle *et al.*, 2001). Briefly, loci from seven housekeeping genes (aspA, glnA, gltA, glyA, pgm, tkt, and uncA) were amplified using gene specific primers by PCR and anticipated sizes of the amplicons were confirmed by agarose gel electrophoresis. The *Campylobacter* MLST oligonucleotides (Dingle *et al.*, 2001) were obtained from Integrated DNA Technologies (Coralville, IA). PCR products were purified using QIA quick 96 PCR purification kit (QIAGEN, Valencia, CA), sequenced in both directions as described previously (Thakur and Gebreyes, 2005). The forward and reverse sequences were aligned using ClustalW (www.ebi.ac.uk/clustalw). Allelic profiles were determined by performing BLAST analysis using the single-locus query function, while sequence types (STs) were assigned using the allelic profile query function available in the MLST *Campylobacter* database (http://pubmlst.org/campylobacter/). STs were then traced to their respective clonal complexes using BURST at http://pubmlst.org/.
Invasion and Intracellular survival potential of *Campylobacter* isolates in INT407 cells

Ovine and bovine-associated *Campylobacter* isolates were further tested for their virulence associated phenotypes by assessing their ability to invade and survive within human intestinal epithelial cells. The invasion studies were conducted as described earlier (Sanad et al., 2011, Konkel and Cieplak, 1992, Prasad et al., 1996). Briefly, 10^5 cells ml\(^{-1}\) of INT407 (human embryonic intestine, ATCC CCL 6) were seeded into each well of a 24-well tissue culture plates after suspension in Eagle’s Minimum Essential Medium (MEM, Fisher scientific, Pittsburgh, PA) supplemented with 10% fetal bovine serum (FBS, Fisher scientific, Pittsburgh, PA). The plates were then incubated at 37 °C in a humidified incubator with 5% CO\(_2\) until semi-confluent mono-layers were obtained. In preparation for infection with *Campylobacter*, the INT407 mono-layers were washed three times and covered with MEM supplemented with 1% FBS. Similarly, *Campylobacter* cultures were washed three times and suspended in MEM supplemented with 1% FBS to obtain 10^7 bacteria ml\(^{-1}\). One ml of bacterial suspension was added to each well containing the INT407 cell monolayer, achieving a 1:100 multiplicity of infection (MOI) then incubated for 3 h. After 3 h of incubation with bacteria, cells were treated with gentamicin (150 µg/ml) and incubated for an additional 2 h. The infected mono-layers were then washed three times with MEM, lysed using 0.1% Triton X-100 (Fisher scientific, Pittsburgh, PA), serially diluted (10-fold) in MEM and 100 µl of each dilution were spread on MH agar plates. The agar plates were then incubated for 48 h at 42° C under microaerobic conditions, after which colony forming units (CFU) were
counted to determine the number of Campylobacter that invaded the monolayers. Each isolate was tested in duplicate per assay, and the experiment was repeated three times on separate occasions. C. jejuni 81-176 (highly invasive) (Bacon et al., 2000), C. jejuni NCTC11168 (poorly invasive) (Bacon et al., 2000) and C. coli (ATCC 33559) were used as controls in all invasion assays.

To assess intracellular survival (Konkel and Cieplak, 1992), Campylobacter cultures and the INT407 cells were processed as described above. However, after treatment with gentamicin, MEM containing 3% FBS and bacteriostatic concentration of gentamicin (10 µg ml⁻¹) were added to each well to allow strict quantitation of intracellular bacteria. After further incubation at 37 ºC for 24 h, the monolayers were washed three times, lysed and serially diluted in MEM as described earlier and plated on MH agar to determine CFU of Campylobacter surviving within the intestinal cells. In addition, we also cultured the supernatant of gentamicin treated monolayers to ensure the quality of the gentamicin protection assay. The experiment was repeated three times on separate occasions. C. jejuni 81-176, C. jejuni NCTC11168, and C. coli (ATCC 33559) were used as controls in all intracellular survival assays.

**Challenge of pregnant ewes with the ovine and bovine abortion C. jejuni isolates.**

For this experiment three groups of ewes in the third trimester of pregnancy were used. These ewes were selected from a commercial herd which had no history of abortions and was vaccinated only against Chlamydia and Toxoplasma. Fecal samples were collected from all ewes used in this experiment a week before inoculation and
examined for the presence of *Campylobacter* infection. Each group consisted of four ewes. Two of which were challenged by the abortion *C. jejuni* isolate via oral route and the other two ewes were challenged intravenously. The first and second groups were challenged by (Bovine abortion) and (Ovine abortion-II) isolates, respectively, while the 3rd group, which consisted of two ewes, was used as a control group. Ewes were infected with $10^9$ CFU/ml in 50 ml sterile saline solution (SSS) for oral inoculation and $10^6$ CFU/ml in 1.5 ml SSS for the I/V route. These doses were selected based on previous studies (Miller *et al.*, 1959; Hedstrom *et al.*, 1987). The ewes were monitored after infection until abortion or the end of pregnancy. Fecal and blood samples were collected frequently following inoculation to check for infection. The ewes were euthanized; samples were collected from the intestine, mesenteric lymph nodes, liver, spleen, blood, placenta, and uterus for microbial culture and histological examination. Also, samples were collected from lambs after delivery or from aborted fetuses.

**Necropsy of ewes and bacteriological examination**

Ewes and fetuses were necropsied immediately following abortion or normal delivery, and control ewes were necropsied concurrently with normal delivery. The maternal and fetal tissues were aseptically collected using separate sterilized sets of necropsy tools for each organ. One g of each tissue was enriched in Preston broth for 48 h at 42 °C under microaerobic conditions (5% O₂, 10% CO₂, and 85% N₂) (13). A (100 µl) inoculum from the enrichments was spread onto modified Cefoperazone Charcoal Deoxycholate Agar (mCCDA) plates, which were then incubated for an additional 48 h at
42°C under microaerobic conditions (Engberg et al., 2000). Maternal tissues cultured included: caruncles and placenta, liver, spleen, lungs, uterus, small intestine, blood, mesenteric lymph nodes, and cecal and rectal feces. Fetal tissues cultured included: small intestine, cecal contents, and blood.

**Histopathological studies**

A Histopathological examination was conducted to detect the pathological changes in infected ewes or fetal tissues. The Histopathological examination was done as previously described by Hald et al. (Hald et al., 2001). Briefly, the target tissues (small intestine, liver, lungs, spleen, mesenteric lymph nodes, placenta, and uterus) were aseptically collected from euthanized ewes and aborted fetuses. Tissues were placed in 10% phosphate buffered formaldehyde (pH 7.0). Tissues were dehydrated in graded alcohol then embedded in paraffin, and cut in 3-μm sections. The fixed tissues were placed on microscope slides and stained by hematoxylin and eosin (H&E) then analyzed for histopathological changes.

**Statistical analysis**

The data derived from cell culture assays were analyzed using one-way ANOVA followed by Tukey's Multiple Comparison Test. A P value of < 0.05 was considered statistically significant for all experiments. Measurements expressed as mean ± SE (standard error) were averages of three replicas.
5.4 Results

**Identification and confirmation of Campylobacter isolates**

Three isolates (Ovine abortion-I, Ovine abortion-II, and Bovine abortion) were identified as *C. jejuni* using the genus as well as species specific PCR. However, one isolate obtained from ovine feces (identified as *C. jejuni* by the ODA) was identified as *C. coli*. These results were further confirmed by MLST analysis (Fig 5.1).

**Genotyping using Pulsed Field Gel Electrophoresis (PFGE) and Multi Locus Typing (MLST) analysis**

In order to determine the genotypic properties of these isolates and the relationship between the isolates used in this study, PFGE analysis was performed on bovine and ovine abortion-associated *C. jejuni* isolates as well as ovine *C. coli* isolated from feces. Analysis of the PFGE profiles showed that both ovine and bovine abortion isolates possessed 100% similar genotypes (Fig 5.1).

MLST analysis was performed on all the aforementioned isolates to further determine the genotypic properties and their relationship to those of human origin. The ovine and bovine abortion *C. jejuni* isolates were identified as ST-8 which belonged to clonal complex (CC) ST-21.

**Antimicrobial susceptibility of Campylobacter isolates**

To better assess the phenotypic properties as well as to confirm whether these abortion-associated isolates were also tetracycline resistant similar to a single clone that
has been attributed to abortions in sheep in several states in the USA (Sahin et al., 2008), antimicrobial susceptibility was assessed on all isolates included in this study using commercially available Sensititre Campy plates. All isolates were resistant to tetracycline similar to previously identified sheep abortion isolates (Sahin et al., 2008); however, they exhibited different antimicrobial resistance patterns. Both ovine and bovine abortion isolates were resistant to multiple antimicrobials including, ciprofloxacin (MIC: 4-64 µg ml⁻¹), tetracycline (MIC: 16-64 µg ml⁻¹), nalidixic acid (MIC: ≥64 µg ml⁻¹), florfenicol (MIC: ≥8 µg ml⁻¹) and telithromycin (MIC: ≥8 µg ml⁻¹) (Fig 5.1). However, all isolates except for ovine abortion-II exhibited resistance to erythromycin, while resistance to gentamicin was observed only for the bovine abortion isolate. Furthermore, both ovine abortion isolates were susceptible to clindamycin (Fig 5.1).

**Invasion and intracellular survival potential of ovine and bovine abortion associated *Campylobacter***

To investigate the virulence-associated potential of the abortion associated sheep and cattle *C. jejuni*, we tested for their invasion and intracellular survival potential in human INT407 intestinal cells. All tested *Campylobacter* isolates invaded INT407 (Fig. 2A); however, the invasion potential varied between isolates, ranging from an average of $5.3 \times 10^2$ to $1.1 \times 10^5$ CFU ml⁻¹ (Fig 5.2A). Interestingly, the Ovine abortion-II isolate exhibited higher invasion potential that was similar to the invasion of *C. jejuni* 81-176, a highly invasive strain (see star in Figure 5.2A). However, the invasion capability of the other two isolates was significantly lower than that of the *C. jejuni* 81-176 ($P<0.01$).
There was no significant difference between the invasion capacities of these 3 isolates compared to that of *C. jejuni* NCTC11168. Although all tested *C. jejuni* isolates were variably capable of intracellular survival, their intracellular survival potential were significantly lower than that of *C. jejuni* 81-176 (Fig 5.2B).

**In-vivo experiment in pregnant ewes**

The primary signs of abortion, which included partial and complete uterine prolapse with retained placenta, occurred in some ewes 6 to 18 days post-inoculation of *C. jejuni* (Fig 5.3A). Interestingly, three ewes (one from bovine abortion-Gr-A and two from ovine abortion-Gr-B) died 17-18 days after oral inoculation (see highlighted ewes in Table 5.1). Rectal temperatures of inoculated ewes were elevated (105-105.5 F) 3 to 4 days post intravenous inoculation and 10-12 days in orally inoculated ewes. Weak lambs were observed after normal delivery in 3 cases from both groups (Fig 5.3B1). Feces changed from pasty to severe diarrheic in most of the infected ewes from both groups 4-12 days after inoculation (Fig. 5.3A.4). *C.jejuni* was intermittently retrieved from all of the inoculated ewes throughout the experiment. No abortions or clinical signs were observed in control ewes (Table 5.1).

**Post mortem, bacteriological, and histopathological examination**

Gross lesion of placentitis occurred in the majority of the *C.jejuni* inoculated ewes. Pin head superficial spots and petechial hemorrhages were occasionally noticed on liver and spleen of intravenously inoculated ewes in Gr-A (bovine abortion) and the
orally inoculated ewes in Gr-B (ovine abortion II) (Fig. 53B.3&4). Gross lesions with enlarged caruncles were present in most of the \textit{C. jejuni} inoculated ewes which were more prominent among ewes in Gr-A (Ovine abortion-II isolate). Interestingly, in addition to the aforementioned observations, an excessive accumulation of thoracic fluids around lungs was observed in three ewes that died (Fig. 5.3B.2). However, no clear gross lesions were observed on lungs of the inoculated or control ewes. No gross lesions were seen in tissues from control ewes.

Bacteriologically, \textit{C. jejuni} was retrieved from feces of all the inoculated ewes at the time of necropsy except from one ewe (Table 5.1). \textit{C. jejuni} was also occasionally retrieved from several organs of euthanized ewes including liver, spleen, uterus, placenta, blood, small intestine. No \textit{C.je}juni was retrieved from lungs of any ewes. Further, \textit{C.je}juni was also occasionally retrieved from lambs’ feces and fetuses’ meconium as well as small intestine and blood (Table 5.1).

Histologically, placentitis was observed in the majority of inoculated ewes, with severe suppurative, necrotizing placentitis along with accumulation of a large number of neutrophils and necrotic cells, including neutrophils, trophoblasts, and cryptal epithelial cells, within the chorionic villi (Fig 5.4A and 5.4B). In addition, severe necrotizing placentitis was observed in some cases with bacterial colonies around the necrotic lesions (Fig 5.4C). Moreover, uterine lesions were also seen ranging from moderate endometritis (Fig 5.5A) to severe necrotizing suppurative endometritis with heavy infiltration of neutrophils and necrotic cells which indicated pyometra (Fig 5.5B). The mesenteric lymph nodes in the orally inoculated ewes in both groups occasionally showed diffuse
suppurative lymphadenitis (Fig 5.7A). Further, variable hepatic lesions like hepatitis with interstitial infiltration of polymorpho nuclear neutrophils in Ewe 1-V-GrA (Table 5.1), and acute severe multifocal coalescing suppurative hepatitis in liver tissues collected from Ewe 1-V-GrB were observed (Fig 5.6A; Table 5.1). In control ewes no abnormal lesions were observed in cotyledons (Fig 5.4D), endometrial epithelium (Fig 5.5A), liver (Fig 5.6B) or spleen (Fig 5.7B). Histological examination of intestinal tissues was not performed as these tissues underwent severe autolysis.

5.5 Discussion

It has been historically known that Campylobacter fetus subsp. fetus is the major cause of most of the ovine abortions all over the world (Grogono-Thomas et al., 2003; Kirkbride, 1993, Mannering et al., 2003; Mannering et al., 2004; Skirrow, 1994). However, recently it was shown in several studies that Campylobacter jejuni was increasingly associated with sheep abortion (Delong et al., 1996; Kirkbride, 1993, Sahin et al., 2008). In addition to different farms in Iowa, Idaho, South Dakota, and California where C. jejuni was isolated from aborted sheep in multiple lambing seasons (Sahin et al., 2008), it was also isolated from Ohio. Recently, three C.jejuni isolates were isolated at the Animal Disease Diagnostic Lab, Ohio Department of Agriculture (ODA) two from aborted twin Suffolk sheep fetuses from ewes in their third trimester and one isolate from bovine abortion. To investigate the genotypic characteristics of these abortion-associated isolates, two different genotyping techniques, PFGE and MLST, were performed. PFGE
analysis showed that both the ovine and bovine abortion-associated C. jejuni isolates possessed identical fingerprints (Fig 5.1). Interestingly, these fingerprints indistinguishably matched the SmaI macro restriction profile that was possessed by the vast majority (66 of 71) of previously described abortion associated isolates (Sahin et al., 2008). The genotypic similarity between our isolates and those that were tested by Sahin et al., regardless of the differences in isolation times and locations, strongly indicates the widespread nature of this clone as a potential cause of abortion in sheep in the USA. Further, the identical genotype of bovine abortion isolates suggests that this C. jejuni can readily colonize different hosts and induce abortion. Furthermore, MLST analysis revealed that both ovine and bovine abortion isolates were identical and classified as ST-8 which belonged to Clonal Complex (CC) ST-21 (Fig 5.1). The ST-8 was also previously recorded in different hosts including humans (Sahin et al., 2008; Sanad et al., 2011, unpublished data). These findings strongly suggest that these abortion inducing C. jejuni strains may have zoonotic potential.

Similar to previously described sheep abortion C. jejuni strains, both ovine and bovine isolates studied here were resistant to tetracycline. However, these isolates exhibited different antimicrobial resistance patterns and were resistant to multiple antimicrobials including, ciprofloxacin, tetracycline, nalidixic acid, florfenicol and telithromycin (Fig 5.1). In contrast, Sahin et al., in their study, which was conducted on 71 sheep abortion-associated isolates obtained from different locations across the USA from 2003-2007, showed that abortion associated C. jejuni were susceptible to most of the antimicrobials tested (Sahin et al., 2008); however, most of the antimicrobial tested
were different from these used in this study and thus no conclusion can be drawn based on the antimicrobial profiles on the relatedness of these isolates. Based on shared fingerprints, sequence types, as well as resistance to tetracyclines it is likely that abortion associated \textit{C. jejuni} isolates studied here belong to the same clonal lineage as described previously (Sahin et al., 2008).

\textit{Campylobacter} pathogenesis studies are hindered by the lack of appropriate animal models. \textit{In vitro} cell culture methods may provide a useful alternative to investigate the interactions between \textit{Campylobacter} and the host epithelium that occur during human infection. Additionally, the existing evidence suggest that the sheep abortion clone may be pathogenic to the human host, but this possibility remains to be examined in future studies (Sahin et al., 2008). Therefore, to better understand the contributions of ovine and bovine abortion-associated isolates to human infections, we examined their invasion and intracellular survival potential in human intestinal cells (INT407). Interestingly, although all tested isolates were capable of invading the human INT407 cells with variable efficiency, one isolate (ovine abortion-II) exhibited an invasive potential that was comparable to a hyper-invasive strain \textit{C. jejuni} 81-176 (Bacon et al., 2000) (Fig 5.2). Though, the intracellular survival potential of all tested isolates was significantly lower than that of \textit{C. jejuni} 81-176 ($P<0.01$), the ovine abortion-II isolate showed a significantly higher survival potential than that of the NCTC11168 ($P<0.05$), the poorly invasive strain (Bacon et al., 2000). These findings clearly highlight the potential public health impact of the abortion associated \textit{C. jejuni} strains.
Pathogenesis of *C. jejuni* induced abortion is not well understood. Recently, it was shown that the abortion associated *C. jejuni* can effectively induce abortion in pregnant guinea pigs. However, no studies have characterized the virulence of these strains in a natural host, sheep. These studies are necessary to develop effective control measures. Therefore, an *in-vivo* study was conducted on pregnant ewes using the ovine and bovine abortion *C. jejuni* isolates. Though these strains were not able to induce clear abortion in pregnant ewes, clinical signs such as uterine prolapse and retained placenta were observed (Fig 5.3A). Interestingly, three ewes, which were inoculated orally, died 17 to 18 days post inoculation from both groups (see highlighted ewes in Table 1). Similar findings were suggested previously by Skirrow (Skirrow, 1994) indicating that the death of pregnant ewes sometimes may occur due to septicemia and uterine sepsis with no clear clinical signs. In a previous study, inoculation of pregnant ewes during the third trimester using abortion-associated *C. jejuni* strain resulted in abortion in 100% of ewes 7 to 12 days post I/V inoculation (Hedstrom *et al.*, 1987). Similarly, in a recent study inoculation of pregnant guinea pigs using abortion-associated *C. jejuni* isolate by oral or intraperitoneal (IP) routes resulted in 100% and 60% abortion, respectively (Burrough *et al.*, 2009). In the current study we used $10^6$ CFU/ml in 1.5 ml of SSS for I/V inoculation, which was less than the dose used in a previous study (Hedstrom *et al.*, 1987) that may explain the lack of complete abortion in the inoculated ewes though they exhibited clear signs of abortion. Further, differences in the stages of pregnancy of different ewes used in this study may have resulted in the variable results. Future studies using different doses
of bacterial cultures and pregnancy synchronized ewes are needed to clearly establish the link to abortion.

The gross lesions and histopathological findings in our study were in agreement with the findings in previous studies (Hedstrom et al., 1987; Sahin et al., 2008). Specifically, gross lesion of placentitis occurred in the majority of the C. jejuni inoculated ewes along with uterine endometritis and enlarged caruncles. This was also observed by Hedstrom et al., on transverse section, swollen caruncles with hemorrhagic streaks covered fibrinous exudates were seen after necropsy of infected ewes (Hedstrom et al., 1987). Furthermore, multiple pin head and petechial hemorrhages were mostly seen on liver and spleen of intravenously inoculated ewes from both groups. In contrast, multiple round and white foci with variable size were identified in 90% of inoculated pregnant guinea pigs with ovine abortion inducing C. jejuni strain (Burrough et al., 2009).

Abortion associated C. jejuni resulted in clear pathological changes in the uterine tissues of inoculated ewes. Placentitis was observed in the majority of inoculated ewes, with severe suppurative, necrotizing placentitis (Fig 5.4A and 5.4B) with bacterial colonies around necrotic areas (Fig 5.4C). In agreement with our findings, necrotic lesions with numerous large bacterial colonies around the placental villi and adjacent stroma were detected from inoculated ewes (Hedstrom et al., 1987). The pathogenic effect of this ovine C. jejuni abortion-associated clone was also confirmed when the hemorrhagic suppurative inflammation and necrosis occurred in the majority of the inoculated pregnant guinea pigs with that clone in comparison to the occurrence of these
lesions in one animal which was inoculated with NCTC 11168 strain (Burrough et al., 2009). Moreover, uterine lesions were observed ranging from moderate endometritis (Fig 5.5A) to severe necrotizing suppurative endometritis with pyometra (Fig 5.5B). Additionally, occasional lymphadenitis (Fig 5.7A) and hepatitis with interstitial infiltration of polymorphonuclear neutrophils were also observed (Fig 5.6A). Furthermore, our bacteriological findings revealed that *C. jejuni* was occasionally retrieved, with variable numbers from liver, spleen, uterus, placenta, blood, small intestine of infected ewes, as well as from offspring’s feces, fetuses’ meconium, small intestine and blood as shown in Table 5.1. In a previous study using pregnant guinea pigs, *C. jejuni* was mainly retrieved from placental and uterine tissues in high numbers, while moderate to low numbers of bacteria were recovered from fecal swabs and heart blood as well as fetal liver and lung samples (Burrough et al., 2009). Similarly, high counts of *C. jejuni* were detected from uterine and placental tissues from stomach content, bile, and feces of inoculated ewes with ovine abortion *C. jejuni* as well as from their aborted fetuses (Hedstrom et al., 1987).

The association of ovine abortion *Campylobacter* isolates to human infection during pregnancy is not established. *Campylobacter* enteritis can occur from aborting ewes in animal caretakers (Duffell and Skirrow, 1987). The zoonotic potential of the *C. jejuni* clone from sheep abortions is still unknown. However, placental infection and preterm labor with delivery of a female infant, who died within an hour, has likely occurred in a 31-year-old farm working lady at 22 weeks of gestation who was admitted to a community hospital with a one week history of diarrhea, abdominal pain, fever, and
shivering. The bacteriological examination of the patient's stool revealed *C. jejuni* infection, which was treated with erythromycin. It was suggested that she had contact with infected animals or ingested contaminated food (Mcdonald and Gruslin, 2001).

Together, the potential of ovine and bovine abortion-associated *Campylobacter* to resist antibiotics including the ones which are of choice for human treatment, invade and persist in human cells, and their genetic classification into clinically important clonal complexes in addition to their ability to induce early abortion symptoms in pregnant ewes, strongly suggests the possible public health threat and contribution of this clone to human infection. Our future studies will include determining the host gene expression profiles using microarray to further understand the molecular basis of pathogenesis of ovine abortions.

5.6 References


**Table 5.1:** Isolation of *Campylobacter jejuni* retrieved from experimentally challenged pregnant ewes.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Animals*</th>
<th><strong>Ewe</strong></th>
<th><strong>Lamb</strong></th>
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<tr>
<td></td>
<td></td>
<td>Feces</td>
<td>Placenta</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Ewe 1</td>
<td></td>
<td>+ve</td>
<td>+ve(HG)</td>
</tr>
<tr>
<td>Ewe 2</td>
<td></td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td><strong>Group-A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Bovine)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ewe 1-I/IV</td>
<td></td>
<td>+ve</td>
<td>+ve(HG)</td>
</tr>
<tr>
<td>Ewe 2-I/IV</td>
<td></td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Ewe 3-I/IV</td>
<td></td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Ewe 4-I/IV</td>
<td></td>
<td>-ve</td>
<td>+ve</td>
</tr>
</tbody>
</table>

*Highlighted ewes indicating the ewes which died after inoculation.

*Heavy *Campylobacter* culture growth.

+ve and –ve indicate positive and negative *Campylobacter* isolation, respectively.
Figure 5.1: Macro Restriction Profiles, Sequence Types and antimicrobial resistance profiles of ovine and bovine *C. jejuni* abortion-associated isolates. Similarity analysis was performed using the Dice coefficient, and clustering was performed by the unweighted pair-group method with arithmetic averages UPGMA (optimization, 1% and position tolerance, 1.5%). Numbers on bootstraps represent Cophenetic correlations. Black boxes indicate resistance to different antimicrobials tested.
Fig 5.2: Invasion and intracellular survival of ovine and bovine C. jejuni isolates in INT407 cells. (A) CFU ml⁻¹ representing the number of the internalized bacteria which could be retrieved after treatment of cells with gentamicin. (B) Intracellular survival of Campylobacter isolates in INT407 cells. CFU ml⁻¹ representing the numbers of internalized bacteria retrieved after 24 h of incubation following the gentamicin treatment. The INT407 cells were infected with 1:100 MOI of Campylobacter strains. C. jejuni 81-176 and C. jejuni NCTC11168 were used as controls. The detection limit of the assay is represented by the dashed line. Each bar represents the mean ± SE of three independent experiments done in duplicates for each sample (P<0.01).
Figure 5.2: Invasion and intracellular survival of ovine and bovine *C. jejuni* isolates in INT407 cells.
Figure 5.3.A: Clinical symptoms which observed in some of the ewes inoculated with ovine and bovine abortion-associated *C. jejuni*.

(1) Uterine prolapse with retained placenta of a ewe from Gr-A, which was inoculated intravenously (I/V). (2) Magnification view of the same ewe showing uterine prolapse with severe inflammatory signs along with enlarged and inflamed cotyledons. (3) Early abortion sign in a ewe from Gr-A, which died 17 days post oral inoculation. (4) Signs of severe diarrhea on the udder of the same ewe.
Figure 5.3.B: Gross signs and lesions were seen on aborted fetuses, delivered lambs, and different harvested organs from inoculated ewes with ovine and bovine abortion-associated *C. jejuni*.

(1) A weak lamb was normally delivered after 3 weeks of I/V inoculation of a ewe from Gr-B. (2) Hydrothorax with excessive accumulation of fluids around lungs of a ewe from Gr-B, which died after 18 days post oral inoculation. (3) An example of petechial hemorrhages which were detected on the liver of some infected ewes. (4) An example of excessive pin head petechial hemorrhages which were observed on the spleens of some infected ewes. (5) & (6) Fetuses in different stages of growth which were obtained from ewes that died after oral inoculation.
Fig 5.4: Cotyledon and caruncles of ewes experimentally infected with *C. jejuni*.

(A) (Ewe 2-O-GrA), showing severe suppurative necrotizing placentitis. (B) A higher magnification of Panel A. Note accumulation of a large number of neutrophils (asterisks) and necrotic cells, including neutrophils, trophoblasts, and cryptal epithelial cells, within the chorionic villi (asterisks). (C) (Ewe 2-O-GrA) showing severe necrotizing placentitis. Note bacterial colonies (arrows) around a necrotic lesion (n). (D) Caruncle of a ewe (Ewe 2-Control) after normally delivering a lamb, showing no abnormality. Note normal appearance of maternal endometrial septa (m) and fetal villi (asterisk) mainly covered by trophoblasts and frequently, trophoblast giant cells (arrows), some of which undergo apoptosis (arrowhead). O=Oral, GrA= group A.
Figure 5.4: Cotyledon and caruncles of a ewe experimentally infected with *C. jejuni*.
Fig 5.5: Uterus of control ewes and an experimentally infected with *C. jejuni*.

(A) (Ewe 1-V-GrB) moderate suppurative endometritis. (B) (Ewe 2-V-GrB) severe necrotizing suppurative endometritis. Note severe necrosis of endometrium (asterisk) and a large accumulation of neutrophils and necrotic cells in the lumen of uterus (L), i.e. pyometra. (C) (Ewe 3-O-GrA) necrosis of maternal endometrial septa (asterisk), including vascular endothelial cells. (D) Uterus of a ewe (Ewe 2-Control) after normal delivery, showing no abnormality in endometrial epithelium. V= Intravenous, O= Oral, GrA= group A, GrB= group B.
Figure 5.5: Uterus of control ewes and an experimentally infected with *C. jejuni*.
Figure 5.6: Liver of control ewe and an experimentally infected with *C. jejuni*.

(A) Liver of an aborted ewe (Ewe 1-V-GrB) experimentally infected with *C. jejuni*, showing moderate focal suppurative cholangiohepatitis. Note interstitial infiltration of polymorphnuclear neutrophils (PMNs) (asterisk) around the bile duct (b). (B) Liver of a control non-infected ewe (Ewe 2-Control) after normal delivery of a lamb showing no inflammatory lesions but mild foamy vacuolation of hepatocytes. V= Intravenous, GrB= group B.
Figure 5.7: Lymph node and Spleen of a ewe experimentally infected with *C. jejuni*.

(A) Lymph node of a ewe (Ewe 2-O-GrA) experimentally infected with *C. jejuni*, showing mild suppurative lymphadenitis. (B) Spleen of a ewe (Ewe 3- O-GrB) experimentally infected with *C. jejuni*, showing mild diffuse suppurative splenitis. O= Oral, GrA= group A, GrB= group B.
CHAPTER 6

SUMMARY AND CONCLUSIONS
Cattle and their byproducts (beef and dairy) are major sources of human food. Consequently, *Campylobacter* contaminated beef or dairy products would pose a significant public health concern. *Campylobacter* is a leading cause of bacterial gastroenteritis in the US and worldwide. Moreover, *Campylobacter* has been increasingly causing economical losses to the sheep industry by inducing abortion in herds throughout the USA. Further, preliminary evidence suggests that abortion associated *C. jejuni* may have public health importance. Thus, this study was conducted; 1) to gain more insights on cattle as a potential source of *Campylobacter* infection in humans and to better understand the epidemiology of cattle-associated *Campylobacter*. 2) to understand the pathogenesis of *C. jejuni* induced abortion in sheep.

The characterization of the prevalence, genotypic and phenotypic properties of *Campylobacter* isolated from cattle fecal samples collected from four different locations across the USA; revealed that; 1) the prevalence of *Campylobacter* varied regionally. 2) *C. jejuni* and *C. coli* isolates were genotypically diverse and possessed some Sequence Types (STs) which belonged to clonal complexes (CC) that are also common in humans. 3) Many cattle associated *Campylobacter* isolates showed increased resistance to several antimicrobials including ciprofloxacin, erythromycin, and gentamicin. Altogether, these results suggested that cattle in the USA constitute a suitable reservoir for the persistence of *Campylobacter* and a potential source for human infections with these pathogens. The potential of cattle-associated *Campylobacter* to resist antibiotics, invade and persist in human cells, and their genetic classification into clinically important clonal complexes highlights the importance of cattle to the epidemiology of these pathogens in the USA.

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The invasion associated marker (*iam*) has been detected in the majority of human and poultry *C. jejuni* strains in previous studies suggesting that the presence of this locus may have a role in the *C. jejuni* colonization of multiple hosts. In this study, we determined the contribution of cattle reservoir to human infection with invasive *C. jejuni* by investigating the occurrence of the *iam* in *C. jejuni* isolated from cattle as well as their potential to colonize multiple hosts, chicken and human intestinal epithelial cells. Our data showed that; 1) the prevalence of *iam* in cattle *C. jejuni* is relatively lower compared to isolates occurring in humans and chickens. 2) *iam* was polymorphic and certain alleles occurred in cattle isolates that were capable of colonizing chickens and invading human intestinal cells. 3) *iam* did not appear to contribute to the invasion, intracellular survival, and antibiotic resistance potential of cattle-associated *C. jejuni*. We propose that the inclusion of the *iam* as a virulence determinant for epidemiological purposes should be carefully considered.

Further, based on our findings we showed that; 1) the prevalence of *C. jejuni* in birds was significantly ($P<0.01$) higher than that in dairy cattle in Northeastern Ohio, 2) several shared genotypes between dairy and starlings’ isolates were observed by PFGE analysis as well as shared clonal complexes (CC) like CC ST-45, and CC ST-21 by MLST analysis, and several of these clonal complexes are also previously recorded in human. 3) certain genotypes and clonal complexes were host restricted to each species. 4) cattle and starlings *Campylobacter* showed high resistance to multiple antimicrobials including ciprofloxacin, erythromycin, and gentamicin. These results highlight starlings
as potential reservoirs for *C. jejuni* and may play important role in the epidemiology of clinically important *C. jejuni* in dairy population.

Finally, it was revealed from our studies on abortion associated *C. jejuni* strains that these strains are 1) genotypically identical. 2) tetracycline resistant similar to previously described and widely distributed clone of *C. jejuni* involved in abortion. 3) exhibit invasion and intracellular survival potential similar to the highly invasive strain 81-176. 4) induce abortion like symptoms in pregnant ewes with histopathological changes in placenta, uterus, liver and lymph nodes suggesting that these strains are invasive and can cause systemic infection.

**Future directions**

Though several epidemiological studies were conducted outside the USA on *Campylobacter* in cattle, this information is still lacking in the US. The data on sources of transmission of infection within the cattle populations and to their byproducts are lacking. Further extensive prevalence studies on *Campylobacter* spp. involving diverse geographical locations within the US and studies incorporating seasonal variations and different management practices are required to better understand the epidemiology of *Campylobacter* spp. in beef and dairy cattle populations.

More studies are required on the abortion-associated *C. jejuni* to better understand the pathogenesis of abortion and the zoonotic implications that these strains may have. Though our preliminary studies using pregnant ewes have produced promising results, additional studies are required to clearly define the virulence characteristics of these
isolates in sheep. Further, determining changes in host gene expression in response to infection would provide better understanding of the host-pathogen interactions. This would aid in the development of effective control measures.
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Institute of Child Health, University of Cape Town, Cape Town, South Africa.


## Appendix A: *C. jejuni* and *C. coli* primers used to amplify and sequence housekeeping genes for MLST analysis.

<table>
<thead>
<tr>
<th><em>Campylobacter jejuni</em> primers</th>
<th><em>Campylobacter coli</em> primers</th>
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<tbody>
<tr>
<td><strong>Name</strong></td>
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<td>aspA F A9</td>
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</tr>
<tr>
<td>aspA R A10</td>
<td>5'-ATTCTACATTGCTTGGC-3'</td>
</tr>
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<td>glnA R A2</td>
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<td>glyA R S4</td>
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<tr>
<th><em>Campylobacter coli</em> primers</th>
<th><em>Campylobacter coli</em> primers</th>
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<tbody>
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<td>pgm R S2</td>
<td>5'-TTGTTATGATGCTATTG-3'</td>
</tr>
<tr>
<td>tkt F S3</td>
<td>5'-GCATGATTATGATACTTCAGG-3'</td>
</tr>
<tr>
<td>tkt R S6</td>
<td>5'-GCATGATTATGATACTTCAGG-3'</td>
</tr>
</tbody>
</table>

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Appendix A: *C. jejuni* and *C. coli* primers used to amplify and sequence housekeeping genes for MLST analysis.