Abstract

Gharst, G., D. J. Hanson and S. Kathariou. Prevalence and Antibiotic Resistance of C*ampylobacter* in Mature Cattle at Harvest

Campylobacter is considered to be a leading bacterial cause of acute enteritis in the United States. *Campylobacter* is found in the fecal material and the gastrointestinal tract of a broad range of animals. It has been suggested that the greatest cause of human infection is cross-contamination and/or the consumption of undercooked meat and poultry products. In the United States there are limited data on the presence of *Campylobacter* in cattle. This study investigated the prevalence of *Campylobacter (C. jejuni and C. coli)* as well as the presence of antibiotic resistant strains in mature cattle at slaughter. Representative fecal samples (n = 610) of the day's harvest were taken from the colon of mature cattle older than 30 months of age, over a period of 17 months. Species of *Campylobacter* isolates were determined by polymerase chain reaction. Pure cultures of *Campylobacter* were obtained from 143 of the 610 samples (23.4%, SE 1.7%). Of the confirmed *Campylobacter*-positive samples, the majority (93.0%) yielded *C. jejuni*, with *C. coli* recovered from the rest (7.0%). Seasonal data showed that *Campylobacter* prevalence is somewhat greater during the winter (29.4%) as compared to summer months (20.7%) ($P \leq 0.05$). Some of the isolates were resistant to selected antibiotics, with the greatest incidence being resistance to ampicillin (49.7%). The data imply that there may be a human health risk from the colonization of mature cattle at harvest by *Campylobacter*. Further research needs to be done to evaluate the food safety impact of *Campylobacte*r colonization of cattle.

PREVALENCE AND ANTIBIOTIC RESISTANCE OF *CAMPYLOBACTER* IN MATURE CATTLE AT HARVEST

by

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Biography

Gregory Allen Gharst was born in Rota, Spain on February 1, 1968. He was raised, for the most part of his young life in Fredericksburg, VA with the guidance of his parents, Doug and Jane, older brother Mike, and older sister Linda. He graduated from Stafford High School in 1986 where he earned a varsity letter in basketball and was the captain of the team. He then joined the United States Army and was an Airborne Medic while achieving the rank of E-4 before finishing his contractual obligation. At North Carolina State University, he studied Biological Life Sciences and received his B.S. in May, 1994. After graduating, Greg was hired as the microbiologist for McLane/MarketFare Foods in Fredericksburg, VA, then he was promoted to Quality Assurance Manager and finally to Technical Services Manager where he re-located to the corporate headquarters in Austin, TX. In August of 2002, Greg began his Master's work in the food pathogen laboratory under the direction of Dr. Sophia Kathariou.

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Chapter 1

Literature Review

Colonization of cattle with *Campylobacter* and food safety implications

INTRODUCTION

Food microbiologists have long been concerned with well-characterized foodborne pathogens, including *Staphylococcus aureus, Salmonella, and Clostridium botulinum*. In recent years, however, several emerging microorganisms, including *Listeria monocytogenes, Campylobacter,* and *Escherichia coli* O157:H7 have gained widespread recognition as agents of foodborne disease (Palumbo 1992). *Campylobacter jejuni* is a remarkable example of an emerging pathogen. It is not evident whether the growing prevalence of this pathogen in foodborne illness is caused by genetic changes within its populations, the food supply becoming more diverse and global, changes in animal management practices, consumer trends in eating habits and lifestyles, development of advanced and more sensitive recovery procedures, or a combination of some or all of the above (Solomon and Hoover 1999).

Campylobacter was first recognized as a cause of human foodborne disease in 1977, and has since become one of the leading sources of acute gastroenteritis in industrialized nations, including the United States (Tauxe 1992). *Campylobacter* accounts for 17.3% of all the food related bacterial infections that are reported (Mead et al. 1999). Most (85-95%) human campylobacteriosis cases are due to *C. jejuni*, with *C. coli* accounting for the majority of the remainder (5-15%) (Friedman et al. 2000).

Campylobacteriosis is an acute gastrointestinal infection with severe abdominal pain, fever, nausea, headache, muscle pain, and diarrhea. The length of the incubation period is 3–5 days with symptoms lasting 5–7 days. Infections are

typically self-limiting. When needed, antibiotics of choice are erythromycin and fluoroquinolones, although the effectiveness of the latter has been recently compromised by increasing resistance to these drugs among *Campylobacter* isolates from clinical cases (Altekruse et al. 1999). Treatment with antibiotics has been shown to reduce the length of time that the pathogen is shed in the feces of the infected individual.

A serious public health concern regarding campylobacteriosis is the occurrence of severe sequelae. One out of every one thousand cases of campylobacteriosis develops serious autoimmune sequelae 1–3 weeks after infection. These include Guillain-Barre Syndrome (GBS), associated with acute flaccid paralysis and weakness of limbs and respiratory muscles, and reactive arthritis. *Campylobacter* infection is the most common prelude to GBS. Furthermore, *Campylobacter* has a low infectious dose (ca. 500 bacteria) for humans (Robinson 1981), and this may contribute to its frequent involvement in human infection.

Campylobacter is a slender gram-negative spiral rod that is vigorously motile by way of polar flagella. This pronounced motility allows the microorganism to be motile in viscous environments (Alm et al 1993) and also colonize mucus within the intestinal and caecal crypts of the host (Lee et al. 1986). *C. jejuni* and *C. coli* are fastidious and require microaerobic conditions of 3-5% oxygen and 2–10% carbon dioxide, as well as elevated temperatures (optimally 37-42°C) for optimal growth. Growth of *C. jejuni* and *C. coli* abruptly stops below 30-31°C.

Campylobacter frequently colonizes avian hosts, including commercial poultry, but is also found in the gastrointestinal tract of other warm-blooded animals,

including commodity animals such as swine, sheep, and cattle. Human infections are primarily foodborne, or transmitted through raw milk and surface water. Handling and consumption of raw or undercooked poultry has been frequently implicated in human illness (Tauxe 1992). However, increasing evidence suggests that food sources other than poultry also contribute significantly to human infection (Altekruse et al. 1999). Most cases of human campylobacteriosis are sporadic, with outbreaks being rare, and often attributed to raw milk or water (Stanley and Jones 2003).

The purpose of this research was to determine the prevalence of *Campylobacter* in fecal samples of cattle at slaughter. Strain fingerprinting studies have revealed the presence of common strain types of *C. jejuni* in cattle and in human infections (Nielsen et al. 1997). In addition, the potential of cattle-derived *Campylobacter* to contribute to human illness was suggested by the Walkerton outbreak, where, in addition to the highly publicized *E. coli* O157:H7, *C. jejuni* was also implicated in several of the illness cases, likely through water contamination by cattle manure (Clark et al. 2003).

Currently there is limited information on cattle as a source of this pathogen in the United States. Thus the purpose of this study was to investigate the prevalence, antibiotic resistance, and strain types of the pathogen in mature cattle at the time of slaughter. It is hoped that the findings from this work will contribute to the establishment of a body of baseline data that may be used to better evaluate the food safety and public health risk posed by *Campylobacter* strains that colonize cattle at the time of harvest.

Food Safety and Public Health Concerns associated with *Campylobacter* in cattle

Epidemiological evidence for contribution of bovine *Campylobacter* to human illness.

Several data suggest that *Campylobacter* of bovine origin contributes significantly to human illness. In a Danish study (Nielsen et al. 1997), the serotype distribution of human isolates overlapped with that of isolates from cattle, and similar strain subtypes were identified among bovine isolates and strains isolated from human clinical samples, leading the authors to conclude that cattle may be an important source of *Campylobacter* for humans. In a subsequent study, application of six testing strain subtyping methods indicated close similarity between certain human clinical isolates and isolates from cattle (Nielsen et al. 2000).

Speciation of *Campylobacter* isolates from cattle feces revealed that the majority is *C. jejuni*, although *C. coli* and *C. hyointestinalis* have also been frequently isolated (Newell and Davison 2003). Recent studies, however, that have employed direct, DNA-based detection of bacteria in fecal samples, have provided evidence for frequent colonization of the animals by several additional *Campylobacter* species, especially *C. lanienae*, which were not readily recovered by culture. Campylobacters of uncertain taxonomic designation, potentially representing new species, were also identified in these studies (Colles et al. 2003; Inglis and Kalischuk 2003[A], 2003[B], and 2004). Isolation of these organisms with currently employed selective enrichment and culture protocols is difficult, and in some cases not feasible, thus preventing their adequate detection in clinical samples. Thus, species other than *C. jejuni*,

which is currently the leading cause of human campylobacteriosis, may contribute to human infections. *C. lanienae* and other, currently poorly characterized campylobacters which may commonly colonize bovines may be associated with human infections which currently would be considered of "unknown etiology."

Contamination of milk.

Epidemiological studies have identified raw milk consumption as a risk factor for endemic campylobacteriosis (Hopkins et al. 1984; Scmidt et al. 1987; Eberhart-Philips et al. 1997; Studahl and Anderson, 2000). In addition, consumption of raw milk is one of the most frequent causes of *C. jejuni* gastroenteritis outbreaks (Friedman et al. 2000). There have been several milk-associated campylobacteriosis outbreaks over the years in the United States. For example, in 1983 57 people became ill in Pennsylvania after consuming raw milk (CDC 1983). In Whittier, California, nine children and three adults became ill after consuming raw milk from a certified bottling plant (CDC 1984). Additionally, in the 1985 San Joaquin County dairy outbreak 23 (46%) of 50 attendees who drank raw milk became ill with *C. jejuni* infection (CDC 1985).

The raw milk implicated in such outbreaks may become contaminated in two ways. The first involves *C. jejuni* from the udder of cows with mastitis, a scenario that may be rare but has been documented (Hutchinson et al. 1985; Orr et al. 1995). The second, which seems to be more likely, involves contamination of milk through cattle feces or other environmental sources due to lack of adequate sanitation during milking (Jacobs-Reitsma 2000). It is relatively easy for the pathogen to enter the milk via inadequate hygiene in the milk production facility, for instance if the milking

equipment were to come in contact with the production facility floor and then used to continue pumping the milk from the cow. Even though *C. jejuni* has been shown to be unable to proliferate in milk, it can survive at refrigeration temperatures for as long as 21 days (Doyle and Roman 1982). Since the pathogen has such a low infectious dose for humans (only about 500 bacteria) (Robinson 1981), even low-level contamination can result in illness if the milk is not subsequently pasteurized.

Carcass and retail meat contamination.

Consumption of beef, pork or lamb has been identified as a risk factor for human campylobacteriosis in case control studies, although clearly of lesser magnitude than sources such as poultry, raw milk, and surface water (Rodrigues et al. 2001; Kapperud et al. 2003; Neimann et al. 2003; Newell and Davison 2003). Relatively few recent studies have addressed cattle carcass and meat contamination by *Campylobacter*, but the available data suggest that contamination of beef carcasses and beef at retail is rather limited. The Food Safety and Inspection Service (FSIS) reported that only 4% of 2,064 representative tissue samples from steer or heifer carcasses tested positive for *Campylobacter* during the period between October 1992 – September 1993 (FSIS 1994). Also, in a recent study carcass contamination of feedlot cattle with *Campylobacter* was only 2%, and was undetectable in pasture-fed cattle (Beach et al. 2002). In studies from other countries, *Campylobacter* was isolated from 0.9% of the carcasses before chilling in Poland (Kwiatek et al. 1990) whereas isolation after chilling was 10% in one study in Belgium (Korsak et al. 1998.) and 0.3% in an Australian survey (Vanderlinde et al. 1998).

In agreement with relatively low recovery of the organism from carcasses, most studies indicated limited recovery from raw meat at retail. *Campylobacter* was isolated from only 1 of the 182 tested raw beef samples at retail (0.5%) (Stern et al. 2001). Isolation from beef at retail was unsuccessful in several studies in Japan (Tokumaru et al. 1991) and N. Ireland (Madden et al. 1998). However, a study in the United Kingdom indicated that ca. 24% of beef meat samples at retail were positive for *Campylobacter* (Fricker and Park 1989), whereas in a Canadian survey 77% of the beef at retail was positive (Brooks et al. 1986). Intrinsic differences in colonization of cattle in different countries as well as variable efficiency of different isolation methods may account for the observed differences in isolation of the pathogen from beef at retail.

The relatively low incidence of *Campylobacter* in cattle carcasses and meat at retail is in strong contrast with data from poultry, which suggest frequent (70.7%) contamination of carcass at the slaughter plant (Zhao et al. 2001), and of raw chicken at retail which has been reported to be as high as 98% in one retail study (Altekruse et al. 1999). *Campylobacter* is unusually sensitive to dehydration (Doyle and Roman 1982), and it has been frequently postulated that overnight forced-air chilling, routinely employed in cattle and pig slaughterhouses, limits the number of organisms that can be recovered from the carcasses (Oosterom et al. 1985; Stern et al. 1988). A three-step wash process of the carcass, including a hot water wash, steam pasteurization, and a lactic acid wash are commonly used in cattle slaughter and processing plants, and this may also contribute to low prevalence of the pathogen on cattle carcasses.

Stress conditions such as dehydration, refrigeration, or washes may injure the organisms or induce them to enter a nonculturable state (Rollins and Colwell 1986; Saha et al. 1991; Hald et al. 2001), preventing their isolation on selective media. *Campylobacter* in the viable but nonculturable (VBNC) state survived for a significant length of time at 4°C far below the optimal (37-42°C) growth range for campylobacters (Rollins and Colwell 1986). The extent to which beef carcasses or meat at retail may be contaminated by *Campylobacter* that is in the VBNC state has not been investigated, and conflicting data exist in the *Campylobacter* literature concerning infectious potential of bacteria in such a state. *C. jejuni* that was injured and viable, but not culturable could be converted to a fully viable, culturable form when nonculturable stocks of freeze-thaw-injured campylobacters were used to infect the gut of rats (Saha et al. 1991). On the other hand, colonization of chicks by *C. jejuni* stopped even before the nonculturable state had been reached (Hald et al. 2001). Moreover, when *C. jejuni* in the VBNC state was given to day-old chicks via direct introduction into the stomach, culturable campylobacters could not recovered from the ceca of the birds after one or two weeks (Ziprin et al. 2003[B]). Thus the food safety threat posed by *Campylobacter* in the VBNC state remains difficult to estimate at the present time.

Contamination of surface water.

There are growing environmental concerns over the disposal of wastewater effluents from feedlots on land or in the water. The large amount of waste produced by mature cattle suggests that high numbers of *Campylobacter* can be released daily by a single colonized animal in the feedlot or at the farm, and result in

contamination of surface or well water. The Walkerton outbreak in May 2000 involved numerous cases of human campylobacteriosis, in addition to infections by *Escherichia coli* O157:H7, and was traced to contaminated well water from a nearby cattle farm. It was speculated that heavy rainfall and the runoff of cattle feces into the local drinking water contributed to the contamination (Clark et al. 2003). Water contamination with manure from cattle was also implicated in another *Campylobacter* outbreak, associated with a cultural fair in New York in 1999 (Bopp et al. 2003). Surface or ground drinking water contamination has been implicated in numerous outbreaks of campylobacteriosis (Friedman et al. 2000), although the source of the organisms has been determined in relatively few. In addition to cattle, water can be contaminated with feces from wild birds or other animals and by human sewage, and several such sources have been documented in waterborne outbreaks (Friedman et al. 2000).

Other food safety and public health implications of *Campylobacter* colonization of cattle.

The colonization of cattle with *Campylobacter* may be of epidemiological relevance for other commodity animals, such as poultry. The presence of other farm animals on broiler farms has been identified as a risk factor for colonization of the birds by *Campylobacter* (Kapperud et al. 2003), and bovine strains of the organism can readily colonize broiler chicks (Ziprin et al. 2003[A]). Such findings are significant, considering that poultry currently are considered a leading contributor to foodborne *Campylobacter* infections for humans. Current production systems may enhance the potential of transfer of *Campylobacter* between cattle and poultry. In our experience

growers of turkeys in western N. Carolina often grow cattle, and cattle are frequently seen in proximity to turkey houses. Although actual transfer of *C. jejuni* between bovines and poultry has not been rigorously investigated and documented, such scenarios suggest that transfer is likely, and of food safety and public health concern.

In summary, the available data suggest that the food safety and public health impact of *Campylobacter* from bovines is currently poorly characterized, but may be significant. Contamination of carcass or of meat at retail may be limited, but is clearly not negligible. In addition, cattle may contribute to human campylobacteriosis through contamination of milk and surface or well water, or through their potential role as reservoirs of the pathogen for other commodity animals, especially poultry.

The significance of cattle as a reservoir of *Campylobacter* is strengthened by the fact that the thermophilic *Campylobacter* species most commonly implicated in human illness, *C. jejuni* (Friedman et al. 2000), is also the species most frequently isolated from cattle. However, recent direct detection studies by DNA-based approaches have shown that cattle may be frequently colonized by additional *Campylobacter* species, several of which appear to be difficult or impossible to isolate with media and conditions currently employed (Inglis and Kalischuk 2003[A], 2003[B], and 2004). Such species may still contribute to human infections, which currently would be described as having unknown etiology. Overall, the available data suggest that the food safety and public health impact of *Campylobacter* colonization of cattle may be currently underestimated, and is clearly in need of further study.

Prevalence and Detection of *Campylobacter* in Cattle

The commercially available culture media tend to select for the species most frequently implicated in human illness, *C. jejuni* and *C. coli*, and may inhibit the growth of other *Campylobacter* species such as *C. lanienae*, which was frequently found in bovine fecal samples by PCR-based detection (Inglis and Kalischuk 2004). Such findings suggest that the true prevalence of *Campylobacter* in bovine feces may be underestimated, since species that cannot be readily cultured with traditional culture-based methodologies would not be identified (Inglis and Kalischuk 2004). With the use of real-time quantitative (RTQ) PCR as well as the use of nested primers, the direct detection of such species that has now become possible (Inglis and Kalischuk 2004). Furthermore, such new techniques even allow detection of species of *Campylobacter* that have not yet been discovered with traditional enrichment and/or culture based methodologies (Inglis and Kalischuk 2003[A] and 2003[B]).

A wide range (5-46.7%) in prevalence of *Campylobacter* in mature cattle has been reported in various studies done in Europe, Japan, and the U.S. (Cabrita et al. 1992; Giacoboni et al. 1993; Wesley et al. 2000; Hoar et al. 2001; Nielsen 2002; Al-Saigh et al. 2004). It is not clear whether the differences in prevalence values are due to regional differences in colonization, factors specific to the animal populations that were surveyed, or the methodology that was employed for isolation and identification of the organism.

Prevalence of *Campylobacter* in cattle is strongly influenced by the age of the animals, being greatest (42-97%) in calves (from birth to one year of age) and decreasing significantly as the animals age (Grau 1988; Giacoboni et al. 1993;

Nielsen 2002). These data suggest that young animals may be more susceptible to *Campylobacter* colonization than adults, possibly due to their special diet and their still immature immune and digestive systems. It has been suggested that the organism may be protected by the milk consumed by calves, thus having enhanced ability to pass through the rumen and colonize the small intestine (Grau 1988). In addition, an undeveloped rumen may make colonization with *Campylobacter* easier in calves (Stanley et al. 1998).

Prevalence in beef cattle vs. dairy cows, and the possible impact of season and diet.

A wide range in prevalence of *Campylobacter*-positive animals has been noted both for beef cattle (5-89.4%) (Garcia et al. 1985; Stanley et al. 1998; Hoar et al. 2001; Beach et al. 2002; Minihan et al. 2004) and for dairy cows (6-37.7%) (Meanger and Marshall 1988; Wesley et al. 2000; Beach et al. 2002; Nielsen 2002; Baily et al. 2003). Several studies have reported seasonal influence on *Campylobacter* prevalence in dairy cattle. Fecal shedding of dairy cows was reported to peak in spring, summer, or autumn, with prevalence in winter being distinctly reduced (Robinson 1982; Maenger and Marshall 1988; Stanley et al. 1998). The peak times coincide with parturition, and accompanying changes in housing and diet of the cows. In contrast, no consistent impact of season has been reported for beef cattle (Stanley et al. 1998).

Prevalence of *Campylobacter* has been reported to be higher in feedlot cattle than in pasture-fed cattle (58-68% - vs. 2–7%, respectively) (Grau 1988; Beach et al. 2002: Bailey et al. 2003). However, it is not clear whether the higher prevalence of

the organism in feedlot cattle is due to diet, crowding and its effects on the immune and gastrointestinal systems of the animal, number of animals (which may influence infection pressure), or a combination of these and other factors. Similarly limited information exists on the possible impact of diet on colonization of dairy cows with *Campylobacter*. Although some dairy cows graze in pastures, this is typically not the practice for large dairy producing facilities. The feed of non-grazing dairy cattle is based on roughage with other components added, such as vitamins, amino acids, corn, etc. The impact of different dairy cow feed formulations and components on *Campylobacter* colonization and/or shedding has not been reported yet. No significant differences in *Campylobacter* prevalence were found between dairy cows grazing in conventional vs. organic pastures (ca. 27 vs. ca. 29%, respectively) (Sato et al. 2004).

Horizontal transmission of *Campylobacter* in cattle.

Colonization of cattle with *Campylobacter* is mediated by horizontal transmission of the organism, and factors implicated in such transmission have been discussed in a recent report (Potter et al. 2003). Contaminated surface water has been implicated in transmission of the organism to cattle (Hanninen et al. 1998), and a bovine-water-bovine infection cycle has been postulated (Newell and Davison 2003). Birds may also contaminate feed used for cattle, and wild migratory birds that travel long distances may be able to introduce new strain types into a herd of cattle. The use of manure spreaders to spread slurry from tank onto land that is being used for the cattle has been found to be associated with *Campylobacter* positivity of the herds (Wesley et al. 2000).

Once introduced, the bacteria are likely to be transmitted from animal to animal in the pasture, feedlot, and other housing types through fecal contamination of the environment, water and feed. Relatively large herd size (more than 100 animals) has been found to be a risk factor for colonization of dairy cows (Wesley et al. 2000), suggesting that a minimum number of animals and sufficient proximity between animals are required to maintain the infection in the herd. Cattle shed the organism intermittently, although chronic shedders have been described (Newell and Davison 2003). Such animals may act as "hot spots" within a herd and can facilitate transmission via the hide, water troughs, pasture and other food sources (Stanley and Jones 2003).

Environmental contamination may also contribute to infection of animals during transport, or in holding pens. Vectors such as flies and other insects may harbor and transmit the pathogen, especially in feedlots where fly density is often very high (Zurek, personal communication). Currently information is lacking on this and other risk factors, such as human and vehicular traffic among different cattle operations, and accessibility to other animals (especially commodity animals such as poultry). The epidemiology of cattle infection with *Campylobacter* clearly needs to be further investigated.

Antibiotic resistance of *Campylobacter* from cattle.

Recently significant attention has been paid by the scientific community to the possible impact of antibiotics given to animals, either for growth promotion or therapeutically, on the emergence of resistance in zoonotic pathogens such as *Salmonella* and *Campylobacter*. Of special concern is the emergence of

fluoroquinolone resistance in *Campylobacter*, which has been reported to be correlated with the use of these antibiotics in poultry (Endtz et al. 1991). Currently erythromycin is the leading drug of choice for treatment of human campylobacteriosis, when treatment is indicated, followed by fluoroquinolones.

Incidence of fluoroquinolone-resistant *C. jejuni* from cattle ranges from 0.5% in a survey in Switzerland (Al-Saigh et al. 2004) to 14% in Denmark (Aarestrup et al. 1997) and 25% in Northeastern Italy (Pezzotti et al. 2002). It is not clear whether these differences reflect different antibiotic administration regimes for cattle in different nations. A common finding from these studies was that quinolone resistance was more frequent among *C. coli* than *C. jejuni* (Aarestrup et al. 1997; Pezzotti et al. 2002). Resistance to erythromycin has also been found to be significantly more frequent among *C. coli* than among *C. jejuni* isolates of bovine origin (Aarestrup et al. 1997). The reasons for the greater incidence of antibiotic (especially erythromycin) resistance in *C. coli* from cattle, in comparison to *C. jejuni*, are not clear but have also been observed with *Campylobacter* from other animals, such as poultry (Aarestrup et al. 1997; Kathariou and Carver, unpublished).

C. jejuni resistance to antibiotics was compared between isolates from cattle, broilers, and swine (Aarestrup et al. 1997). Cattle isolates had limited (≤ 10%) resistance to most of the antibiotics that were tested, except nalidixic acid where resistance was 14%. Swine isolates showed moderate (33%) resistance to erythromycin and streptomycin. Broilers had the lowest (≤ 6%) resistance to all of the antibiotics tested (Aarestrup et al. 1997). In another study, tetracycline resistance was detected in 45% of dairy cattle isolates of *Campylobacter* (Sato et al.

2004). Also, calves were more resistant to tetracycline than cows and there was no difference in the frequency of tetracycline resistant *Campylobacter* between conventional farms and organic farms (Sato et al. 2004). Additional studies need to be done to clarify the mechanisms underlying resistance to these and other antibiotics in *Campylobacter* of bovine origin, and to identify risk factors for the emergence of antibiotic resistant strains that colonize cattle.

CONCLUSIONS

- Consumption of raw milk is a leading cause of *C. jejuni* gastroenteritis outbreaks.
- Limited contamination has been documented on cattle carcass and raw meat at retail, likely due to intervention strategies at the slaughter plant.
- *Campylobacter* has been implicated in waterborne outbreaks, such as the Walkerton outbreak of 2000, and cattle may serve as a possible vector for infection via manure or runoff of manure into the local water supply after heavy rainfall.
- Younger cattle are colonized more frequently than older cattle, possibly due to their immature immune system, and prevalence drops significantly as the cow gets older.
- Dairy cows tend to be more frequently colonized with *Campylobacter* than beef cattle but many factors influence such colonization including season, geography, and diet.
- Feedlot-fed cattle tend to be more frequently colonized with *Campylobacter* than pasture-fed cattle. The use of high-energy feed additives such as amino acids and proteins may enhance the colonization of cattle with *Campylobacter.*

- Methods used to detect/culture *Campylobacter* have an impact on prevalence data. True prevalence of *Campylobacter* may be underestimated with culture-based methods. Direct detection of *Campylobacter* by non-culture based methods have been shown to detect other species than *C. jejuni* and *C. coli*. Stress conditions in the harvesting environment may cause injury to *Campylobacter* or induce them to enter a nonculturable state that will impact prevalence.
- Antibiotic resistant strains do occur in cattle, although less frequently than other animals such as poultry and swine, possibly reflecting antibiotic use in the feed causing *Campylobacter* to develop resistance.
- Horizontal transmission of *Campylobacter* is likely to be through exposure to contaminated water and pests such as insects and birds. Wild migratory birds that travel long distances may be able to introduce new genotypes into a given population of organisms. “Chronic shedders” may act as “hot spots” within a cattle herd and facilitate transmission via contamination of the hide, water troughs, and pasture.

Chapter 2

The impact of direct culture versus selective enrichment on the isolation of thermophilic *Campylobacter* from mature cattle at harvest

INTRODUCTION

Campylobacter is one of the leading causes of acute human gastroenteritis in the United States and other industrialized nations, and is found in the gastrointestinal tract and fecal material of a broad range of animals, including poultry, cattle, swine, and sheep (Tauxe 1992). The handling and consumption of raw or undercooked poultry has been identified as a leading risk factor for human infections (Tauxe 1992). *Campylobacter* infections have also been connected with severe autoimmune sequelae such as Guillain-Barre Syndrome (in ca. one in one thousand campylobacteriosis cases), characterized by temporary paralysis (Altekruse et al. 1999). Human illness is primarily due to the thermophilic species *C. jejuni* and, less frequently, *C. coli*. It is not clear whether the growing prevalence of this pathogen in foodborne illness is caused by genetic changes within its populations, the food supply becoming more diverse and global, changes in animal husbandry practices, consumer trends in eating habits and lifestyles, development of advanced and more sensitive recovery procedures, or a combination of some or all of the above (Solomon and Hoover 1999).

Growth of *C. jejuni* and *C. coli* in the environment is restricted by the need for a microaerobic environment and by the narrow temperature range for growth (32-44°C), with optimal growth between 37 and 42°C. Hence, the intestines of host animals are a critical site for amplification of these pathogens (Stanley et al. 1998).

Bovine colonization with *Campylobacter* may have significant food safety and public health impact through contamination of milk, meat, and surface or well water (Friedman 2000; Clark et al. 2003). In addition, dairy cattle may be asymptomatic

carriers for *Campylobacter* and can serve as potential sources of contamination for neighboring poultry farms by possible transmission through fecal shedding, water source contamination, and possibly insects as a vector for carrying this pathogen (Ziprin et al. 2003[A]).

Bacteriological and epidemiological studies of *Campylobacter* in cattle are relatively limited, and few reports describe comparisons of different isolation schemes. The available data, based on samples from animals in Europe and Canada, suggest that enrichment is superior to direct plating for recovery of *Campylobacter* from positive bovine samples, possibly due to relatively low numbers of bacteria in such samples (Garcia et al. 1985; Manser and Dalziel 1985; Stanley et al. 1998). However, exclusive reliance on enrichment for recovery of the microorganism may compromise the accuracy of prevalence and molecular epidemiological data. In particular, certain isolates may be unable to be recovered by enrichment, due to their poor competitiveness in the presence of other microflora, including other campylobacters, in the enrichment cultures. Although direct, DNA-based detection of *Campylobacter* in fecal samples of bovines has been successfully implemented (Inglis and Kalischuk 2003[A]), for many studies it is essential to isolate and characterize the bacteria from the samples. It is therefore important to accurately determine the impact of isolation procedures on recovery of *Campylobacter* from cattle, especially with culture media and conditions that are currently commonly employed.

In this study, we examined the relative efficiency of direct plating vs. selective enrichments for recovery of thermophilic campylobacters form fecal samples of

mature cattle (beef and dairy) immediately after slaughter. Our results indicate that, in agreement with the surveys mentioned above, the use of selective enrichments enhanced recovery of *Campylobacter* from bovine fecal samples, relative to direct platings. However, recovery of the organism from a number of samples (16% and 6% of samples from dairy and beef cattle, respectively) was only successful with direct platings. Almost all isolates that could be obtained only by direct plating were *C. jejuni*. The findings suggest that for optimized surveillance of *C. jejuni* in fecal material from cattle, especially from dairy cows, both direct plating and selective enrichment protocols may need to be employed.

MATERIALS AND METHODS

Animals and Sample collection.

The plant was visited over two years (2001-2003), with 300 samples obtained in the summer (June, July, August) with an average temperature of 29.8°C (range 25-34.4°C) and 180 samples obtained in winter (December, January, February) with an average temperature of 9.4°C (range 7-12°C). A total of 610 animals (358 dairy cows and 252 beef cattle) were sampled immediately after slaughter in a commercial beef packing plant in the southeastern United States. The plant harvested 250-300 animals per day, originating from different areas in the Eastern United States. Typically 30 samples representative of the day's harvest were obtained during each visit, with a total of ten visits made in the summer months, five visits in the winter months, two visits in the fall months, and two visits in the spring months. During each visit, animals were sampled in sequence as they were processed, typically within the time period between 8:00 and 10:00 am. One fecal sample per animal was

taken from the colon immediately after slaughter in the tripe room adjacent to the kill floor. The colon was cut approximately 12 – 18 inches from the distal end. A sterile cotton swab was then inserted about 3-4 inches into the colon and used to collect the fecal sample, or to swab the lining of the colon in the rare occasions where fecal material was scarce. The swabs were placed in 15 ml polypropylene tubes (Corning) containing 3 ml Cary-Blair Medium (Oxoid, Ogdensburg, NY), placed immediately on ice, and transported to the laboratory where they were held under refrigeration until they were processed, typically within 24 hours.

***Campylobacter* isolation.**

Isolations by direct plating.

To directly isolate *Campylobacter* from the fecal samples, approximately 0.05 g of the sample was streaked directly onto *Campylobacter* blood-free selective agar (CCDA; Oxoid, Ogdensburg, NY) with the corresponding supplement (SE 155, Oxoid, Ogdensburg, NY). Plates were incubated in anaerobic jars containing a CampyPak^{TM} Plus microaerobic system (Becton Dickinson, Sparks, MD) at 42° C for 48 hours. Areas of growth with the appearance typical of *Campylobacter* were examined by phase contrast microscopy for typical shape and motility of the microorganisms, and the bacteria were subcultured for purification as needed on sheep blood agar (SBA) (Remel, Lenexa, KS), under microaerobic conditions at 42° C for 48 hours. Typically 3-4 serial transfers were necessary for isolation of pure cultures. Bacteria were confirmed by phase contrast microscopy and absence of aerobic growth at 42°C in Mueller Hinton Agar (Difco, Sparks, MD). Typically one

pure culture, derived from a single colony, was obtained from each fecal sample by direct plating.

Isolations by selective enrichment.

A swab with approximately 0.1 g of fecal material was put into 2 ml of Bolton Broth (Oxoid, Ogdensburg, NY) containing the corresponding supplement (SR183E, Oxoid, Ogdensburg, NY) and Laked Horse Blood (SR048C, Oxoid, Ogdensburg, NY) in a 10 ml Falcon tube (Beckton Dickinson, Sparks, MD). The tubes were vortexed slightly to disperse the fecal material from the swab, covered loosely with caps, and incubated in anaerobic jars containing a CampyPak™ Plus microaerobic system at 37°C for 24 hours. A 20 µl sample of the selective enrichment was streaked for isolation onto CCDA plates, which were then incubated in anaerobic jars containing a CampyPak™ Plus microaerobic system at 42°C for 48 hours. Subsequent purifications and confirmation were done as described above. Pure cultures (obtained either by direct plating or by enrichment) were preserved at -80°C in Brain Heart Infusion (BHI) (Difco, Sparks, MD) broth with 15% sterile glycerol. Typically one pure culture was isolated from each enrichment.

DNA extraction.

DNA was extracted from SBA-grown cells (ca. ½ of a confluent 15 cm-diameter plate) using the DnEasy™ Tissue Kit (Qiagen, Valencia, CA) following the procedures suggested by the vendor. DNA was resuspended in 200 µl of the elution buffer provided with the kit.

Polymerase Chain Reaction (PCR).

PCR employed the *C. jejuni*-specific *hip* primers (5' ATG ATG GCT TCT TCG GAT AG 3' and 5' GCT CCT ATG CTT ACA ACT GC 3') (Marshall et al. 1999) and the *C. coli*-specific *ceu* primers CC1 (5' GAT TTT ATT ATT TGT AGC AGC G 3') and CC2 (5' TCC ATG CCC TAA GAC TTA ACG 3') (Houng et al. 2001). Reactions used Ex-Taq DNA polymerase (Fisher Scientific, Hampton, NH) and were carried out in 20 µl with 1.0 µl of genomic DNA as template. The PCR conditions used were 95°C for 5 min, followed by 30 cycles (each consisting of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min), with a final extension at 72°C for 5 min. PCR products (176 bp and 900 bp for *hip* and *ceu* primers, respectively) were detected following electrophoresis on a 1.5% tris borate-EDTA gel (60 min at 85 volts) with DNA molecular marker XIV (100 bp ladder, Roche). Genomic DNA from *C. jejuni* strain NCTC 11168 and *C. coli* strain D124 (strains provided by Dr. D. Threadgill, Univ. of N. Carolina at Chapel Hill) were included each time as positive controls for the *hip* and *ceu* PCR, respectively.

Statistical analysis.

Statistical analyses for *Campylobacter* prevalence and the impact of season (winter vs. summer) and animal type (dairy vs. beef) were done (with the variables alone and in combination) using Continuity Adjustment Chi-Square. Statistics for analyzing prevalence in summer beef cattle vs. winter beef cattle were analyzed by Chi-Square. The impact of direct plating vs. selective enrichment for recovery of thermophilic *Campylobacter* was analyzed using McNemar's Test. All statistical analyses were performed using SAS statistical software (version 8.0; SAS Institute, Cary, NC).

RESULTS

Prevalence of *Campylobacter* in the samples.

Of the 610 samples, 258 (42.3%) were considered *Campylobacter*–positive on the basis of the appearance of characteristic growth on the CCDA plates, and by phase contrast microscopy which revealed bacteria with characteristic morphology and motility. However, pure cultures could only be obtained from 143 (95 from dairy cattle and 48 from beef cattle, representing 23.4% of the total samples (Table 1). One isolate was preserved and speciated from each pure culture, with 53 samples that were positive by both direct plating and enrichment therefore yielding two isolates (one each through direct plating and selective enrichment), leading to a total of 196 isolates (182 *C. jejuni* and 14 *C. coli*). As indicated in Table 1, more samples were positive for *Campylobacter* from dairy cows (26.5%) than from beef cattle (19.0%), and all isolates from beef cattle samples were *C. jejuni*. Other *Campylobacter* species, which would be negative with the species-specific tests that we employed, were not identified. Furthermore, in 53 cases where two different isolates were characterized from the same sample (one isolate obtained by direct plating and one by selective enrichment) we found that each pair of isolates was of the same species (either *C. jejuni* or *C. coli*). In the case of five different cultures (3 from selective enrichment and 2 from direct plating), bacteria from multiple (2-5) single colonies were speciated by PCR. In each case, the bacteria from different colonies from the same direct plating vs. selective enrichment cultures were found to be of the same species, either *C. jejuni* (3 cultures from selective enrichment) or *C.*

coli (2 cultures from direct plating). Thus, the available data did not provide evidence for simultaneous presence of *C. jejuni* and *C. coli* in the same sample.

Seasonal prevalence of *Campylobacter* in the samples.

The data suggest that seasonality had an influence on *Campylobacter* as summarized in Table 2. Beef cattle sampled in the winter (28.5%) were more likely to harbor *Campylobacter* than beef cattle sampled during the summer (11.8%) ($P \leq 0.01$). There was no statistical significant seasonal impact seen with the dairy cattle samples. Overall, prevalence of culture-confirmed positive was greater during the winter (29.4%) than during the summer (20.7%) ($P \leq 0.05$).

Impact of isolation protocol (direct plating vs. selective enrichment) on recovery of *Campylobacter* from the fecal samples.

Of the 143 samples from which pure cultures of *Campylobacter* could be isolated, 72 (50.3%) were positive only with selective enrichment, while 18 (12.6%) were positive only with direct plating and 53 (37.1%) were positive with both methods (Table 3). Thus, enrichment led to recovery of the organism from 125 (87.4%) of the 143 confirmed *Campylobacter*-positive samples, and direct plating from 71 (49.7%). The difference in recovery of *Campylobacter* by selective enrichment vs. direct plating was statistically significant ($P \leq 0.001$). Although these results indicated that exclusive use of direct plating for isolations would significantly reduce the overall recovery of *Campylobacter* from these samples, they also revealed 18 samples (12.6%) from which the organism could be recovered by direct plating, but not by selective enrichment (Table 3). Such direct plating-positive, selective enrichment-negative samples were more frequent from dairy cows than from beef cattle (15.8%

and 6.3% of confirmed *Campylobacter*-positive samples from dairy cows and from beef cattle, respectively). The majority (17/18) of these isolates were *C. jejuni* (Table 3), a finding that may reflect the high overall recovery 133/143 (93.0%) of *C. jejuni* in the samples investigated in this study.

DISCUSSION

Prevalence of *Campylobacter* in mature cattle at harvest remains under-reported, especially in the United States, where only few descriptions of colonization of cattle by this pathogen are available (Wesley et al. 2000; Beach et al. 2002). The prevalence found among the 610 animals sampled in this study (presumptive positive 42.3%, with pure cultures isolated and species determined from 23.4%) is within the range (5 – 46.7%) described in other studies (Garcia et al. 1985; Giacoboni et al. 1993; Atabay and Corry 1998; Wesley et al. 2000; Beach et al. 2002; Stanley and Jones, 2003). The majority of the *Campylobacter*-positive samples (93.0%) harbored *C. jejuni*, with *C. coli* recovered from the remainder, and only from dairy cow samples. Other species, such as *C. lanienae*, *C. fetus* and *C. hyointestinalis*, recovered from cattle by others (Garcia et al. 1985; Atabay and Corry 1998; Wesley et al. 2000; Inglis and Kalischuk 2003[B]) may also have been present in the fecal samples that we analyzed but not recovered, possibly because they were in a numerical minority relative to *C. jejuni*, or because their isolation requires alternative media and conditions.

In this study, we found the prevalence of *Campylobacter* higher in winter samples (29.4%) vs. summer samples (20.7%) ($p \leq 0.05$). A study done in the U. K. reported that fecal shedding of *Campylobacter* by dairy cows had a seasonal

periodicity, with peaks in spring and autumn (Stanley et al. 1998). Higher summer prevalence of *Campylobacter* in dairy cattle has been reported by others as well (Meanger and Marshall 1988). The reasons for the higher prevalence in winter than summer in this study may be due to migratory birds, and other unidentifiable factors.

The literature reports a wide range in prevalence of *Campylobacter* from beef cattle (5-89.4%) (Garcia et al. 1985; Stanley et al. 1998; Hoar et al. 2001; Beach et al. 2002; Minihan et al. 2004), and from dairy cows (6-37.7%) (Meanger and Marshall 1988; Wesley et al. 2000; Nielsen 2002; Beach et al. 2002; Baily et al. 2003). Our prevalence data are within these reported ranges.

The media that were used in this study for the direct plating and selective enrichments favor the recovery of *C. jejuni* and *C. coli* (Atabay and Corry 1998). Surprisingly, direct platings have been found to be significantly more effective than selective enrichments for isolating *Campylobacter* from fecal material from broilers and turkeys (Musgrove et al 2000; Kathariou and Carver, unpublished). Direct plating has been found to be relatively inefficient for *Campylobacter* recovery from cattle, leading some investigators to discontinue its use in studies that compared the efficiency of different isolation protocols (Atabay and Corry 1998). Earlier studies, performed with samples from animals in Europe and Canada, have indicated that isolation of *Campylobacter* from cattle is greatly facilitated by the use of selective enrichment protocols (Manser and Dalziel, 1985; Garcia et al. 1985; Atabay and Corry 1998; Stanley et al. 1998). The superiority of enrichment protocols for isolation of *Campylobacter* from bovine fecal samples may reflect the relatively low abundance of the organism in cattle, which have been estimated to harbor on the

average ca. 2-3 $\log_{10}$ CFU/g of fresh fecal material (Stanley et al. 1998; Nielsen 2002). Conversely, *Campylobacter* found in fecal material from poultry is much greater (6-9 $\log_{10}$ CFU/g) than what is found in cattle (Berndtson et al. 1992).

The data suggest that recovery of *C. jejuni* and *C. coli* from bovine samples was overall more efficient with enrichment than with direct plating. Recovery with direct plating, however, was more efficient than previously reported (Garcia et al.1985; Atabay and Corry 1998; Stanley and Jones 2003), possibly due to the high effectiveness of the media employed for direct plating (mCCDA) (Atabay and Corry 1998). In addition, in this study the fecal material was directly taken from the colon, and therefore more likely to harbor viable organisms than samples from the ground (Hoar et al. 1999). This method of sampling was chosen because it was deemed to more accurately reflect colonization of the animals, and assured that each animal was surveyed at the time of slaughter.

In spite of the overall superiority of enrichment over direct plating, our findings also revealed a fraction of confirmed *Campylobacter*-positive samples (ca. 13%) from which the organism could be isolated only through direct plating. Such samples were more likely to be derived from dairy cows than from beef cattle. The underlying reasons do not seem to involve relative abundance of the organisms, since the number of colonies in direct platings from these samples was not noticeably different from that obtained with samples that were also positive with selective enrichments. It is possible that the organisms in these samples had unusually poor competitive ability against other microflora, and became outgrown in

selective enrichments, leading to failure of recovery of *Campylobacter*. Further studies are required to characterize these isolates, genetically and physiologically.

The existence of samples which can yield *Campylobacter* only through direct plating has been suggested in one earlier study (Manser and Dalziel 1985), which surveyed fecal samples of various mammals (primarily cattle, sheep and swine) from a veterinary diagnostic investigations unit. *Campylobacter* (primarily *C. coli*) was isolated only by direct platings from 16% of the *Campylobacter*-positive samples (Manser and Dalziel 1985). The predominance of *C. coli* observed in this earlier study was in contrast to our results, which indicated that the majority of the direct plating-positive (but enrichment-negative) samples harbored *C. jejuni*. The reasons likely reflect the population of animals being surveyed. In our study, the samples were exclusively from cattle, and most animals were colonized by *C. jejuni*, whereas in the study of Manser and Dalziel (1985) a significant number of samples were from swine, and therefore harbored *C. coli*. Thus the requirements of direct plating for isolation of *Campylobacter* from certain fecal samples, appears to be relevant to samples of either bovine or swine origin, and for *C. jejuni* as well as *C. coli*.

In this study one sample was obtained from the fecal material of each animal for analysis. *Campylobacter* may not be homogeneous throughout the fecal material. Therefore, multiple samples would have given a better representation of *Campylobacter* prevalence. Logistical reasons prohibited the use of multiple samples during this study.

In conclusion, our data suggest that selective enrichment is more sensitive than direct plating and, therefore, the protocol of choice for recovery of *C. jejuni* and

C. coli from fecal samples of cattle at slaughter, if resources only allow the use of one method. However, when possible, and especially in the case of dairy cow samples, enrichments should be complemented by direct plating, to allow isolation of the organism from samples harboring campylobacters that are resistant to recovery by enrichment. The combination of these methods would enhance the accuracy of prevalence studies and improve the potential to assess the population diversity of *C. jejuni* and *C. coli* that colonize cattle.

Table 1 Recovery of *C. jejuni* and *C. coli* from dairy and beef cattle fecal samples

Sample type	*C. jejuni*[1]	*C. coli*[1]	Total[1]
Beef cattle (n=252)	48 (19.0%)	0 (0%)	48/252 (19.0%)
Dairy cows (n=358)	85 (23.7%)	10 (2.8%)	95/358 (26.5%)
Total (n=610)	133 (21.8%)	10 (1.6%)	143/610 (23.4%)

[1] Percent positive indicated in parenthesis.

Table 2 Impact of season on recovery of thermophilic *Campylobacter* from beef and dairy cattle fecal samples

Animal Type	Winter[1]	Summer[1]
Dairy cows	37/124 (29.8%)	49/190 (25.8%)
Beef cattle	16/56 (28.6%)	13/110 (11.8%)
Total	53/180 (29.4%)	62/300 (20.7%)

[1] Percent positive indicated in parenthesis.

Table 3 Impact of direct plating vs. selective enrichment on recovery of *C. jejuni* and *C. coli* from dairy cow and beef cattle fecal samples

Isolation method[1]	**Fecal sample**				
	Dairy cows		**Beef cattle**		
	C. jejuni	***C. coli***	***C. jejuni***	***C. coli***	**Total**[2]
DP only	14	1	3	0	18 (18)
SE only	44	5	23	0	72 (72)
Both DP and SE	27	4	22	0	53 (106)
Total[2]	85 (112)	10 (14)	48 (70)	0	143 (196)

[1] DP, direct plating; SE, selective enrichment; DP only, positive by direct plating but negative by selective enrichment; SE only, positive by selective enrichment, but negative by direct plating.

[2] Total number of samples from which pure cultures of *Campylobacter* were recovered. Number of *Campylobacter* isolates indicated in parentheses.

Chapter 3

FlaA strain typing and antibiotic resistance of *Campylobacter* in mature cattle

INTRODUCTION

One of the most common foodborne human illnesses in the United States (U.S.) is campylobacteriosis, estimated at 2,400,000 cases per year (Mead et al. 1999). *Campylobacter* has a low infectious dose of about 500 cells (Robinson 1981). This, along with this organism's ubiquitous presence in foods that come from animals may be responsible for its prevalence in foodborne illness. *Campylobacter* frequently colonizes avian hosts, including commercial poultry, but is also found in the gastrointestinal tract of other warm-blooded animals, including swine, sheep, and cattle. Human infections are primarily foodborne, or transmitted through raw milk and surface water. The majority of campylobacteriosis cases in the U.S. are sporadic and are usually associated with improper handling and consumption of raw or undercooked poultry (Altekruse et al. 1999).

Cattle have been reported to be a reservoir for *Campylobacter* and a number of *Campylobacter* species has been recovered from the gastrointestinal tract of cattle (Minihan et al. 2004). Most studies were done in Europe with relatively few done in the U.S. (Hoar et al. 1999; Wesley et al. 2000). There is a need for further studies to obtain a better understanding of the relationship between *Campylobacter* and cattle.

Subtyping studies suggest that non-poultry sources of *Campylobacter* infection have been underestimated and that some human strains are indistinguishable from strains derived from animals other than poultry such as cattle (Stanley and Jones 2003). In a Danish study, the serotype distribution of human isolates overlapped with that of isolates from cattle, and similar strain subtypes were

identified among bovine isolates and strains isolated from human clinical samples, leading the authors to conclude that cattle may be an important source of *Campylobacter* for humans (Nielsen et al. 1997). In a subsequent study, application of six testing strain subtyping methods confirmed close similarity between certain human clinical isolates and isolates from cattle (Nielsen et al. 2000).

Antibiotic resistance of foodborne pathogens is an important concern to the world today. Use of antibiotics to prevent diseases such as mastitis in cows may contribute to the emergence of microorganisms resistant to antibiotics currently used in the treatment of humans.

Incidence of fluoroquinolone-resistant *C. jejuni* from cattle ranges from 0.5% in a survey in Switzerland (Al-Saigh et al. 2004) to 14% in Denmark (Aarestrup et al. 1997) and 25% in a study in Northeastern Italy (Pezzotti et al. 2002). It is not clear whether these differences reflect different antibiotic administration regimes for cattle in different nations. A common finding from several of these studies was that fluoroquinolone resistance was more frequent among *C. coli* than *C. jejuni* from bovines (Aarestrup et al. 1997; Pezzotti et al. 2002). Resistance to erythromycin is also significantly more frequent among *C. coli* than among *C. jejuni* isolates of bovine origin (Aarestrup et al. 1997). The reasons for the greater incidence of antibiotic (especially erythromycin) resistance in *C. coli* from cattle, in comparison to *C. jejuni*, are not clear, but have also been observed with strains from other animals, such as poultry (Aerestrup et al. 1997; Kathariou and Carver, unpublished). Additional studies need to be done to clarify the mechanisms underlying resistance

to these and other antibiotics in *Campylobacter* of bovine origin, and to identify risk factors for the emergence of antibiotic resistant strains that colonize cattle.

The purpose of this study was to investigate the genotypic characteristics and antibiotic resistance of *Campylobacter* in mature cattle at the time of harvest at the packaging plant. It is hoped that the findings from this work will contribute to the establishment of a body of baseline data that may be used to better evaluate the food safety risk posed by *Campylobacter* strains that colonize cattle at the time of slaughter.

MATERIALS AND METHODS

Animals and Sample collection.

The plant was visited over two years (2001-2003), with 300 samples obtained in the summer (June, July, August) with an average temperature of 29.8°C (range 25-34.4°C) and 180 samples obtained in winter (December, January, February) with an average temperature of 9.4°C (range 7-12°C). A total of 610 animals (358 dairy cows and 252 beef cattle) were sampled immediately after slaughter in a commercial beef packing plant in the southeastern United States. The plant harvested 250-300 animals per day, originating from different areas in the Eastern United States. Typically 30 samples representative of the day's harvest were obtained during each visit, with a total of ten visits made in the summer months, five visits in the winter months, two visits in the fall months, and two visits in the spring months. During each visit, animals were sampled in sequence as they were processed, typically within the time period between 8:00 and 10:00 am. One fecal sample per animal was taken from the colon immediately after slaughter in the tripe room adjacent to the kill

floor. The colon was cut approximately 12 – 18 inches from the distal end. A sterile cotton swab was then inserted about 3-4 inches into the colon and used to collect the fecal sample, or to swab the lining of the colon in the rare occasions where fecal material was scarce. The swabs were placed in 15 ml polypropylene tubes (Corning) containing 3 ml Cary-Blair Medium (Oxoid, Ogdensburg, NY), placed immediately on ice, and transported to the laboratory where they were held under refrigeration until they were processed, typically within 24 hours.

***Campylobacter* isolation.**

Isolations by direct plating.

To directly isolate *Campylobacter* from the fecal samples, approximately 0.05 g of the sample was streaked directly onto *Campylobacter* blood-free selective agar (CCDA; Oxoid, Ogdensburg, NY) with the corresponding supplement (SE 155, Oxoid, Ogdensburg, NY). Plates were incubated in anaerobic jars containing a CampyPak^{TM} Plus microaerobic system (Becton Dickinson, Sparks, MD) at 42° C for 48 hours. Areas of growth with the appearance typical of *Campylobacter* were examined by phase contrast microscopy for typical shape and motility of the microorganisms, and the bacteria were subcultured for purification as needed on sheep blood agar (SBA) (Remel, Lenexa, KS), under microaerobic conditions at 42° C for 48 hours. Typically 3-4 serial transfers were necessary for isolation of pure cultures. Bacteria were confirmed by phase contrast microscopy and absence of aerobic growth at 42°C in Mueller Hinton Agar (Difco, Sparks, MD). Typically one pure culture, derived from a single colony, was obtained from each fecal sample by direct plating.

Isolations by selective enrichment.

A swab with approximately 0.1 g of fecal material was put into 2 ml of Bolton Broth (Oxoid, Ogdensburg, NY) containing the corresponding supplement (SR183E, Oxoid, Ogdensburg, NY) and Laked Horse Blood (SR048C, Oxoid, Ogdensburg, NY) in a 10 ml Falcon tube (Beckton Dickinson, Sparks, MD). The tubes were vortexed slightly to disperse the fecal material from the swab, covered loosely with caps, and incubated in anaerobic jars containing a CampyPak^{TM} Plus microaerobic system at 37°C for 24 hours. 20 µl of the enrichment was streaked for isolation onto CCDA plates, which were then incubated in anaerobic jars containing a CampyPak^{TM} Plus microaerobic system at 42°C for 48 hours. Subsequent purifications and confirmation were done as described above. Pure cultures (obtained either by direct plating or by enrichment) were preserved at -80°C in Brain Heart Infusion (BHI) (Difco, Sparks, MD) broth with 15% sterile glycerol. Typically one pure culture was isolated from each enrichment.

Antibiotic resistance determinations.

A loopfull of a pure culture was grown on SBA for 48 hours at 42°C and resuspended into 1 ml of Brain Heart Infusion (BHI) (Difco, Sparks, MD) by vortexing. Then 200 µl were spread-plated onto SBA aseptically. Antibiotic resistance was determined by the disk diffusion method using commercially available disks (Beckton Dickinson, Sparks, MD) with the following antibiotics: tetracycline (30 µg), streptomycin (10 µg), ampicillin (100 µg), erythromycin (15 µg), kanamycin (30 µg), nalidixic acid (30 µg), and ciprofloxacin (5µg). Plates were

incubated microaerobically at 42°C for 48 hours. Isolates were considered resistant if no zone of inhibition could be seen around the disk.

The NCCLS method was used to determine resistance and Minimal Inhibitory Concentration (MIC) of nalidixic acid for selected strains found to be resistant to this antibiotic through the disk diffusion method. Mueller Hinton Agar (MHA) (Becton Dickinson, Sparks, MD) plates were prepared with different concentrations of nalidixic acid (20µg, 40µg, 80µg, and 160µg). Drops of the culture in question were dispensed in duplicate on plates of each concentration, along with a known negative nalidixic acid control (*C. jejuni* ATCC 33560). The plates were incubated for 48 hours at 42°C microaerobically and scored for growth of the bacteria.

DNA extraction.

DNA was extracted from SBA-grown cells (ca. ½ of a confluent 15 cm-diameter plate) using the DnEasy™ Tissue Kit (Qiagen, Valencia, CA) following the procedures suggested by the vendor. DNA was resuspended in 200 µl of the elution buffer provided with the kit.

Polymerase Chain Reaction (PCR).

PCR employed the *C. jejuni*-specific *hip* primers (5' ATG ATG GCT TCT TCG GAT AG 3' and 5' GCT CCT ATG CTT ACA ACT GC 3') (Marshall et al. 1999) and the *C. coli*-specific *ceu* primers CC1 (5' GAT TTT ATT ATT TGT AGC AGC G 3') and CC2 (5' TCC ATG CCC TAA GAC TTA ACG 3') (Houng et al. 2001). Reactions used Ex-Taq DNA polymerase (Fisher Scientific, Hampton, NH) and were carried out in 20 µl with 1.0 µl of genomic DNA as template. The conditions used were 95°C for 5 min, followed by 30 cycles (each consisting of 95°C for 1 min, 50°C for 1 min, and 72°C

for 2 min), with a final extension at 72°C for 5 min. PCR products (176 bp and 900 bp for *hip* and *ceu* primers, respectively) were detected following electrophoresis on a 1.5% tris borate-EDTA gel (60 min at 85 volts) with DNA molecular marker XIV (100 bp ladder, Roche) Genomic DNA from *C. jejuni* strain NCTC 11168 and *C. coli* strain D124 (strains provided by Dr. D. Threadgill, Univ. of N. Carolina at Chapel Hill) were included each time as positive controls for the *hip* and *ceu* PCR, respectively.

Molecular subtyping of strains.

Subtyping of strains was conducted by *flaA* PCR (Nachamkin et al. 1993). The *flaA* gene was amplified with primers 5' ATG GGA TTT CGT ATT AAC AC 3' and 5' CAA AAT GTT TTA AGA TTA CTA CAG 3', using the PCR conditions described above, except the reactions were in 25 µl, and 25 µl of the PCR product (1.7 kb) was cut using the enzyme DdeI (New England Biolabs, Beverly, MA) following the conditions suggested by the vendor. Restriction fragments were separated on a 3% tris borate-EDTA gel (180 min at 60 volts) and the band patterns were photographed and scanned. Band patterns were analyzed by Bionumerics (version 3.5) (Applied Maths, Inc., Austin, TX). Parameters used for the analysis were similarity coefficient using UPGMA dendrogram type and Dice coefficient (Opt: 1.00%) (Tolerance: ± 1.0%).

Statistical analysis.

Prevalence and resistance to different antibiotics and the distributions of *flaA* subtypes were analyzed statistically using Continuity Adjustment Chi-Square. All statistical analysis was performed using SAS statistical software (version 8.0; SAS Institute, Cary, NC).

RESULTS

Antibiotic resistance.

All antibiotic resistance results are summarized in Table 4. Overall, the antibiotic resistance pattern that was most frequently isolated was the pan-sensitive type (32.5%), i.e. sensitivity to all antibiotics that were tested. An estimated 50% of the isolates were resistant to ampicillin, with ca. 32% being resistant to this antibiotic but sensitive to all other antibiotics that were tested. *Campylobacter* resistance to tetracycline only was 6.4% and to the other antibiotics that were used to screen the bacteria, the resistance was only sporadic.

The number of quinolone-resistant *C. jejuni* isolates (7.5%) identified in this study was within the range (0.5 – 25%) reported in the literature (Aarestrup et al. 1997; Pezzotti et al. 2002; Al-Saigh et al. 2004). Resistance to nalidixic acid only was 3.8% and ciprofloxacin resistance was only detected in the multi-resistant isolates (1.9%) and with one separate antibiotic pattern of tetracycline, ampicillin, and nalidixic acid (0.6%). A total of three such multi-resistant *C. jejuni* strains were isolated from the fecal samples (2 from dairy cows and 1 from beef).

Campylobacter from dairy cows were found to have a large number (66.2%) of observed antibiotic resistance patterns as compared to isolates from beef cattle that exhibited 33.8% of the resistance patterns (Table 4). All fifteen of the antibiotic resistance patterns were detected in *Campylobacter* isolates recovered from dairy cows while only 9/15 (60.0%) antibiotic resistance patterns were detected in the bacteria recovered from beef cattle. *C. jejuni* isolates, recovered from dairy cows, had antibiotic resistance resulting in 12/15 (80.0%) patterns and *C. coli* isolates had

resistance resulting in 7/15 (46.7%) patterns. *C. jejuni* pan-sensitive isolates were recovered with approximately the same frequencies from dairy cows and beef cattle (32.9% and 35.8%, respectively).

***flaA* PCR-RFLP analysis.**

A total of 196 strains were characterized with the flaA subtyping method, yielding 157 different flaA patterns. A dendrogram was constructed based on the analysis of the banding patterns (Figure 1). A total of 38 cattle *flaA* genotype groups (CFGT) were identified, with the largest group comprising 12.2% of the total isolates (Figure 1). Isolates from dairy cows significantly contributed ($P \leq 0.001$) 66.5% (129/194) of all *flaA* banding patterns found from the fecal samples as compared to beef cattle that contributed 33.5% (65/194). The isolation method of selective enrichment only of the fecal material yielded a recovery of 72/194 (37.1%) *flaA* banding patterns while direct plating only, which had fewer total isolates, yielded 18/194 (9.3%). Winter recovery of *flaA* banding patterns was nearly similar to summer recovery with 73 and 80 of 194 (37.6%, 41.2%, respectively) (Table 6).

Several strain types were identified on three or more sampling times, and have been designated as “recurrent.” A prevalent recurrent strain type (CFGT 16), for instance, was isolated from September 2002 through February 2004, on several different visits.

Predominate clusters were identified in 13/38 (34.2%) CFGT groups (Table 5). A predominant cluster is a cluster that has five or more isolates. Within the predominant clusters, four cluster types were especially noticeable. They were CFGT (Cattle *FlaA* Genotype) 1, 17, 35, and 9 & 6. CFTG 1 was the largest

composed of 24 isolates, *C. jejuni* only, and recovered from both dairy cows and beef cattle. The isolates were found in the spring, summer, and winter. They were isolated mostly with selective enrichment but also with one instance of direct plating isolation. CFTG 17 had 16 isolates all composed of *C. jejuni* except for one *C. coli* (BS 618). The isolates were found in the spring, summer, and winter. They were recovered from both dairy cows and beef cattle. The strains were isolated with both selective enrichment and direct plating. CFTG 9 & 6, which had similar *flaA* banding patterns, had 16 isolates, all of which were highly related, that was all *C. jejuni*. The isolates were found in spring, summer, and winter. Dairy cows contained most of the strains isolated but beef cattle were also found to contain the strains as well. The strains were isolated with both selective enrichment and direct plating. CFTG 35 was composed of 15 *C. jejuni* isolates. The isolates were found in summer and winter only. Dairy cows and beef cattle were found to have harbored the isolates, and the strains were isolated with both selective enrichment and direct plating.

An estimated 80% of isolates that were recovered with both isolation methods were the same *flaA* type. The other 20% gave distinct and different *flaA* banding patterns although they were of the same species of *Campylobacter* (*C. jejuni*). Additionally, most strains that were recovered with one medium were also recovered with a second medium except for a few exceptions (Figure 2).

C. coli was found in two related cluster types (CFTG 5 and 28, a total of 7 isolates), while the other four *C. coli* isolates were shared with *C. jejuni* and found in the other 38 cluster types. Of the 38 strain types, 13 (34.2%) were represented only with one or two strains and were designated as unique. Of these 13 unique strain

types, eight were identified among dairy cows and five were from beef cattle. All isolates with unique strain types were *C. jejuni*.

Additionally, strains of the same *flaA* banding patterns had different antibiotic profiles. CFGT cluster 4, comprised of only *C. jejuni* isolates, had antibiotic profiles of ampicillin, pan-sensitive, and ampicillin kanamycin. CFGT cluster 28, comprised of only *C. coli* isolates, had antibiotic profiles of ampicillin, tetracycline ampicillin, and tetracycline ampicillin kanamycin nalidixic acid. Strains with the same antibiotic profile such as ampicillin had different *flaA* banding patterns (CFGT 4 and CFGT 3). Also, the data were examined for antibiotic resistance and strain types that may be common during only one season such as summer or winter, but all were found throughout both summer and winter.

The Simpson's Index of Diversity was calculated to determine the number of strain subtypes per population. The diversity among isolates from dairy cows, and among those from beef cattle was 1 – D (0.05) = 0.95 and 0.95 respectively. This is an indication that there was great sample diversity from all of the strains isolated. Figure 2 details the diversity that one may find using two media methods (CCDA and Karmali agar [KA]) in conjunction with selective enrichment. Three isolates with unique *flaA* types were recovered from the sampling period during which both CCDA and KA were employed, and of these two were isolated from KA and one from CCDA. Overall Figure 2 shows how diverse the isolates from a single sampling may be, and is a snapshot of the total diversity that was found during this study. The diversity of the entire sampling was 0.95 in the Simpson's Index of Diversity.

DISCUSSION

Limited studies have described antibiotic resistance of *Campylobacter* in mature cattle at the time of harvest. In this study, a significant fraction of the isolates were pan-sensitive (32.5%), or resistant to ampicillin but to no other antibiotics (31.8%). However, resistance to certain other antibiotics was noted, at low incidence. This may suggest that there are *Campylobacter*, resistant to some antibiotics used for human treatment, colonizing the colon of cattle at harvest. The implication is that antibiotic resistant campylobacters may be developing in cattle and may pose a concern to public health.

Multiple antibiotic resistance of *C. jejuni* isolated from mature cattle was detected in this study which is in contrast to the literature that showed *C. coli* is more likely to have multiple antibiotic resistance (Pezzotti et al. 2002). A similar result, limited antibiotic resistance within *C. coli*, was also found in another study (Leatherbarrow et al. 2004). This may be attributed to the relative scarcity of *C. coli* strains, limiting the identification of isolates from the colon of mature cattle at harvest that may have antibiotic resistance, or to regional differences in antibiotic resistance.

The isolates from the fecal samples were characterized in terms of their *flaA* patterns. The isolates were recovered via either direct plating and/or selective enrichment. A total of 38 *flaA* patterns were identified and a dendrogram of representative patterns was constructed (Figure 1). Of the 38 representative banding patterns (Figure 1), CFTG 16 was identical to that of NCTC 11168 (Skirrow 1977), a well-characterized isolate from a human clinical case (1977). This may suggest that isolates genetically related to *C. jejuni* NCTC 11168 are found in both beef and dairy

cattle as well as across different seasons (summer and winter) and may be a normal inhabitant in the colon of mature cattle sampled in this study. This also indicates that confirmed human isolates of *C. jejuni* may also be a normal inhabitant in the colon of mature cattle at harvest. This may suggest that the use of multiple methods may give distinct and different isolations of *Campylobacter* strains that may have not been able to be recovered with the use of only one method which has been shown in previous studies (Atabay and Corry 1998; Leatherbarrow et al. 2004). There were 19 isolates that were isolated only through direct plating and two of those had *flaA* types not encountered among other isolates, which may indicate that these strains may not compete well. However, the opportunity to miss minority strains is still possible even with the use of both methods. This can be attributed to the fact that recovering all possible *Campylobacter* from a sample is just not possible and the use of multiple recovery methods is logistically difficult.

The *C. coli* isolates (7) were organized mainly into two strain groups, CFTG 5 and CFTG 28. The isolates were closely related and were from dairy cows. The difference is that CFTG 28 was found in the summer and CFTG 5 was found in the winter. This suggests that although these are not large predominant cluster groups, they may harbor campylobacters within the dairy cows throughout only summer and winter.

FlaA subtyping was used throughout this study in order to determine the relations between isolates obtained during sampling. *FlaA*'s low resolution, the ability for *fla* genes to undergo recombinant events and its ability to be less discriminatory (Nielsen et al. 2000) are limitations in this subtyping procedure. Pulse Field Gel

Electrophoresis (PFGE) would be a more discriminating subtyping method to use as it allows for the examination of polymorphisms throughout the genome and is the current standard operating procedure used by the Center for Disease Control (CDC).

In conclusion, this study was able to identify predominant strain banding patterns of *Campylobacter* from mature cattle at harvest. The use of multiple media methods was shown to allow isolation of additional strains. There was substantial genetic diversity among the isolated *Campylobacter* strains as indicated by Simpson's Index of Diversity. Additionally, four major clusters were identified. The study showed that there is resistance to antibiotics used for human treatment in *Campylobacter* recovered from mature cattle. All of the findings have a real time implication for human public health through the food chain. Further study is needed to determine the host-specificity of these isolates and strain types that may be in common with humans and other animal isolates.

Figure 1 Dendrogram of *flaA* banding patterns of *C. jejuni* and *C. coli*. The strain type designation and frequency within the characterized population is indicated.

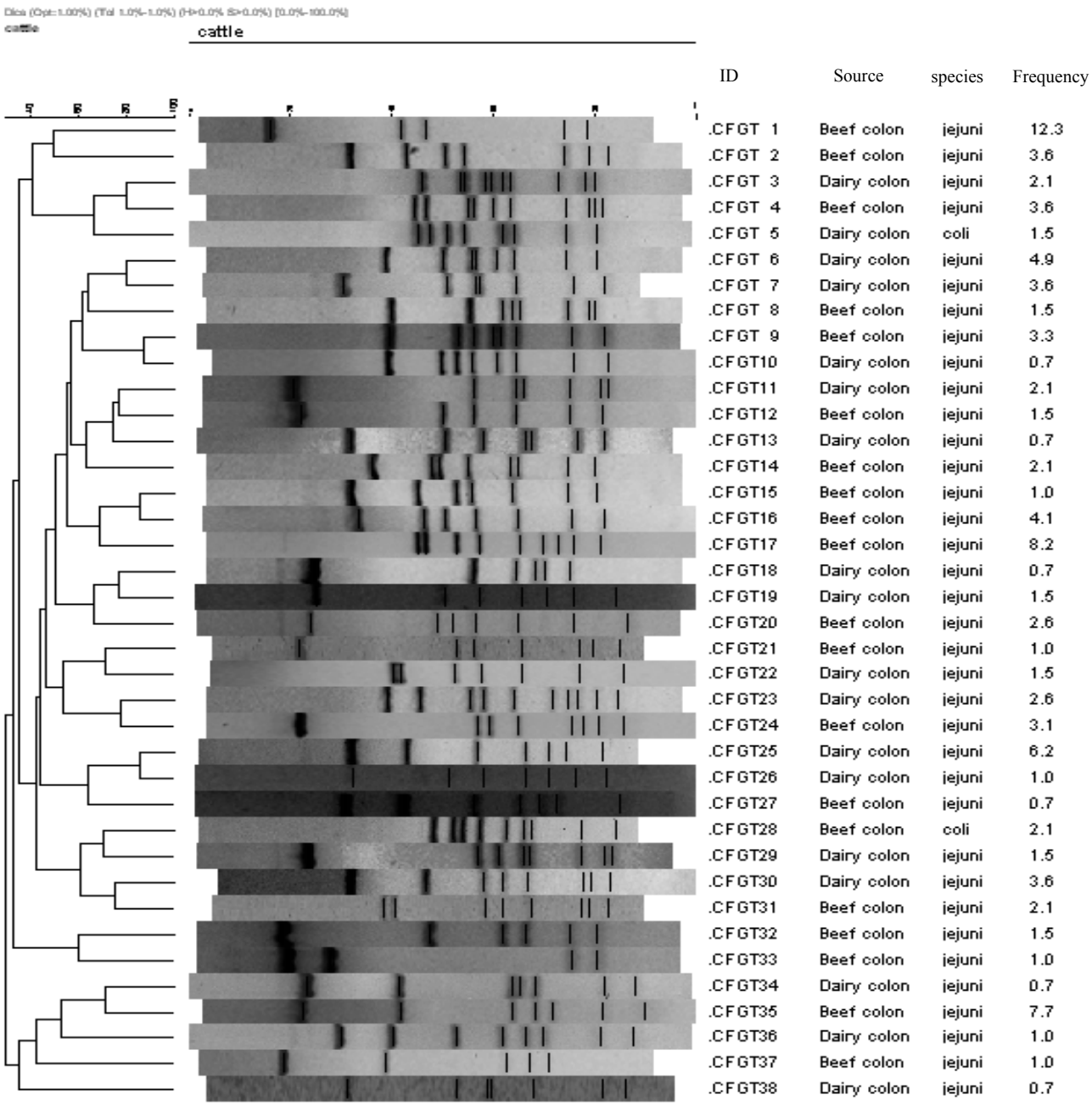
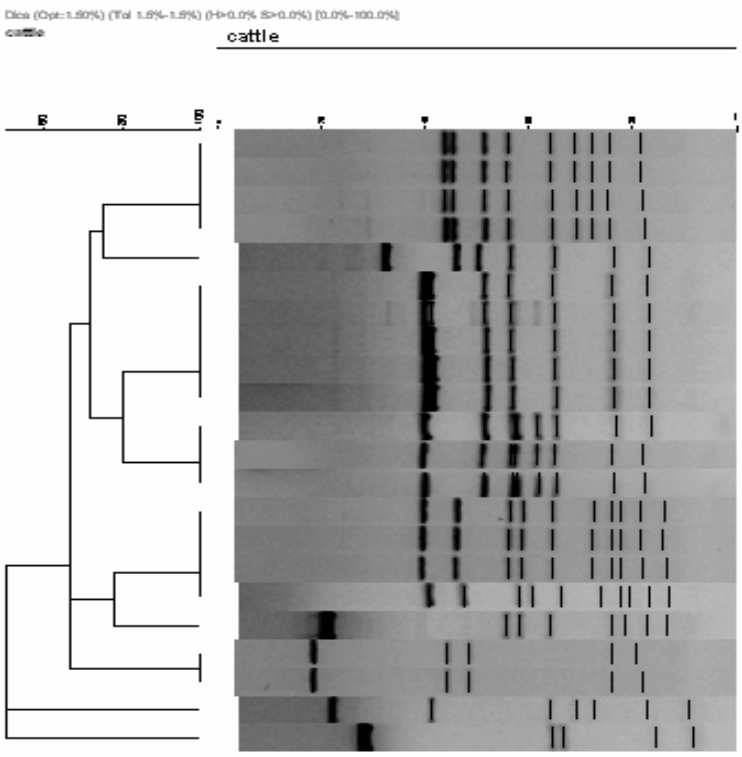


Figure 2 Strain types of *C. jejuni* identified from sampling of mature cattle at harvest using *Campylobacter* blood-free selective agar (CCDA) and Karmali agar (KA).



ID	Source	Species	Date
.BS 657 KA	Dairy colon	jejuni	2/23/04
.BS 657E KA	Dairy colon	jejuni	2/23/04
.BS 657 CCDA	Dairy colon	jejuni	2/23/04
.BS 657E CCDA	Dairy colon	jejuni	2/23/04
.BS 677ECCDA	Dairy colon	jejuni	2/23/04
.BS 677E KA	Dairy colon	jejuni	2/23/04
.BS 678 KA	Dairy colon	jejuni	2/23/04
.BS 678E CCDA	Dairy colon	jejuni	2/23/04
.BS 679E KA	Dairy colon	jejuni	2/23/04
.BS 679E CCDA	Dairy colon	jejuni	2/23/04
.BS 660 CCDA	Dairy colon	jejuni	2/23/04
.BS 660 KA	Dairy colon	jejuni	2/23/04
.BS 660E KA	Dairy colon	jejuni	2/23/04
.BS 655 KA	Dairy colon	jejuni	2/23/04
.BS 655 CCDA	Dairy colon	jejuni	2/23/04
.BS 655E CCDA	Dairy colon	jejuni	2/23/04
.BS 655E KA	Dairy colon	jejuni	2/23/04
.BS 670 CCDA	Dairy colon	jejuni	2/23/04
.BS 654E KA	Dairy colon	jejuni	2/23/04
.BS 654E CCDA	Dairy colon	jejuni	2/23/04
.BS 672E KA	Dairy colon	jejuni	2/23/04
.BS 661EKA	Dairy colon	jejuni	2/23/04

Table 4 Antibiotic resistance profiles of *C. jejuni* and *C.coli* from mature cattle at harvest

Antibiotic resistance profile[1]	**Fecal sample**[2]						
	Dairy cow			**Beef cattle**			
	C. jejuni	***C. coli***	**Total**	***C. jejuni***	***C. coli***	**Total**	**Total *Campylobacter***
T[3]	5 (5.3)	0	5	5 (9.4)	0	5	10 (6.4)
A[3]	32 (34.0)	2 (20.0)	34	16 (30.2)	0	16	50 (31.8)
K[3]	1 (1.1)	0	1	2 (3.8)	0	2	3 (1.9)
Nal	4 (4.3)	0	4	2 (3.8)	0	2	6 (3.8)
T, A	11 (11.7)	2 (20.0)	13	3 (5.7)	0	3	16 (10.2)
T, K	3 (3.2)	0	3	2 (3.8)	0	2	5 (3.2)
T, Nal	1 (1.1)	0	1	0	0	0	1 (0.6)
A, K	1 (1.1)	0	1	0	0	0	1 (0.6)
T, A, K	2 (2.1)	1 (10.0)	3	3 (5.7)	0	3	6 (3.8)
T, S, K	0	2 (20.0)	2	0	0	0	2 (1.2)
T, S, Nal	1 (1.1)	0	1	0	0	0	1 (0.6)
T, S, A, K	0	1 (10.0)	1	0	0	0	1 (0.6)
T, A, Nal, Cip	0	1 (10.0)	1	0	0	0	1 (0.6)
MR	2 (2.1)	0	2	1 (1.9)	0	1	3 (1.9)
Pan-Sensitive	31 (32.9)	1 (10.0)	32	19 (35.8)	0	19	51 (32.5)
Total	94	10	104	53	0	53	157

[1]tetracycline (T); ampicillin (A); kanamycin (K); streptomycin (S); nalidixic acid (Nal); ciprofloxacin (Cip); multi-resistant (MR)

[2]Frequency indicated in parentheses

[3] Resistance was only detected for the indicated antibiotic

Table 5 Predominant strain subtype of *C. jejuni* from cattle fecal samples

Cluster of strain subtypes	Isolation dates	Source	
		Dairy cow	Beef cattle
4	4/30/03, 6/16/03, 6/27/03, 7/1/03	4	3
9 & 6	2/24/03, 5/30/03, 6/2/03, 6/13/03, 7/1/03, 7/8/03, 1/6/04, 2/23/04	15	1
17	2/24/03, 4/30/03, 6/27/03, 7/1/03, 7/8/03, 1/6/04, 2/9/04, 2/23/04	12	4
7	6/20/03, 6/27/03, 1/6/04	5	2
25	6/16/03, 7/1/03, 11/12/03	4	8
16	9/30/02, 2/24/03, 6/20/03, 7/18/03, 11/12/03, 2/9/04, 2/23/04	4	4
2	2/24/03, 4/30/03, 6/20/03	6	1
35	2/24/03, 6/13/03, 6/20/03, 7/1/03, 7/8/03	12	3
30	6/16/03, 1/6/04	3	4
24	2/24/03, 6/13/03, 6/20/03, 2/9/04, 2/23/04	4	2
23	4/30/03, 5/30/03, 2/23/04	4	1
20	7/1/03, 7/18/03	3	2
1	2/24/03, 4/30/03, 6/2/03, 6/13/03, 6/16/03, 6/20/03, 6/27/03, 7/1/03, 7/18/03, 1/6/04, 2/9/04, 2/23/04	15	9

Table 6 Summary of data on *Campylobacter flaA* banding patterns for dairy cows and beef cattle

CFGT[1] cluster	Banding patterns of dairy cows	Banding patterns of beef cattle	Total number of banding patterns/cluster
4	4	3	7
3	2	2	4
28	4	0	4
5	3	0	3
14	3	1	4
10	1	0	1
6 and 9	15	1	16
17	12	4	16
19	3	0	3
12	1	2	3
26	2	0	2
7	5	2	7
25	4	8	12
16	4	4	8
15	0	2	2
2	6	1	7
38	1	0	1
27	0	1	1
13	1	0	1
32	0	3	3
35	12	3	15
34	1	0	1
22	3	0	3
31	1	3	4
8	2	1	3

Table 6 Continued

11	4	0	4
30	3	4	7
29	3	0	3
24	4	2	6
21	0	2	2
36	2	0	2
23	4	1	5
20	3	2	5
18	1	0	1
1	15	9	24
33	0	2	2
37	0	2	2
Total	129	65	194

[1] CFGT (Cattle *flaA* genotype)

Table 7 Frequency of resistance to antibiotics among *C. jejuni* and *C. coli* investigated in this study

Antibiotic resistance profile	***C. jejuni***	***C. coli***	**Total frequency**
Tetracycline	39/157 (24.8%)	7/157 (4.5%)	46/157 (29.3%)
ampicillin	71/157 (45.2%)	7/157 (4.5%)	78/157 (49.7%)
kanamycin	17/157 (10.8%)	4/157 (2.5%)	21/157 (13.4%)
streptomycin	4/157 (2.5%)	3/157 (1.9%)	7/157 (4.5%)
nalidixic acid	11/157 (7.0%)	1/157 (0.6%)	12/157 (7.6%)
ciprofloxacin	3/157 (1.9%)	1/157 (0.6%)	4/157 (2.5%)
Multi-resistant	3/157 (1.9%)	0	3/157 (1.9%)

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APPENDIX 1:

INTRODUCTION

The Karmali agar and CCDA comparison media experiment was conducted to see if another medium might be able to recover campylobacters that might not have been recovered by using only one medium type. Furthermore, in a recent study the use of Karmali agar was shown to recover other species such as *C. lanienae*, in addition to *C. jejuni* and *C. coli*, from bovine feces (Inglis and Kalischuk 2003[1]).

MATERIALS AND METHODS

Isolations using Karmali agar.

For a select number of samples (30 samples obtained during the last sampling visit) both CCDA and an alternate medium, Karmali agar (Oxoid, Ogdensburg, NY) with selective supplement CM935 (Oxoid, Ogdensburg, NY), were used in direct plating and selective enrichments. Enrichments were done as described in Chapter 2, except that Karmali agar was used in place of CCDA.

Results

Impact of isolation protocol (Karmali agar vs. CCDA) on recovery of *Campylobacter* from the fecal samples.

Of the 30 samples, 15 (50.0%) were considered *Campylobacter*–positive on the basis of the appearance of characteristic growth on the CCDA plates, and by phase contrast microscopy which revealed bacteria with characteristic morphology and motility. However, pure cultures could only be obtained from 9 (5 from dairy cattle and 4 from beef cattle, representing 30% of the total population). One isolate was preserved and speciated from each pure culture, with 3 samples that were positive

by both direct plating and enrichment yielding two isolates (one each through direct plating and enrichment), thus leading to a total of 12 isolates (all were *C. jejuni*). Karmali agar recovered 9 pure cultures while 18 (60.0%) were characterized as *Campylobacter*-positive samples. Other *Campylobacter* species were not found. Furthermore, in 3 cases where two different isolates were characterized from the same sample using Karmali agar and CCDA (one isolate obtained by direct plating and one by selective enrichment), we found that each pair of isolates was of the same species (*C. jejuni*). Selective enrichment with Karmali agar recovered 5 of 9 isolates. A side by side comparison study of the isolation medium indicated that only one sample yielded *Campylobacter* with direct plating on CCDA, but with no other method or combination of methods, while two samples yielded *Campylobacter* with selective enrichment on Karmali agar, but with no other method or combination of methods.

Strain subtyping using PCR-RFLP identified 9 cluster groups with cluster group number 3 being the largest with five total strains. Two of the cluster groups were single isolates recovered from selective enrichment onto Karmali agar. A third cluster group was also a single isolate, but was recovered from direct plating on CCDA.

DISCUSSION

The use of Karmali agar recovered 9 pure cultures which was the same as with CCDA. Most of the samples found to be *Campylobacter*-positive with Karmali agar were *Campylobacter*-positive with CCDA as well. Furthermore, the use of multiple media methods was shown to allow isolation of additional strains. The small number

of samples that were collected may play a large role, as additional species of *Campylobacter* might be recovered if a larger sample size was taken. Overall, the use of many types of media when processing samples from mature cattle at slaughter would be optimal, perfect way to go in a perfect world, but this must be balanced with logistic concerns.

APPENDIX 2:

Discontinue of rumen sampling.

The study originally consisted of sampling from the rumen as well as the colon of the cattle that were harvested for that day's production. Upon further research within the literature (Stanley et al. 1998), it was determined to discontinue the sampling of the rumen as it was an unlikely location to recover *Campylobacter*. The decision was to instead focus the time, money, and energy upon sampling the colon where *Campylobacter* would be more likely to be recovered from the harvested animals. A footnote to this decision is that of the 39 samples taken from the rumen during the initial part of the study, one animal yielded a positive recovery from the rumen (BS13) with direct plating.

APPENDIX 3:

Discontinue of carcass sampling.

During the initial phase of the research, samples from inside the carcass (approximately at the 7^{th} rib area) were taken with sterile swabs. Ten harvested animals representative of the day's production, were sampled before the three-wash cycle, after the lactic acid rinse, and after the 24 hrs. of hanging in the cooler. The sample total was 30. The results were all negative. The decision was made to

discontinue sampling the carcass and focus the time, money, and energy upon sampling the colon. A recent study in the literature (Minihan et al. 2004) supports the fact that carcasses can be negative even though the animals had been colonized.

APPENDIX 4:

Table 7 Results of *Campylobacter* prevalence data from mature cattle at harvest. This table indicates prevalence of *Campylobacter* during each sampling time.

Sample date	Sample number	Animal type[1]	Number positives[2]
9.30.02	10	10 DC	1
12.12.02	30	30 DC	2
2.24.03	60	60 DC	22
4.30.03	60	50 BC 10DC	15
5.30.03	30	12 BC 18DC	4
6.2.03	30	6 BC 24 DC	3
6.6.03	30	30 DC	1
6.9.03	30	21 BC 9DC	2
6.13.03	30	9 BC 21 DC	7
6.16.03	30	16 BC 14 DC	5
6.20.03	30	12 BC 18 DC	2
6.27.03	30	30 DC	7
7.1.03	30	30 DC	13
7.8.03	30	23 BC 7 DC	3
7.18.03	30	23 BC 7 DC	9
11.12.03	30	24 BC 6DC	8
1.6.04	30	16 BC 14 DC	14
2.9.04	30	30 BC	6
2.23.04	30	10 BC 20DC	9
Total	610	252 BC 358 DC	143

[1] Beef cattle = BC; Dairy cow = DC

[2] Samples from only the colon that cultures were actually purified and characterized.

Appendix 5:

Table 8 Results of characteristics for all purified cultures recovered from the colon only of mature cattle at harvest.

Sample #	Date of isolation	Cluster group	Type of animal[1]	Mode of isolation[2]	Species[3]	Antibiotic profile[4]
BS12	9.30.02	16	D	DP	CJ	P
BS97*	12.12.02	11, 13	D	BOTH	CJ, CC	M, TAK
BS109	12.12.02	11	D	SE	CC	TA
BS113	2.24.03	9	D	SE	CJ	A
BS115	2.24.03	35	D	BOTH	CJ	TAK
BS120	2.24.03	29	D	BOTH	CJ	TK
BS123	2.24.03	14	D	SE	CJ	A
BS124	2.24.03	2, 17	D	BOTH	CJ	A, TA
BS125	2.24.03	2	D	BOTH	CJ	A, P
BS129	2.24.03	14	D	SE	CJ	A
BS131	2.24.03	34	D	SE	CJ	A
BS132	2.24.03	24	D	SE	CJ	A
BS133	2.24.03	17	D	BOTH	CJ	A
BS138	2.24.03	6 & 9, 14	D	BOTH	CJ	A
BS142	2.24.03	6 & 9	D	BOTH	CJ	A, P
BS145	2.24.03	5	D	BOTH	CC	TSAK
BS154	2.24.03	36	D	BOTH	CJ	A
BS155	2.24.03	3	D	BOTH	CJ	P
BS156	2.24.03	16	D	SE	CJ	P
BS159	2.24.03	1	D	BOTH	CJ	T
BS161	2.24.03	35	D	DP	CJ	K
BS165	2.24.03	16, 35	D	BOTH	CJ	A
BS166	2.24.03	17	D	SE	CJ	TA
BS169	2.24.03	5	D	SE	CC	TSK
BS170	2.24.03	35	D	BOTH	CJ	TA

BS180	4.30.03	15	B	SE	CJ	A
BS185	4.30.03	15	B	SE	CJ	P
BS192	4.30.03	8	B	SE	CJ	P
BS193	4.30.03	8	D	BOTH	CJ	P
BS200	4.30.03	17	B	SE	CJ	P
BS204	4.30.03	2	B	SE	CJ	TAK
BS205	4.30.03	14	B	SE	CJ	T
BS206	4.30.03	4	B	SE	CJ	P
BS207	4.30.03	4	B	BOTH	CJ	A
BS208	4.30.03	1, 23	B	BOTH	CJ	P, A
BS209	4.30.03	1	B	BOTH	CJ	P
BS210	4.30.03	3	B	BOTH	CJ	A
BS217	4.30.03	2	D	BOTH	CC	A
BS223	4.30.03	1	B	BOTH	CJ	P
BS227	4.30.03	37	B	BOTH	CJ	A
BS246	5.30.03	6 & 9	D	SE	CJ	A
BS247	5.30.03	6 & 9	D	SE	CJ	A
BS250	5.30.03	23	D	SE	CJ	TA
BS252	5.30.03	23	D	SE	CJ	TA
BS269	6.2.03	6 & 9	D	DP	CJ	A
BS272	6.2.03	1	D	DP	CJ	TK
BS273	6.2.03	29	D	DP	CJ	A
BS295	6.6.03	18	D	SE	CJ	A
BS344	6.9.03	26	D	SE	CJ	TN
BS345	6.9.03	26	D	SE	CJ	N
BS352	6.13.03	35	B	SE	CJ	TA
BS364	6.13.03	1	D	SE	CJ	P
BS365	6.13.03	1	D	SE	CJ	A
BS367	6.13.03	6 & 9	D	SE	CJ	A
BS371	6.13.03	35	D	BOTH	CJ	P, A

BS373	6.13.03	35	D	SE	CJ	A
BS375	6.13.03	24	D	SE	CJ	A
BS389	6.16.03	25	B	SE	CJ	T
BS397	6.16.03	31	D	SE	CJ	TK
BS403	6.16.03	4	D	SE	CJ	A
BS409	6.16.03	30	D	BOTH	CJ	P
BS410	6.16.03	1, 30	D	BOTH	CJ	P, T
BS418	6.20.03	2, 35	D	BOTH	CJ	P, TA
BS419	6.20.03	1	D	SE	CJ	TA
BS420	6.20.03	7	D	BOTH	CJ	A
BS421	6.20.03	7	D	SE	CJ	A
BS422	6.20.03	11	D	DP	CJ	T
BS423	6.20.03	11	D	DP	CJ	TA
BS426	6.20.03	24	D	DP	CJ	P
BS428	6.20.03	1	D	BOTH	CJ	T
BS429	6.20.03	1	D	SE	CJ	TA
BS433	6.20.03	35	B	BOTH	CJ	P, A
BS435	6.20.03	16	B	SE	CJ	A
BS437	6.20.03	12	B	BOTH	CJ	P
BS446	6.27.03	4	D	SE	CJ	P
BS447	6.27.03	28	D	SE	CC	A
BS448	6.27.03	1	D	SE	CJ	P
BS449	6.27.03	17	D	BOTH	CJ	T, P
BS450	6.27.03	7	D	SE	CJ	A
BS458	6.27.03	7	D	SE	CJ	A
BS462	6.27.03	12	D	SE	CC	TSK
BS473*	7.1.03	28	D	SE	CC	TAKN
BS474	7.1.03	17	D	SE	CJ	TAK
BS477	7.1.03	19	D	SE	CJ	TSN
BS479	7.1.03	19	D	SE	CJ	N

BS480	7.1.03	19	D	SE	CJ	N
BS486	7.1.03	35	D	SE	CJ	TA
BS488	7.1.03	6 & 9	D	BOTH	CJ	A, P
BS489	7.1.03	6 & 9	D	BOTH	CJ	A
BS491	7.1.03	28	D	BOTH	CC	TA
BS492*	7.1.03	20	D	SE	CJ	N
BS494	7.1.03	1	D	SE	CJ	TA
BS495	7.1.03	4	D	BOTH	CJ	AK
BS501	7.1.03	25	D	BOTH	CJ	A, P
BS504	7.8.03	17	B	DP	CJ	A
BS505	7.8.03	9, 17	B	BOTH	CJ	A
BS526	7.8.03	35	D	DP	CJ	A
BS534	7.18.03	32	B	BOTH	CJ	P
BS535	7.18.03	32	B	DP	CJ	P
BS542*	7.18.03	20	B	SE	CJ	N
BS549	7.18.03	20	B	SE	CJ	N
BS552	7.18.03	1, 16	B	BOTH	CJ	P
BS553	7.18.03	33	B	BOTH	CJ	K
BS556	7.18.03	20	D	SE	CJ	P
BS558	7.18.03	20	D	SE	CJ	P
BS560	7.18.03	1	D	SE	CJ	P
BS562	11.12.03	31	B	SE	CJ	TAK
BS563	11.12.03	31	B	SE	CJ	A
BS569	11.12.03	31	B	SE	CJ	TK
BS570	11.12.03	25	B	BOTH	CJ	A
BS572	11.12.03	25	B	BOTH	CJ	P
BS581	11.12.03	25	B	BOTH	CJ	T
BS584	11.12.03	16, 25	B	BOTH	CJ	A
BS590	11.12.03	25	D	BOTH	CJ	P
BS592	1.6.04	21	B	BOTH	CJ	TAK, TK

BS593	1.6.04	7	B	BOTH	CJ	TA, T
BS594*	1.6.04	27	B	SE	CJ	M
BS595	1.6.04		B	SE	CJ	A
BS598	1.6.04	1	B	BOTH	CJ	P
BS599	1.6.04	30	B	BOTH	CJ	T
BS600	1.6.04	30	B	BOTH	CJ	P
BS610	1.6.04	38	D	DP	CJ	A
BS612	1.6.04	1	D	SE	CJ	P
BS614	1.6.04	6 & 9	D	SE	CJ	P
BS615	1.6.04	6 & 9	D	SE	CJ	M
BS616	1.6.04	17	D	SE	CJ	P
BS617	1.6.04	17	D	DP	CJ	P
BS618	1.6.04	17	D	DP	CC	P
BS629	2.9.04	17	B	DP	CJ	P
BS643	2.9.04	1	B	SE	CJ	P
BS644	2.9.04	24	B	SE	CJ	A
BS646	2.9.04	24	B	SE	CJ	A
BS648	2.9.04	16	B	SE	CJ	A
BS651	2.9.04		B	SE	CJ	TA
BS654	2.23.04	1	D	SE	CJ	P
BS655	2.23.04	23	D	BOTH	CJ	P
BS657	2.23.04	17	D	BOTH	CJ	P, A
BS660	2.23.04	6 & 9	D	DP	CJ	P
BS666	2.23.04	10	D	DP	CJ	P
BS670	2.23.04	24	D	DP	CJ	P
BS677	2.23.04	16	B	SE	CJ	K
BS678	2.23.04	22	D	BOTH	CJ	P
BS679	2.23.04	22	B	SE	C	P

[1] Dairy cow (D); Beef cattle (B)

[2] Direct plating (DP); Selective enrichment (SE); Both = selective enrichment and direct plating

[3] *Campylobacter jejuni* (CJ); *Campylobacter coli* (CC)

[4] [1]tetracycline (T); ampicillin (A); kanamycin (K); streptomycin (S); nalidixic acid (N); ciprofloxacin (C); multi-resistant (M); pan-sensitive (P)

* Robin found antibiotic resistance patterns to be different. Her findings were: BS97 TK; BS473 TNC; BS492 P; BS542 P; BS 615 P.