MOLECULAR TYPING AND ANTIMICROBIAL RESISTANCE OF *CAMPYLOBACTER* ISOLATED DURING COMMERCIAL BROILER PRODUCTION

A Thesis

by

CHARLES ANDREW HERNANDEZ JR.

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2010

Major Subject: Poultry Science

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Approved by:

Chair of Committee,	David J. Caldwell
Committee Members,	Michael E. Hume
	James Allen Byrd
	Joseph M. Sturino
Head of Department,	John B. Carey

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ABSTRACT

Molecular Typing and Antimicrobial Resistance of *Campylobacter* Isolated During Commercial Broiler Production. December 2010

Charles Andrew Hernandez Jr., B.S., Texas A&M University

Chair of Advisory Committee: Dr. David J. Caldwell

Campylobacter jejuni is a commensal microorganism of the poultry gastrointestinal tract. Broilers, layers, ducks, turkeys, and quails can be colonized by *Campylobacter* without illness occurring. The vast majority of human *Campylobacter* infections are recognized as being foodborne. For 2008, preliminary FoodNet data showed that the *Campylobacter* incidence of infection, 12.68 per 100,000 of the U.S. population, is the second highest, only behind *Salmonella* at 16.20 per 100,000. To further understand *Campylobacter*'s role as a foodborne pathogen, analysis at the molecular level is needed.

Microbial molecular typing allows for identification and differentiation of bacterial strains beneath the species level. In this study, the "gold standard" method for molecular subtyping, Pulsed Field Gel Electrophoresis (PFGE), along with Diversilab® repetitive element Polymerase Chain Reaction (rep-PCR) and 16S-23S Internal Spacer Region Denaturing Gradient Gel Electrophoresis (ISR DGGE) were used for the molecular typing of *Campylobacter jejuni* isolates obtained during different stages of commercial broiler production and processing. In addition, the *C. jejuni* isolates were

tested for resistance to antimicrobials commonly used in both veterinary and human medicine. Antimicrobial resistance testing was carried out using a broth dilution system. The majority of recovered isolates came from post-harvest carcass rinsates. Carcass rinses were obtained at post-evisceration, post-chill stages. All isolates (n = 46) were identified by the Polymerase Chain Reaction as *Campylobacter jejuni*. Three genotypes (n = 44, n = 1, n = 1) were identified by PFGE. The 46 rep-PCR products grouped into seven clusters and two outliers. Clustering of rep-PCR products by sample source was not observed. No relatedness trends were observed for isolates recovered from the same source. The combination of PFGE and Diversilab rep-PCR methods provides highly discriminatory molecular typing results.

These results provide practical epidemiological information that shows post-evisceration and post-chill stages are still important targets for intervention studies. The very high occurrence of *C. jejuni* isolates exhibiting genotype A suggests it may differentially express certain gene(s) that enable this strain to more favorably survive under the different harsh environmental conditions encountered during production and processing.

In addition, phenotypic testing revealed all of the isolates were not resistant to the antimicrobials azithromycin, ciprofloxacin, erythromycin, gentamycin, tetracycline, florfenicol, nalidixic acid, telithromycin, and clindamycin at any of the concentrations tested. All the *C. jejuni* isolates exhibited an indistinguishable two-band 16S-23S ISR DGGE profile. Overall, these *C. jejuni* commercial broiler pre- and post-harvest isolates exhibited an extremely low degree of molecular and phenotypic variability.

DEDICATION

I dedicate my thesis to my amazing and lovely wife Cindy. Thank you so much for always believing in me, even when I did not believe in myself. Your undying support and love is appreciated more than you realize.

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CHAPTER I

INTRODUCTION

It is estimated that 76 million cases of foodborne illness occur annually in the United States (DuPont, 2007). Bacteria within the genus *Campylobacter* are found in reproductive organs, the gastrointestinal tract, and the oral cavity of humans and other animals (Brenner et al., 2005). Most *Campylobacter* species are pathogenic to humans and other animals. The thermotolerant species *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus* form a distinct subcluster and cause gastroenteritis in humans. *Campylobacter jejuni* is a leading cause of acute bacterial gastroenteritis in the developed world (Allos, 2001). For 2008, preliminary FoodNet data showed that the *Campylobacter* incidence of infection, 12.68 per 100,000 of the U.S. population, is the second highest, only behind *Salmonella* at 16.20 per 100,000 (CDC, 2009). The vast majority of human *Campylobacter* infections are recognized as being foodborne (Jacobs-Reitsma, 2008). Consuming inadequately cooked poultry and drinking contaminated water or unpasteurized milk are risk factors for developing campylobacteriosis. In poultry, the highest numbers are found within the ceca in the lower intestine. Sources of *Campylobacter* during commercial poultry rearing include the environment, litter,

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This thesis follows the style of Poultry Science.

water, wild birds, contaminated cecal and fecal material from flock mates, and insects (Jacobs-Reitsma, 2008), as well as through contaminated feed, rodents, and human handlers. Cross contamination can also take place during poultry feed withdrawal, transit to the processing plant, and the slaughtering, defeathering, scalding, evisceration, and immersion chilling stages of processing (Byrd and McKee, 2005). The use of molecular methods to identify *Campylobacter* from the aforementioned sources has contributed to a better understanding of its epidemiology.

Microbial typing allows for identification and differentiation of bacterial strains beneath the species level. Epidemiological bacterial strain typing methods have evolved quickly over the past few decades. Pulsed Field Gel Electrophoresis (PFGE) is widely considered to be the "gold standard" of molecular subtyping (Olive and Bean, 1999). In addition, PFGE is generally accepted as being one of the most discriminatory methods for subtyping *Campylobacter* (Ribot et al., 2001). It has been demonstrated that repetitive element Polymerase Chain Reaction (rep-PCR) is another highly reproducible and discriminatory process for microbial subtyping and microbial ecology analysis (Hiett and Seal, 2009). In addition to these genotypic methods, the phenotypic analysis of *Campylobacter* antimicrobial resistance provides valuable data.

When antibiotic therapy is indicated for treatment against campylobacteriosis, macrolides are usually selected although fluoroquinolones (FQs) are also recommended frequently. Many countries have reported an increase in the occurrence of human *Campylobacter* infections in which these bacteria are resistant to macrolides and FQs (Aarestrup et al., 2008). Foods that originate from poultry are considered the primary

sources of human infections due to *Campylobacter* spp. Therefore, increased *Campylobacter* antimicrobial resistance due to antibiotic use in food animals is currently a very important issue (Aarestrup et al., 2008). A number of studies have reported a rise in occurrence or frequency in macrolide and FQ resistance among *Campylobacter* from food animals (Endtz et al., 1991; Saenz et al., 2000; Aarestrup et al., 2008). Microbiological and epidemiological evidence support poultry as the primary source of human *Campylobacter* infection as well as fluoroquinolone-resistant *Campylobacter* (Nelson et al., 2007). In comparison with *C. jejuni*, a greater percentage of *C. coli* strains show resistance to erythromycin (Brenner et al., 2005). Phenotypic evaluations, such as antimicrobial resistance testing, provide little to no capability of revealing *Campylobacter* strain differentiation. To better assess strain differences, genotypic evaluations must be carried out.

Members of *Bacteria* and *Archaea* domains typically contain an rRNA operon consisting of a 16S rRNA, a 23S rRNA, and a 5S rRNA gene as well as an internal transcribed spacer region and at least one tRNA gene (Acinas et al., 2004). The number of rRNA operons in *Bacteria* and *Archaea* varies from 1 to 15 per genome (Lee et al., 2009). *Campylobacter jejuni* possesses three copies of the *rrn* operon. The degree of amplicon separation during Denaturing Gradient Electrophoresis (DGGE) is dependent on the double stranded DNA nucleotide sequence. Attempts have been made to use the 16S-23S ISR DGGE method to differentiate strains of environmental *Escherichia coli* isolates (Buchan et al., 2001).

CHAPTER II

LITERATURE REVIEW

Campylobacter Taxonomy

The family *Campylobacteraceae* is made up of the genera *Campylobacter, Arcobacter, and Sulfurospirillum,* and the species *Bacteroides ureolyticus*. The genus *Campylobacter* is taxonomically classified primarily based on phylogeny and has resulted in the grouping of species with very diverse cellular morphologies and a DNA base ratio range which goes beyond that of almost all clearly defined genera (Brenner et al., 2005). Currently, 18 accurately named *Campylobacter* species exist: *C. fetus*, *C. hyointestinalis*, *C. lanienae*, *C. sputorum*, *C. mucosalis*, *C. consisus*, *C. curvus*, *C. rectus*, *C. gracilis*, *C. showae*, *C. hominis*, *C. jejuni, C. coli*, *C. lari*, *C. insulaenigrae*, *C. canadensis*, *C. upsaliensis*, *and C. helveticus*.

Most of the *Campylobacter* species are spirally curved, slim, bacilli with dimensions 0.2- 0.8 μm wide and 0.5-5 μm long. The outer cell membrane has two layers, is loosely arranged over the cell wall and has a wavy shape. *Campylobacter* species are nonsporeformers, Gram-negative, and have a multilaminar polar membrane at each end of the cell. Most of the species are motile and display distinctive corkscrew-like darting movement. *Campylobacter* are microaerophilic and have a respiratory metabolism. They are chemoorganotrophs that do not ferment nor oxidize carbohydrates. All of the campylobacters grow in microaerobic environments. Energy is obtained from amino acids and tricarboxylic acid intermediates.

Campylobacter jejuni and *C. coli* are commensal microorganisms in the poultry gastrointestinal tract. Broilers, layers, ducks, turkeys, and quails can be colonized by *Campylobacter* without illness occurring (Wagenaar et al., 2008). The thermotolerant species *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus* form a well-defined subcluster and cause gastroenteritis in humans (Brenner et al., 2005). *Campylobacter* does not grow outside of its host (Wagenaar et al., 2008).

Campylobacter jejuni subspecies *jejuni* and *C. coli* are extremely similar phenotypically and genotypically and are frequently located in the same ecological areas. Housekeeping genes of these two species share 86.5% nucleotide sequence identity (Sheppard et al., 2008). Genetic exchange within and between these two species occurs (Sheppard et al., 2008).

Campylobacter and Human Foodborne Illness

Campylobacter jejuni is the leading cause of acute bacterial gastroenteritis in the developed world (Allos, 2001) and continues to be a major cause of human foodborne illness in the United States. In the U.S., it is estimated that *Campylobacter* causes 2 million illnesses each year (Ailes et al., 2008). Most of the infections are sporadic. In terms of diagnosed bacterial diarrheal infections, greater than 95% are caused by *C. jejuni* (Aarestrup et al., 2008).

The USDA Economic Research Service has calculated annual costs associated with camplyobacteriosis to be several billions of dollars (Chinivasagam et al., 2009). Incubation time in the human gastrointestinal tract ranges from 24 to 72 hours.

Campylobacteriosis is usually self-limiting (Allos, 2001). Characteristic symptoms include diarrhea and abdominal pain for 1 to 7 days. Illness seriousness in humans is influenced by age and the condition of the immune system. Guillain-Barré Syndrome is a rare but very serious sequela of campylobacteriosis (Janssen et al., 2008). In this disease state, *Campylobacter* lipooligosaccharides mimic human gangliosides and induce an autoimmune response that targets the gangliosides and in extremely rare cases results in death (Allos, 2001; Janssen et al., 2008). Consumption of inadequately cooked poultry and drinking contaminated water or unpasteurized milk are risk factors for developing campylobacteriosis.

In milk, the infective dose of *C. jejuni* is 500 cells (Robinson, 1981). Experimental human infection with 800 *C. jejuni* strain A3249 cells was enough to cause infection and diarrheal illness (Black et al., 1988). The 50% infective dose for humans is about 900 cells (Gormley et al., 2008). The vast majority of human *Campylobacter* infections are recognized as being foodborne (Jacobs-Reitsma, 2008). According to the World Health Organization, the widespread incidence of *Campylobacter* presents an obstacle to strategy development for controlling it in the food supply "from farm to fork" (WHO, 2000). The duration that *Campylobacter* can survive in food is dependent on the initial number of cells, the specific food product, and environmental conditions such as storage temperature (Jacobs-Reitsma, 2008). It's clear to see that the aforementioned facts contribute to *Campylobacter*'s standing as one of the leading causes of foodborne illness.

For 2008, preliminary FoodNet data showed that the *Campylobacter* incidence of infection, 12.68 per 100,000 of the U.S. population, is the second highest, only behind *Salmonella* at 16.20 per 100,000 (CDC, 2009). In 2006, the frequency of culture-confirmed *Campylobacter* infection in FoodNet dropped 30% compared to 1996-1998 baseline numbers (Ailes et al., 2008). In the US, campylobacteriosis occurrence is low compared to several other countries (Ailes et al., 2008). Incidence rate may be attributed to the implementation by the Food Safety and Inspection Service in 1996 of the Pathogen Reduction: Hazard Analysis and Critical Control Point System (Ailes et al., 2008). Also, differences may be partly due to different surveillance systems (Ailes et al., 2008).

In Scotland, *Campylobacter* infection incidence was 90.2 cases per 100,000 people in 2005 (Gormley et al., 2008). From 1997 through 2005, reported campylobacteriosis cases in Sweden numbered 69-96 per 100,000 (Lindqvist and Lindblad, 2008). In the EU, the overall campylobacteriosis incidence level for 2005 was 51.6 per 100,000 (De Cesare et al., 2008). Beginning in 2008, the Zoonoses Directive 2003/99/EC made the monitoring of *Campylobacter* mandatory in the European Union (EU) (Wagenaar et al., 2008).

Poultry Production and Processing

In 2006, broilers accounted for 99% of all chicken produced in the US and for 86% of all poultry produced (MacDonald, 2008). The broiler slaughter volume in 2006 was 8.84 billion (MacDonald, 2008). In 2002, US market share of worldwide broiler

production was 27% and the EU's was 13% (Adkin et al., 2006). Commercial poultry in the US are primarily reared using the broiler system where water and feed are provided *ad libitum*. Most of the commercial poultry rearing houses in the US are tunnel-ventilated (Chinivasagam et al., 2009).

For control of *Campylobacter* and *Salmonella* during pre-harvest, implementing biosecurity measures, controlling litter moisture, carrying out a well-timed feed withdrawal, and using acids in drinking water during feed withdrawal are effective (FSIS, 2008). The United Kingdom poultry industry regularly implements poultry house cleaning and disinfection practices between flocks (Shreeve et al., 2002). Intervention methods currently in development include probiotic use, competitive exclusion, vaccination, and genetic resistance (Wagenaar et al., 2008).

The stages of poultry processing are live receiving and live hanging, stunning and bleeding, scalding, picking, eviscerating, chilling, on-line/off-line reprocessing, and packaging. Cross contamination can take place during feed withdrawal, transport to the processing plant, and the slaughtering, defeathering, scalding, evisceration, and immersion chilling stages (Byrd and McKee, 2005).

The amount and impact of cross contamination is highly dependent on slaughter practices (Normand et al., 2008). Processing may lead to carcass contamination with fecal matter and digested feed. During automated processing, rupturing of the viscera is common due to carcass size variation and the fixed size of evisceration machinery (Rosenquist et al., 2006). In the US, there is a zero tolerance policy for visible fecal carcass contamination. Some plants have inside-outside bird washers (IOBWs) on the

processing line to ensure proper compliance. Chlorine is mostly used in IOBWs. The USDA requires poultry carcasses to be cooled rapidly in order to stop bacterial growth. The majority of broiler processing plants in the US use cold water immersion to reduce carcass temperature at the end of the first processing while in Europe most use air chilling (Berrang et al., 2008). Chemical methods (organic and inorganic acids and bases, and/or chlorine and similar compounds), physical methods (cold water, hot water, and high pressure), and irradiation are most commonly used in attempting to reduce microbial loads on poultry carcasses (Keener et al., 2004). Interventions showing the most potential in the processing plant include reducing fecal leakage during processing, separating contaminated and non-contaminated flocks, and decontaminating contaminated flocks (Molbak and Havelaar, 2008).

Campylobacter and Poultry

Poultry such as broilers, layers, turkeys, fowl, ducks, ostriches, and quails are all susceptible to colonization by *Campylobacter* (Wagenaar et al., 2008). The poultry gastrointestinal tract is a natural reservoir for *C. jejuni* and *C. coli*. *Campylobacter jejuni* grows optimally at 42° C (Park, 2002). The normal core body temperature of chickens is 42° C while that of humans is 37° C. Inner body temperature may be an important stimulus for *C. jejuni* to either colonize or invade cells (Stintzi, 2003). Colonization in chicks mostly occurs within the ceca and is mainly limited to the intestinal mucous layer in the cecal and cloacal crypts (Beery et al., 1988). Generally, in chicken and turkey intestinal contents, approximately 10^4 to 10^6 *C. jejuni* CFU/g are

present (Beery et al., 1988). Usually, chickens empty their cecal contents two times a day and feces ten to sixteen times per day (Dhillon et al., 2006). This leads to higher numbers of *C. jejuni* being able to accumulate in the ceca versus the intestine.

In all research studies of "intensively reared broilers", *C. jejuni* was found to be the most common colonizer while *C. coli* and other species were found at low levels (Wagenaar et al., 2008). In addition, *Campylobacter* has been isolated from tray liners of day of hatch broilers (Byrd et al., 2007).

Sources of *Campylobacter* during production include the environment, litter, water, other birds, cecal and fecal transmission, and insects (Jacobs-Reitsma, 2008). Climate, number of animals per farm, and distance between farms may all affect the colonization rate. Chickens are coprophagic and so fecal shedding of *Campylobacter* will contribute to its spread throughout a flock. Flock colonization can be delayed through thorough disinfection and cleaning of poultry houses, utilizing disinfectant foot baths, and other biosecurity procedures (Stern et al., 2001).

There is a seasonal variation in the rate of *Campylobacter* colonization in poultry. During summer, reported colonization rates are higher than in the fall (Wagenaar et al., 2008). Free range *Campylobacter*-negative flocks rarely occur due to their considerable environmental exposure (Huneau-Salaün et al., 2007). No significant difference in *Campylobacter* incidence between organically and conventionally raised broilers in Belgium was observed (Overbeke et al., 2009). But at slaughter there was a significantly higher occurrence of *Campylobacter* in organic broiler cecal and duodenum samples.

Orally challenged one-day-old chickens were highly susceptible to *Campylobacter* colonization (Wagenaar et al., 2008). Cecal colonization peaked at five days then declined very slightly at about four weeks (Wagenaar et al., 2008). *Campylobacter* is rarely isolated from broiler flocks in rearing houses until at least two to three weeks after hatch (Jacobs-Reitsma et al., 1995; Hiett et al., 2002; Bull et al., 2006). Usually around 3 weeks of age, broilers start shedding *Campylobacter* and within 2 to 4 days occurrence in the flock can reach 90-100% (Chinivasagam et al., 2009). Within a week of the first isolation of *Campylobacter* in a flock, most of the broilers were colonized by *Campylobacter* (Bull et al., 2006).

Some broiler producers will separate a flock into different batches that are physically separated within the same house (e.g. males and females). Batches are removed for slaughter at different times. Broiler house batch depletion increased the prevalence of *Campylobacter*-colonized chickens in surveyed flocks due to the introduction of the bacterium during the catching of the first batch of chickens and within a week *Campylobacter* spread throughout the flock (Hald et al., 2001). Risk factors for *Campylobacter*-positive flocks include multispecies farming, thinning, increasing median flock size, spreading manure, and increasing the number of broiler houses (Guerin et al., 2007). Thinning is a method of partly depopulating broiler houses in order to make more space available for the remainder of the flock on the basis of economical and ethical reasons.

Even after disinfection of cleaning crates, *Campylobacter* could still be isolated from them (Hansson et al., 2005). *Campylobacter* contamination may be reduced 100 to

1,000 times during processing (Jacobs-Reitsma, 2008). Application of an 8 to 12% trisodium phosphate (TSP) solution on chicken carcasses decreased *Campylobacter* by about 1.0 to 2.0 $\log_{10}$ (Riedel et al., 2009). Carcass washing using TSP or acidified sodium chloride (ASC) is more effective in reducing *Campylobacter* than carcass washer systems consisting of multiple washers (Bashor et al., 2004). Post-chill use of ACS on carcasses resulted in a significant decline in *Campylobacter* spp. numbers (Oyarzabal et al., 2004). As an intervention step in poultry processing, freezing has been documented to decrease *Campylobacter* levels and as such Norway, Iceland, and Denmark have implemented freezing as a strategy to reduce carcass contamination (Rosenquist et al., 2006).

In the United Kingdom, up to 90% of broiler flocks are colonized with *Campylobacter* at slaughter (Newell et al., 2001). In 2006, 25.9% of Swiss poultry flocks tested positive for *Campylobacter* (Zweifel et al., 2008). About 35% of broiler flocks in Denmark were *Campylobacter* positive in 2003 (Rosenquist et al., 2006). In Germany, almost 46% of chicken carcasses were contaminated with *Campylobacter* (Klein et al., 2007). In Scotland between 2001 and 2006, human infection decreased but in retail chicken *Campylobacter* occurrence and numbers did not (Gormley et al., 2008). From the aforementioned data, it's clear that a target for intervention research is *Campylobacter* colonization of chickens.

Presently, there are two experimental methods aimed at lowering *Campylobacter* shedding in colonized chickens: lytic bacteriophage administration and bacteriocin treatment (Wagenaar et al., 2008). Lytic phage therapy significantly reduced *C. jejuni*

numbers in the ceca of previously colonized chickens (Wagenaar et al., 2005). Administration of a bacteriocin isolated from *Lactobacillus salivarius* strain NRRL B30514 resulted in a statistically significant drop in *C. jejuni* isolated from chickens (Stern et al., 2006).

Molecular Identification of *Campylobacter*

Culture-dependent methods of identifying or characterizing *Campylobacter* have significant limitations (Hayden, 2004). Frequently, these methods do not have the sensitivity or specificity preferred in a clinical assay (Hayden, 2004). The interest in nucleic acid amplification techniques has been due to its overall increased sensitivity, accuracy, and speed of detection and identification of pathogens compared to culture-based methods (Hayden, 2004). Additionally, standard culturing of environmental samples has been observed to result in poor recovery rates of *Campylobacter* (Ridley et al., 2008). *Campylobacter* identification using standard culture-dependent isolation methods is challenging due to the fastidious growth requirements of the bacterium and "biochemical inertness" (Lubeck et al., 2003; On and Jordan, 2003). Phenotypically, differentiating between *C. coli* and *C. jejuni* subspecies *jejuni* is difficult. The hippurate hydrolysis test is commonly used as a phenotypic test to differentiate the two species. *Campylobacter coli* are negative for this test. The problem with this test is that some strains of *C. jejuni* subspecies *jejuni* also produce a negative result. The use of molecular methods such as the Polymerase Chain Reaction (PCR) increase sensitivity and specificity in *Campylobacter* speciation studies.

PCR produces copies of a targeted double stranded DNA molecule through a repeated series of three steps: denaturing, annealing, and extension. Theoretically, at the end of each three step cycle, the number of copies of the targeted DNA segment that were present at the beginning of the cycle will be doubled.

PCR gene targets that are frequently used for genus and species differentiation of *Campylobacter* spp. include: *ceuE*, *hipO*, *mapA*, *asp*, 16S rRNA, *lpxA*, and 23S rRNA (Fitzgerald et al., 2008). Real-time PCR targeting the *flaA* short variable region has been used to identify *C. jejuni* and *C. coli* strains isolated from broilers (Ridley et al., 2008). In an evaluation of 11 PCR assays, not a single PCR test identified all the *C. jejuni* strains examined (On and Jordan, 2003). Because none of these PCR assays was 100% specific nor sensitive, On and Jordan (2003) recommended using a polyphasic approach for *C. jejuni* identification by PCR. Lubeck et al. (2003) tested 26 combinations of primers for an international validation of a PCR that could rapidly, effectively, and selectively detect *C. jejuni*, *C. coli*, and *C. lari* in foods.

Molecular Epidemiology and Typing of *Campylobacter*

Molecular epidemiology has been defined as "the definition, identification, and tracking of relevant pathogen species, subspecies, strains, clones, and genes by means of molecular technology and evolutionary biology; and the evaluation of the impact of a pathogen's genetic diversity on its relevant medical properties" (Tibayrenc, 2009). Typing has been defined as "phenotypic and/or genetic analysis of bacterial isolates, below the species/subspecies level, performed in order to generate strain/clone-specific

fingerprints or data sets that can be used, for example, to detect or rule out cross-infections, elucidate bacterial transmission patterns and find reservoirs or sources of infections in humans" (Belkum et al., 2007).

Campylobacter species are found extensively throughout the environment and there are many possible sources of infection. Molecular epidemiology can be used to track the source of isolated *Campylobacter* colonizer strains from poultry by comparing molecular types to those of recovered isolates from the farm environment. Understanding of the epidemiology of *Campylobacter* infections remains incomplete even though research has provided insights (Hartnack et al., 2009). *Campylobacter* epidemiology in broiler production is yet to be entirely understood (Zweifel et al., 2008). Various molecular epidemiology results suggest that commercially reared chickens are colonized with one or two main strains of *Campylobacter* (Ridley et al., 2008).

Microbial typing allows for identification and differentiation of bacterial strains beneath the species level. According to On et al. (2008), there are three main difficulties in human campylobacteriosis source tracing: "the scale of the problem, the possible route or routes of infection, and the population biology of the major pathogenic *Campylobacter* species." Additionally, decisions concerning relatedness between isolates are made difficult due to genetic diversity (On et al., 2008). Molecular methods that have been used to type *Campylobacter* include: Pulsed Field Gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST), Amplified Fragment Length Polymorphism (AFLP) Profiling, *fla* (flagellin) typing, *fla*-restriction fragment length polymorphism, *fla*-short variable region (SVR) sequencing, REP-PCR, ERIC-PCR,

single nucleotide polymorphism (SNP) PCR, DNA microarrays and high resolution melt analysis (On et al., 2008).

Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis is generally considered one of the most discriminatory methods for typing bacteria (On et al., 2008). Distinctively, PFGE is considered the molecular typing "gold standard" for the majority of clinically relevant bacterial pathogens "because PFGE provides a sense of global chromosomal monitoring" (Goering, 2004). Genomic DNA is digested with a rare cutting restriction endonuclease that yields a small number of fragments. PFGE is able to separate high molecular weight DNA fragments; separation of 20 kb to 1,000 kb fragments can be achieved. The electric field orientation relative to the gel is changed and results in DNA molecules relaxing when the current is momentarily switched off and elongating when the electric field is reapplied (Peters, 2009). Contour-clamped homogenous electric field (CHEF) electrophoresis is the type of PFGE that is by far the most utilized (Goering, 2004). The comparability of PFGE results has considerably improved due to extensive acceptance worldwide of the CDC PulseNet protocol (On et al., 2008). During the 1980's and early 1990's, PFGE proved to be a highly discriminatory subtyping method that was valuable in defining foodborne pathogen-associated outbreaks throughout the world (Gerner-Smidt et al., 2008).

A standardized PulseNet PFGE protocol has been developed for *C. jejuni* (Ribot et al., 2001). In order to further standardize PFGE analysis, the size standard strain

Salmonella enterica subspecies *enterica* serovar Braenderup H9812 was introduced for common use (Hunter et al., 2005). For *C. jejuni* PFGE, the restriction enzyme *Sma*I, which usually yields 4 to 10 restriction fragments, has been primarily used (On et al., 2008). Additionally, *Kpn*I has been used for secondary enzyme analysis in a move to increase the discriminating depth of PFGE in situations where the primary enzyme fails to yield distinctive restriction patterns. For *C. jejuni*, the order of enzymes yielding highest to lowest restriction fragment numbers is usually *Kpn*I, *Bam*HI, *Sma*I, *Sal*I (On et al., 2008).

In 2003, the USDA Agricultural Research Service started USDA VetNet to combine antimicrobial resistance monitoring of bacterial isolates obtained from animals with PFGE molecular subtyping surveillance (Jackson et al., 2007). VetNet uses slaughter, processing, and diagnostic isolates that have been obtained by the National Antimicrobial Resistance Monitoring System (NARMS) animal division (Jackson et al., 2007). VetNet aims to utilize the PFGE data for surveillance and investigation of foodborne illness outbreaks (Jackson et al., 2007).

Currently, there is no established agreement of what constitutes a different PFGE pattern (On et al., 2008). PulseNet USA isolates are assigned a common pattern number, but other laboratories and networks use their own pattern assignment criteria and designations. In many instances, digestion with a single enzyme is adequate to show differences among isolates, but is insufficient to demonstrate similarity among isolates (On et al., 2008). On et al. (2008) argued that what is more important than the enhanced discrimination yielded by adding a second digestion with a different enzyme is the

improved confidence in interpretation that it allows. Results from a secondary enzyme digestion can provide a strong indication of a close clonal relationship. It has been stated that when the goal is to show that two isolates are genotypically indistinguishable, primary and secondary enzyme digestions should be carried out (On et al., 2008). Currently, PulseNet recommends its members "use *Kpn*I on isolates that are indistinguishable by *Sma*I before inferring strain relatedness between isolates" (Gerner-Smidt et al., 2008).

Additional problem areas inherent to the genomic character sometimes make PFGE data difficult to interpret. Results from PFGE do not yield phylogenetic information (Gerner-Smidt et al., 2008). This means that restriction fragments of the same size from different isolates do not necessarily originate from the same site on the genome. So isolates that have similar restriction patterns may not be as related as they appear. Even though it is considered the "gold standard", PFGE separation of similar sized bands remains hard to accomplish (Healy et al., 2005). Isolates exhibiting dissimilar restriction patterns may have a clonal relationship (On et al., 2008). Transformation, point mutation, and plasmid gain or loss can result in major or minor differences of PFGE patterns (On et al., 2008). Genetic instability can arise as a result of genomic rearrangements in *Campylobacter* that are colonizing the chick GI tract (Denis et al., 2008).

Subtyping isolates is necessary to relate human illness to a particular strain (Gormley et al., 2008). Each group of tetracycline resistant and tetracycline susceptible *C. jejuni* isolates from organically raised broilers displayed a distinct PFGE fingerprint

pattern (Luangtongkum et al., 2008). Fluoroquinolone (FQ)-resistant and FQ-susceptible *C. jejuni* isolates from chickens had identical PFGE fingerprint patterns (Luo et al., 2003).

The first use of PFGE to type *C. jejuni* and *C. coli* was reported in 1991 (Yan et al., 1991). Since these initial efforts, PFGE studies have been described for *C. concisus*, *C. fetus*, *C. hyointestinalis*, *C. upsaliensis*, and *C. sputorum* (On et al., 2008). The first *C. upsaliensis* PFGE study revealed genetic heterogeneity among human and animal isolates (Bourke et al., 1996). One study found that a predominant *C. jejuni* PFGE genotype was stable over the course of 10 rounds of *in vitro* subculturing and passage through the chicken GI tract (Wassenaar et al., 1998). Typing by PFGE was used to compare *Campylobacter* environmental and fecal isolates in order to evaluate the specificity of a real-time PCR method to detect environmental sources of *Campylobacter* strains which had colonized broiler flocks (Ridley et al., 2008).

Zweifel et al. (2008) found identical PFGE genotypes in consecutive broiler flocks and absence of these PFGE genotypes in the environments of four farms and concluded that this pointed to the presence of persistent contamination in these broiler houses of these farms. By using *Kpn*I, Normand et al. determined there were 39 *C. jejuni* PFGE genotypes present among post-evisceration carcass rinse and cecal content samples from 21 broiler flocks (2008). An additional finding was that carcass isolates from consecutively slaughtered flocks had common genotypes. Because *C. jejuni* genotypes from pooled ceca were similar to those of carcasses, Normand et al. (2008)

asserted that this result indicated "contamination effectively happened at the slaughterhouse."

Berrang et al. (2008) found that neither air chilling nor immersion chilling selected for different PFGE genotypes. Genotyping by PFGE was carried out on *Campylobacter* isolates from German poultry slaughterhouse carcasses to determine subtypes present and to show whether "predominant stable strains relevant for human infection were present" (Klein et al., 2007). In this study, *Sma*I and *Kpn*I were used as the primary and secondary enzymes, respectively.

The different PFGE restriction patterns of four epidemiologically related *C. jejuni* strains was found to be due to the presence, absence, or translocation of a temperate bacteriophage(s) (Hanninen et al., 1999).

Repetitive Sequence PCR-Based Microbial Typing

The REP (repetitive extragenic palindromic) sequence consists of 30-35 conserved bases and an extremely conserved inverted repeat (Stern et al., 1984). An inverted repeat refers to close by sequences in DNA which are the identical or nearly identical when read in the 5′ to 3′ direction on the opposite strands. *Escherchia coli* had 563 REP sequences in 295 clusters present throughout its genome (Lupski and Weinstock, 1992). A high degree of conservation is present among different bacterial species. These sequences could have a role in genome evolution (Gilson et al., 1984). In *E .coli*, these palindromic units are present for the most part in extragenic areas either individually or in clusters (Gilson et al., 1984). Additionally, REP sequences can be

present in up to four tandem copies, which are always inverted. Repetitive extragenic palindromic PCR was optimized for subtyping *Campylobacter* spp. from the following sources: chicken, mice, wild birds, a fly, pre- and post-transport crates, humans, ducks, hogs, production facility, a turkey, and a calf (Hiett et al., 2006). The repetitive extragenic palindromic PCR separated the isolates "into spatially and temporally epidemiological relevant groups" (Hiett et al., 2006).

The Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences are a second group of conserved repetitive DNA and can be found in several copies throughout many enterobacterial genomes (Hulton et al., 1991). The ERIC consensus sequence is 126 bp long. These ERIC sequences have been found in *E. coli*, *S. typhimurium*, *Yersinia pseudotuberculosis*, *Klebsiella pneumoniae*, and *Vibrio cholarae* (Hulton et al., 1991). Every one of the ERIC sequences contains the same central core inverted repeat and is situated external to an open reading frame. The position of ERIC sequences varies among species. In *E. coli* and *S. typhimurium*, ERIC copy numbers have been proposed to be about 30 and 150, respectively (Hulton et al., 1991).

The similarities between ERIC and REP elements are: location on the chromosome is variable among species, but is in non-coding transcribed areas, having a conserved inverted repeat, and can be present in each direction in respect to transcription (Hulton et al., 1991). Differences between REP and ERIC are: REP sequences are around 75% shorter than ERIC sequences, often several REP sequences are found in one area of a genome, while ERIC sequences only occur individually, and in *E. coli* the

number of REP sequences has been estimated to be ten times more than that of ERIC sequences (Hulton et al., 1991).

Sequences for REP and ERIC have been found in genomes of several prokaryotes (Versalovic et al., 1991). Bacterial genomic fingerprinting has been carried out by using PCR and consensus primers (outwardly directed) that specifically anneal to highly conserved REP and ERIC sequences (Versalovic et al., 1991). PCR amplification of DNA between consensus repetitive element primers repetitive elements generates amplicons that differ in base pair length. Species-specific REP amplification patterns have been observed. There are REP and ERIC primer sets used in PCR which have been validated to yield clear-cut species- and strain-specific genomic fingerprints (Versalovic et al., 1991). This study by Versalovic et al. (1991) showed that REP- and ERIC-like sequences were found mainly in Gram-negative enterics and their close relatives in the same phyla. Versalovic et al. (1991) provided the first demonstration of ERIC-like sequences in eubacteria through techniques other than computer-assisted DNA sequence analysis. In addition, this study by (Versalovic et al., 1991) provided "the first documented use of extragenic repetitive sequences to directly fingerprint bacterial genomes" . REP- and ERIC-PCR yield distinctive results among different bacterial species and strains that have these particular repetitive elements. Furthermore, differentiation and classification of highly related strains of *Rhizobium* can be accomplished through the use of REP- and ERIC- PCR (de Bruijn, 1992).

Repetitive element sequence-based PCR (rep-PCR) utilizes primers "matching the consensus sequence for REP and ERIC sequences, with the base inosine placed at the

nonconserved positions" (Lupski and Weinstock, 1992). Validation of the DiversiLab automated rep-PCR system for strain typing has been carried out successfully and convincingly showed its usefulness for molecular epidemiology (Healy et al., 2005). DiversiLab rep-PCR amplicons can range in size from 50 to 4000 bp. Microfluidic detection of rep-PCR products increased sensitivity 50 times compared to that of agarose gel electrophoresis. A National Center for Biotechnology Information (NCBI) PubMed database search using the term "DiversiLab" yielded 28 results. The DiversiLab automated rep-PCR system is discriminatory at the strain level for mycobacteria and *Aspergillus* and *Candida* spp. (Healy et al., 2005). The DiversiLab system is useful for *Aspergillus* subspecies discrimination (Hansen et al., 2008).

16S-23S Internal Transcribed Spacer Denaturing Gradient Gel Electrophoresis

The Internal Transcribed Spacer (ITS), also referred to as Internal Spacer Region (ISR) and the Intergenic Spacer Region (ISR), separates bacterial 16S and 23S ribosomal RNA genes. Its use for microheterogeneity assessment has been increasing (Stewart and Cavanaugh, 2007). Compared to other bacterial phylogenetic markers, "the ITS region experiences low selective constraint, evolves rapidly, and provides a high resolution estimate of gene flow and genetic structuring at the population scale" (Stewart and Cavanaugh, 2007).

Members of *Bacteria* and *Archaea* domains typically contain an rRNA (*rrn*) operon consisting of a 16S rRNA, a 23S rRNA, and a 5S rRNA gene as well as an ITS region and at least one tRNA gene (Acinas et al., 2004). The ITS region is more

variable than the neighboring 16S and 23S ribosomal RNA genes (Khan and Edge, 2007) and has been used to differentiate closely related microorganisms (Lee et al., 2009), as well as a target for PCR-identification of bacteria (Jensen et al., 1993; Christensen et al., 1999). *Campylobacter jejuni* strains 269.97, 81-176, 81116, NCTC 11168, and RM1221 each have three copies of the *rrn* operon (Lee et al., 2009). The *C. jejuni* ITS class is tRNA-alanine + tRNA- isoleucine (Stewart and Cavanaugh, 2007). Each of the three copies of *C. jejuni* strain NCTC 11168 16S-23S ISR is 806 bps.

It has been demonstrated that a multiplex PCR assay based on sequence variability in the 16S-23S ITS region could simultaneously identify and differentiate *C. jejuni*, *C. coli*, and *C. lari* (Khan and Edge, 2007). A PCR-ELISA method targeting the *Campylobacter* 16S-23S ISR for the detection and differentiation of *C. coli* and *C. jejuni* on broiler neck skin has been described (Grennan et al., 2001).

Strain differentiation based on 16S-23S ISR DGGE has been used to type environmental *E. coli* isolates (Buchan et al., 2001), the highly phylogenetically related microorganisms *Rhizobium leguminosarum*, *Rh. Tropici*, and *Agrobacterium rhizogenes* (Oliveira et al., 1999) as well as for characterization of environmental *Streptomyces* samples (Park and Ii, 2006). The principle of DNA separation in denaturing gradient gel electrophoresis (DGGE) is based on the electrophoretic movement of partly denatured DNA in polyacrylamide gels (Muyzer et al., 1993) containing chemical denaturants. Sequence differences will result in different melting domain temperatures. Nearly 100% of all potential sequence differences can be observed by adding a GC clamp to a sequence of interest (Muyzer et al., 1993).

Campylobacter Antimicrobial Resistance

In commercial poultry production, antimicrobial usage is for therapeutic treatment or for growth promotion. Antimicrobial resistance can be acquired, innate, or result from mutation. *Campylobacter jejuni* and *C. coli* resistance mechanisms include: production of antibiotic inactivating or modifying enzymes, antimicrobial target modification and protection, efflux transportation of antimicrobials out of the cell, and unique cell membrane structures decreasing permeability to antimicrobials (Zhang and Plummer, 2008).

The fluoroquinolones ciprofloxacin, enrofloxacin, and levofloxacin are synthetic compounds which exhibit strong bactericidal activity against Gram-positive and Gram-negative bacteria (Zhang and Plummer, 2008). Adding a fluorine atom to position 6 of the core quinolone molecule increases activity against Gram-negative bacteria and drug penetration into the bacterial cell (Bolon, 2009). The target in Gram-negative bacteria is DNA gyrase. Fluoroquinolone treatment of *Campylobacter*-colonized flocks leads to a rapid increase in numbers of FQ-resistant *Campylobacter* (Price et al., 2005). Using veterinary-specific FQs in chickens leads to a quick rise in *C. jejuni* ciprofloxacin minimum inhibitory concentrations (MICs) required to kill the microorganism (McDermott et al., 2002). *Campylobacter jejuni* exhibits a "hypermutable phenotype in infected chickens under the selection pressure of FQ antibiotics" (Luo et al., 2003). In *C. jejuni* and *C. coli*, genetic evidence has revealed CmeABC is a significant efflux pump involved in antimicrobial resistance and that the single point mutation Thr-86-Ile can make them resistant to FQs (Ge et al., 2005).

When antibiotic treatment for humans is indicated, macrolides are usually selected, although FQs are also frequently recommended. A rise in occurrence or frequency in macrolide and FQ resistance among *Campylobacter* from food animals has taken place (Aarestrup et al., 2008). The macrolides erythromycin, tylosin, spiramycin, tilmicosin, and roxithromycin are used for growth promotion, improving feed efficiency and therapeutic treatment in chickens (Lin et al., 2007; Zhang and Plummer, 2008). Transversion mutations in the 23S rRNA gene are primarily responsible for macrolide resistance in *C. jejuni* and *C. coli* (Gibreel et al., 2005).

In one study, all *in vivo*-selected mutants which exhibited high levels of erythromycin resistance had the A2074G mutation present in all three copies of the 23S rRNA gene (Lin et al., 2007). When a growth promoting dose of tylosin was fed to chickens experimentally infected with *C. jejuni*, macrolide-resistant *Campylobacter* were detected several weeks later (Lin et al., 2007). In comparison with *C. jejuni*, a greater percentage of *C. coli* strains show resistance to erythromycin (Brenner et al., 2005). Erythromycin resistance in *C. coli* ranges from 0 to 50% and in *C. jejuni* 0 to 12% (Fitzgerald et al., 2008). Macrolide resistance in food animals is usually higher in isolates from poultry and swine production locations (Aarestrup et al., 2008).

Aminoglycosides are widely used in human and animal medicine. The most commonly used aminoglycosides for therapeutic purposes are kanamycin, neomycin, gentamycin B, streptomycin, spectinomycin, and streptothricins. Most of the aminoglycoside modifying enzymes in *Campylobacter* are reported to be 3′-aminoglycoside phosphotransferases (APH (3′)) (Zhang and Plummer, 2008). The APH

(3′) type III gene has been identified in many different *Campylobacter* clinical isolates and can be found on transmissible plasmids or the chromosome. In *Campylobacter*, aminoglycoside resistance mechanisms are based on the inactivation of the antimicrobial through chemical modification.

Tetracycline is regarded as a second-line treatment for *Campylobacter* (Aarestrup et al., 2008). In *Campylobacter*, the mechanisms responsible for tetracycline resistance identified so far involve an efflux system and a ribosomal protection protein referred to as Tet(O) (Zhang and Plummer, 2008). The frequency of *C. jejuni* and *C. coli* tetracycline resistance has risen considerably throughout the world (Luangtongkum et al., 2008). Transfer of the *tet*(O) gene happens quickly and without antimicrobial selective pressure among *C. jejuni* strains in the GI tract of chickens (Avrain et al., 2004). A *C. jejuni* cell that has a conjugative plasmid containing the resistance gene *tet*(O) gene will only be transferred between *Campylobacter* species (Habib et al., 2009). Results strongly suggest that horizontal transfer of the *tet*(O) gene from Gram-positive bacteria to *Campylobacter* has occurred (Zhang and Plummer, 2008).

Not including carbapenems, most strains of *C. jejuni* and *C. coli* are resistant to β- lactam compounds. *Campylobacter jejuni* and *C. coli* porin proteins form pores that only permit the passage of solutes that have a molecular weight of 342 or less (Zhang and Plummer, 2008). In general, *Campylobacter* is less susceptible to β-lactams due to the fact that most of them have molecular weights larger than 360 (Zhang and Plummer, 2008).

In *Campylobacter*, CmeABC and CmeDEF are currently the only functionally characterized antimicrobial efflux pumps (Zhang and Plummer, 2008). It has been reported that "CmeABC contributes significantly to the intrinsic and acquired resistance of *Campylobacter* to structurally diverse antimicrobials" (Zhang and Plummer, 2008).

The US National Antimicrobial Resistance Monitoring System was established in 1996 "to prospectively monitor the occurrence of antimicrobial resistance of zoonotic pathogens from human diagnostic specimens, retail meats, and food animals." The NARMS animal division handles testing of isolates obtained from food-producing animals at slaughter, diagnostic animal specimens, and healthy on-farm animals. The antimicrobials chosen reflect antimicrobials commonly used in both veterinary and human medicine. Those screened antimicrobials are: azithromycin, ciprofloxacin, erythromycin, gentamycin, tetracycline, florfenicol, nalidixic acid, telithromycin, and clindamycin.

Many studies have shown that antimicrobial use will select for resistance, though this relation is not always linear (Aarestrup et al., 2008). In September 2005, the US banned FQ use in poultry. Data from NARMS showed that in 2005 the *Camplylobacter jejuni* ciprofloxacin resistance rate in broilers peaked at 15%. From 2005 to 2006, NARMS data showed that the *Campylobacter jejuni* ciprofloxacin resistance rate of broiler carcass rinse isolates dropped 41% down to 8.8% (USDA-ARS, 2008).

Berrang et al. (2008) showed that air-chilling did not select for different antimicrobial resistance in *Campylobacter* more than immersion chilling. *Campylobacter* isolates from immersion-chilled broilers had a higher nalidixic acid

resistance rate compared to air-chilled carcasses (Sanchez et al., 2002). Furthermore, *Campylobacter* air-chilled broiler isolates had a higher rate of tetracycline resistance than immersion-chilled broilers.

The current research project will involve a molecular survey and characterization of *Campylobacter* spp. isolated from commercial broiler production pre- and post-harvest sampling points. In addition, the degree of *Campylobacter* strain diversity will be evaluated through the use of specific molecular methods. Research objectives include determining if *Campylobacter* species trends exist at specific pre- and post-harvest stages during commercial broiler production and in patterns and prevalence of genomic fingerprints. Samples obtained will be representative of specific time points during grow-out cycles of two broiler flocks. Molecular identification by PCR will be used for typing to genus and species. Genomes of recovered isolates will be analyzed by PFGE and rep-PCR to examine trends in genotype distribution at pre- and post-harvest commercial production stages. The degree of correlation between PFGE and rep-PCR subtype groups and the numbers of isolates that fall into each group will be determined. Comparisons of speciation results and those from other studies reported in the literature will be evaluated. An additional objective is to investigate the usefulness of 16S-23S ISR DGGE as a *Campylobacter* molecular typing tool. Antimicrobial resistance profiles will be determined for all the *Campylobacter* isolates. An additional objective is to determine whether any isolate genotype group(s) has specific antimicrobial resistant characteristics. Furthermore, identification of areas where potential valuable intervention strategies might be implemented will be proposed.

CHAPTER III

MOLECULAR IDENTIFICATION, PULSED FIELD GEL ELECTROPHORESIS, AND DIVERSILAB® REPETITIVE ELEMENT (REP)-PCR TYPING OF *CAMPYLOBACTER* PRE- AND POST-HARVEST POULTRY ISOLATES

Background

The thermotolerant species *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus* form a distinct subcluster within the genus and cause gastroenteritis in humans. *Campylobacter jejuni* is a leading cause of acute bacterial gastroenteritis in the US as well as in other countries of the developed world (Allos, 2001). *Campylobacter jejuni* are found in the gastrointestinal tract of a wide range of animals. In poultry, it is a commensal microorganism and the highest numbers are found within the ceca.

It has been estimated that 76 million occurrences of foodborne illness occur annually in the US (DuPont, 2007). The vast majority of human *Campylobacter* infections are recognized as being foodborne (Jacobs-Reitsma, 2008). Consuming inadequately cooked poultry and drinking contaminated water or unpasteurized milk are risk factors for developing campylobacteriosis. The US Department of Agriculture Economic Research Service has calculated annual costs connected with camplyobacteriosis to be several billions of dollars (Chinivasagam et al., 2009).

Sources of *Campylobacter* during commercial broiler production include: environment, litter, water, other birds, cecal and fecal transmission, and insects (Jacobs-

Reitsma, 2008). Cross contamination can also take place during feed withdrawal, transit to the processing plant, and the slaughtering, defeathering, scalding, evisceration, and immersion chilling stages of commercial processing (Byrd and McKee, 2005).

Molecular epidemiology can be used to track the source of isolated *Campylobacter* colonizer strains from poultry by comparing molecular types to those of recovered isolates from the farm environment (Wagenaar et al., 2008). Microbial typing allows for identification and differentiation of bacterial strains beneath the species level. Molecular bacterial strain typing methods have evolved quickly over the past few decades. Pulsed Field Gel Electrophoresis (PFGE) is widely considered to be the "gold standard" of molecular subtyping (Olive and Bean, 1999). In addition, it is generally accepted as being one of the most discriminatory methods for typing *Campylobacter* (Ribot et al., 2001). A PFGE restriction pattern is representative of greater than 90% of the bacterial genome (Goering, 2004).

It has been demonstrated that repetitive element (rep)-PCR is a reproducible and discriminatory process for microbial strain typing and microbial ecology analysis (Hiett and Seal, 2009). The DIVERSILAB automated rep-PCR system has been successfully validated for strain typing and has also convincingly exhibited its usefulness for molecular epidemiology (Healy et al., 2005).

For each isolate, expectations are that the typing results will be more discriminatory when the results of each method are combined. Examination of whether the PFGE and rep-PCR fingerprint types cluster according to respective sample sources was assessed. Determination of whether or not the number of different rep-PCR types

corresponded to the number of PFGE genotypes was carried out. The presence of any trends in the data was determined. Clonality between isolates with similar PFGE restriction patterns needed to be confirmed so a secondary PFGE was carried out using *Kpn*I restricted genomic DNA.

Materials and Methods

Sample Collection

Five different commercial broiler flocks (designated as 1, 2, 3, 4, and 5) were followed from each of two rearing houses (designated as 25 and 26) throughout a 49-day grow-out period. Pre-harvest samples were previously collected at the hatchery (fluff) and rearing houses. Random sampling of cecal content, water, feed, and house litter had been previously carried out. Flock sampling was carried out on day 0 (placement of birds), 14, 28, and 42 of grow-out. Flock 4 pre-harvest sampling of cecal content, water, feed, and house litter was carried out on day 45 instead of day 42. Post-harvest sampling occurred on day 49 at a commercial poultry processing plant during the first processing run of the day. Ceca were aseptically excised for analysis of content. Carcass rinsates were collected at pre-evisceration, post-evisceration, and post-chill processing stages. Presumptive *Campylobacter* cells that had been previously collected were stored at -80° C in a mixture of sterile glycerol and sterile Butterfield's buffer (0.62 m*M* of KH_2PO_4, pH 7.2) that yielded a final glycerol concentration of 20% (v/v). The total number of samples of presumptive *Campylobacter* was 181 from the 5 flocks.

Pure Culture Preparation

The first step in preparing Campy-Cefex agar for culturing of *Campylobacter* was to mix and bring to a boil the following components: 43 g of Brucella Agar (C5301, Hardy Diagnostics, La Jolla, CA), 0.5 g of ferrous sulfate (F8048-250G, Sigma-Aldrich, St. Louis, MO), 0.2 g of sodium bisulfite (243973-5G, Sigma-Aldrich), 0.5 g of sodium pyruvate (P8574-25G, Sigma-Aldrich), and 950 ml of distilled deionized H_2O. This mixture was then autoclaved for 15 min at 121° C and 15 psi and subsequently cooled to 50° C in a water bath. Next, 50 ml of lysed horse blood cells (7233402, Lampire Biological Laboratories, Pipersville, PA), 0.3325 mL of 100 mg/mL of cefoperazone (C4292-5G, Sigma-Aldrich), and 1 mL of 200 mg/mL of cycloheximide (C7698-5G, Sigma-Aldrich) were added to the cooled agar and mixed. Molten agar was dispensed into sterile petri plates (08-757-13, Fisher Scientific, Houston, TX).

After quadrant-streaking the presumptive *Campylobacter* cells onto Campy-Cefex agar, petri plates were placed in ZipLoc plastic bags and filled with a gas mixture consisting of 5% oxygen, 10% carbon dioxide, and 85% nitrogen (BotCo, Bryan, TX). After microaerobic incubation for 48 h at 42°C, a single colony was picked with a sterile inoculating needle and inoculated into 5 mL of autoclaved Brucella Broth (C5311, Hardy Diagnostics, La Jolla, CA) and then incubated at 42°C for 24 h under microaerobic conditions as stated above. Pure culture seed stock was prepared for storage at -80°C by mixing 1.6 mL of overnight culture with 400 µl of sterile glycerol (49769, Fluka, Sigma-Aldrich, St. Louis, MO) in 2-mL sterile cryovial tubes (03-374-21, Fisher Scientific, Pittsburg, PA) to yield a final concentration of 20% (v/v) glycerol.

Working stocks were prepared in the same fashion and stored in separate cryovial storage boxes at -80 °C.

DNA Isolation and Quantification

To obtain genomic DNA for PCR, *Campylobacter* cells were scraped from Campy-Cefex plates and placed in sterile 1.5-mL lock-top microcentrifuge tubes (02-681-284, Fisher Scientific, Houston, TX) containing 250 µL of sterile TE (10 m*M* Tris-1mM EDTA, pH 8.0) (Sigma-Aldrich). After resuspension of the cells, the tubes were placed in a boiling water bath for 10 min to lyse the cell membranes. Lysed cells were placed on ice for 1 min and then centrifuged for 10 min at 8,000 rpm. Supernatants were transferred to 0.2-mL sterile PCR tubes and stored at -20°C until DNA concentrations could be measured.

A NanoDrop™ 1000 (ND-1000, Wilmington, DE) spectrophotometer was used to obtain DNA concentrations and purity by measuring absorbance at 260 nm and 280 nm. Two microliters of DNA-containing solution was used for each measurement. Each of the respective DNA-containing solutions was diluted in sterile TE to a concentration of 50 ng/µL.

Polymerase Chain Reaction

Molecular identification of isolates to genus and species level was conducted utilizing PCR. The *Campylobacter* genus PCR was carried out using a primer pair that generated an 816-bp amplicon. The sequences of the forward and reverse primers were

5′-GGATGACACTTTTCGGAGC-3′ and 5′-CATTGTAGCACGTGTGTC-3′, respectively (Linton et al., 1996). These primers anneal to complimentary bases present in the *Campylobacter* 16S ribosomal RNA (rRNA) gene.

Each PCR sample mixture consisted of 12.5 µL of 2x Jumpstart RedTaq Ready Mix (09802-100, Sigma-Aldrich), 0.4 µM each of forward and reverse primers (Integrated DNA Technologies, Coralville, IA), 0.5 µL of purified 10 mg/ml bovine serum albumin (B9001S, New England BioLabs Inc., Ipswich, MA), 8.0 µL of PCR water (17000-10, MoBio, San Diego, CA), and 2 µL (50 ng/µl) of genomic DNA template. A positive (previously identified *Campylobacter* field isolate) control and a no template control were prepared for each PCR. The genus PCR program consisted of the following steps: 1) 95.0° C for 3 min, 2) 94.0° C for 1 min, 3) 55.0° C for 30 s, 4) 72.0° C for 1 min, repeating a cycle consisting of steps two through four 39 more times, and a final extension step at 72.0° C for 5 min.

A multiplex PCR that can identify and differentiate *C. jejuni* and *C. coli* based on DNA sequence differences of a segment of the *ceuE* gene was carried out (Gonzalez et al., 1997). The *ceuE* gene codes for a portion of a siderophore protein, which is involved in iron transfer and is also a putative virulent determinant. The forward and reverse *C. jejuni* primer sequences were 5′-CCTGCTACGGTGAAAGTTTTGC-3′ and 5′-GATCTTTTTGTTTTGTGCTGC-3′, respectively, while the *C. coli* primer sequences were 5′- ATGAAAAAATATTTAGTTTTTGCA-3′ and 5′-ATTTTATTATTTGTAGCAGCG-3′, respectively (Gonzalez et al., 1997). These

primers anneal to complimentary bases within the *ceuE* gene. The expected length of the PCR product for *C. jejuni* was 793 bp and for *C. coli* 894 bp.

Each PCR sample mixture consisted of 12.5 µL of 2x Jumpstart RedTaq Ready Mix, 0.4 µM each of *C. jejuni* forward and reverse primers, 0.4 µM each of *C. coli* forward and reverse primers, 3.5 mM $MgCl_2$ (M-8787, Sigma-Aldrich), 0.5 µL of purified 10 mg/ml bovine serum albumin (New England BioLabs Inc.), 5.5 µL of PCR water, and 2 µL (50 ng/µL) of genomic DNA template. A *C. jejuni* and *C. coli* positive (previously identified field isolates) control and a no template control were prepared for each PCR. The multiplex PCR consisted of the following steps: 1) 95.0° C for 4 min, 2) 94.0° C for 1 min, 3) 55.0° C for 30 s, 4) 72.0° C for 1 min, repeating a cycle consisting of steps two through four 34 more times, and a final extension step at 72.0° C for 5 min.

Agarose Gel Electrophoresis

The PCR products were electrophoresed in precast 2% agarose E-Gels (G5018-02, Invitrogen, Carlsbad, CA) containing ethidium bromide. Two microliters of each PCR product sample was mixed with 18 µL of TE and added to its respective well. A 100 bp DNA molecular weight ladder (G2101, Promega, Madison, WI) was added to one well of each E-Gel. Electrophoresis settings were 25 min using an iBASE power supply (G6400, Invitrogen, La Jolla, CA) under pre-programmed setting number 1. Gels were then subjected to ultraviolet light exposure using an AlphaImager HP digital imaging system (92-13823-00, AlphaInnotech, San Leandro, CA) and digital images were captured.

Pulsed Field Gel Electrophoresis Plug Preparation

The Centers for Disease Control PulseNet USA "One Day Standardized Laboratory Protocol for Molecular Subtyping of *Campylobacter jejuni* by Pulsed Field Gel Electrophoresis (PFGE)" was followed (Ribot et al., 2001). First, a 10 µL loopful of each *C. jejuni* pure culture isolate was quadrant streaked onto Campy-Cefex agar and incubated for 24 h at 42° C under microaerobic conditions. Next, cells were suspended in 2-mL of sterile phosphate-buffered saline (BP399-4, Fisher Scientific, Pittsburg, PA). Cell suspension absorbances were measured at a wavelength of 610 nm using a NanoDrop 1000 spectrophotometer. Each cell suspension was adjusted to an absorbance value of 0.680.

To prepare the agarose plugs, 400 µL of cell suspension was mixed with 400 µL of 1% SeaKem Gold (50152, Lonza, Rockville, MD) molten agarose cooled to 50°C. The cell-agarose mixture was transferred to plug molds and allowed to solidify at 4°C. The agarose-embedded cells were lysed with *N*-Lauroylsarcosine sodium salt (L9150, Sigma-Aldrich, St. Louis, MO). Recombinant PCR grade proteinase K (03115836001, Roche Applied Science, Indianapolis, IN) was added to degrade proteins. Screened caps (170-3711, BioRad, Hercules, CA) were used to keep the plugs in the tubes when pouring out buffer.

Lysis buffer was discarded from each tube and 15 mL of preheated (54°C) sterile reagent grade water was added to each tube and then poured out. Next, 15 mL of preheated (54°C) sterile reagent grade water was added to each tube and they were placed for 15 minutes in a shaking water bath set at 54°C and 175 rpm. After water was

discarded, 15 mL of preheated (54°C) sterile TE was added to each tube and placed for 15 min in a shaking water bath set at 54°C and 175 rpm. This procedure was repeated two more times. After the TE from the final wash was discarded, 5 mL of room temperature sterile TE was added to each conical tube and the plugs were stored at 4°C until ready for the restriction digestion step.

For each sample, incubation with 40 Units of the restriction endonuclease *Sma*I (R0141S, New England BioLabs Inc., Ipswich, MA) in 1X NE Buffer 4 for 4 hours at 25° C was used to digest intact genomic DNA embedded in the agarose plug. Secondary endonuclease restriction was carried out on selected samples. Each agarose plug was incubated with 40 Units of *Kpn*I (R0142S, New England BioLabs) in 1X NE Buffer 1 for 4 hours at 37° C.

Pulsed Field Gel Electrophoresis

A 1% SeaKem Gold agarose gel was prepared with TE. A CHEF Mapper (170-3670, BioRad, Hercules, CA) was used to carry out PFGE. CHEF Mapper settings for *Sma*I digested DNA were selected as follows: auto algorithm, 50 kb – low MW, 400 kb – high MW, gradient of 6 V/cm, two-state included angle = 120°, and run time 18 h. The default initial switch time was 6.76 s and the final switch time was 35.38 s. For *Kpn*I digested DNA, the following settings were selected: two-state included angle = 120°, gradient of 6 V/cm, 18-h run time, initial switch time = 5.2 s, final switch time = 42.3 s and default values for all other settings. Once the electrophoresis was complete, the gel was transferred to a staining container, stained with 500 µL of 0.5 µg/mL

ethidium bromide (161-0433, BioRad, Hercules, CA) solution for 30 min, and then destained 90 min with slow agitation in reagent grade water. The water was changed every 20 min. The gel was then exposed to ultraviolet light and a digital image was captured using an AlphaImager HP digital imaging system.

PFGE Analysis

In order to normalize banding patterns for intragel and intergel comparisons restricted DNA from the reference strain *Salmonella enterica* subspecies *enterica* serovar Braenderup H9812 was uniformly applied to lane wells on every pulsed field gel. Comparison of the normalized PFGE macro-restriction banding patterns was based on the Dice Coefficient of Correlation. A dendrogram was created based on the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using Molecular Analyst Fingerprinting Software (170-7561, BioRad, Hercules, CA). Position tolerance is the variation where two bands are considered the same and was set at 1%. Optimization value is the allowance for movement of the whole pattern up and down a gel and was set at 0.5%. Band pattern comparisons were expressed as a percentage similarity coefficient (%SC).

DiversiLab® Repetitive Sequence-Based PCR

The rep-PCR strain typing procedure was carried out using the Diversilab Microbial Typing System (270700, bioMérieux, Inc, Durham, NC). The DiversiLab® *Campylobacter* Fingerprinting Kit (270607, bioMérieux Inc.) contained genus-specific

repetitive element primers. Genomic DNA was isolated and purified using a QIAamp DNA Mini Kit (51306, Qiagen, Valencia, CA). The purified DNA was quantified using a NanoDrop 1000 spectrophotometer. DNA solutions were each diluted to 50 ng/µL in AE Buffer (Tris-EDTA, pH 8.0; Qiagen).

Per the Diversilab protocol, the rep-PCR thermocycler program consisted of an initial denaturation at 94° C for 2 min, 35 repeated three-step cycles consisting of 94° C for 30 s, 55° C for 30 s, and 70° C for 90 s, followed by a final extension step at 70° C for 3 min and ultimately a holding step at 4°C at the ∞ setting. DiversiLab LabChip Kits (270670, bioMérieux Inc.) were used to separate the rep-PCR products by microfluidic electrophoresis in an Agilent 2100 Bioanalyzer. The rep-PCR fingerprints were analyzed using Diversilab version 3.4 software.

The DiversiLab rep-PCR reports were used in this study to understand the relationships between the *C. jejuni* isolates. The report included a dendrogram, interactive similarity matrix, scatterplot and individual sample graphs. The rep-PCR fingerprints were analyzed in a two step process using the Diversilab® software, version 3.4. The electropherograms that resulted from the microfluidic electrophoresis runs were transformed into virtual gel images. These banding patterns made up the fingerprints used for the comparison.

The actual similarity percentage among corresponding data points was calculated using the Pearson correlation. Next, the software calculated a correlation coefficient from the Pearson correlation and transformed it to a distance value. This distance value was then assembled into a distance matrix. In the next analysis step, a dendrogram was

made from the previously mentioned distance matrix through an iterative procedure using the unweighted pair group method with arithmetic mean (UPGMA).

The dendrogram gives a general view of the relationships between groups of samples. It may put a sample within or near a cluster due to its high average similarity to the cluster even though the sample may really be more related to another individual isolate not contained in the cluster.

The scatterplot shows a broad view of data clustering. The similarity matrix shows similarity percentages among each pair of samples in the report and explains the averaging that is observed in the dendrogram. Similarity matrices frequently provide more individual details about cluster relationships. Even though the dendrogram and similarity matrix reveal percent similarities between samples, final interpretation decisions will be based on the banding differences between the fingerprint patterns because percentages can change based on the number of samples compared.

Frequently, related sample groups appear in the same cluster. A cluster is a group of samples which are more similar to each other than any other isolates.

Results

Of the 181 presumptive *Campylobacter*-positive samples that had previously been stored at -80° C, only 46 of the cryopreserved samples contained viable cells that grew when incubated for these analyses. All 46 of the isolates were identified by PCR as *Campylobacter jejuni*. Electrophoresis in 2% agarose E-gels and subsequent UV

exposure revealed the presence of a 796-bp band, which is characteristic of the length of the expected *C. jejuni* PCR product.

Three distinct *Sma*I-digested PFGE genomic fingerprints were revealed among the 46 recovered *C. jejuni* isolates. These genotypes were arbitrarily designated as A, B, and C. Genotype A was most prevalent. To confirm the clonality of 39 of the genotype A isolates, a secondary PFGE analysis was conducted using the restriction endonuclease *Kpn*I. All 39 genotype A fingerprints were identical following *Kpn*I digestion, thus confirming the clonal relationship among these particular isolates. Genotypes B and C each only had one isolate exhibiting a distinct genomic fingerprint. A dendrogram of the representative PFGE fingerprints was created using UPGMA (Figure A-3). The Genotype A fingerprint had an 89%SC to the Genotype C fingerprint. Genotype B fingerprint had, on average, a 77.9%SC to samples in the Genotype A-C cluster.

Figure A-4 shows the pair-wise relationships of the Diversilab rep-PCR products. Evaluation of the virtual gel images dendrogram revealed that the 46 rep-PCR products grouped into seven clusters and two outliers. In 4 of the 7 clusters, the respective members of each of these were on average greater than 97% similar. The average similarity within cluster 1 was greater than 96.9%. The average similarity within cluster 6 was greater than 93.2%.

The average similarity within cluster 2 was greater than 97.2%. Within cluster 2, there were two distinct sub-clusters. The average similarity within sub-clusters 2a and 2b was greater than 97.4% and 97.8%, respectively. The average similarity within cluster 4 was greater than 97.6%. Within cluster 4, there were two distinct sub-clusters.

The average similarity within sub-clusters 4a and 4b was greater than 97.6% and 97.8%, respectively.

Cluster 3 only contained two samples and they were 98.6% similar. Cluster 5 only contained two samples and they were 98.8% similar. Cluster 7 only contained two samples and they were 94.4% similar. A higher percent similarity indicates that samples are more alike.

Samples in cluster 1 were, on average, 96% similar to samples in cluster 2. Samples in cluster 2 were, on average, 94.9% similar to samples in cluster 3. rep-PCR clustering by sample source was not observed. There were no relatedness trends observed for isolates recovered from the same source.

Discussion

In the current study, pre- and post-harvest isolates obtained from two commercial broiler flocks were examined for trends in *Campylobacter* species presence. Isolates were analyzed by Pulsed Field Gel Electrophoresis (PFGE) and repetitive element (rep-) PCR to examine trends in genotype and strain distribution at pre- and post-harvest stages.

Culturing and preserving environmentally stressed *Campylobacter* presents a challenge and, therefore, recovery from the rearing farm environment may be extremely insensitive (Wagenaar et al., 2008). A total 29.28% of the 181 presumptive *Campylobacter* samples originally isolated were able to be cultivated following storage. The low cultivable *Campylobacter* rate was surprising initially and several attempts were made to cultivate these samples. These repeated attempts never were successful. After

considering its fastidious nature and biochemical characteristics, there was not any concern about the recovery rate of these wild type microorganisms. The majority of recovered isolates came from post-harvest carcass rinsate samples.

In general, culture-independent methods are more specific and take less time to carry out than culture-dependent methods. Culture-dependent methods of identifying or characterizing *Campylobacter* have significant limitations (Hayden, 2004). The low discrimination of phenotypic tests limits the identification of *C. jejuni* and *C. coli* (Christensen et al., 1999). The hippurate hydrolysis test to phenotypically differentiate *C. jejuni* and *C. coli* can be problematic. *Campylobacter coli* are negative for this test and some strains of *C. jejuni* subspecies *jejuni* also produce a negative result. In the case of human infection, it is important to be able to differentiate *C. jejuni* from *C. coli* as the causative agent, because erythromycin treatment is not as effective against *C. coli* due to the trait that *C. coli* is more likely to be resistant to this antimicrobial (Grennan et al., 2001).

Difficulties associated with culture-based methods to differentiate these two species have been suitably addressed by highly discriminating molecular-based PCR techniques. An evaluation of 11 PCR assays has led to a polyphasic approach being recommended for *Campylobacter* identification (On and Jordan, 2003). In contrast, *ceuE* multiplex PCR results in the current study indicated that specificity and the targeting of a single gene were not issues of concern.

The finding that 100% of the isolates identified within these two commercial broiler flocks were *C. jejuni* was not surprising. Wagenaar et al. (2008) stated that in all

research studies of intensively reared broilers, *C. jejuni* was found to be the most common colonizer, while *C. coli* and other species were found at low levels. Out of 5,154 samples from 14 Swiss farms, 228 were identified as *C. jejuni* and 92 as *C. coli* by PCR (Zweifel et al., 2008). The ratio of *C. jejuni* to *C. coli* isolates found to be colonizing poultry varies between countries (Wagenaar et al., 2008).

Pulsed field gel electrophoresis is widely considered to be the "gold standard" of molecular subtyping due to its extensive applicability, high discriminating power, and epidemiologic concordance (Barrett et al., 2006). This molecular technique allowed greater than 90% of the *Campylobacter* genome to be analyzed. In the current study, genotyping of PFGE *Sma*I-digested genomic DNA revealed three different genomic fingerprints among the 46 recovered *C. jejuni* isolates. These genotypes were arbitrarily designated as A, B, and C. The genotype A fingerprints were indistinguishable and most prevalent (n= 44 or 95.7%). Genotypes B and C each were represented by one isolate (2.1%) and each exhibited unique genomic fingerprints. Each of the three genotypes had *Sma*I macrorestriction profiles consisting of nine bands. Based on molecular weight, the three genotype fingerprint patterns had seven restriction bands in common. Among 26 *C. jejuni* poultry isolates, DE Boer et al. (2000) reported fingerprint patterns of *Sma*I macrorestriction profiles consisting of 4, 5, 6, 7, 8, 9, and 10 bands. To confirm clonality among genotype A isolates, a secondary PFGE analysis using the restriction endonuclease *Kpn*I was conducted on 39 selected genotype A isolates. All 39 genotype A fingerprints were indistinguishable, thus confirming the clonal relationship among these particular isolates.

In general, the four types of mutations that can alter restriction sites are: insertions, deletions, single-base substitutions, and rearrangements (Goering, 2004). When an insertion occurs in a restriction site, it can result in its maintenance or disruption. Specific guidelines for interpreting restriction patterns have been proposed (Tenover et al., 1995).

The genotype C isolate had an insertion of DNA which did not disrupt the restriction site. This is evident from the only difference in the restriction profile between genotypes A and C is a loss of a band in genotype A and the addition of a higher molecular weight band in genotype C. The genotype B isolate appeared to have undergone a rearrangement of DNA which did not alter the number of the restriction sites. This is evident from the only difference in the restriction profile between fingerprints A and B is the loss two bands in fingerprint A and the addition of a higher molecular weight band and a lower molecular weight band in fingerprint B.

Simpson's Index of Diversity was determined to estimate *Campylobacter jejuni* genetic diversity (Denis et al., 2008).

$$\text{Simpson's Index of Diversity} = 1\text{-}\ \frac{1}{N(N-1)}\sum_{j=1}^{S} nj(nj-1)$$

N = number of isolates tested, S = number of different PFGE genotypes, and nj = number of isolates belonging to genotype$j$. The Simpson's Index of Diversity for this study was 0.086. This number corresponds to the probability that two individuals randomly selected from a sample will belong to different genotypes.

Currently, the biological explanation of rep-PCR peak and intensity differences is not as defined as it is with PFGE (bioMerieux, 2009). The combination of PFGE and DIVERSILAB rep-PCR provides highly discriminatory molecular typing results. The results provide practical epidemiological information that shows post-evisceration and post-chill stages are still important targets for intervention studies. The high occurrence of *C. jejuni* isolates exhibiting genotype A suggests it may differentially express certain gene(s) that enable this strain to more favorably survive under the different harsh environmental conditions it encountered during processing and production.

CHAPTER IV

ANTIMICROBIAL RESISTANCE PROFILING OF PRE- AND POST-HARVEST *Campylobacter jejuni* ISOLATES

Background

The foodborne pathogen *Campylobacter jejuni* remains one of the most common causes of acute bacterial diarrhea throughout the entire world (Allos, 2001). *Campylobacter jejuni* are microaerobic and found in the gastrointestinal tract of a wide range of animals. For 2008, preliminary US Foodborne Diseases Active Surveillance Food Network (FoodNet) data has shown that the *Campylobacter* incidence of infection, 12.68 per 100,000 of the US population, is the second highest, only behind *Salmonella* at 16.20 per 100,000 (CDC, 2009). In the US, it's estimated that *Campylobacter* causes 2 million illnesses each year (Ailes et al., 2008). In terms of diagnosed bacterial diarrheal infections, greater than 95% are caused by *C. jejuni* (Aarestrup et al., 2008).

The vast majority of human *Campylobacter* infections are recognized as being foodborne (Jacobs-Reitsma, 2008). Food that originates from poultry is considered to be the primary source of human infections due to *Campylobacter* spp. (Aarestrup et al., 2008). It is evident how the potential impact of increased antimicrobial resistance could affect humans being treated for campylobacteriosis.

In the United States, over 50% of the antibiotics manufactured are used for agricultural purposes (Lipsitch et al., 2002). In commercial poultry production, antimicrobial administration is performed for therapeutic treatment or growth promotion.

In regards to human health, a rise in *Campylobacter jejuni* antimicrobial resistance is cause for concern (Aarestrup et al., 2008). Antimicrobial resistance can be acquired, innate or result from mutation. Because the availability of helpful antimicrobials is necessary for human health needs, measures for usage in animals should be taken in order for human health not to be negatively compromised (Aarestrup et al., 2008). The procedures for antimicrobial usage in animals would necessitate "continuously monitoring antimicrobial resistance, creating models to analyze risks associated with different selection pressures, and assessing the public health impact of resistant *Campylobacter* infections" (Aarestrup et al., 2008)

Two different broiler flocks in the current study were followed from each of two rearing houses throughout the grow-out period. Bacitracin was used as a growth promoter and administered in the broilers' feed. Pre-harvest sampling took place at the hatchery and at the two commercial rearing houses. Post-harvest sampling occurred on day 49 at the poultry processing plant during the first processing run of the day. The objective of this study was to determine antimicrobial resistance profiles for all the *Campylobacter jejuni* isolates obtained from specific commercial broiler production pre- and post-harvest sampling points. Additionally, comparison of antimicrobial resistance results between pre- and post-harvest samples to assess potential trends was carried out.

Materials and Methods

Sample Collection

Samples had been obtained from the same two rearing houses during five successive broiler flock grow-out cycles, each of which lasted 49 days. Pre-harvest sampling occurred on days 0, 14, 28, 42 and post-harvest on day 49. Day 0 represents the day of hatch. On day 49, poultry processing plant sampling occurred during the first run of the day. Random pre-harvest samples were taken from cecal content, water, feed, house litter, and live bird. Post-harvest samples were collected at the processing plant at pre-evisceration, post-evisceration, and as post-chill carcass rinsates. The previously identified *Campylobacter jejuni* isolates were stored at-80° C as pure cultures and were subsequently thawed in a 42° C incubator.

Antimicrobial Susceptibility Testing

Sensititre 96-well susceptibility plates for *Campylobacter* (CAMPY, TREK Diagnostic Systems, Cleveland, Ohio) were used to conduct the antimicrobial resistance studies. The antimicrobials (respective Clinical and Laboratories Institute antibiotic subclass names are listed in parentheses) dosed on the plate were: azithromycin (macrolide), ciprofloxacin (fluoroquinolone), erythromycin (macrolide), gentamycin (aminoglycoside), tetracycline, florfenicol (phenicol), nalidixic acid (quinolone), telithromycin (ketolide), and clindamycin (lincosamide). All dilution units are in micrograms per milliliter (µg/mL).

For azithromycin and ciprofloxacin, the dilution concentrations for testing were: 0.015, 0.03, 0.06, 0.12, 0.25, 0.5, 1, 2, 4, 8, 16, 32, and 64. For erythromycin and florfenicol testing, the dilution concentrations were: 0.03, 0.06, 0.12, 0.25, 0.5, 1, 2, 4, 8, 16, 32, and 64. For gentamycin, the dilution concentrations were: 0.12, 0.25, 0.5, 1, 2, 4, 8, 16, and 32. For tetracycline, the dilution concentrations were: 0.06, 0.12, 0.25, 0.5, 1, 2, 4, 8, 16, 32, and 64. For nalidixic acid, the dilution concentrations were: 4, 8, 16, 32, and 64. For telithromycin, the dilution concentrations were: 0.015, 0.03, 0.06, 0.12, 0.25, 0.5, 1, 2, 4, and 8. The clindamycin dilution concentrations were: 0.03, 0.06, 0.12, 0.25, 0.5, 1, 2, 4, 8, and 16.

For each thawed pure culture isolate, a 10-µL loopful of *Campylobacter* cells was streaked for colony isolation on Campy-Cefex agar. The plates were incubated at 42° C for 24 h in a microaerobic environment made up of 85% N_2, 10 % CO_2, and 5% O_2. From each sample plate, several colonies were picked and placed into respective tubes containing 5 mL of Sensititre cation-adjusted Mueller-Hinton broth with TES (T3462-5mL, TREK Diagnostic Systems, Cleveland, Ohio). Using the Sensititre nephelometer (V3011, TREK Diagnostic Systems, Cleveland, Ohio), cell suspensions were adjusted to a 0.5 McFarland standard and mixed well. Next, 100 µL of each cell suspension was transferred into a tube containing 11 mL of cation-adjusted Mueller-Hinton broth with TES + Lysed Horse Blood (CP112, TREK Diagnostic Systems, Cleveland, Ohio) to yield inoculum of 5×10^5 cfu/mL. One-hundred microliters of the inoculum was added to each well using the Sensititre AutoInoculator (V3010, TREK Diagnostic Systems, Cleveland, Ohio). The inoculated plates were incubated at 42° C

for 24 h under microaerobic conditions as stated above. Minimum inhibitory concentrations (MICs) were determined using a Sensititre SensiTouch plate reader (TREK Diagnostic Systems, Cleveland, Ohio).

Results and Discussion

Turbidity or a clump of cells appearing at the bottom of a well indicated cell growth. For each 96-well plate analyzed, the positive control well displayed visible cell growth and as such the experimental results observed in all wells were considered valid. Each of the *Campylobacter jejuni* isolates was not resistant to any of the nine antimicrobials tested. The erythromycin MICs of all the tested isolates indicated intermediate susceptibility. The ratio of isolates having MICs indicating intermediate susceptibility to florfenicol was 29 of 50 (58%). The clindamycin MIC for sample 151(drag swab on day 42) indicated intermediate susceptibility. All of the isolates had MICs which indicated susceptibility to azithromycin, ciproflaxicin, gentamycin, nalidixic acid, telithromycin, and tetracycline.

This study demonstrated that numerous *Campylobacter jejuni* multidrug nonresistant isolates can be recovered from a wide variety of commercial poultry production environments from the same rearing farm houses and processing plant over the course of several months. The antimicrobial resistance results of the pre-harvest isolates were not indicative of what can be found in the literature. The manually read results were interpreted according to the "2007 NARMS Veterinary Isolates Final Report- Slaughter Isolates" and the guidelines of the Clinical and Laboratories Standards

Institute (CLSI) (USDA-ARS, 2008). The MIC is designated as the lowest antimicrobial concentration which inhibits visible growth.

The NARMS animal division is responsible for the testing of isolates obtained from food-producing animals at slaughter, diagnostic animal specimens, and healthy on-farm animals. The group of antimicrobials selected for NARMS antimicrobial resistance testing is indicative of those commonly used in both veterinary and human medicine. In 2005, the micro-broth dilution system began to be used by NARMS. Resistance results obtained from using this system are available for 2005, 2006, and 2007. In 2005, 2006 and 2007, *Campylobacter jejuni* isolates from poultry carcass rinsates accounted for 59.9% (n = 567), 65.0% (n = 228), and 68.6% (n = 166), respectively, of *Campylobacter* species identified among the NARMS slaughter isolates. In 2005, 2006 and 2007, *Campylobacter coli* isolates from poultry carcass rinsates accounted for 40.1% (n = 380), 35.0% (n = 123), and 31.4% (n = 76), respectively, of *Campylobacter* species identified among the NARMS slaughter isolates. In comparison, 100% (n = 50) of the isolates from poultry carcass rinsates obtained in this study were identified by PCR as *Campylobacter jejuni* and 70% (n = 35) of the isolates tested were obtained from carcass rinsates.

The tetracycline resistance gene is mainly encoded on a plasmid, but can be chromosomally encoded in some *Campylobacter jejuni* strains. The loss of a plasmid due to extended storage time at -20° C and -80° C (Calcott, 1986) prior to preparing pure cultures for resistance testing may have been a contributing factor to the 0% tetracycline resistance rate observed in this study.

None of the 2006 and 2007 NARMS *Campylobacter jejuni* slaughter isolates were resistant to florfenicol. This is in exact agreement with the florfenicol results of the current study. In 2007, none of the NARMS *Campylobacter jejuni* slaughter isolates were resistant to gentamycin, clindamycin, azithromycin, telithromycin, and erythromycin. The resistance rates for 2007 NARMS *Campylobacter jejuni* against ciprofloxacin and nalidixic acid were each 21.7% and tetracycline resistance was 56.6%. No antimicrobial resistance was detected in 46.9% (2005), 39.9% (2006), and 34.3% (2007) of NARMS *Campylobacter jejuni* isolates, respectively.

All of the isolates in this current study displayed intermediate susceptibility to erythromycin. In contrast, the NARMS slaughter isolates from 2005, 2006, and 2007 did not exhibit intermediate susceptibility to erythromycin. Reported erythromycin resistance in *Campylobacter jejuni* has been shown to range from 0% to 12% (Fitzgerald et al., 2008).

The finding of no resistance to any of the antimicrobials for all isolates was somewhat surprising. Given the variety of sources of the isolates and the number of antimicrobials tested, the expectation would be to find some level of antimicrobial resistance. But considering there wasn't any antimicrobial selective pressure other than bacitracin exposure during the broiler rearing, the total lack of resistance is easily understood.

CHAPTER V

EVALUATION OF 16S-23S INTERNAL SPACER REGION DENATURING GRADIENT GEL ELECTROPHORESIS FOR THE MOLECULAR TYPING OF *Campylobacter jejuni*

Background

Molecular-based methods for bacterial identification and typing are more sensitive and specific than traditional culture-based methods. The Internal Transcribed Spacer (ITS), also referred to as the Internal Spacer Region (ISR), separates bacterial 16S and 23S ribosomal RNA genes. Its use for microheterogeneity assessment has been increasing (Stewart and Cavanaugh, 2007). Compared to other bacterial phylogenetic markers, "the ITS region experiences low selective constraint, evolves rapidly, and provides a high resolution estimate of gene flow and genetic structuring at the population scale" (Stewart and Cavanaugh, 2007). The ITS region has been used to differentiate closely related microorganisms (Lee et al., 2009).

Members of *Bacteria* and *Archaea* domains typically contain an rRNA operon consisting of a 16S rRNA, a 23S rRNA, and a 5S rRNA gene as well as the ITS and at least one tRNA gene (Acinas et al., 2004). The number of rRNA operons in *Bacteria* and *Archaea* varies from 1 to 15 per genome (Lee et al., 2009). *Campylobacter jejuni* possesses three copies of the *rrn* operon per genome.

In parallel Denaturing Gradient Electrophoresis (DGGE), the linear gradient is parallel to the electrophoresis direction. The degree of amplicon separation during

DGGE is dependent on a double stranded DNA molecule's nucleotide sequence and relative GC content. Parallel DGGE is highly sensitive and has mutation detection rates near 100%. Attempts have been made to use the 16S-23S ITS DGGE method to differentiate strains of environmental *E. coli* isolates (Buchan et al., 2001). Rapid identification of *Salmonella* serovars by PCR amplification of the 16S-23S ITS has also been described (Bakshi et al., 2002).

Campylobacter epidemiology in broiler production is yet to be entirely understood (Zweifel et al., 2008). Various molecular epidemiology results suggest that commercially reared chickens are colonized with one or two main strains of *Campylobacter* (Ridley et al., 2008).

The objective of this current study was to investigate the potential of 16S-23S ITS DGGE to be used as a molecular typing comparison tool for the foodborne pathogen *Campylobacter jejuni*. The molecular typing evaluation was conducted using isolates that were obtained from two consecutive commercial broiler flocks, which spanned several months.

Materials and Methods

16S-23S Internal Spacer Region Polymerase Chain Reaction

All the isolates (n = 53) used in this study were previously identified by PCR as *Campylobacter jejuni*. The forward and reverse primer sequences that were used in amplification of the *Campylobacter jejuni* 16S-23S ITS were 5′-CTAGAGTACAAACTAATAAGTCTC-3′ and 5′-ATTCTAAAACGCATCTCCTTG-

3′, respectively (Khan and Edge, 2007). These primers are specific for conserved DNA regions which flank the 16S-23S internal transcribed spacer region of the genus *Campylobacter* (Khan and Edge, 2007). For DGGE PCR, the forward primer was synthesized with a GC clamp attached to its 5′ end (Muyzer et al., 1993). The sequence of the GC clamp was 5′-CGCCCGCCGCGCGCGGCGGGCGGIGCGGIGGCACGGGIGG- 3′. The forward primer was HPLC-purified and the reverse primer was PAGE-purified. Primers were synthesized and purified by Integrated DNA Technologies (Coralville, IA).

Initially a gradient PCR was run to optimize the annealing temperature. The annealing temperatures (° C) tested were: 56, 56.2, 56.5, 57, 57.7, 58.6, 59.6, 60.5, 61.1, 61.6, 61.9, and 62. Based on these results, another gradient PCR with different annealing temperatures was run. The temperatures (° C) were: 62, 62.2, 62.5, 63, 63.7, 64.6, 65.6, 66.5, 67.1, 67.6, 67.9, and 68.0.

All PCRs were run using a PTC-100 thermocycler (MJ Research, Boston, MA). The reaction components consisted of 12.5 µL of 2x Jumpstart RedTaq Ready Mix (Sigma-Aldrich), 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 0.5 µL of 10 mg/mL of purified bovine serum albumin (New England BioLabs), 8 µL of PCR-certified H_2O (MoBio), and 2 µL of genomic template DNA (50 ng/µL). The PCR program that was run consisted of the following steps: 1) 94.0° C for 3 min, 2) 94.0° C for 1 min, 3) 63.7° C for 1 min, 4) 72.0° C for 1 min, 5) Go to step 2, 39 times, 6) 72.0° C for 5 min, 7) hold at 4.0° C at the ∞ setting, 8) end. Amplicons were electrophoresed using 2% agarose E-gels (Invitrogen) for 25 min using an iBASE power supply

(Invitrogen). E-gels were exposed to UV light and a digital image of the gel was captured.

Denaturing Gradient Gel Preparation

A 100% denaturant solution consists of 7 M urea and 40% deionized formamide. One-hundred forty mL of 50x TAE buffer (2 M Tris base, 1 M glacial acetic acid, 50 mM EDTA, pH 8.0) was mixed with 6,860 mL of deionized H_2O for use as running buffer. A 40% acrylamide/bis (37.5:1) (161-0148, BioRad, Hercules, CA) solution is made by combining 38.93 g acrylamide and 1.07 g bis-acrylamide, and then mixing with deionized H_2O to a volume of 100 mL.

A 6% acrylamide solution containing 35% denaturants was prepared by mixing 15 mL of 40% acrylamide/bis, 2 mL of 50x TAE, 14 mL of deionized formamide, (F9037-100ML, Sigma-Aldrich, St. Louis, MO) and 14.7 g of urea (161-0731, BioRad, Hercules, CA) and bringing the volume to 100 ml with deionized H_2O. Preparation of the 45% denaturing solution is the same except 18 mL of deionized formamide and 18.9 g of urea are used instead. Using a vacuum pump, these solutions were degassed in order to remove dissolved oxygen and then filter-sterilized through a 0.45 μ*M* syringe filter. A final solution concentration of 0.9% ammonium persulfate (161-0700, BioRad, Hercules, CA) and 0.09% TEMED (161-0801, BioRad, Hercules, CA) for each denaturant solution was used to initiate and catalyze the polymerization reaction. A 1-mm thick polyacrylamide denaturing gel having a 35% to 45% linear denaturing gradient was cast. Next, a 4% stacking gel was cast after polymerization of the 6% gel.

16S-23S Internal Spacer Region Denaturing Gradient Gel Electrophoresis

Separation by DGGE was carried out using the BioRad DCode™ Universal Mutation Detection System (170-9080, BioRad, Hercules, CA). The PCR products were electrophoresed for 17 h at 60 V in 7 L of 1xTAE running buffer at a constant temperature of 59° C.

After DGGE was complete, the double stranded DNA-binding dye SYBR Green I (S7567, Invitrogen, Carlsbad, CA) was diluted 1:10,000 in TAE and the gels were stained for 15 min on an orbital shaker. Next, an AlphaInnotech HP digital imaging device was used to capture an image of the stained gels as they were exposed to UV light.

Results and Discussion

Campylobacter jejuni possesses three copies of the *rrn* operon. The primers used in this study anneal to DNA at conserved positions that flank the entire 16S-23S ITS. All 46 of the *C. jejuni* samples yielded the expected 840-bp amplicon characteristic of the *Campylobacter jejuni* 16S-23S ITS as reported by Khan et al. (2007). Standard agarose gel electrophoresis separates molecules based on molecular weight, while DGGE separates the same-sized DGGE amplicons based on GC content and distinctive melting domains within the denaturing gel. All 46 of the *Campylobacter jejuni* 16S-23S ITS PCR products exhibited the same banding pattern when electrophoresed in DGGE gels. The pattern consisted of two distinct bands spaced fairly close together near the top of the gel.

The 16S-23S ISR DNA sequence length is the same in each of the three copies of the respective published *C. jejuni* genomes. However, *C. jejuni* ITS lengths differ between the published strains. The results of the current study show that all the *C. jejuni* isolates exhibited the same two band DGGE profile. The two bands were slightly separated and only migrated a short distance into the denaturing gel. This limited migration into the denaturing gradient indicated that the amplicons, without the GC clamp, had a high AT base composition. As indicated by these results, additional studies utilizing different defined *C. jejuni* strains (such as American Type Culture Collection strains) would need to be carried out before additional assessments could be made of 16S-23S ITS DGGE. Additionally, DNA sequencing of the DGGE separated ITS amplicons to determine what DNA sequence differences exist, if any, would be very useful. Since DGGE separation is dependent on DNA nucleotide sequence differences and GC content of the amplicons, the use of a proofreading thermostable DNA polymerase instead of *Taq* DNA polymerase may improve typing results.

CHAPTER VI

CONCLUSION

The low cultivable rate of 25.4% can be possibly explained by the fact that *Campylobacter jejuni* is a fastidious microorganism. In addition, *Campylobacter* is sensitive to oxygen concentrations above 10% and to dry environments. All isolates in the current study were identified by PCR as *Campylobacter jejuni*. Three distinct genotypes were identified by PFGE. The combination of PFGE and Diversilab repetitive element PCR provided highly discriminatory molecular typing results. The majority of recovered cultivable isolates originated from post-harvest carcass rinsates (30 of 46). Clustering of rep-PCR data by sample source was not observed. There were no relatedness trends observed for isolates recovered from the same source.

The finding that 100% of the isolates were not resistant to any of the nine antimicrobials was unexpected. This research study demonstrated that several *Campylobacter jejuni* multidrug nonresistant isolates can be cultivated from a wide selection of commercial poultry production environments from the same rearing farm houses and processing plant over the course of several months. From a human perspective, no resistance is welcome information. The antimicrobial resistance research findings provide some evidence that challenges the widespread view that antimicrobial usage during intensive poultry production will lead to or increase antimicrobial resistance in *Campylobacter jejuni*.

The effectiveness of 16S-23S Internal Transcribed Region DGGE method for molecular typing of *Campylobacter jejuni* remains unclear. After evaluation of the results, the data is neither supportive nor non-supportive for the use of this method for the molecular strain typing of *Campylobacter jejuni*. Additional DGGE studies utilizing various defined *C. jejuni* strains (such as the American Type Culture Collection strains) would need to be carried out before additional evaluations could be made about the effectiveness of 16S-23S ITS DGGE typing.

The results provide practical epidemiological information that shows post-evisceration and post-chill stages are still important targets for intervention studies. The high occurrence of *C. jejuni* isolates exhibiting PFGE genotype A suggests it may differentially express certain gene(s) that enable this strain to more favorably survive under the different harsh environmental conditions it encountered during processing and production.

Overall, these *C. jejuni* commercial broiler pre- and post-harvest isolates exhibited an extremely low degree of molecular and phenotypic variability. The paradox of *Campylobacter* being able to survive in areas where, based on its intrinsic characteristics, it should not be surviving is quite amazing. Gene expression studies on chill and scald tank water along with carcass rinsates would be a possible next step in attempting to understand *Campylobacter* survivability during processing.

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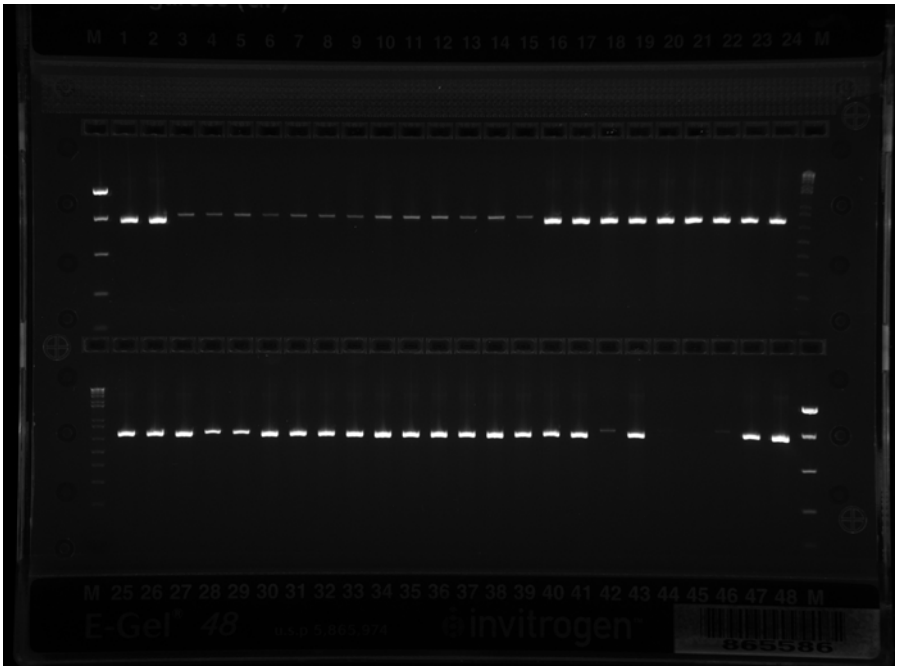
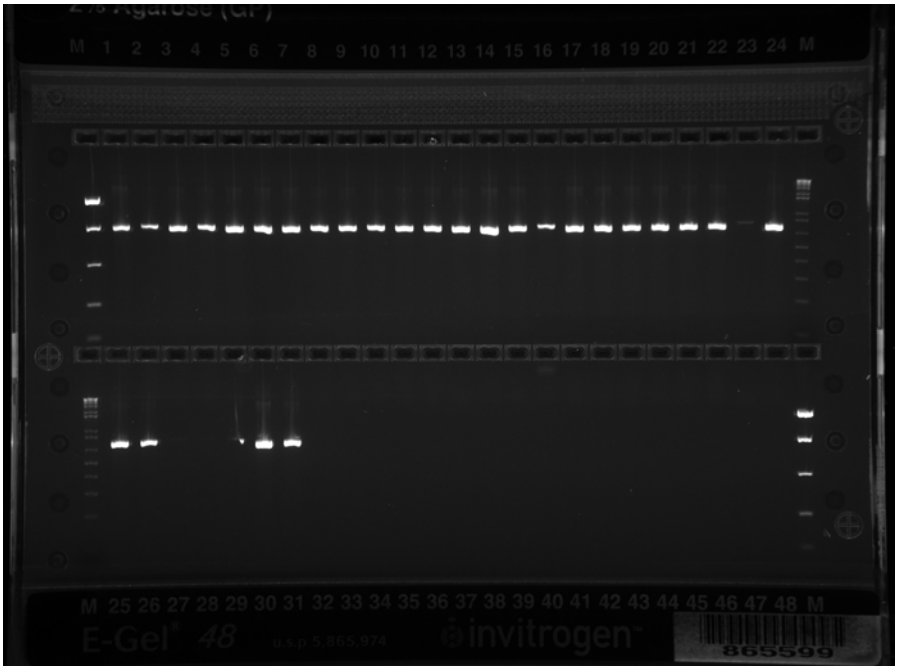
APPENDIX A

Figure A-1 Electrophoresis results of *Campylobacter* genus PCR amplicons in 2% agarose gels. The top row left and bottom row right lanes labeled as "M" in gels (a) & (b) represent a 100 bp DNA molecular weight ladder. A low range quantitative DNA ladder is represented by the "M" lanes on the top row right and bottom row left lanes of gels (a) & (b). Isolate number and (lane) in gel (a) were as follows: *C. jejuni* positive control (1), *C. coli* positive control (2), 125 (16), 126 (17), 127 (18), 128 (19), 129 (20), 136 (21), 132 (22), 133 (23), 133A (24), 134 (25), 134a (26), 135 (27), 135A (28), 136 (29), 137 (30), 138 (31), 139 (32), 140 (33), 140A (34), 141 (35), 142 (36), 143 (37), 144 (38), 145 (39), 146 (40), 147 (41), 148 (42), 150 (43), 151 (44), 152 (45), 152a (46), *C. jejuni* positive control (47), and *C. coli* positive control (48). Isolate number and (lane) in gel (b) were as follows: *C. jejuni* positive control (1), *C. coli* positive control (2), 152A (3), 153 (4), B26153A (5), 154 (6), 159 (7), 160 (8), 161 (9), 162 (10), 163 (11), 167 (12), 169 (13), 170 (14), 171 (15), 171a (16), 173 (17), 174 (18), 175 (19), 176 (20), 177 (21), 178 (22), 179 (23), 180 (24), 181 (25), *C. jejuni* positive control (30), and *C. coli* positive control (31).


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 M
M 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 M
E-Gel 48
invitrogen
865586

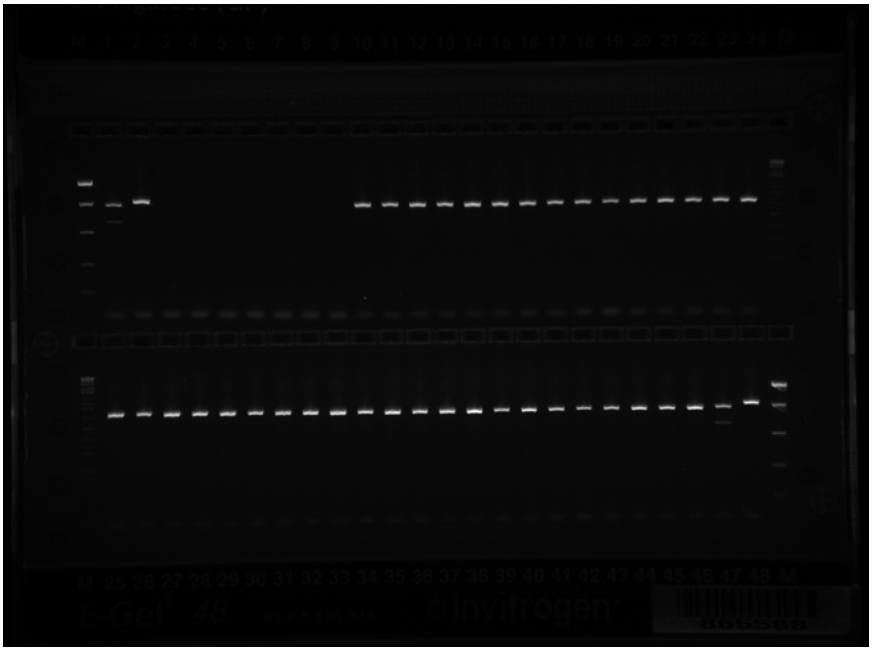
(a)


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 M
M 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 M
E-Gel 48
invitrogen
865599

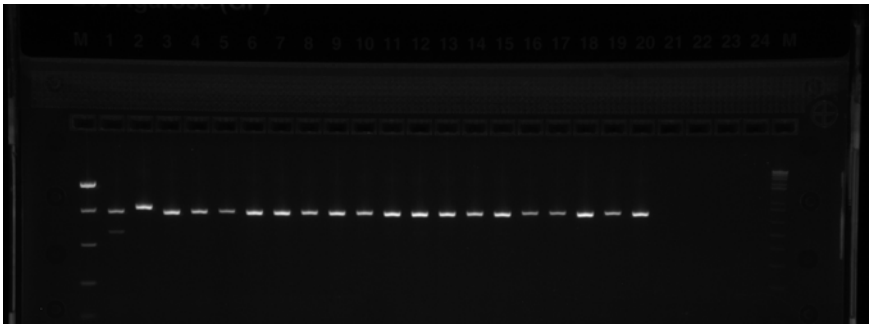
(b)

Figure A-1

Figure A-2 Electrophoresis results of *Campylobacter ceuE* multiplex species PCR amplicons in 2% agarose gels. The top row left and bottom row right lanes labeled as "M" in gels (a) & (b) represent a low range quantitative DNA ladder. A 100 bp DNA molecular weight ladder is represented by the "M" lanes on the top row right and bottom row left lanes of gels (a) & (b). Isolate number and (lane) in gel (a) were as follows: *C. jejuni* positive control (1), *C. coli* positive control (2), 125 (10), 126 (11), 127 (12), 128 (13), 129 (14), 136 (15), 132 (16), 133 (17), 133A (18), 134 (19), 134a (20), 135 (21), 135A (22), 136 (23), 137 (24), 138 (25), 139 (26), 140 (27), 140A (28), 141 (29), 142 (30), 143 (31), 144 (32), 145 (33), 146 (34), 147 (35), 148 (36), 150 (37), 151 (38), 152 (39), 152a (40), 152A (41), 153 (42), B26153A (43), 154 (44), 159 (45), 160 (46), *C. jejuni* positive control (47), and *C. coli* positive control (48). Isolate number and (lane) in gel (b) were as follows: *C. jejuni* positive control (1), *C. coli* positive control (2), 161 (3), 162 (4), 163 (5), 167 (6), 169 (7), 170 (8), 171 (9), 171a (10), 173 (11), 174 (12), 175 (13), 176 (14), 177 (15), 178 (16), 179 (17), 180 (18), 181 (19), and 150 (20).



(a)



(b)

Figure A-2

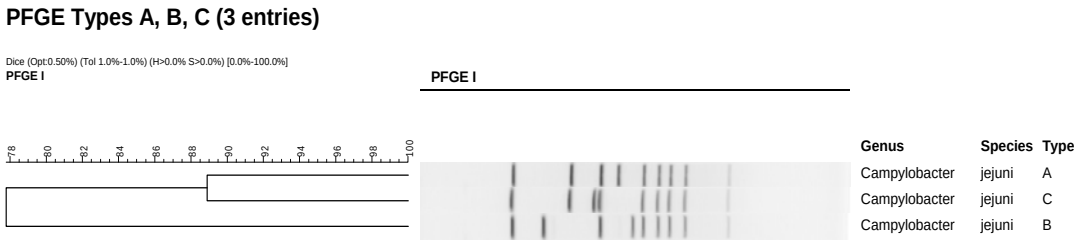


Figure A-3 Dendrogram of PFGE representative fingerprints for *Campylobacter jejuni* genotypes A, B, and C. The horizontal bar indicates percentage similarity coefficient (%SC).

Figure A-4 A dendrogram representing the *Campylobacter jejuni* DIVERSILAB rep-PCR fingerprints generated using the Pearson Correlation Coefficient. The bar indicates the percentage similarity coefficient (%SC). The vertical gray line on the dendrogram is the 97% SC cutoff.

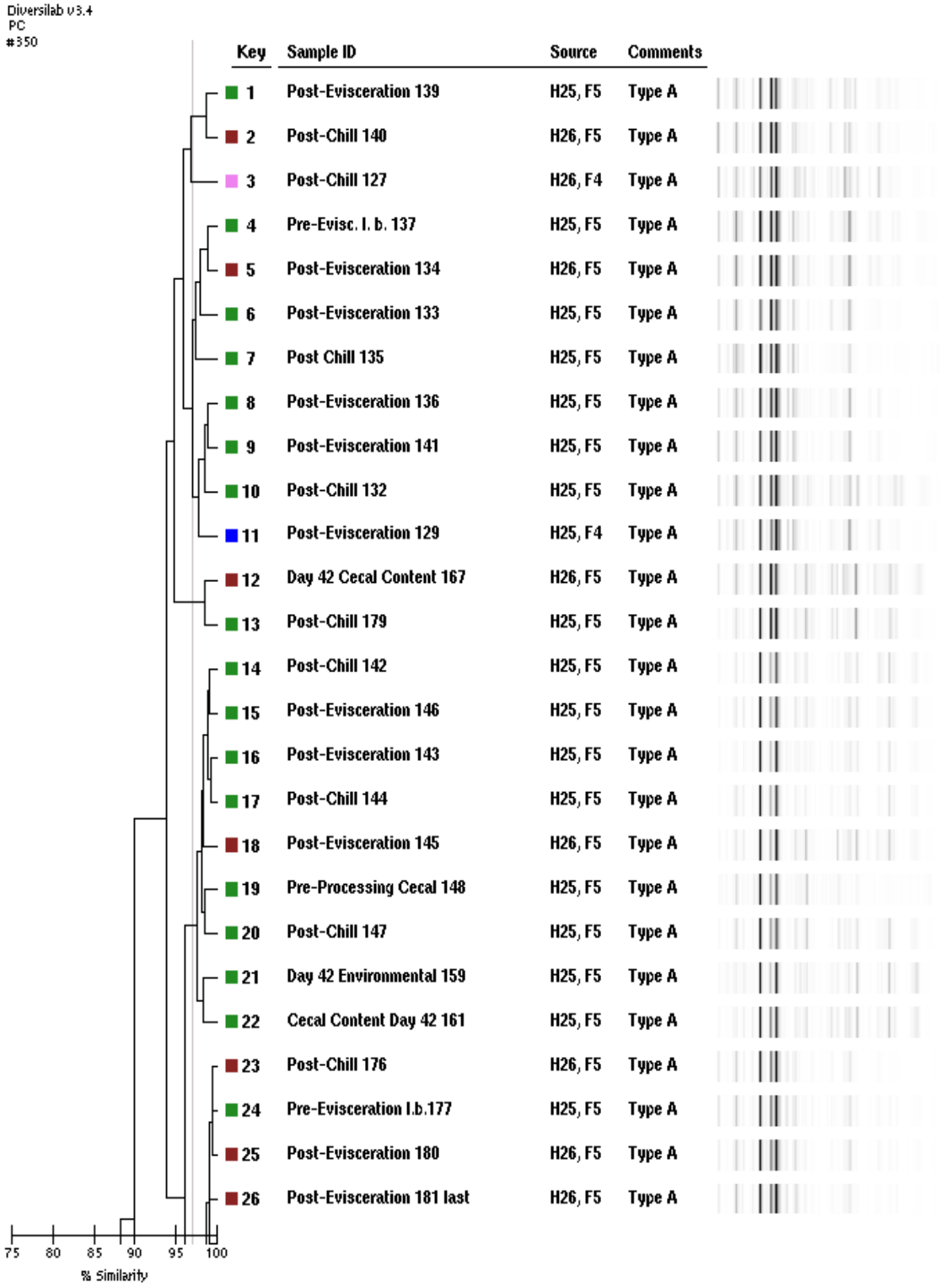

Diversilab v3.4
PC
#350
Key
Sample ID
Source
Comments
1 Post-Evisceration 139 H25, F5 Type A
2 Post-Chill 140 H26, F5 Type A
3 Post-Chill 127 H26, F4 Type A
4 Pre-Evisc. I. b. 137 H25, F5 Type A
5 Post-Evisceration 134 H26, F5 Type A
6 Post-Evisceration 133 H25, F5 Type A
7 Post Chill 135 H25, F5 Type A
8 Post-Evisceration 136 H25, F5 Type A
9 Post-Evisceration 141 H25, F5 Type A
10 Post-Chill 132 H25, F5 Type A
11 Post-Evisceration 129 H25, F4 Type A
12 Day 42 Cecal Content 167 H26, F5 Type A
13 Post-Chill 179 H25, F5 Type A
14 Post-Chill 142 H25, F5 Type A
15 Post-Evisceration 146 H25, F5 Type A
16 Post-Evisceration 143 H25, F5 Type A
17 Post-Chill 144 H25, F5 Type A
18 Post-Evisceration 145 H26, F5 Type A
19 Pre-Processing Cecal 148 H25, F5 Type A
20 Post-Chill 147 H25, F5 Type A
21 Day 42 Environmental 159 H25, F5 Type A
22 Cecal Content Day 42 161 H25, F5 Type A
23 Post-Chill 176 H26, F5 Type A
24 Pre-Evisceration I.b.177 H25, F5 Type A
25 Post-Evisceration 180 H26, F5 Type A
26 Post-Evisceration 181 last H26, F5 Type A
75 80 85 90 95 100
% Similarity

Figure A-4

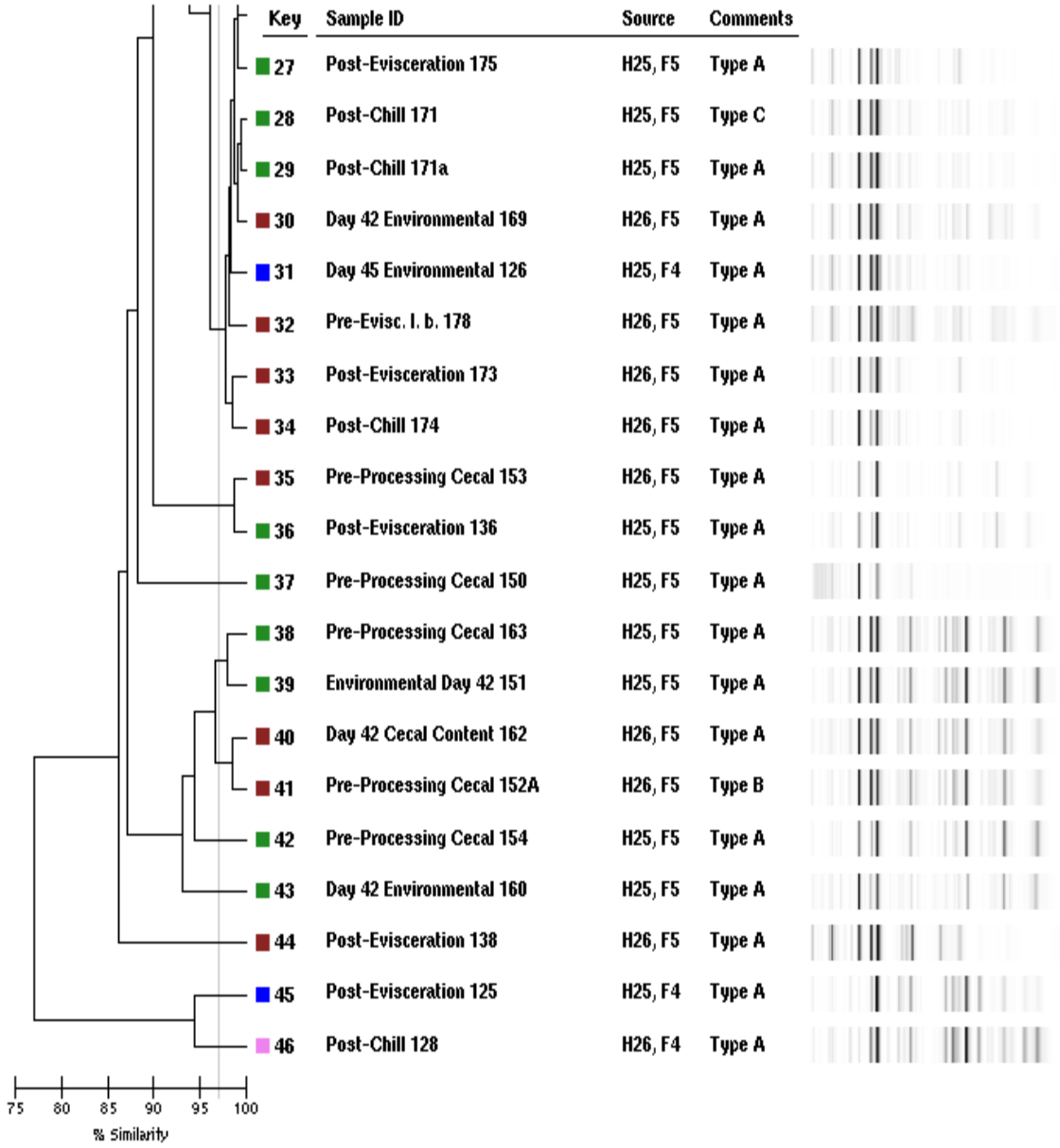


Figure A-4, continued

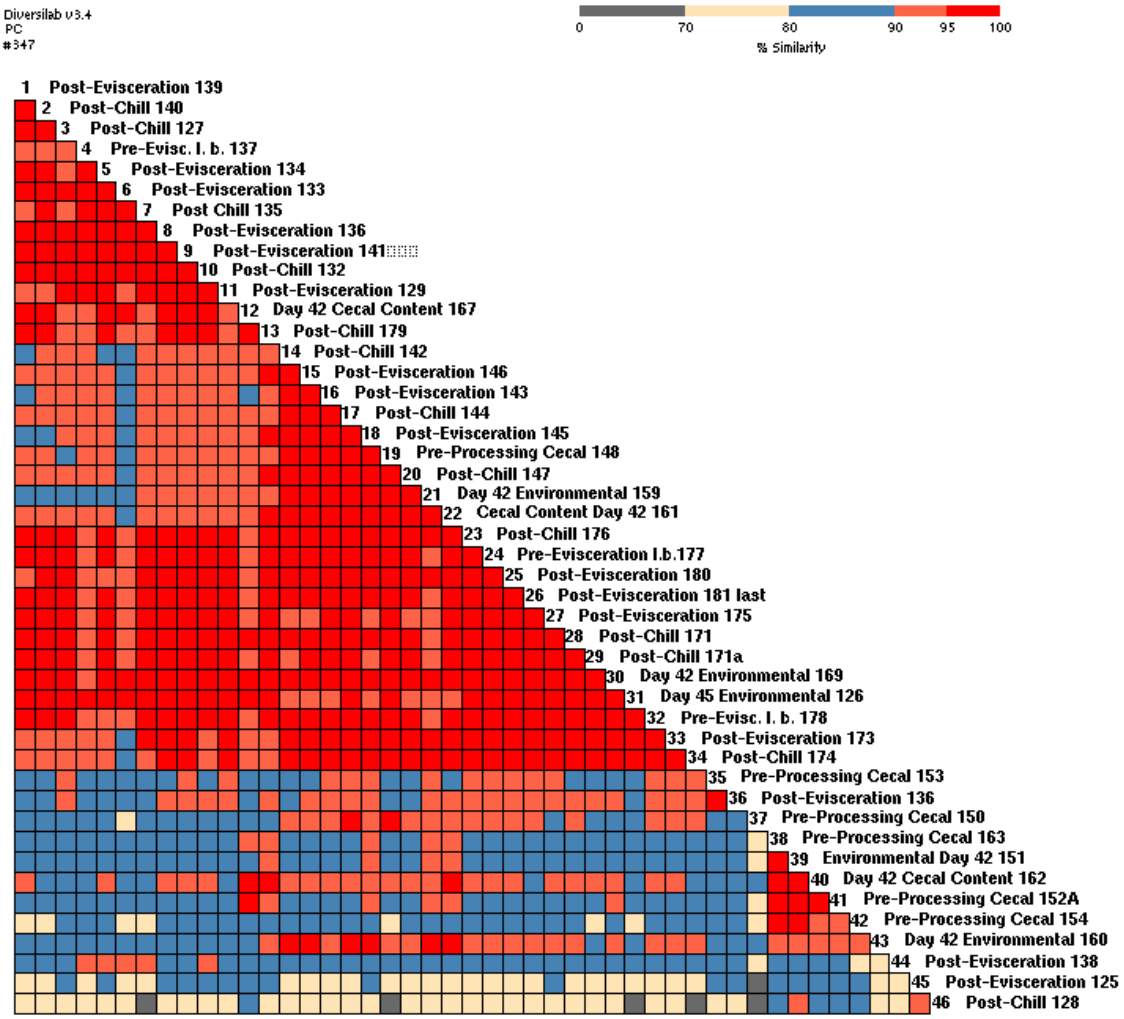
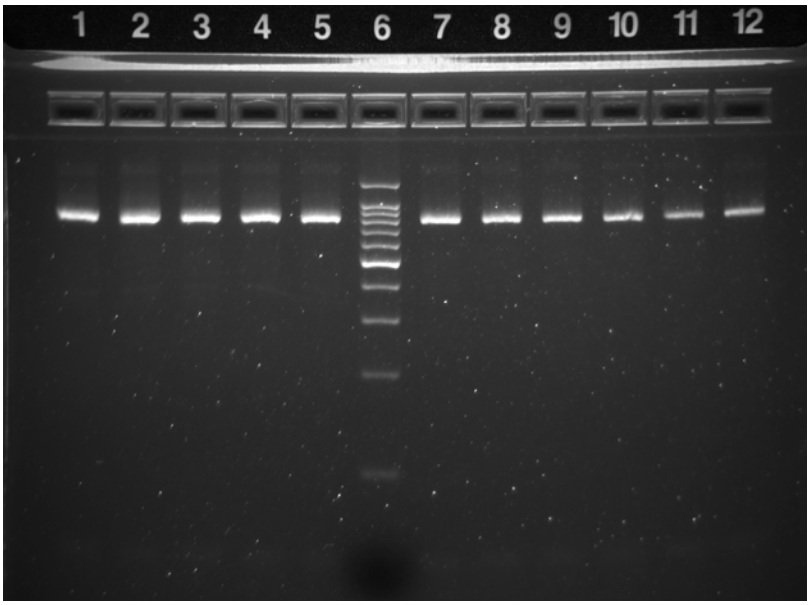
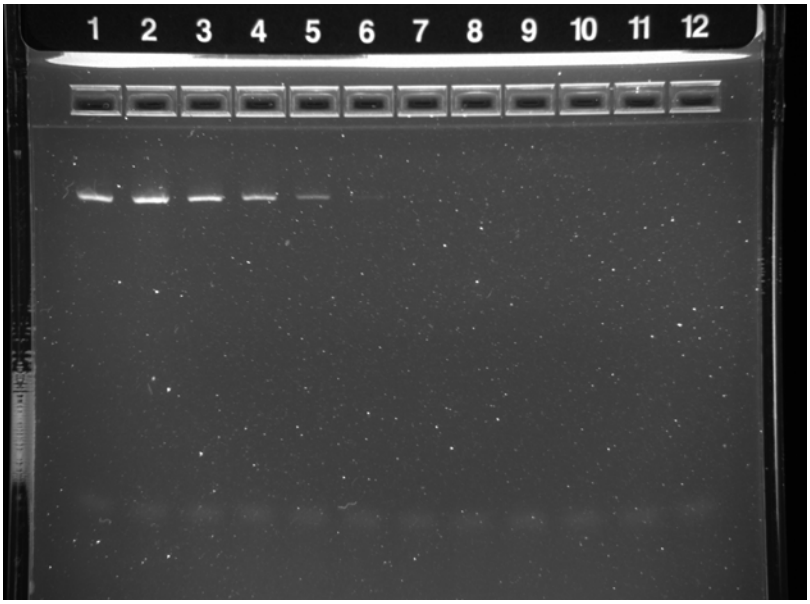


Figure A-5 Similarity matrix comparing individual isolate pairs of rep-PCR products.

Figure A-6 Electrophoresis results of annealing temperature optimization of *Campylobacter jejuni* 16S-23S Internal Spacer Region PCR in 2% agarose gels. Isolate 132 genomic DNA was used as template in all the optimization PCRs. Annealing temperature (° C) and (lane) in gel (a) were as follows: 56 (1), 56.2 (2), 56.5 (3), 57 (4), 57.7 (5), 100 bp DNA molecular weight ladder (6), 58.6 (7), 59.6 (8), 60.5 (9), 61.1 (10), 61.6 (11), and 61.9 (12). Annealing temperature (° C) and (lane) in gel (b) were as follows: 62 (1), 62.2 (2), 62.5 (3), 63 (4), 63.7 (5), 64.6 (6), 65.6 (7), 66.5 (8), 67.1 (9), 67.6 (10), 67.9 (11), and 68 (12).



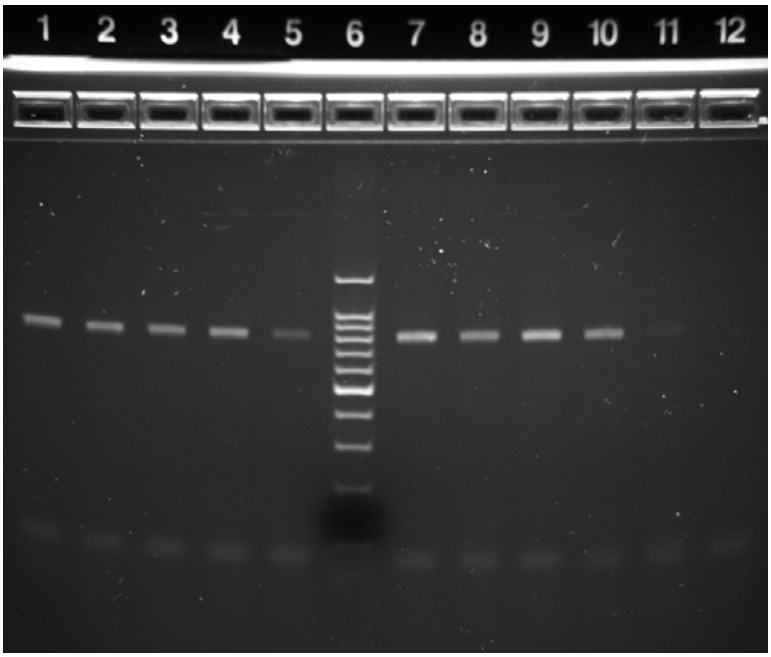
(a)



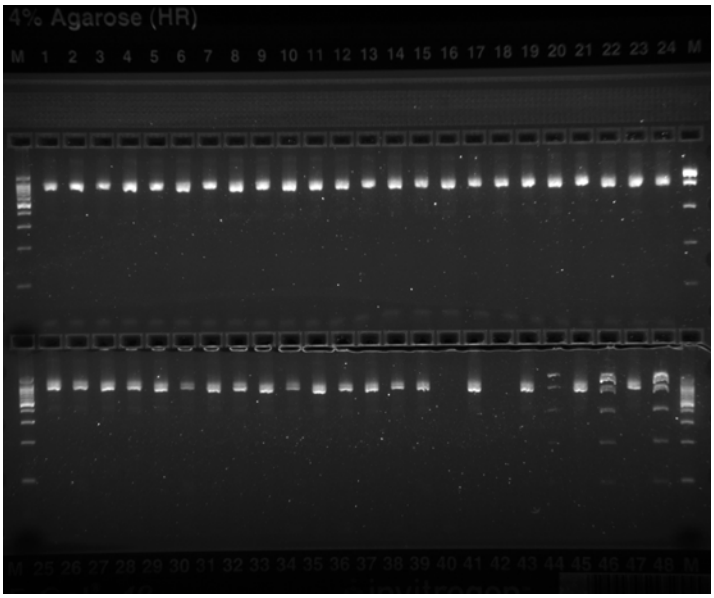
(b)

Figure A-6

Figure A-7 Electrophoresis results of Guanine Cytosine-clamped *Campylobacter jejuni* 16S-23S Internal Spacer Region amplicons in 2% agarose gels. A 100 bp DNA molecular weight ladder is represented in (a) in lane 6. The lanes labeled as "M" on the far left hand side and bottom row on the far right side of gel (b) represent a 100 bp DNA molecular weight ladder. A low range quantitative DNA ladder is represented by the "M" lane on the top row on the far right side of gel (b). Isolate number and (lane) in gel (a) were as follows: 125(1), 126 (2), 127 (3), 128 (4), 129 (5), 174 (7), 175 (8), 178 (9), 179 (10), 136 (11), no template control (12). Isolate number and (lane) in gel (b) were as follows: 136 (1), 143 (2), 132 (3), 144 (4), 133 (5), 145 (6), 134 (7), 146 (8), 135 (9), 147 (10), 137 (11), 148 (12), 138 (13), 150 (14), 139 (15), 151 (16), 140 (17), 152 (18), 140A (19), 152a (20), 141 (21), B26153A (22), 142 (23), 159 (24), 160 (25), 181 (26), 161 (27), 133A (28), 162 (29), 134a (30), 163 (31), 152A (32), 167 (33), 153 (34), 169 (35), 154 (36), 171 (37), 170B (38), 171a (39), blank (40), 173 (41), no template control (42), 176 (43), 177 (45), 180 (47), and low range quantitative DNA ladder (44, 46, 48).


1 2 3 4 5 6 7 8 9 10 11 12

(a)


4% Agarose (HR)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 M

(b)

Figure A-7

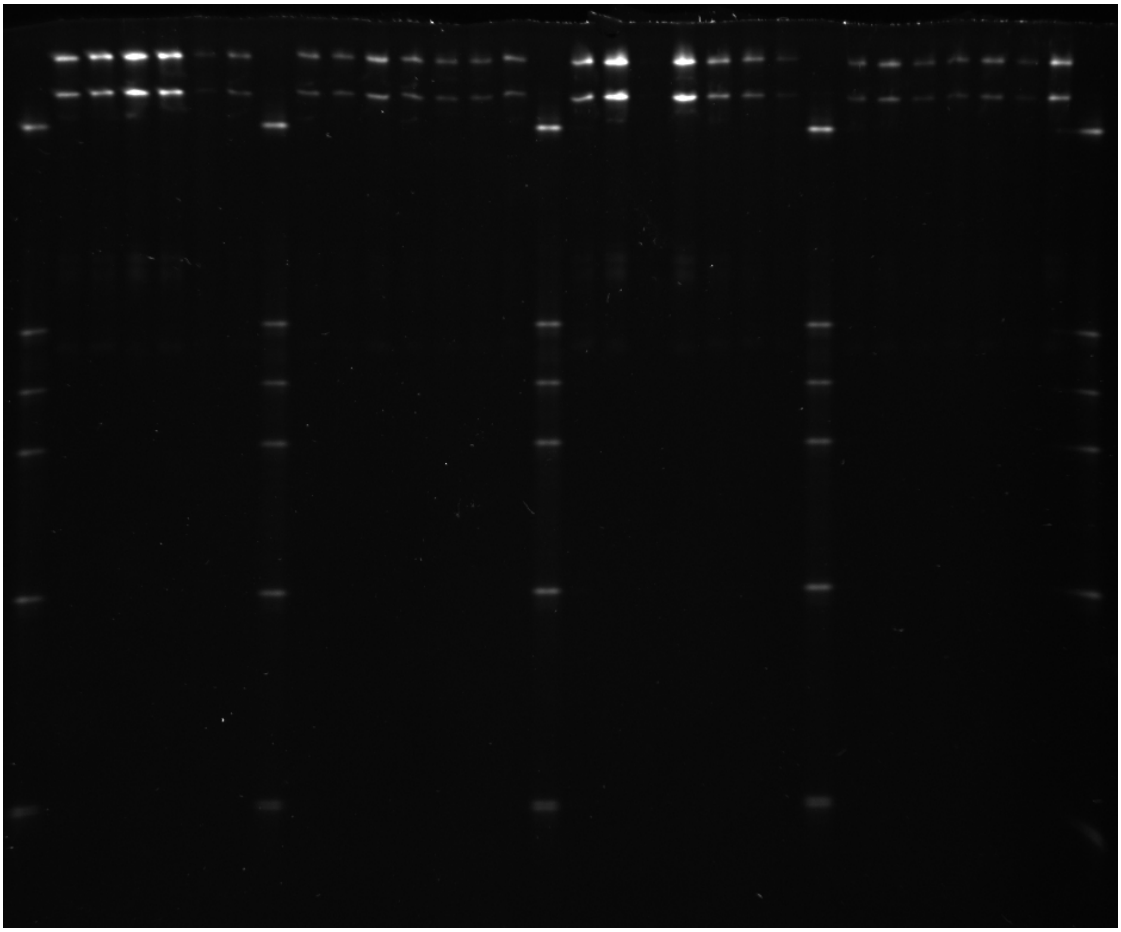


Figure A-8 *Campylobacter jejuni* 16S-23S Internal Spacer Region DGGE banding patterns of isolates 1 - 27. Lanes 1, 8, 16, 24, and 32 consist of reference standards. Isolate number and (lane) were as follows: 125(2), 126 (3), 127 (4), 128 (5), 129 (6), 132 (7), 133 (9), 133A (10), 134 (11), 134a (12), 135 (13), 136 (14), 136 (15), 137 (17) , 138 (18), 139 (19), 140 (20), 140A (21), 141 (22), 142 (23), 143 (25), 144 (26), 145 (27), 146 (28), 147 (29), 148 (30), and 150 (31).

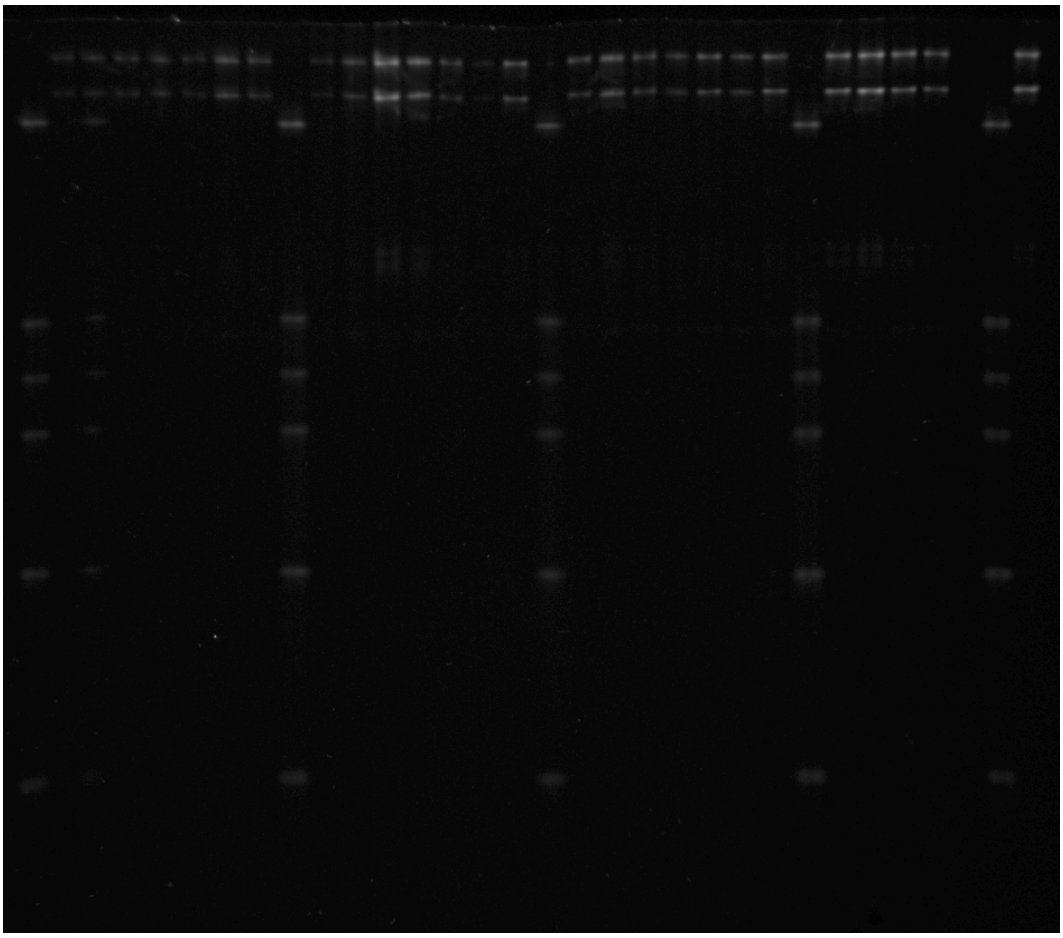


Figure A-9 *Campylobacter jejuni* 16S-23S Internal Spacer Region DGGE banding patterns of isolates 28 - 53. Lanes 1, 9, 17, 25, and 31 consist of reference standards. Isolate number and (lane) were as follows: 151(2), 152 (3), 152a (4), 152A (5), 153 (6), B26153A (7), 154 (8), 159 (10), 160 (11), 161(12), 162 (13), 163 (14), 167 (15), 169 (16) , 170B (18), 171 (19), 171a (20), 173 (21), 174 (22), 175 (23), 176 (24), 177 (26), 178 (27), 179 (28), 180 (29), blank (30), and 181 (32).

APPENDIX B

Table B-1. Sources of cultivable *Campylobacter jejuni* isolates

Sample	Type	Time	Flock
126	Litter	Day 45	4
151	Litter	Day 42	5
159	Litter	Day 42	5
160	Litter	Day 42	5
169	Litter	Day 42	5
137	Live Bird	Processing	5
177	Live Bird	Processing	5
178	Live Bird	Processing	5
148	Cecal Content	Processing	5
150	Cecal Content	Processing	5
152A	Cecal Content	Processing	5
153	Cecal Content	Processing	5
154	Cecal Content	Processing	5
161	Cecal Content	Day 42	5
162	Cecal Content	Day 42	5
163	Cecal Content	Processing	5
167	Cecal Content	Day 42	5
125	Post Evisceration	Processing	4
129	Post Evisceration	Processing	4
133	Post Evisceration	Processing	5
134	Post Evisceration	Processing	5
136	Post Evisceration	Processing	5

Table B-1, continued.

Sample	Type	Time	Flock
139	Post Evisceration	Processing	5
141	Post Evisceration	Processing	5
143	Post Evisceration	Processing	5
145	Post Evisceration	Processing	5
146	Post Evisceration	Processing	5
136	Post Evisceration	Processing	5
173	Post Evisceration	Processing	5
175	Post Evisceration	Processing	5
180	Post Evisceration	Processing	5
181	Post Evisceration	Processing	5
127	Post Chill	Processing	4
128	Post Chill	Processing	4
132	Post Chill	Processing	5
135	Post Chill	Processing	5
140	Post Chill	Processing	5
142	Post Chill	Processing	5
144	Post Chill	Processing	5
147	Post Chill	Processing	5
171	Post Chill	Processing	5
171A	Post Chill	Processing	5
174	Post Chill	Processing	5
176	Post Chill	Processing	5
179	Post Chill	Processing	5

Table B-2. Distribution of cultivable *Campylobacter jejuni* isolates

FLOCK 4			
Sample Type	**House 25**	**House 26**	**Combined Houses**
Litter Day 45	1	0	1
Post Evisceration	2	0	2
Post Chill	0	2	2
Total	3	2	5

FLOCK 5			
Sample Type	**House 25**	**House 26**	**Combined Houses**
Cecal content Day 42	1	2	3
Litter Day42	3	1	4
Cecal Content Processing	4	2	6
Pre-Evisceration Live Bird	2	0	2
Post-Evisceration	8	7	15
Post-Chill	8	3	11
Total	26	15	41

Table B-3. Sources and collection points of cultivable *Campylobacter jejuni* isolates

Sample	Source	Collection Point	House	Flock
126	Litter	Day 45	25	4
151	Litter	Day 42	25	5
159	Litter	Day 42	25	5
160	Litter	Day 42	25	5
169	Litter	Day 42	26	5
137	Live Bird	Processing	25	5
177	Live Bird	Processing	25	5
178	Live Bird	Processing	26	5
148	Cecal Content	Processing	25	5
150	Cecal Content	Processing	25	5
152A	Cecal Content	Processing	26	5
153	Cecal Content	Processing	26	5
154	Cecal Content	Processing	25	5
161	Cecal Content	Day 42	25	5
162	Cecal Content	Day 42	26	5
163	Cecal Content	Processing	25	5
167	Cecal Content	Day 42	26	5
125	Post Evisceration	Processing	25	4
129	Post Evisceration	Processing	25	4
133	Post Evisceration	Processing	25	5
134	Post Evisceration	Processing	26	5
136	Post Evisceration	Processing	25	5
138	Post Evisceration	Processing	26	5

Table B-3, continued.

Sample	Type	Time	House	Flock
139	Post Evisceration	Processing	25	5
141	Post Evisceration	Processing	25	5
143	Post Evisceration	Processing	25	5
145	Post Evisceration	Processing	26	5
146	Post Evisceration	Processing	25	5
136	Post Evisceration	Processing	25	5
173	Post Evisceration	Processing	26	5
175	Post Evisceration	Processing	25	5
180	Post Evisceration	Processing	26	5
181	Post Evisceration	Processing	26	5
127	Post Chill	Processing	26	4
128	Post Chill	Processing	26	4
132	Post Chill	Processing	25	5
135	Post Chill	Processing	25	5
140	Post Chill	Processing	26	5
142	Post Chill	Processing	25	5
144	Post Chill	Processing	25	5
147	Post Chill	Processing	25	5
171	Post Chill	Processing	25	5
171A	Post Chill	Processing	25	5
174	Post Chill	Processing	26	5
176	Post Chill	Processing	26	5
179	Post Chill	Processing	25	5

Table B-4. *Campylobacter jejuni* antimicrobial minimum inhibitory concentrations[1]

	A[2]	C	Cl	E	F	G	NA	T	Tet
Sample	**MIC**	**MIC**	**MIC**	**MIC**	**MIC**	**MIC**	**MIC**	**MIC**	**MIC**
125	0.12	0.12	0.5	2	4	0.5	8	2	2
126	0.12	0.12	0.5	2	2	0.5	8	2	2
127	0.12	0.12	0.5	2	4	0.5	8	2	2
128	0.25	0.12	0.5	2	4	0.5	8	1	2
129	0.12	0.12	0.5	2	4	0.5	8	1	2
132	0.12	0.12	0.5	2	4	0.5	8	1	2
133	0.12	0.12	0.5	1	2	0.5	8	1	1
133A	0.12	0.12	0.25	1	4	0.5	8	1	1
134	0.12	0.12	0.5	1	4	0.5	8	1	2
134A	0.12	0.12	0.5	2	4	0.5	8	2	2
135	0.12	0.12	0.5	2	4	0.5	8	2	2
136	0.12	0.12	0.5	2	4	0.5	8	2	2
137	0.12	0.12	0.5	1	2	0.5	8	1	2
138	0.12	0.12	0.5	1	4	0.5	8	1	2
139	0.12	0.12	0.5	2	4	0.5	8	2	2
140	0.12	0.12	0.25	1	2	0.5	8	1	2
140A	0.12	0.12	0.25	1	2	0.5	8	1	1
141	0.06	0.12	0.25	1	2	0.5	8	1	1
142	0.12	0.12	0.25	1	2	0.5	8	1	1
143	0.12	0.12	0.25	1	2	0.5	8	1	1
144	0.12	0.12	0.5	1	2	0.5	8	1	1
145	0.12	0.12	0.5	1	4	0.5	8	2	2
146	0.12	0.12	0.5	2	4	0.5	8	2	1
147	0.12	0.12	0.5	2	4	0.5	8	1	2
148	0.12	0.12	0.25	1	2	0.5	8	1	1

[1]MIC in μg/mL.

[2]A = Azithromycin, C = Ciprofloxacin, Cl = Clindamycin, E = Erythromycin, F = Florfenicol, G = Gentamycin, NA = Nalidixic Acid, T = Telithromycin, Tet = Tetracycline

Table B-4, continued.

	A	C	Cl	E	F	G	NA	T	Tet
Sample	**MIC**	**MIC**	**MIC**	**MIC**	**MIC**	**MIC**	**MIC**	**MIC**	**MIC**
150	0.25	0.12	0.5	2	4	0.5	8	2	2
151	0.12	0.12	2	2	4	0.5	8	1	2
152A	0.12	0.12	0.25	2	2	0.5	8	2	1
153	0.12	0.12	0.25	1	2	0.5	8	2	1
154	0.12	0.12	0.25	2	4	0.5	≤ 4	2	1
B26 153	0.12	0.12	0.25	1	2	0.5	8	2	1
159	0.12	0.12	0.25	1	2	0.5	8	1	1
160	0.12	0.12	0.25	1	2	0.5	8	1	1
161	0.12	0.12	0.5	1	2	0.5	8	1	2
162	0.12	0.12	0.5	1	4	0.5	8	1	1
163	0.06	0.12	0.25	1	2	0.25	8	2	1
167	0.12	0.12	0.5	1	4	0.5	8	1	2
169	0.12	0.12	0.5	1	4	0.5	8	1	2
170	0.12	0.12	0.25	1	4	1	8	1	1
171	0.06	0.12	0.25	1	2	1	8	1	1
171A	0.06	0.12	0.25	1	2	0.5	8	2	2
173	0.12	0.12	0.5	1	4	0.5	8	1	2
174	0.12	0.12	0.5	2	4	0.5	8	2	2
175	0.12	0.12	0.5	2	4	0.5	8	1	2
176	0.12	0.12	0.25	1	2	0.5	8	1	1
177	0.12	0.12	0.5	1	2	0.5	8	1	2
178	0.12	0.12	0.5	2	4	0.5	8	2	2
179	0.12	0.12	0.5	2	4	0.5	8	1	2
180	0.12	0.12	0.5	1	4	0.5	8	1	2
181	0.12	0.12	0.5	1	4	0.5	8	1	2

Table B-5. *Campylobacter jejuni* antimicrobial susceptibility [1]

	A[2]	C	Cl	E	F	G	NA	T	Tet
Sample	**Su**	**Su**	**Su**	**Su**	**Su**	**Su**	**Su**	**Su**	**Su**
125	S	S	S	I	I	S	S	S	S
126	S	S	S	I	S	S	S	S	S
127	S	S	S	I	I	S	S	S	S
128	S	S	S	I	I	S	S	S	S
129	S	S	S	I	I	S	S	S	S
132	S	S	S	I	I	S	S	S	S
133	S	S	S	I	S	S	S	S	S
133A	S	S	S	I	I	S	S	S	S
134	S	S	S	I	I	S	S	S	S
134A	S	S	S	I	I	S	S	S	S
135	S	S	S	I	I	S	S	S	S
136	S	S	S	I	I	S	S	S	S
137	S	S	S	I	S	S	S	S	S
138	S	S	S	I	I	S	S	S	S
139	S	S	S	I	I	S	S	S	S
140	S	S	S	I	S	S	S	S	S
140A	S	S	S	I	S	S	S	S	S
141	S	S	S	I	S	S	S	S	S
142	S	S	S	I	S	S	S	S	S
143	S	S	S	I	S	S	S	S	S
144	S	S	S	I	S	S	S	S	S
145	S	S	S	I	I	S	S	S	S
146	S	S	S	I	I	S	S	S	S
147	S	S	S	I	I	S	S	S	S
148	S	S	S	I	S	S	S	S	S

[1] Su = Susceptibility, S = Susceptible, I = Intermediate.

[2]A = Azithromycin, C = Ciprofloxacin, Cl = Clindamycin, E = Erythromycin, F = Florfenicol, G = Gentamycin, NA = Nalidixic Acid, T = Telithromycin, Tet = Tetracycline

Table B-5, continued.

	A	C	Cl	E	F	G	NA	T	Tet
Sample	**Su**	**Su**	**Su**	**Su**	**Su**	**Su**	**Su**	**Su**	**Su**
150	S	S	S	I	I	S	S	S	S
151	S	S	I	I	I	S	S	S	S
152A	S	S	S	I	S	S	S	S	S
153	S	S	S	I	S	S	S	S	S
154	S	S	S	I	I	S	S	S	S
B26153	S	S	S	I	S	S	S	S	S
159	S	S	S	I	S	S	S	S	S
160	S	S	S	I	S	S	S	S	S
161	S	S	S	I	S	S	S	S	S
162	S	S	S	I	I	S	S	S	S
163	S	S	S	I	S	S	S	S	S
167	S	S	S	I	I	S	S	S	S
169	S	S	S	I	I	S	S	S	S
170	S	S	S	I	I	S	S	S	S
171	S	S	S	I	S	S	S	S	S
171A	S	S	S	I	S	S	S	S	S
173	S	S	S	I	I	S	S	S	S
174	S	S	S	I	I	S	S	S	S
175	S	S	S	I	I	S	S	S	S
176	S	S	S	I	S	S	S	S	S
177	S	S	S	I	S	S	S	S	S
178	S	S	S	I	I	S	S	S	S
179	S	S	S	I	I	S	S	S	S
180	S	S	S	I	I	S	S	S	S
181	S	S	S	I	I	S	S	S	S

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