A POTENTIAL ROUTE OF *CAMPYLOBACTER* SPP. DISSEMINATION TO INTERNAL TISSUES OF BROILERS AND SENSITIVITY OF *CAMPYLOBACTER* ENRICHMENT PROCEDURES

by

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(Under the Direction of Mark A. Harrison)

ABSTRACT

The overall aims were to 1) investigate the circulating blood as a potential route of *Campylobacter* spp. dissemination to internal tissues and organs within broilers, 2) evaluate the sensitivity of cultural procedures for recovery of *Campylobacter* spp., and 3) determine whether *Campylobacter* strains enter into a viable but non-culturable state after subjection to dry-atmospheric-temperature stress. Circulating blood was demonstrated to contain viable *Campylobacter* spp. and a potential dissemination route of *Campylobacter* spp. to internal tissues and organs. Seven *Campylobacter* subtypes were aseptically recovered from the circulating blood of broilers from 9 of 19 broiler flocks at a rate of 11.5%. Isolates differed in invasiveness into polarized Caco-2 cells, ranging from no invasiveness to 1.25%. Blood and ceca isolates from the same bird differed in invasiveness but were similar by *flaA* SVR DNA sequencing. In separate studies, two enrichment broths (TECRA® and Bolton enrichment broth) and twelve different enrichment procedures (using the two broths) were evaluated for their sensitivity

to recover *Campylobacter* spp. from rehang and post-chill carcass rinses. Sensitivity of recovery was influenced by enrichment method, enrichment broth, plant, and sample type. Carcass rinses at rehang contained higher numbers of *Campylobacter* and non-*Campylobacter* cells than at post-chill. The antibiotics in TECRA® broth significantly reduced background microflora when compared to the antibiotics in Bolton broth, which influenced sensitivity. The best procedure for rehang samples used TECRA® broth, incubated microaerophilicly at 42ºC for 5 h without antibiotics followed by the addition of antibiotics for an additional 43 h incubation. The best procedure at post-chill used TECRA® broth incubated microaerophilicly at 37ºC for 5 h without antibiotics followed by the addition of antibiotics and incubation at 42ºC for an additional 43 h incubation. The best Bolton broth procedure for rehang and post-chill was microaerophilic incubation at 42°C for 48 h with antibiotics and 5% lysed horse blood. An increase in *Campylobacter* spp. recovery from samples were observed when using the best TECRA® and Bolton enrichment procedure together for each sample. When marker *Campylobacter* spp. were exposed to dry-atmospheric-temperature stress for 24 h, *C. jejuni* and *C. coli* could not be significantly recovered after 2 h and was unrecoverable after 6 h of exposure using the best methods from the above studies. However, using a chick bioassay, *Campylobacter* strains were still viable from the culture negative (6 h and 24 h) samples.

INDEX WORDS: *Campylobacter*, broiler, blood, molecular phylogeny, invasion, enrichment methodology, carcass rinses, stresses, VBNC

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DEDICATION

I would like to dedicate this dissertation to my beloved family. Achieving such a goal scholastically could not have been possible if not for my loving parents, Larry and Marilyn Richardson, my loving sister, Jan Brown and lastly but most significantly my loving wife Anna Richardson. Throughout my tenure as a graduate student here at The University of Georgia, my mother, father, sister and wife have always been there for encouragement, and constantly reminding me of the bigger picture in life. To my beloved family, thank you.

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Sincerely,

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CHAPTER 1

INTRODUCTION

Reducing and eliminating foodborne human pathogens associated with poultry has received much attention. A goal of the poultry industry is to produce a safe, edible and economical product for consumers. The USDA Food Safety and Inspection Service stated in the 1996 Federal Register "…the FSIS food safety goal should be to reduce the risk of foodborne illness associated with the consumption of meat and poultry products to the maximum extent possible by ensuring that appropriate and feasible measures are taken at each step in the food production process where hazards can enter…" (Federal Register, 1996). The United States total farm income from broiler production grew from approximately 1 billion dollars in 1960 to over 20 billion dollars in 2005 (GDA and USDA, 2006; Cunningham, 1993). The number of broilers being commercially processed has seen significant growth over this period with over 9 billion broilers being processed in 2005 (GDA and USDA, 2006). Just a decade ago, the per capita consumption in the U.S. of poultry was 90 pounds and that has significantly increased to over 102 pounds today (GDA and USDA, 2006, 2002).

A problem the poultry industry and government regulatory agencies have encountered with the boost in production and consumption of poultry is an

amplified likelihood of a foodborne illness associated with its product. The two major human bacterial pathogens of concern in regards to poultry are *Salmonella* and *Campylobacter*. A goal set by U.S. and other countries is to reduce the level of infection in the human population by focusing interventions pre- and post-harvest (CDC, 2003; Mallinson, 2001; Rose *et al.*, 1996; USDA, 1996).

The Centers for Disease Control and Prevention estimates that in the United States, human campylobacteriosis accounts for more than 2.4 million cases of gastroenteritis annually, and 80% of the cases are considered foodborne (Friedman *et al.*, 2000; Mead *et al.*, 1999). The two major species associated with illness are *C. jejuni* and *C. coli*. In addition to gastroenteritis, controlled epidemiological studies have also linked infection of humans with *C. jejuni* to post-infectious immune diseases (Winer, 2001). A commonly known example is Guillain-Barré syndrome which is usually associated with a multifocal demyelinating disorder of the peripheral nerves in close association of macrophages and is believed to occur in 2/100,000 people (Ang *et al.*, 2001; Winer, 2001).

Campylobacter spp. are commonly carried in the alimentary tract of poultry in levels up to 10^8 – 10^9 cfu/g of fecal content and can be isolated frequently from the ceca of poultry (Grant *et al.*, 1980). The prevalence rate of broilers contaminated with *Campylobacter* spp. entering the processing plant can reach 100% (Sahin *et al.*, 2002). The average prevalence rate of infected flocks is 44% to 59% (Nauta and Havelaar, 2008; Humphrey *et al.*, 2007). The number of contaminated broilers account for the high incidence of *Campylobacter* spp. in

poultry processing plants and on processed carcasses (Allen *et al.*, 2007; Jacobs-Reitsma, 2000; Oosterom *et al.*, 1983). The average *Campylobacter* spp. prevalence rate on chicken at retail is 57% with a range of 23% to 100% (Humphrey *et al.*, 2007). The high colonization prevalence of poultry and the resultant clinical infection in humans have prompted a number of investigations focused upon identifying and subsequently eliminating sources of *Campylobacter* spp. contamination in poultry flocks (Solomon and Hoover, 1999).

Numerous approaches have been developed with some success to control/prevent *Salmonella* serovars from entering and colonizing poultry flocks, thereby reducing the number of *Salmonella*-positive flocks entering the processing plant. The measures put into place for *Salmonella* serovars pre- and post-harvest have had success in controlling *Campylobacter* spp., but incidence post-processing remains high. This may be due to differences in the ecology, physiology, and epidemiology of these two human pathogens (Newell and Fearnley, 2003). In order for adequate development of intervention and a reduction in *Campylobacter* spp. colonization rate of broiler flocks, an improved understanding of the ecology and physiology of *Campylobacter* spp. in poultry is needed. Several key factors hinge on the ability for the above to be accomplished: 1) optimization of recovery media and methodology procedures 2) more standardized and advanced molecular typing methods 3) better understanding of the organism's ability to disseminate within a bird 4) role of vertical transmission and 5) better understanding of the viable but non-culturable state (VBNC).

Campylobacter spp. are believed to be more sensitive and less robust to certain conditions than organisms in the *Enterobacteriaceae* family that are associated with poultry. Studies are beginning to show some *Campylobacter* spp. may be more vigorous than previously considered and certain routes of transmission into poultry flocks more significant than once believed (Byrd *et al*., 2007; Humphrey *et al.*, 2007; Buhr *et al.*, 2002; Cox *et al.,* 2002). Horizontal transmission plays a role in the introduction of *Campylobacter* spp. into a broiler flock (Humphrey *et al.*, 2007; Bull *et al*., 2006; Ramabu *et al*., 2004; Newell and Fearnley, 2003; Sahin *et al.*, 2002). The role of vertical transmission of *Campylobacter* spp. into poultry flocks has been dismissed by the majority of the research community mainly due to the inability to routinely culture *Campylobacter* spp. from the hatchery environment or from newly hatched chicks. Using molecular and cultural techniques are beginning to show that vertical transmission of *Campylobacter* spp. from the breeder hen to its progeny (the broiler) can occur (Byrd *et al.*, 2007; Acevedo, 2005; Hiett *et al*., 2003, 2002; Cox *et al*., 2002; Chuma *et al*., 1997, 1994; Pearson *et al*., 1996). The significance of this transmission in relation to inter-flock contamination and post-processing contamination has not been shown.

The source of broiler flock infections, modes of transmission and environmental factors that contribute to the spread of *Campylobacter* spp. on poultry farms are beginning to be determined (Buhr *et al.*, 2002; Carmarda *et al.*, 2000; Cox *et al.*, 2002; Sahin *et al.*, 2002). Understanding *Campylobacter* spp.

ecology within broiler breeders and broilers; developing improved methodology procedures and determining when and how *Campylobacter* spp. contaminates broiler flocks may provide information needed by the poultry industry, researchers, and regulatory agencies. This should lead to the development of intervention strategies to reduce *Campylobacter* spp. contamination rates pre- and post-harvest, thus reducing the level of contaminated carcasses entering and exiting the processing operation.

The objectives of this dissertation are to investigate a possible dissemination route of *Campylobacter* spp. to internal organs and tissues of broilers, evaluate the sensitivity of enrichment methodology for recovery of *Campylobacter* spp., and evaluate the VBNC state. The goals were to:

a) Develop methodology to determine whether *Campylobacter* strains can be isolated from the internal contents of the spleen.

b) Determine whether *Campylobacter* spp. can be aseptically recovered from the circulating blood of commercial broilers.

c) Evaluate the invasiveness of *Campylobacter* strains from the blood and the molecular phylogeny of the *fla*A short variable region of those isolates compared to isolates recovered from the ceca within the same bird.

d) Evaluate the performances of enrichment broths and methodology procedures for *Campylobacter* spp. recovery from poultry samples.

e) Evaluate the sensitivity of improved methods and procedures for *Campylobacter* strain recovery after dry-atmospheric-temperature stress.

f) Determine whether dry-atmospheric-temperature stressed *Campylobacter* strains, which have been determined to be culture negative are viable using a chick bioassay.

The dissertation is divided into 8 chapters. In chapter 2, a review of the literature on *Campylobacter* spp. is discussed. The focus of the discussion is on the characteristic, illnesses, sources and incidences, transmission in poultry, ecology inside birds, molecular and invasion analysis techniques, media and methodology, and viable but non-culturable states. Chapter 3 describes the ecology of *Campylobacter* spp. in birds. Chapters 4 and 5 describe studies evaluating enrichment broths and procedures for recovery of *Campylobacter* spp. from poultry carcasses. Chapter 6 describes a study evaluating enrichment broths and procedures for recovery of dry-atmospheric-temperature stressed *Campylobacter* populations and the sensitivity of those procedures using a chick bioassay. A summary and conclusion of the studies from Chapters 2-6 are included in Chapter 7. The appendices are intended to provide readers with additional data that was not extensively discussed in the chapters on two preliminary conducted studies. Appendix A describes studies on a method that was developed to aseptically sample the external and internal contents of the spleen after the birds have been inoculated with *C. jejuni*. Appendix B describes a study to recovery inoculated *C. jejuni* in the circulating blood of broilers.

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CHAPTER 2

LITERATURE REVIEW

Characteristics of *Campylobacter* spp.

The taxonomy of the *Campylobacteraceae* family has changed considerably over the years as understanding of organisms within the family evolves. To date the family *Campylobacteraceae* includes the genera: *Campylobacter*, *Arcobacter*, *Sulfurospirillum* and generically misclassified *Bacteroides ureolyticus* (Vandamme, 2000). In the genus *Campylobacter*, there are 18 species and of these species, several are considered pathogenic to humans, causing enteric and extra-intestinal illnesses (Humphrey *et al.*, 2007). *Campylobacter* spp. are Gram-negative, non-spore forming; curved or small spiral-shaped cells that have characteristic rapid, darting reciprocating motility (corkscrew-like motion by means of a single polar flagellum at one or both ends of the cell) and can occur in short or long chains. Cells range in width from 0.2 to 0.9 µm and in length from 0.5 to 5 µm and most species have an optimum temperature range for growth of 30 to 37°C (Penner, 1988). A few strains can grow aerobically or anaerobically (Vandamme, 2000). *C. jejuni* and *C. coli* are microaerophilic and thermophilic, therefore requiring 3-15% O_2 and 2-5% CO_2 concentrations and grow optimally at 42ºC (Park, 2000, Ketley, 1997). An atmosphere containing increased nitrogen may be needed for optimum growth of other strains (Portner *et al.*, 2007; Lastovica, 2006;

Lastovica and Le Roux, 2000). The cells can form spherical or coccoid bodies as cultures age and certain species can have the characteristics of a viable, but not culturable state (Moran and Upton, 1987; Rollins and Colwell, 1986).

Campylobacter spp. have a chemoorganotrophic metabolism and energy is derived from amino acids or tricarboxylic acid cycle intermediates due to their inability to oxidize or ferment carbohydrates (Penner, 1988). The majority of *Campylobacter* spp. reduce nitrate and nitrite. *Campylobacter* spp. are able to reduce fumarate to succinate; have a negative reaction to methyl red, acetone, and indole; negative hippurate hydrolysis (except for most *C. jejuni*); and positive for oxidase activity (Sellars *et al.*, 2002; Vandamme, 2000). *Campylobacter* spp. can be either catalase positive or negative. Broadly speaking, catalase positive *Campylobacter* spp. are most often associated with human disease, but not in all cases.

The pathogenic mechanisms for campylobacteriosis are not totally understood and information on species other than *C. jejuni* is scarce. Some important virulence factors include motility, translocation, chemotaxis, and production of toxins (Ketley, 1997; Wassenaar, 1997; Walker *et al.*, 1986). The virulence factors involved in the infection may influence the symptoms of the disease, and pathogenesis results from several different bacterial properties and host defenses. Motility, which is achieved by means of a single flagellum at one (monotrichous) or both ends (amphitrichous) of the bacteria, has an extremely important role in virulence because it is required for the bacteria to reach the attachment sites and penetrate into the intestinal cells (Fernando *et al.*, 2007; Carrillo *et al.*, 2004; Grant *et al.*, 1993). Motility of *Campylobacter* spp. can be described as rapid or darting in a corkscrew

fashion (Karmali and Fleming, 1979). If the bacterium loses its motility, colonization of the gastrointestinal tract is believed to be lost (Morooka *et al.*, 1995).

C. jejuni contains two flagellin genes, *flaA* and *flaB*; the wild-type bacteria express *flaA* only, but *flaB* can be expressed under certain conditions. A highly conserved *flaC* gene is present in the *C. jejuni* NCTC11168 (Song *et al.*, 2004). The flagellum of *Campylobacter* spp. plays a much more important function than just motility. *C. jejuni* flagella may also play a role in the dissemination and internalization of the organism (Song *et al.*, 2004; Day *et al.*, 2000). The flagellum has been proposed as an adhesin in the binding to culture cells (Byrne *et al*., 2007; McSweegan and Walker, 1986). *C. jejuni* is able to become internalized within human intestinal epithelial cells and traverse monolayers of polarized human colonic carcinoma cells allowing access to submucosal tissue which leads to tissue inflammation and damage (Ketley, 1997; Grant *et al.*, 1993; Walker *et al.*, 1986). Toxin production has also been hypothesized to play a role in pathogenicity. In regards to *C. jejuni*, the organism synthesizes several toxins, mainly classified as enterotoxins or cytotoxins (Wassenaar, 1997). Spikes in levels of all immunoglobulin classes occur in humans after infection. Even though the synthesis of several toxins has been reported, their mechanism of action and importance in regards to disease still remain unclear.

Type of illness

For approximately four decades, the genus *Campylobacter* has had increased focus as a threat to food safety, due to the rise in enteritis in humans caused by consumption or handling of foods contaminated with the organism. The infectious dose for campylobacters can be a few hundred cells (Gormley *et al.*, 2008; Black *et al.*, 1988). Even though *Campylobacter* spp. are sensitive to drying, high oxygen concentration, and low pH (less than or equal to 4.7) they are still a major cause of bacterial gastroenteritis. In the U.S., approximately 2.5 million people are infected each year due to consumption of foods containing campylobacters (Mead *et al.*, 1999). Four species (*C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*) are known as "thermophilic campylobacters" and are clinically significant due to their association as dominant causative agents of human campylobacteriosis (Keener *et al.*, 2004; Jacobs-Reitsma, 2000; Blaser *et al.*, 1982). *C. jejuni* is the predominant species that causes bacterial gastroenteritis in the U.S. and in many other developed countries, with *C. coli* being second (Lastovia, 2006). In the U.S., campylobacteriosis and salmonellosis alternate as the leading cause of bacterial foodborne illness.

Transmission of *Campylobacter* spp. to humans generally occurs by either ingestion of contaminated food or water or by direct contact with fecal material from infected animals or persons. In humans, there are two types of illnesses associated with *Campylobacter* spp. infections, intestinal and extraintestinal. Two types of diarrhea are usually observed with campylobacteriosis: (1) an inflammatory diarrhea, with slimy, bloody stools containing leukocytes (2) noninflammatory diarrhea, with watery stools and

the absence of blood and leukocytes (Wassenaar, 1997). In some cases, intense abdominal pain, headaches, fever, cramping and vomiting can occur. Serious complications can arise such as Reiter's syndrome, Gullain- Barré syndrome, osteomyelitis, pancreatitis, nephritis, myocarditis, cystitis, septic abortion, and bacteremia in certain cases (Keener *et al.*, 2004; Winer, 2001; Altekruse *et al.*, 1999). Although campylobacteriosis does not usually lead to death, approximately 700 people in the U.S. die annually, often due to secondary complications (Saleha *et al.*, 1998).

Campylobacteriosis is primarily a self-limiting bacterial gastroenteritis and recovery occurs in approximately 8 days. Using antibiotics as a treatment for the common infection is rarely performed. Symptoms can persist longer than 2 weeks in some cases. Antibiotic resistant strains to clinically important drugs are becoming more common. An infection with a resistant strain can exhibit more intense symptoms than infection from a sensitive strain (Helms *et al.*, 2005). The population that is most susceptible to illness includes children less than 1 year of age, young adults aged 15 to 25 years of age, and immunosuppressed individuals (Keener *et al.*, 2004). A concern for those suffering from campylobacteriosis is that they will suffer from neurological sequelae months or even years afterwards. Two neuropathies associated with *C. jejuni* infections are: Gullain-Barré syndrome (GBS) and Miller Fisher syndrome (MFS). Both of these syndromes are characterized by being acute or sub–acute, immune mediated neuropathies.

GBS is characterized by alexia, motor paralysis, acellular increase in the total protein content in the cerebrospinal fluid and inflammatory demyelinating polyradiculoneuropathy (Winer, 2001; Willison and O'Hanlon,

2000; Lindsay, 1997). GBS occurs in approximately 1 out of 100,000 people (Winer, 2001). The prevalence within infected people is estimated to be 1 out of 1,000 and can be even less depending on strain (Nachamkin, 2002). GBS cases are associated with nerve roots, causing mononuclear infiltration of peripheral nerves, and this eventually leads to primary axonal degeneration or demyelination. Molecular mimicry is believed to be the cause of GBS because a few peripheral nerves of the human neurological system share similar molecules with antigens on the surface of *C. jejuni* cells (Nachamkin, 2002; Winer, 2001)**.** *C. jejuni* contain a lipopolysaccharide structure (LPS) attached to the outer membrane, the core oligosaccharides of its LPS contain ganglioside-like structures, which are similar to certain human gangliosides (Ang *et al.*, 2001; Perez and Blaser, 1985; Logan and Trust, 1982). The LPS structure is highly antigenic and upon exposure to *C. jejuni*, the immune system produces antibodies against the LPS structure as an attempt to fight the infection. Due to the similarity of the core oligosaccharides of the LPS and the gangliosides, after the infection, antibodies attack the gangliosides on the neuromuscular junction contributing to the appearance of neurological symptoms (Godschalk *et al.*, 2004; Ang, 2001; Lindsay, 1997).

Sources and incidence

Unlike many other enteric pathogens, *Campylobacter* spp. have limited spread from host to host. *Campylobacter* spp. may not be recovered by available conventional cultural methods outside of the host if exposed to dry conditions or atmospheric oxygen levels for extended periods of time (Cox et al., 2001). *Campylobacter* spp. can be classified as zoonoses, because

animals are the main reservoir of this organism. Pathogenesis of the organisms differs in humans compared to animals and the differences could be due to differential gene expression within the hosts (Humphrey *et al*., 2007).

Campylobacter spp. exist as commensals in many wild and domestic animals (Keener *et al.*, 2004). This presents a risk to food safety due to the contamination of animal carcasses at slaughter and other foodstuffs due to cross-contamination when raw or undercooked meat is handled. Contamination with this pathogen can occur at numerous stages along the food chain. This includes, but not limited to production, processing, distribution, handling and preparation. *Campylobacter* spp. have been sporadically recovered from rivers, coastal waters, shellfish, and vegetables but are routinely recovered from sheep, cattle, swine, rodents and avian species (Jacobs-Reitsma, 2000; Kemp *et al.*, 2005). Certain species of *Campylobacter* are routinely associated with certain species of animals. In poultry and cattle, *C. jejuni* is the predominant species and in swine *C. coli* is the common species recovered. *Campylobacter* infections are sporadic and outbreaks are rare, but contaminated water, raw milk, poultry, beef, eggs, fruits, and contact with farm animals and pets have been identified through epidemiological trace-back (Friedman *et al.*, 2004; Altekruse *et al.*, 1999). The primary source of contamination of the environment and foods is believed to be from animal feces (Brown *et al.*, 2004).

Avian species, particularly poultry are the most common host for *Campylobacter* spp. and poultry is considered the main source of human illness (Mackiw *et al.*, 2008; Vellinga and Van Loock, 2002). Approximately

70% of human illnesses due to *Campylobacter* spp. are caused by the consumption or handing of raw or undercooked poultry or poultry products (Friedman *et al.*, 2000; Mead *et al.*, 1999). Increased attention has been given to reducing the level of *Campylobacter* spp. in poultry pre- and post-harvest to reduce the level and incidence of raw product contamination (Friedman *et al.*, 2004; Keener *et al.*, 2004; Allos, 2001).

The decimal reduction time for *Campylobacter* spp. varies depending on the type of food product but survival kinetics generally involve a rapid decline in numbers followed by a slower rate of inactivation. This may explain the increased survival rate of *Campylobacter* spp. on poultry carcasses due to the high levels of the organisms in the bird's digestive tract at the time of processing. The potential for survival decreases to a few hours at temperatures of 37ºC and increases to a few days at temperatures of 4ºC. Prevalence of *Campylobacter* spp. contamination of carcasses and poultry products can vary greatly depending on the sensitivity of the cultural procedures used and by the point along the process chain where sampling is being conducted. The type of methodology employed significantly affects incidence rates of *Campylobacter* spp. from carcasses at the final stages of processing. For example, if a survey is being conducted on the incidence of *Campylobacter* spp. on poultry carcasses post-chill and enrichment of the sample is used, then 70% to 100% of the samples can be positive for *Campylobacter* spp. If a less sensitive method is used, such as direct plating onto selective agar, which may exclude sublethally injured cells, then the number of samples detected as positive could be greatly reduced. Including both direct plating and enrichment often allows the best probability for

recovery. Even though the enrichment used is designed to be selective for *Campylobacter* spp., the organism can be culturally fragile to the extent that it can be overgrown by organisms that were meant to be suppressed (Musgrove *et al.*, 2001). Whether the injured or stressed cells could have the ability to infect humans and cause illness is still unknown. This is one reason why incidence of *Campylobacter* spp. in poultry processing plants vary between reports, and it is critical to consider the cultural procedures used and the impact those choices have on sensitivity to recover or detect the organism.

A significantly high prevalence rate of *Campylobacter* spp. contamination can be found in retail poultry and poultry products and is often directly related to the prevalence rate at the farm (Skovgaard, 2007). The average prevalence rate of infected flocks is 44% to 59% with a range of 3% to 100% (Nauta and Havelaar, 2008; Humphrey *et al.*, 2007). The number of contaminated broilers accounts for the high incidence of *Campylobacter* spp. in poultry processing plants and on processed birds (Lindqvist and Lindblad, 2008; Allen *et al.*, 2007; Jacobs-Reitsma, 2000). The average *Campylobacter* spp. prevalence rate on chicken at retail is 57% with a range of 23% to 100% (Humphrey *et al.*, 2007). In supermarkets, *Campylobacter* spp. have been isolated from 82%, 82%, and 71% of whole chickens, breast with skin attached, and pieces, respectively (Harrison *et al.*, 2001). The correlation of *Campylobacter* spp. incidence at pre-harvest does not generally relate to an increase in retail positives in other types of animal products. *Campylobacter* spp. have been recovered at a rate of 47% in cattle and 46% in swine pre-harvest (Nielsen *et al.*, 1997). Comparatively low prevalence

rates (less than 2%) of *Campylobacter* spp. have been found in these meat products at the retail level (Zhao *et al.*, 2001). The difference in the prevalence between meat types could be due to a number of factors such as: commensal level in poultry, skin removal from the carcasses of other animals during processing unlike poultry, processing procedures used for poultry carcasses, and the sheer number of poultry carcasses being processed in a plant each day.

Transmission to birds

Campylobacter spp. may enter into poultry horizontally via vertical transmission.

Horizontal transmission: Epidemiological studies of poultry flocks have shown that *Campylobacter* spp. resides in numerous vectors in the environment of a poultry farm (Sahin *et al.*, 2002). Horizontal transmission is a potential source of flock contamination, but it has been difficult to implicate a single transmission route as the primary means of flock contamination. Implementation of biosecurity programs pre-harvest have had limited success in eliminating *Campylobacter* spp. infection of broilers (Gibbens *et al*., 2001; Shreeve *et al.*, 2000). A plethora of studies have produced conclusive evidence that horizontal (i.e. litter, feed, drinking water, insects, rodents, equipment, bird to bird contact, and wildlife) transmission of *Campylobacter* spp. into and within a poultry flock does occur (Bull *et al.*, 2006; Line, 2006; Brown *et al.*, 2004; Newell and Fearnley, 2003; Herman *et al.*, 2003; Sahin *et al.*, 2002; Wedderkopp *et al.*, 2001; Ketley, 1997; Pearson *et al.*, 1996; Kazwala *et al.*, 1990; Lindblom *et al.*, 1986). It has not been conclusively shown that horizontal transmission is the primary or only route of poultry flock

contamination (Sahin *et al.*, 2002). Horizontal transmission is generally regarded by the majority of the research community as the main source of *Campylobacter* spp. introduction into poultry flocks.

Fresh feed and litter are not usually considered as a means for *Campylobacter* spp. introduction into broiler flocks due to the low water activity (Berndston *et al.*, 1996). Spent litter may be a means of transmission. Line (2006) found that in experimental environments with very high relative humidity, *Campylobacter* spp. could survive in the litter between flocks. A difference in *Campylobacter* spp. colonization rate of broilers grown on fresh litter compared to spent litter has not been found and most conclude that litter plays a very limited role in contamination (Sahin, 2003a; Payne *et al*., 1999). Water may play a role in transmission. *Campylobacter* spp. have been shown to survive in water in experimental conditions (Newell and Fearnley, 2003) and have been recovered from coastal plain streams (Vereen *et al*., 2007). In commercial poultry settings, water is usually not considered the initial cause of contamination of a broiler flock but plays a role in the cross-contamination within the flock (Lindblom *et al.*, 1986). Poultry houses contaminated by a previous flock may contribute to the contamination of subsequent poultry flocks (Lee *et al*., 2005; Petersen and Wedderkopp, 2001). Correlating the status of the next flock from evaluation of the past flock has not been successful (Evans and Sayers, 2000). Domestic and wild animals harbor *Campylobacter* spp. and can infect poultry flocks either directly or indirectly (Sahin *et al*., 2002). Insects can be carriers of *Campylobacter* spp. and increases of insect numbers in or around a poultry

house can relate to increased flock contamination (Bull *et al.*, 2006; Templeton *et al.*, 2006).

Vertical transmission: *C. jejuni* can be experimentally infected in fertile hatching eggs and at hatch the chicks carry this organism. Doyle (1984) demonstrated that *C. jejuni* could penetrate the outer membranes of refrigerated table eggs but did not find that it penetrated into the egg contents. Shanker *et al.* (1986) found that *C. jejuni* could penetrate into the inner membranes of eggs in which hens were naturally infected. Neill *et al.* (1985) showed that *C. jejuni* could penetrate into the egg and they did not find that the organism could infect the albumin or yolk. Chaudhary and others (1989) found that *C. jejuni* could penetrate the inner and outer membranes and the egg contents of cracked eggs. Using a luminescent *C. jejuni*, the organism penetrated the eggshell in 4.2% of eggs (Allen and Griffiths, 2001). Scanning electron microcopy enabled the researchers to visualize *C. jejuni* on the exterior and interior surfaces of the egg membranes. A survey within a commercial egg laying operation, found that 0.5% (1/180) of the egg shell samples were positive for *Campylobacter* spp. (Jones and Musgrove, 2007).

C. jejuni can survive within un-hatched chick contents and from the hatched chicks when the egg is experimentally infected by a temperature differential method at day 18 of incubation (Clarke and Bueschkens, 1985). Eleven percent of the hatched chicks had the organism in their intestinal tract. *C. jejuni* can survive in chicken egg yolk for 14 days at 18°C (Sahin *et al*., 2003a). Viability in the air sac and albumin was not detected at 8 days post-inoculation. *C. jejuni* was able to survive in the egg yolk of Japanese quail for up to 86 days at 4°C. *C. jejuni* has been recovered from eggs that were laid

by experimentally challenged laying hens (Sahin *et al*., 2003b). Using the chicken embryo to study *Campylobacter* invasion, injecting 11 day-old embryos intravenously resulted in invasion of some *C. jejuni* strains into the liver and the levels within the liver increased over 24 h (Field *et al.*, 1986). An increase in the percentage of highly motile strains was observed in the liver compared to the initial inoculum after inoculating the chorioallantoic membrane. The survival rate was strain dependant and even though certain strains were not recoverable after 72 h, one strain was viable after 6 days post-inoculation. Viable but nonculturable *C. jejuni* cells can be recovered in embryonated eggs (Cappelier *et al*., 1999). Thirty day-old starved *C. jejuni* cells determined to be non-culturable by the methodology used were recovered after inoculating the cells into the yolk sacs of embryonated eggs.

Vertical transmission of *Campylobacter* spp. from the hen to its progeny in commercial settings is presently being debated. The issue exists due to the inability to isolated *Campylobacter* spp. by conventional cultural techniques from the commercial hatchery environment, difficulty in measuring rates of egg transmission, variability of results between studies, and inability to recover *Campylobacter* spp. in young broilers in commercial settings. Conflicting evidence in the published literature exist as to whether *Campylobacter* spp. can pass from broiler breeder hen to progeny but some of the reports should be viewed subjectively. The sample numbers tested in certain studies are very small and then strong conclusions against vertical transmission are stated. Herman *et al*. (2003) the number of each type of hatchery sample evaluated was not discussed in the report and the only hatchery data was a statement that “no *Campylobacter* spp. were isolated in

any of the samples". Callicott *et al*. (2006) stated that they could not find any evidence for vertical transmission of *Campylobacter* spp. from grandparent flocks in Sweden and their progeny in Iceland. In the study only 13 hatchery fluff samples were tested and 86 cecal contents out of 121,926 pullet birds (progeny). This correlates to only 4 cecal samples per flock averaging 6,100 birds. In that study some of the *Campylobacter* alleles from the grandparent birds and the parent birds were similar, but they concluded that this was from genetic exchange between nations by possible migrating birds. Fonseca *et al*. (2006) sampled the meconium and a pooled sample of the heart, spleen, and liver from 117 chicks and could not isolate *Campylobacter* spp., concluding vertical transmission is a rare event. This leaves the impression that transmission *via* the egg is highly improbable. Studies with larger sample sizes support just the opposite conclusion.

Recent evidence has conclusively shown that vertical transmission can occur by cultural isolation of *Campylobacter* spp. from commercial hatchery trayliners (Byrd *et al*., 2007). Byrd *et al*. (2007) using a modified methodology procedure, incorporated a pre-enrichment step, isolated *Campylobacter* spp. from three different commercial hatcheries out of the eight hatcheries sampled. Trayliners (n=2,000) were evaluated which represented a screening of approximately 200,000 broiler chicks. Breeder lots were positive for *Campylobacter* spp. in 2 of the 3 hatcheries. The third hatchery's breeders were not sampled. In the five hatcheries that were negative for *Campylobacter* spp., the breeder lots sampled were also negative. Overall, *Campylobacter* spp. were recovered from 0.75% (15/2,000) of the trayliners sampled. Taking into account only samples in which the breeder lots were

positive, 1.6% (15/920) of the trayliners were positive. From this study an incidence rate of vertical transmission could be as high as 1/133 eggs or as low as 1/13,333 eggs. Hatcheries that have eggs being laid by positive broiler breeder lots may have a much higher incidence rate (Byrd *et al.*, 2007). When evaluating just positive breeder lots, the incidence rate could range from 1/61 to 1/6,133 eggs. Overall, the incidence rate is more likely on the low end and this may explain why in some studies the recovery of *Campylobacter* spp. from the hatchery or the fertile egg has been unsuccessful. Sahin *et al.* (2003b) sampled 500 eggs obtained from actively shedding broiler breeders and 1,000 commercial eggs obtained from a hatchery and did not detect *Campylobacter* spp. Acevedo *et al.* (2005) sampled 960 commercial breeder eggs for *Campylobacter* spp. and 1.6% of the interior egg contents and 3.8% of the egg surfaces were contaminated with the organism. *Campylobacter* spp. were culturally isolated from one of the commercial incubators but chicks were not evaluated.

Since approximately 9 billion fertile eggs are hatched per year in the U.S., this could hypothetically mean that approximately 675,000 broilers have acquired *Campylobacter* spp. through vertical transmission by a conservative evaluation of the results obtained in the Byrd *et al.* (2007) study. If a single bird in a flock is colonized then the spread to adjacent rearing mates may be rapid and within a week *Campylobacter* prevalence in the flock could reach 100% (Nauta and Havelaar, 2008; Wallace *et al.*, 1998; Gregory *et al.*, 1997; Beery *et al.*, 1988). A transmission rate of even 1/13,333 should not be considered insignificant since over 20,000 broilers represent a flock within a

single broiler house. This transmission rate may explain why *Campylobacter* spp. are not recovered within a broiler flock until 2 to 3 weeks-of-age.

Campylobacter DNA has been isolated from hatchery fluff, developing intestinal tract of embryos and newly hatched chicks (Hiett *et al*., 2003a, 2002; Lu, 2003b; Chuma *et al*., 1997, 1994). Chuma *et al.* (1994) found that 35% of the chicks sampled contain *Campylobacter* DNA. Samples of fluff and eggshells were positive for *Campylobacter* DNA using a primer set specific for the *fla*A short variable region which detected 100% of the fluff and 70% of the eggshells positive (Hiett *et al*., 2002). Neither study was able to detect *Campylobacter* spp. by conventional culture techniques. Isolation of similar clonal isolates suggests that vertical transmission of *Campylobacter* spp. occurs between broiler breeder parent flocks and their progeny broiler flocks (Cox *et al*., 2002a; Pearson *et al*., 1996). Dissemination of a fluoroquinolone-resistant *C. coli* from broiler breeder hens to their progeny (broilers) has been observed in a commercial poultry production system (Idris *et al*., 2006).

How, when, and to what extent *Campylobacter* spp. are transferred between broiler breeder flocks and its progeny (the broiler) is still not well understood or defined. Increased studies on the ecology of *Campylobacter* spp. within birds may provide valuable information in developing an understanding of the aforementioned. Studies need to be conducted to determine when and how *Campylobacter* spp. gains entry into broiler flocks and how the organism is vertically transmitted. A possible route of vertical transmission is that *Campylobacter* spp. can reside in the circulatory system and contaminate the immature and mature ovarian follicles. If *Campylobacter* spp. are present inside these ovarian follicles and survive until incubation,

they could develop reservoirs within organs and tissues of the developing embryo and resulting chick.

Ecology of *Campylobacter* inside Birds

In chickens, the main known colonization site of *Campylobacter* spp. is the mucus layer within the lower intestinal tract overlaying the epithelial cells in the ceca and cloacal crypts (Meinersmann *et al.*, 1991). *Campylobacter* spp. once colonized within the digestive tract can be found in levels up to 10^9 CFU/g of fecal content (Altekruse *et al.*, 1999). Once a broiler is colonized with *Campylobacter* spp., the organisms are usually present throughout the production cycle. Colonization of these sites in natural environments is not usually observed until 14 to 21 days-of-age (Evans and Sayers, 2000). It has been surmised that this age dependency could be due to *Campylobacter*-specific maternal antibodies (Sahin *et al*., 2003b; 2001). *Campylobacter* colonization rate in 3 day-old chicks was compared to the colonization rate in 21 day-old chicks at 1, 3, 5, 7-28 days post-inoculation (Sahin *et al.*, 2003b). The inoculation level of the two age groups was 300 CFU/bird and analysis of colonization was performed using cloacal swabs. Shedding of *C. jejuni* occurred at 3 days post-inoculation in the 21 day-old chicks but was not observed until 7 days post-inoculation in the 3 day-old chicks. The difference was believed to be due to elevated *Campylobacter*-specific maternal antibodies in the 3 day-old chick which provides protection from *Campylobacter* spp. colonization of the ceca or cloacal crypts.

A number of changes occur in the gut microflora of chicks from day-of-age until about two weeks of age. At day-of-age, the gut microflora is still rudimentary, not fully developed and the complexity of the microflora is

different from older birds (van der Wielen *et al*., 2000). Lu *et al*. (2003a) evaluated the microbial diversity of the intestinal bacterial community of maturing broilers and isolated *Campylobacter* spp. from the ileum in 3 day-old broiler chickens but not from the ceca. They surmised that the natural colonization of *Campylobacter* spp. during the early life of broilers may initially occur in the ileum or possibly other sites within the bird rather than the ceca and cloacal crypts as often seen in commercial settings. Introduction of high levels of *Campylobacter* cells into the intestinal tract could influence site colonization in young broilers. The *Campylobacter* inoculum level needed to colonize the ceca of 2 or 14 day-old chickens, 1 day post-inoculation were 50,000 CFU/chicken and strain dependant (Ringoir *et al.*, 2007). This might explain why *Campylobacter* spp. are usually readily isolated from the ceca of birds within hours after being artificially inoculated with high levels of inoculum.

Campylobacter colonization of a chick intestinal tract has been mainly limited to the gastrointestinal tract (i.e., the ceca). Evidence is emerging that suggest *Campylobacter* spp. may not be limited to the gastrointestinal tract and in fact possible colonization may occur in tissues and organs throughout the bird's body. In broiler breeder hens, *C. jejuni* and *C. coli* was isolated naturally from numerous internal sites within a bird. Cox *et al.* (2005) isolated *Campylobacter* spp. naturally from 26% of the mature and 12% of the immature ovarian follicles of commercial broiler breeder hens. *Campylobacter* spp. can be recovered from all segments of the reproductive tract of actively laying commercial breeder hens (Buhr *et al*., 2002; Carmarda *et al*., 2000; Jacobs-Reitsma, 1997). Buhr *et al*. (2002) found that an

increased number of positive samples were observed as sampling moved down the different segments of the reproductive tract towards the vagina and concluded that fecal contamination could be playing an important role in this trend. *Campylobacter* spp. have been recovered from primary (thymus) and secondary (spleen) lymphoid organs as well as from the liver/gallbladder of commercial broiler breeder laying hens (Cox *et al*., 2006a). Naturally occurring *Campylobacter* spp. were recovered from 26% of the thymuses, 19% of the spleens, and 9% of the liver/gallbladders.

Campylobacter spp. have not been culturally isolated from the testes of broiler breeder roosters, but detected by PCR from the vas deferens (Hiett *et al*., 2003b). *Campylobacter* spp. were culturally isolated from the testes and vas deferens of turkey toms (Cole *et al*., 2004). The semen of commercial broiler breeder roosters has been found to be contaminated with *Campylobacter* spp. (Cox *et al.*, 2002b). Contamination of semen by feces is thought to be the main source of contamination and not the male reproductive organs. Using electron microscopy, *C. jejuni* has been shown to attach to chicken spermatozoa (Thaxton *et al*., 2006).

In the broiler, *C. jejuni and C. coli* were naturally recovered from 20% of the spleen, 17% of the liver/gallbladder, and up to 32% of the unabsorbed yolks of commercial broilers at market age (Cox *et al*., 2007, 2006b). The presence of *Campylobacter* spp. in the internal tissues and organs has been observed in other avian species such as quail. *C. jejuni* has been recovered in inoculated quail from the lung, liver and spleen between the 7^{th} and 17^{th} d after oral inoculation (Maruyama and Katsube, 1988).

Molecular typing techniques for strain differentiation

The strain diversity within *Campylobacter* spp. has been well documented (Hiett *et al*., 2007; Rivoal *et al*., 2005; Meinersmann *et al.* 1997). Genotyping methods provide a means to discriminate one strain of *Campylobacter* from another within a species and provide a means to study the epidemiology and ecology of *Campylobacter* spp. in poultry at the molecular level (On and Jordan, 2003; Burnett *et al.*, 2002). Examples of genotyping methods for strain differentiation of *Campylobacter* spp. are ribotyping, pulsed-field gel electrophoresis (PFGE), flagellin typing (*fla*), and random amplified polymorphic DNA analysis (RAPD). The discriminatory power varies between the above genotyping methods and PFGE and *fla* typing have been shown to have adequate discriminatory power for strain differentiation (Wassenaar and Newell, 2000). PFGE evaluates the whole chromosomal DNA but is labor intensive. Organisms are embedded into an agarose gel, subjected to protease digestion and infrequent restriction enzyme digestion. The plugs are inserted into an agarose gel, electrophoresis performed, and the bands interpreted (Yan *et al*., 1991; Olive and Bean, 1999). Using more than one restriction enzyme allows for greater discriminatory power between strains (Gibson *et al*., 1994).

Flagellin typing, entails probing the flagellin gene (*flaA*) for differences or similarities between strains (Nachamkin *et al.*, 1996). The method uses a polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). Several *fla* typing procedures have been developed (Wassenaar and Newell, 2000). Meinersmann *et al.* (1997) described a system that allows differentiation of *Campylobacter* strains based on

differences in *flaA* gene sequencing. Comparison of the entire coding sequence in *flaA* in the study revealed two regions of high variability and using the short variable region and categorizing the isolates by *fla* SVR DNA sequence allowed for discrimination of *C. jejuni* isolates.

The two methods are considered standards for determining outbreak strains and for more in-depth analysis of epidemiological data but genotype instability has been demonstrated (Wassenaar *et al*., 1998). Multi-locus sequence typing showed that *C. jejuni* is partly non-clonal, having a natural ability to take up DNA (Wassenaar, 2002). This genome plasticity which allows for recombination within loci used in typing, makes for complex population genetics, and can complicate interpretations of typing data (Wassenaar, 2004).

Strain invasiveness and techniques used

Motility plays an important role in *Campylobacter jejuni* attachment and invasion into human intestinal cells (Fernando *et al*., 2007; Morooka *et al*., 1985). *Campylobacter* spp. are commensal organisms in chickens and an intense immune response is not generally observed. *In vitro* studies have shown that *Campylobacter* spp. can adhere to and invade human and chicken epithelial cells (Byrne *et al*., 2007; Hanel *et al*., 2004; Knokel and Joens, 1989). Using a *Campylobacter* shuttle vector (green fluorescent protein) provided a means to evaluate *Campylobacter* adherence and invasion *in vivo* (Mixter *et al*., 2003; Konkel *et al*., 2000). Konkel *et al*. (2000) used GFP as a means to detect *Campylobacter* binding to cultured epithelial cells. *Campylobacter* cellular attachment and invasion has not been observed *in vivo* (Meinersmann *et al*., 1991; Beery *et al*., 1988). Invasion into

chicken epithelial cells may occur because invasiveness of *Campylobacter* strains has been shown to be highly variable (Byrne *et al*., 2007; Hanel *et al*., 2004; Carvalho *et al*., 2001; Biswas *et al*., 2000). *C. jejuni* can be recovered from the thymus, spleen, liver/gallbladder, and bursa of day-of-age chicks within 1 hour post-inoculation by either the oral or intracloacal route of inoculation (Cox *et al*., 2005b). The findings suggest that some type of invasion and/or dissemination into the tissues and organs does occur. Hanel *et al.* (2004) determined that *Campylobacter* strains that strongly colonized the gastrointestinal tract of chickens were more invasive by *in vitro* cell cultures than poor colonizers of the tract. *Campylobacter* strains that were more invasive were able to be recovered from the liver of the chickens. *C. jejuni* strains have adhered to and invaded chicken intestinal cells *in vitro* (Byrne *et al*., 2007).

In vitro methods using epithelial cells have been beneficial in evaluating host-bacterial interactions and bacterial virulence (Friis *et al*., 2005; McCormick, 2003; Elsinghorst, 1994). A method commonly used to evaluate the ability of a bacterial strain to penetrate into epithelial cells is the gentamicin resistance assay (Friis *et al*., 2005). The principle is based on the mechanism of action of gentamicin on eukaryotic cells. Gentamicin does not have the ability to penetrate into these cells, but not killing any bacterial cells internalized. The assay involves incubating the *in vitro* cell culture with a bacterial cell suspension for a period of time. Then a washing step to remove non-adhered bacteria is conducted and the number of adhered bacteria can be determined. The cells are then treated with gentamicin and incubated for a period of time. After incubation, the cells are washed, lysed with a

detergent and the number of bacteria that invade determined. By evaluating the initial, adhered, and invaded bacterial cells, the invasiveness of the bacterial strain can be determined. The percent of bacterial cells that invade are very low and 1% invasion is usually interpreted as a highly invasive strain (Wassenaar *et al*., 1991; DeMelo *et al*., 1989).

Media and methodology for *Campylobacter*

Campylobacter spp. are fastidious organisms that require special growth temperatures, gaseous environments and a nutrient rich basal medium. Current conventional methods for detecting *Campylobacter* spp. in samples involve selective enrichment followed by plating onto a selective medium, microscopic observation, and biochemical confirmation or confirmation through latex agglutination. There is still no standard medium or procedure for *Campylobacter* spp. recovery (Correy *et al*., 1999). Numerous growth media (i.e., Skirrow formulation, Preston, semisolid blood-free selective medium, charcoal cefoperazone deoxycholate agar; Campy-Brucella agar (CBA), Abeyta-Hunt-Bark agar, Campy-Line agar and Campy-cefex agar) for *Campylobacter* spp. have been developed over the last 30 years (Habib *et al*., 2008; Rosenquist *et al*., 2007; Paulsen *et al*., 2005; De Mello, 2002; Denton, 2002; Line, 2001; Corry *et al*., 1995; Bolton and Coates, 1983; Skirrow, 1977). Membrane filtration techniques and semisolid enrichment media have also been developed for recovery of *Campylobacter* spp. (Potturi-Venkata *et al*., 2007; Hanninen *et al*., 2003; Jeffrey *et al*., 2000; Correy *et al.*, 1995; Shimuda and Tsuji, 1986).

Most of the media contain ingredients intended to protect *Campylobacter* spp. from the toxic effects of substances that form in the presence of oxygen and light (Bolton *et al*., 1984). This includes, but not limited to: blood, ferrous suphate, sodium metabisulphite, sodium pyruvate, haematin and charcoal (Corry *et al*., 1995). A problem observed with most of the media is that *Campylobacter* spp. can be overgrown by other organisms such as coliform bacteria, yeast and molds, and *Proteus* spp. making it difficult to evaluate whether *Campylobacter* spp. are present in the sample (Line, 2001; Corry *et al*., 1995). To combat the overgrowth in or on media, antibiotics such as vancomycin, rifampicin, polymyxin, trimethoprim, amphotericin B, cephalosporins and cycloheximide are used (Martin, 2002; Nachamkin *et al*., 2000; Correy *et al*., 1995; Humphrey, 1990). Vancomycin and rifampicin are incorporated to inhibit Gram-positives; trimethoprim inhibits *Proteus* spp. and Gram-positives; amphotericin B and cycloheximide inhibit yeasts and molds; and cephalosporins inhibit *Pseudomona aeruginosa*, *Enterobacter* spp., and *Proteus* spp. (Martin *et al*., 2002; Humphrey, 1990; Humphrey and Cruikshank, 1985). Some media are better for recovery of *Campylobacter* spp. and exclusion of background microflora than others (Potturi-Venkata *et al*., 2007; Oyarzabal *et al*., 2005; Line, 2001; Merino *et al*., 1986). The media used can also affect the number of *Campylobacter* colonies that grow on the plate (Corry *et al*., 2007; Line, 2001). The most used medium worldwide is charcoal cefoperazone deoxycholate agar (CCDA) or modified versions (Potturi-Venkata *et al*., 2007). Numerous other media are also used including Campy-cefex or modified versions. Oyarzabal *et al*. (2005) evaluated Campy-cefex, modified Campy-cefex, modified CCDA,

Karmali, CAMPY, and Campy-Line agars for recover of *Campylobacter* spp. from poultry carcass rinses and found that Campy-cefex and its modification had the best recovery rates. No statistical difference was observed compared between CAMPY, mCCDA, Karmali media, and Campy-cefex.

Selective enrichment broths are used to improve the recovery of *Campylobacter* cells within a sample (Humphrey, 1989; Shimada and Tsuji, 1986). Most enrichment broths differ in their nutrient composition, oxygen-quenching agent, antimicrobial supplements, and incubation time, temperature, and environment. Examples of selective enrichment broths that have been evaluated are Preston broth, Bolton broth, *Campylobacter* enrichment broth, blood-free enrichment broth, and Hunt enrichment broth (Corry *et al*., 1995). Using a selective enrichment broth in methodology procedures has increased sensitivity for recovery of *Campylobacter* spp. compared to direct plating (Park *et al*., 1983). Humphrey (1989) investigated the efficacy of enrichment for isolation of *C. jejuni* from water and food and found that an increase in the number of damaged cells due to heat and freezing could be recovered by enrichment in basal or selective media. Baylis *et al*. (2000) compared Bolton broth, Preston broth and *Campylobacter* selective broth (CEB) plated onto CBA and found that CEB and Bolton broth resulted in more *Campylobacter* spp. growth than Preston broth from foods. A newly developed enrichment broth called TECRA® broth may allow for better recovery of *Campylobacter* spp. than some of the above enrichment broths (Bailey *et al.*, 2008). The TECRA® CAMVIA protocol; enrichment in a proprietary TECRA® *Campylobacter* enrichment broth followed by an ELISA, was compared to a conventional cultural method, with enrichment in Bolton

medium (containing lysed horse blood), followed by plating to Campy-cefex agar. Significantly more samples were positive for *Campylobacter* spp. by the ELISA assay than the conventional cultural method. This increased recovery was believed to be due to the enrichment broth.

Several Incubation temperatures and methods have been suggested for recovery of *Campylobacter* spp. (Jones *et al*., 1999; Correy *et al*., 1995). The optimal growth temperature for thermotolerant *Campylobacter* spp. ranges from 37°C to 42°C (Corry *et al*., 1995). Incubation in an atmosphere containing 5-7% oxygen, 10% carbon dioxide and 80% nitrogen may be necessary for optimal recovery of *C. jejuni* and *C. coli* (Corry *et al*., 1995). *Campylobacter* spp. may also adapt to grow in a normal air atmosphere (Jones *et al*., 1993; Fraser *et al*., 1992). Incubating samples believed to harbor injured or stressed *Campylobacter* cells at 37°C increases recover sensitivity (Humphrey, 1989). Byrd *et al.* (2007) recovered *Campylobacter* spp. from hatchery samples after enrichment of the samples in a non-selective enrichment (buffered peptone) for 24 h at 37°C then transferring an aliquot of the sample into Bolton broth for 48 h incubated at 42°C. Selective supplements may negatively influence sensitivity of enrichment broths (Martin, 2002; Nachamkin *et al*., 2000; Humphrey, 1986; Humphrey and Cruikshank, 1985; Ray and Johnson, 1984). Enrichments containing no selective supplements or enrichments where selective supplements are added after a period of incubation have been suggested as a means to recover low levels of injured or stressed *Campylobacter* cells (Byrd *et al*., 2007; Scotter *et al*., 1993; Humphrey, 1989; 1986b).

Numerous media, enrichment broths, and procedures have been developed for recovery of *Campylobacter* cells. The majority were developed for recovery of *Campylobacter* spp. from fecal samples where large populations of *Campylobacter* cells exist and are not adequate for recovery of injured or stressed cells (Cox *et al*., 2001). In order to better evaluate the ecology of *Campylobacter* spp. within poultry flocks and routes of transmission, more sensitive enrichment broths, media and methodology procedures are needed.

Viable but non-culturable states

When environments become unfavorable for growth, *Campylobacter* spp. can enter into a viable, but non-culturable state. *Campylobacter* cells can transition from a curved, spiral morphology to a coccoid morphology when subjected to conditions that are not optimum for growth (Tholozan *et al*., 1999; Thomas *et al*., 1999; Giffiths, 1999; Ng *et al.*, 1985). The cells are metabolically active and show signs of respiratory activity, but are unable to be cultured using conventional methodology. The viable, but nonculturable (VBNC) stage was first described by Rollins and Colwell (1986) who postulated they could play a role in human infection and illness. The VBNC state arises from exposure to sublethal adverse environmental conditions and recovery occurs by passage of the organism through a susceptible host. Several studies have explored the recovery of VBNC forms of *Campylobacter* cells (Chaveerach *et al.*, 2003; Talibart *et al*., 2000; Cappelier *et al*., 1997; Bovill and Mackey, 1997; Jones *et al.*, 1991; Saha *et al.*, 1991). Non-culturable *C. jejuni* and C. *coli*, after subjection to acid stress, were shown to

be viable by injecting the cultures into the amniotic fluid and yolk sac of fertilized eggs (Chaveerach *et al.*, 2003). *C. jejuni* and *C. coli* strains which were in aqueous microcosm and became non-culturable after 30 days were determined viable after injection into 9-day fertilized eggs (Talibart *et al*., 2000). Freeze-thaw-injured *C. jejuni* cells that were non-culturable were converted back to culturable after passage through the rat gut (Saha *et al.*, 1991). Stern *et al*. (1994) found that non-culturable *Campylobacter* cells that were stored in liquid at 4°C for eight weeks were viable using a chick bioassay. Studies have produced contrary results on the ability of *Campylobacter* spp. to become VBNC and could be strain dependent (van de Giessen *et al*., 1996; Medema *et al*., 1992; Beumer *et al*., 1992). The significance of the VBNC state remains unclear and controversial, but as the understanding of this phenomenon unfolds, this could provide answers to better understand the epidemiology and ecology of *Campylobacter* spp. transmission within poultry.

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CHAPTER 3

Isolation of *Campylobacter* spp. from circulating blood of commercial broilers *via* aseptic vena-puncture of the brachial vein and the genetic relatedness and invasiveness of those isolates compared to the ceca isolates[1]

[1] L. J. Richardson, R. J. Buhr, N. A. Cox, K. L. Hiett, D. E. Akins, and M. A. Harrison. To be submitted to Applied and Environmental Microbiology. 2008

Abstract

Campylobacter spp. have been recovered from the internal organs and tissues of broiler breeders and broilers. *Campylobacter* spp. dissemination has also been shown to occur rapidly to these organs and tissues. The objectives of this study were threefold: 1) determine whether naturally occurring *Campylobacter* spp. could be recovered from the circulating blood of market age commercial broilers using aseptic techniques 2) evaluate the genetic relatedness of the *Campylobacter* isolates and 3) determine the invasiveness of those isolates. In Experiment 1, ten broilers per day were acquired from three commercial processing facilities live haul areas on 10 separate days. Feathers where removed from the ventral surface of the wing and 70% alcohol applied onto the surface of the skin, then Betadine® applied to the skin area for one min before vena-puncture (brachial vein) with a sterile needle and syringe. Five ml of circulating blood was collected and immediately added to 45 ml of Bolton broth without lysed horse blood or antibiotics. Direct plating onto aerobic plate count agar was performed to verify that the skin had been disinfected. In Experiment 2, 12-25 broilers per day were acquired from a commercial processing facilities live haul area on seven separate days. The broilers were given 20 mg of ketamine, the above procedures for sterilization perfomed, and the skin was reflected exposing the brachial vein, enabling blood collection by inserting a needle directly into the exposed blood vessel. Genotyping of the isolates was performed using the *flagellinA* short variable region (*flaA*-SVR) DNA sequence analysis. A gentamicin resistant assay was used for evaluation of invasiveness of the isolates into polarized Caco-2 cells. In Experiment 1, *Campylobacter* spp.

were recovered from 12% of the blood and 42% of the ceca sampled. In Experiment 2, *Campylobacter* spp. were recovered from 11% of the blood and 50% of the ceca sampled. A total of 7 subtypes by *flaA*-SVR DNA sequencing were found within 9 flocks. The predominant subtype (*flaA*-SVR type 1) contained isolates recovered from three flocks and from all three integrators. Closely related subtypes of *Campylobacter* spp. were naturally present among flocks and poultry integrators. From the invasion assays, 2 blood isolates were found to be highly invasive, 1 showed medium invasiveness, 6 marginal invasiveness, and 13 no invasiveness into Caco-2 cells. The ceca isolate was noninvasive while the blood isolate highly invasive in two birds, but similar by *flaA* SVR DNA sequencing. *Campylobacter* spp. being recovered from the circulating blood provides insight into a possible means by which this organism rapidly disseminates to tissues and suggests that *Campylobacter* spp. are not strictly limited to the lumens of the digestive and reproductive tracts.

Keywords: *Campylobacter*, blood, broilers, ceca, invasiveness, Caco-2 cells, genotyping, f*laA*

Introduction

Campylobacter spp. exist as commensals in many wild and domestic animals (35). The presence of this organism in the digestive tract of animals presents a risk of contamination or cross-contamination of animal products being produced for human consumption. Contamination with this pathogen can occur at numerous stages along the food chain. This includes, but not limited to production, processing, distribution, handling and preparation. Avian species, particularly poultry are the most common host for *Campylobacter* spp. and are considered to be the main source of human illness (38, 52, 56). In the U.S., it has been estimated that 2.5 million people are infected each year due to consumption of foods containing *Campylobacter* spp. (40). Seventy percent of human illnesses due to *Campylobacter* spp. are thought to be caused by the consumption or handling of raw or undercooked poultry or poultry products (23, 40). Increased attention has been given to reducing the level of *Campylobacter* spp. in poultry pre- and post-harvest to reduce the level and incidence of raw product contamination (1, 22, 35).

In order for better intervention strategies to be implemented pre-harvest, an increased understanding of *Campylobacter* spp. ecology in poultry flocks and within birds has to be attained. *C. jejuni* has been shown to disseminate to the lymphoid organs of day-of-age chicks one hour after both oral and intra-cloacal inoculation and remain seven days later in tissues and organs (15). *Campylobacter* spp. within birds has been thought to be mainly limited to the colonization of the gastrointestinal tract (i.e., the ceca). Evidence is emerging that suggest *Campylobacter* spp. may not be limited to

the gastrointestinal tract and colonization or presence does occur in tissues and organs throughout the bird's body.

In broiler breeder hens, *C. jejuni and C. coli* has been recovered naturally from numerous internal organs and tissues. *Campylobacter* spp. have been recovered from 26% of the mature and 12% of the immature ovarian follicles of commercial broiler breeder hens at various ages (14). *Campylobacter* spp. can be naturally occurring in all segments of the reproductive tract of actively laying commercial breeder hens (5, 9, 33). Through DNA sequencing of the *flaA* short variable region, different subtypes of *Campylobacter* spp. are present in the reproductive tracts of broiler breeder hens compared to in the ceca (28). *C. jejuni* and *C. coli* have been recovered from 26% of the primary (thymus) and 19% of the secondary (spleen) lymphoid organs and 9% of the liver/gallbladder of commercial broiler breeder laying hens of various ages (12). Two predominant subtypes were recovered from each flock (representing four different age groups) tested (30). Isolates that grouped in the *C. jejuni* specific subtype were recovered from the liver/gallbladder, spleen, and thymus. This group did not contain any isolates recovered from the ceca. Isolates that comprised the *C. coli* specific subtype were recovered from all locations tested. In the broiler, *C. jejuni* and *C. coli* were recovered from 20% of the spleen, 17% of the liver/gallbladder, and up to 32% of the unabsorbed yolks of commercial broilers at market age (11, 13). The diversity of subtypes within these tissue sites was evaluated by *flaA*-SVR DNA sequencing and different *Campylobacter* subtypes were recovered from the tissue sites (29).

The above studies did not determine whether *Campylobacter* spp. were located on the inside or the outside of the organs and tissues. A follow-up study (Appendix A) was conducted to determine whether *Campylobacter* spp. could be recovered from the internal contents of the spleen. Day-of-age chicks were inoculated with a characterized strain of *C. jejuni* either orally, intracloacally, or through the ocular and then the external and internal contents of the spleen sampled 1 week post-inoculation. *C. jejuni* was isolated from the internal tissues of the spleen of the inoculated broilers, suggesting that *Campylobacter* spp. could travel in the circulating blood (46). A preliminary trial was conducted in which eight and ten-week old broilers were orally inoculated with a characterized strain of *C. jejuni* and the blood sampled one week later (Appendix B). The inoculated strain was recovered from the circulating blood. The objectives of the current study were threefold: 1) determine whether naturally occurring *Campylobacter* spp. could be recovered from the circulating blood of market age commercial broilers using aseptic techniques with a modified enrichment procedure 2) evaluate the genetic relatedness of the *Campylobacter* isolates recovered from the blood and ceca, and 3) evaluate the invasiveness of the *Campylobacter* isolates obtained from the blood and compare the most invasive and least invasive to *Campylobacter* isolates recovered from the ceca of the same bird.

Materials and Methods

Experimental design

Commercial market age broilers were used. Broilers were obtained from three different poultry integrator sources over a 20-month period. A total of 19 flocks over two experiments were evaluated. The broilers from each

flock were obtained from the live haul area at the processing plant. From the dump coops on the holding area, broilers were randomly removed from several of the dump coop compartments in the holding area and placed into disinfected transport crates. The broilers were carried back to the laboratory and placed in a necropsy room. In experiment 1, for the 12 flocks evaluated, an assistant held the birds on their side with the wing extended. The feathers were removed from the ventral side of the wing over the humoral and surrounding area. Ethanol (70%) was sprayed on the skin and allowed a 30 s contact time. Betadine® was then applied to the area and allowed a 1 min contact time before vena-puncture (brachial vein) with a sterile needle. Using sterile 5 ml syringes and 20 gauge needles, 5 ml of circulating blood was collected from each broiler. The blood from each bird was then added into 50 ml sterile conjugal centrifuge tubes which contained 45 ml of modified Bolton enrichment broth (broth without the addition of lysed horse blood and supplements). Furthermore, the ceca from each bird was aseptically removed and placed into individual sterile stomacher bags. Blood and ceca samples were then placed on ice before microbiological analysis.

In experiment 2, for the seven flocks evaluated the broilers were administered 20 mg of ketamine HCL (a disassociate) into their breast muscle. The broilers were placed back into transport coops for approximately 5-7 min. to allow for the drug to enter into the broiler's system and take effect. The aforementioned procedures were used, but in addition the skin was aseptically reflected exposing the brachial vein of each broiler. Betadine® was applied to the outer area of the exposed brachial vein and allowed to sit for 1 min. The sterile needle was inserted directly into the exposed blood

vessel. In experiment 1 and 2, a total of 125 broilers and 123 broilers, respectively, were sampled. All *Campylobacter* cultural data from the experiments are expressed as number of positive isolates from each sample site over the number of sample sites tested from each carcass or as a percentage of the aforementioned. All experiments were conducted within standards of the animal care and use guidelines.

Campylobacter laboratory procedures for cultural analysis

From the individual blood samples which contained blood and modified Bolton's enrichment broth, a 0.1ml aliquot was directly streaked onto Campy-cefex agar plates and the plates were incubated at 42ºC for 48 h in a microareophilic environment containing 5% O_2, 10% CO_2 and 85% N_2. The minimum detection level by direct plating was 100 CFU/ml. The remaining blood sample containing the enrichment was then incubated for 48 h at 42°C in the above microareophilic environment. After 48 h of incubation, the direct plates were evaluated for *Campylobacter* colony forming units and results recorded. For the samples that were negative by direct plating, 0.1ml from the enriched sample was streaked onto Campy-cefex agar plates and incubated at 42ºC for 48 h in a microareophilic environment. Presumptive presence of *Campylobacter* colony forming units on the plates from all samples was confirmed using phase-contrast microscopy and observing the characteristic rapid, darting motility under wet mount. In addition, for further confirmation latex slide agglutination (Microgen Bioproducts Limited, Camberley, Surrey, U.K.) was performed. An isolate from each *Campylobacter* positive blood sample was then selected and placed into vials

containing treated beads in a cryopreservative fluid (PROTECT, Heywood, Lancashir, UK) and stored at -80°C.

The ceca were individually weighed, macerated with a rubber mallet and 3 times the weight-to-volume of Bolton broth containing lysed horse blood and supplements added. The samples were stomached for 1 min. A 0.1 ml aliquot from the ceca sample was then direct plated onto Campy-cefex agar and the enrichment and direct plates incubated for 48 h at 42°C in microareophilic conditions. The minimum detection level by direct plating was 30 CFU/g of ceca. After incubation, the above laboratory methods used for the blood samples were conducted for isolation and confirmation. An isolate from each *Campylobacter* positive ceca sample was then selected and also placed into vials containing treated beads in a cryopreservative fluid (PROTECT, Heywood, Lancashir, UK) and stored at -80°C.

Molecular subtype analysis of *Campylobacter* isolates from the blood and ceca

Campylobacter isolates (n=76) from blood and ceca samples that could be recovered from beads within the cryopreservative were used for subtype analysis. DNA analysis of the *flaA* short chain variable region was performed as previously described (31). Briefly, isolated colonies were resuspended in 300 µl of sterile H_2O and placed at 100°C for 10 min. For each boiled cell suspension, 10 µl was used as template for *flaA* SVR PCR with the following primers: FLA242FU: $^{5'}$CTA TGG ATG AGC AAT TWA AAA T$^{3'}$ and FLA625RU: $^{5'}$CAA GWC CTG TTC CWA CTG AAG$^{3'}$ (42). A 35-cycle reaction was used with 1 min denaturing at 96°C, annealing at 52°C, and extension at 72°C. This resulted in a product of approximately 425 bp.

Sequence data was generated using either the FLA242FU primer or the FLA625RU primer with the Big-Dye Dye-Terminator Cycle Sequencing Kit (ABI-PE, Foster City, CA). Data was assembled with Sequencher 4.2 (GeneCodes Corp., Ann Arbor, MI) and aligned using ClustalX (54). The aligned sequences were compared and dendograms generated using the Neighbor-Joining algorithm with HKY85 distance measurements in PAUP*4.0 (Phylogenetic Analysis Using Parsimony) (53).

Adherence and invasion assays of *Campylobacter* isolates

Experimental design/control strains: Invasiveness of 22 blood isolates into polarized Caco-2 cells was evaluated using a gentamicin resistant assay by the method of Biswas and others (2000) with some modifications (4). Select isolates from the ceca of broilers where the blood isolates were highly invasive and non-invasive were also evaluated. *E. coli* DH5α was used as the non-invasive strain and served as the negative control. *Campylobacter jejuni* 81-176 which has been shown to be highly invasive was used as a positive control strain. For the invasion assays, two replications per *Campylobacter* blood or ceca isolate were conducted and the average percent adhesion and invasion into Caco-2 cells recorded.

Inoculum preparation: *Campylobacter* blood and ceca isolates were obtained from the $^{-}$80°C refrigerator and grown for 24 h under microaerobic conditions. The negative control strain (*E. coli* DH5α) was also obtained and incubated at 37°C on Mueller-Hinton (MH) agar plates for 24 h under microaerobic conditions. Isolates were then grown in MH biphasic broth (5 ml of broth over 10 ml of agar) at 37°C under microaerobic conditions for an additional 24 h. At mid log phase (10^6-10^8 cfu/ml), 1 ml of each isolate was

centrifuged at 6,000 x g for 10 min and resuspended in 1.5 ml of MEM+1% FBS. The positive control strain (*Campylobacter jejuni* 81-176) was obtained and incubated aerobically at 37°C on Mueller-Hinton agar plates for 24 h.

Cell culture: The Caco-2 cells (human colonic carcinoma) were cultured as cell monolayers in modified Eagle medium (MEM) supplemented with nonessential amino acids, sodium pyruvate and 20% fetal bovine serum (FBS) and grown routinely in a 75-cm^2 flask at 37°C in a 5% CO_2 humidified incubator. The Caco-2 cells were seeded into 24 well plates at semiconfluency (~1 x 10^5), approximately 16 h prior to infection and incubated at 37°C in a 5% CO_2 humidified incubator to allow for monolayer reformation. Prior to the assay, the cell monolayers were washed with phosphate-buffered saline (PBS) at a pH 7.2.

Adherence and invasion assay: The invasion assays were performed by co-incubating mid-log-phase *Campylobacter* blood and ceca (grown in biphasic MH medium) isolates along with the two control isolates in triplicate with semiconfluent Caco-2 cells. A 0.5 ml aliquot of each resuspended isolate was inoculated into duplicate wells containing monolayers of Caco-2 cells. The actual number of *Campylobacter* and *E. coli* DH5α cells in the inoculum added to monolayers was determined by serial dilutions and spread plating in duplicate onto MH agar plates. The MH agar plates from the *Campylobacter* inoculums were incubated at 37°C in a microaerophilic environment. The MH agar plates from the *E. coli* DH5α were incubated at 37°C in an aerobic environment. After incubation, the colonies on the plates were counted and recorded for the appropriate dilution.

After inoculation of the Caco-2 cells with the *Campylobacter* blood and ceca isolates and the two control isolates, the Caco-2 cells were incubated for 3 h at 37°C in a 5% CO_2 humidified incubator to allow bacterial adhesion and internalization. For determination of adherence after the 3 h of incubation, half of the Caco-2 cells were washed three times with PBS and the cell monolayer lysed with 0.1% Triton X-100 in phosphate buffered saline. The total number of *Campylobacter* cells recovered from the blood and ceca along with the control isolates associated with Caco-2 cells (intracellular and extracellular bacteria) were determined by spread plating serial dilutions (10^{-1}, 10^{-3},10^{-5}) in duplicate onto MH agar plates and the above procedures used for incubation and analysis. The remaining wells containing different bacterial cells along with the Caco-2 cells were used to measure bacterial invasion. The infected cells were washed two times with PBS and incubated in fresh culture medium (MEM + FBS+supplements) containing 250 µg per ml of gentamicin for 3 h to kill remaining viable extracellular bacteria. Quantification of viable intracellular *Campylobacter* cells and invasive control cells were performed by washing the infected Caco-2 cells with PBS twice and subsequent lysing with 0.1% Triton x-100 in PBS. The total number of *Campylobacter* cells recovered from the blood and ceca along with the control invasive isolate associated with invasion into the Caco-2 cells were determined by spread plating serial dilutions (10^{-1}, 10^{-3}) in duplicate onto MH agar plates and the above procedures used for incubation and analysis.

Statistical analysis

The Chi-Square test for independence calculated by InStat 3.0 (GrahPad Software, Inc., San Diego, CA) were used to evaluate differences

in the total number of positive samples recovered from the blood and ceca of birds by flock, integrator, season, and blood collection technique. A 95% confidence interval was used with a p-value less than 0.05.

Results

Cultural analysis

In experiment 1, *Campylobacter* spp. were not recovered from the blood nor the ceca by direct plating or enrichment from broilers obtained from 6 flocks (Table 3.1). From 5 of the flocks, *Campylobacter* spp. were recovered from approximately 33% of the blood samples and 96% of the ceca samples (Table 3.1). In only one flock were *Campylobacter* spp. isolated from the blood but not the ceca. The non-recovery from the ceca could have been due to overgrowth of background microflora on the agar plates or the organism may not have originated from the ceca. Within the 5 flocks that were positive for *Campylobacter* spp., none of the blood samples were positive by direct plating and being only detected after enrichment. Levels of *Campylobacter* spp. of less than 100 CFU's/ml must have been present in the positive blood samples. The majority (66%) of positive ceca samples were detected by direct plating.

In experiment 2, *Campylobacter* spp. were not recovered from the blood nor the ceca from 2 of the flocks (Table 3.2). From 1 flock, only one ceca was positive. From 4 of the flocks, *Campylobacter* spp. were recovered from approximately 23% of the blood samples and from all of the ceca samples (Table 3.2). On no occasions were *Campylobacter* spp. recovered from the blood of a bird without being recovered from the ceca.

Campylobacter spp. were also not detected by direct plating as observed in experiment 1.

A significant difference in the overall recovery rate of *Campylobacter* spp. from the blood compared to the ceca of positive birds was observed (Table 3.3). No significant difference was observed in recovery between the three integrators (Table 3.3). In regards to the differences in recovery of *Campylobacter* spp. from the blood of broilers by the method used in either experiment 1 or 2, no significant difference was found. A significant difference was noted by season (Table 3.4). Isolation of *Campylobacter* spp. from the blood and ceca was more frequent during the summer season than all other seasons.

Molecular genotyping

The type and number of isolates analyzed from each flock is shown in Table 3.5. The dendogram of the molecular phylogeny of the isolates is shown in Figure 3.1. Isolates recovered from the blood and ceca of 9 flocks contained 7 different subtypes. Overall, different subtypes were recovered from the blood and ceca of only five broilers across three flocks. Closely related subtypes of *Campylobacter* spp. were naturally present among flocks and integrators.

The predominant subtype (*flaA*-SVR type 1) was subtype 1 which contained 47% of the isolates analyzed and the isolates in this subtype were from six flocks within integrators 2 and 3. In this subtype, six of the isolates were from the blood samples of 4 flocks and 30 isolates were from the ceca of six flocks. The blood and cecal isolates of 2 birds were the same within

this subgroup. The blood isolates from 2 birds had corresponding cecal isolates in subtype 2 and 5.

The second most dominate subtype was *flaA*-SVR type 5 which contained 21% of the isolates analyzed. This subtype contained isolates from 3 flocks sampled during the summer from integrators 2 and 3. In this subtype, 5 of the isolates were from blood samples of 3 flocks and 11 isolates were from the ceca of the same 3 flocks. The blood and cecal isolates of 2 birds were the same within this subgroup. The blood isolates from 2 birds had corresponding cecal isolates in subtype 1 and 3.

The third and fourth most predominant subtypes were *flaA*-SVR type 4 and 6, respectively. Subtype 4 contained 10 isolates sampled during the summer from integrator 2. One of the isolates in this group was a blood isolate and the rest were cecal isolates. The blood and cecal isolate of one bird were both from this subtype. Subtype 6 contained 8 isolates sampled during the summer from integrator 1 and winter from integrator 2. The blood and cecal isolates of 4 birds were the same within this subgroup and were from integrator 1.

The fifth and sixth most prominent subtypes were *flaA*-SVR type 3 and 7. Subtype 3 contained 4 isolates from a single flock sampled during the winter. The blood and ceca of 1 bird were the same within this group. Subtype 7, contained 2 isolates from a single flock sampled during the winter. The least prominent subtype was *flaA*-SVR type 2, which only contained 1 isolate.

Invasion assays

The invasiveness of the blood isolates is shown in Table 3.6. The *Campylobacter* isolates that adhered to the polarized Caco-2 cells ranged from 0.001-3.22% of the starting inoculums. Sixteen (55%) of the *Campylobacter* strains adhered to the cells at a range of 0.001-<0.2%. Nine (31%) of the *Campylobacter* strains adhered to the cells at a range of 0.2-<1%. Four (14%) of the *Campylobacter* strains adhered to the cells at a range of 1-3.22%. The invasiveness of the *Campylobacter* isolates into polarized Caco-2 cells ranged from 0.000-1.255%. Fourteen (48%) of the *Campylobacter* strains did not or invaded at a very low level into the cells and ranged from 0.000-<0.01%. Twelve (41%) of the *Campylobacter* strains that invaded showed low to medium invasiveness and ranged from 0.01-<0.1%. Three (10%) of the *Campylobacter* strains were medium to highly invasive and invasion ranged from 0.1-1.255%. The non-invasive control *E. coli* DH5α adhered to the Caco-2 cells at a rate of 0.090 and did not invade into the Caco-2 cells, while the invasive control *C. jejuni* 81-176 adhered at a rate of 0.958 and invaded at a rate of 0.951%. From the invasion assays, 2 blood isolates (I2-W-8-9B and I2-Sp-14-25B) were found to be highly invasive but the ceca isolate (I2-W-8-9C and I2-Sp-14-25C) from the same bird were not found to be invasive. The invasive isolates (I2-W-8-9B and I2-Sp-14-25B) that were recovered from the blood and the non-invasive ceca isolate ((I2-W-8-9C and I2-Sp-14-25C) recovered from the same bird were similar by *flaA*-SVR DNA sequence analysis even though differences were observed in invasiveness (Figure 3.1).

Discussion

The ability of *Campylobacter* spp. to reside in the blood of commercial market age broilers was demonstrated in this study. *Campylobacter* spp. were isolated from the blood of broilers obtained from all three poultry integrators evaluated. The number of flocks and broilers sampled within each integrator varied but no significant difference in the incidence rate between the three was found. All three integrators used different genetic parent lines in their breeder operations. Presence of *Campylobacter* spp. in the blood could be surmised not to be breeder line dependant. A difference in recovery rate of *Campylobacter* spp. from blood or ceca samples was noted by the season in which the sample was taken. The difference in recovery by season has been documented in numerous other studies (32, 57). Studies have shown an increase in *Campylobacter* spp. incidence in poultry flocks during warmer months. A study evaluated the prevalence rate of *Campylobacter* spp. in 10,803 broiler flocks over a 3 year period found that a significant spike in the prevalence rate of *Campylobacter* spp. in broiler flocks was observed during the summer months (32). No recovery of *Campylobacter* spp. in the fall was observed in our study. This could have been due to the low number (n=20) of samples and not from seasonality effects.

Evaluation of the recovery rate of *Campylobacter* spp. between the two experiments revealed no significant differences. A concern in the first experiment was contamination of the blood sample with possible *Campylobacter* spp. present on the skin. The possibility of *Campylobacter* spp. along with other bacteria being on the skin of the wing had to be considered (34, 39). It has been shown that levels of greater than $\log_{10} 4$

CFU/cm^2 is found on chicken skin (26). When a phlebotomist collects blood from humans, they disinfect the area with alcohol. However, the skin of a human and chicken differ. Betadine® is an effective bactericide and has some penetration ability. Therefore, in experiment 1, Betadine® was also rubbed onto the skin where the blood sample was going to be taken. When insertion of the needle through the skin was performed, special attention was given not to penetrate the skin through a feather tract. If *Campylobacter* contamination of the blood was coming from the skin then differences in recovery rate would have been expected between the two experiments.

No *Campylobacter* spp. were isolated from the blood by direct plating. Since the minimum detection level by direct plating was 100 CFU/mL of blood, the level of *Campylobacter* spp. in the blood was probably low. Whether the *Campylobacter* cells in the blood were in an injured or stressed state was not determined. *Campylobacter* enrichment broths may not be sensitive enough to recover low levels, injured or stressed cells due to the possible negative effects of the antibiotics in the enrichment. Background microflora in the blood was not a concern and a modified Bolton enrichment broth was used which incorporated Bolton broth without lysed horse blood or antibiotic supplements. This may have increased the sensitivity of the enrichment procedure and subsequent recovery rate of *Campylobacter* cells from the blood.

A significantly greater number of positive isolates were recovered from the ceca samples than from the blood samples. In almost every case, *Campylobacter* spp. were recovered from the blood and from the ceca. In the majority of the broilers, the isolates obtained from the blood were similar to

the isolates obtained from the ceca of that same bird by DNA sequencing of the *flaA* short variable region. Within five birds, different *flaA* (SVR) types were observed. The strain diversity within *Campylobacter* spp. has been well documented (6, 17, 31, 45, 47). In the present study, a total of seven subtypes were observed in nine broiler flocks from three poultry integrators and similar subtypes were found between flocks and different integrators.

In chickens, the main known colonization site of *Campylobacter* spp. is the mucus layer within the lower intestinal tract overlaying the epithelial cells in the ceca and cloacal crypts (41). *Campylobacter* spp. that are colonized within the digestive tract can be found in levels up to 10^9 CFU/g of fecal content (2). Greater recovery of *Campylobacter* spp. in the ceca was expected in this study. Colonization of the ceca in natural environments is not usually observed until 14 to 21 days of age (19). Age dependency could be due to *Campylobacter*-specific maternal antibodies (49, 50). *Campylobacter* colonization rate in 3 day-old chicks was compared to the colonization rate in 21 day-old chicks at 1, 3, 5, 7-28 days post-inoculation (48). The inoculation level of the two age groups was 300 CFU/bird and analysis of colonization was performed using cloacal swabs. Shedding of *C. jejuni* occurred at 3 days post-inoculation in the 21 day-old chicks, but was not observed until 7 days post-inoculation in the 3 day-old chicks. The difference was believed to be due to elevated *Campylobacter*-specific maternal antibodies in the 3 day-old chick which may have provided protection from *Campylobacter* colonization of the ceca or cloacal crypts.

A number of changes occur in the gut microflora of chicks from day-of-age until about two weeks-of-age. At day-of-age, the gut microflora is still

rudimentary, not fully developed and the complexity of the microflora is different from older birds (55). In a study evaluating the microbial diversity of the intestinal bacterial community of maturing broilers, *Campylobacter* spp. were isolated from the ileum in 3 day-old broiler chickens but not from the ceca (37). In commercial settings the natural colonization of *Campylobacter* spp. during the early life of broilers may initially occur in the ileum or other sites within the bird other than the ceca and cloacal crypts. *Campylobacter* strains have been found to be invasive when 11 day-old chicken embryos were inoculated intravenously and invasion into the liver occurred and the levels within the liver increased over 24 h (21).

Motility plays an important role in *Campylobacter* spp. attachment and invasion into human intestinal cells (20, 44). *Campylobacter* spp. are considered commensal in chickens and an intense immune response is not generally observed. *In vitro* studies demonstrated that *Campylobacter* spp. can adhere to and invade human and chicken epithelial cells (8, 25, 36). Using a *Campylobacter* shuttle vector (green fluorescent protein) has provided a means to evaluate *Campylobacter* adherence and invasion *in vivo* (36, 43). *Campylobacter* cellular attachment and invasion into chicken epithelial cells has not been observed *in vivo* (3, 41). Invasion into chicken epithelial cells may occur because invasiveness of *Campylobacter* strains is variable (4, 8, 10, 25). *C. jejuni* can be recovered from the thymus, spleen, liver/gallbladder, and bursa of day-of-age chicks within 1 h post-inoculation by either the oral or intracloacal route of inoculation (15). This suggests that invasion and/or dissemination into the tissues and organs occurs. Hanel *et al.* (2004) determined that *Campylobacter* strains that strongly colonized the

gastrointestinal tract of chickens were more invasive by *in vitro* cell cultures than poor colonizers of the tract (25). *Campylobacter* strains that were more invasive were recovered from the liver of the chickens. *C. jejuni* strains have also adhered to and invaded chicken intestinal cells *in vitro* in another study (8).

In vitro methods using epithelial cells have been beneficial in evaluating host-bacterial interactions and bacterial virulence (18, 24). A method commonly used to evaluate the ability of a bacterial strain to penetrate into epithelial cells is the gentamicin resistance assay (18). The principle is based on the mechanism of action of gentamicin on eukaryotic cells. Gentamicin does not have the ability to penetrate into these cells therefore not killing any bacterial cells internalized. The percentage of bacterial cells that invade are very low and 1% invasion is usually interpreted as a highly invasive strain (16, 56). In our study, differences in the invasiveness of isolates were observed. In two of the birds in which the blood isolates were highly invasive, the ceca isolate was non-invasive but the isolates from each sample type within the birds were determined to be similar by DNA sequencing of the *flaA* short variable region. Analysis of invasiveness using the DNA sequencing of the *flaA* short variable region may not be reliable. Only a few isolates were examined and additional study is needed.

Increased studies on the ecology of *Campylobacter* spp. within birds may provide valuable information in developing an understanding of the aforementioned. If *Campylobacter* spp. are invading into the epithelial cells then this could be one route the organisms are entering into the circulating

blood and a possible route of *Campylobacter* spp. entry into tissues and organs within birds. The significance of *Campylobacter* spp. in the circulating blood and the ability of these organisms to invade chicken epithelial cells are unknown. Vertical transmission of *Campylobacter* spp. from the broiler breeder hen to its progeny the broiler through the mature ovarian follicles could be occurring. *C. jejuni* and *C. coli* have been cultured from the ovarian follicles of commercial broiler breeder hens of various ages (14). *C. jejuni* survives in chicken egg yolk for 14 days at 18°C but survival in the air sac and albumin not detected at 8 days post-inoculation (48). The presence of *Campylobacter* spp. inside ovarian follicles and its survival time in yolk, *Campylobacter* spp. may be transmitted trans-ovarian and developing reservoirs within organs and tissues of the embryo.

Vertical transmission of *Campylobacter* spp. from the hen to its progeny is debated. This stems from the inability to isolate *Campylobacter* spp. by conventional cultural techniques from the commercial hatchery environment, difficulty in measuring rates of egg transmission, variability of results between studies, and inability to recover *Campylobacter* spp. in young broilers in commercial settings. The inability to culture *Campylobacter* spp. from young commercial broilers may be due to the insensitive methods of sampling flocks and inadequate number of samples taken. Conflicting evidence in the published literature exist as to whether *Campylobacter* spp. can pass from breeder hen to progeny in commercial settings. Recent evidence has conclusively shown that vertical transmission can occur by cultural isolation of *Campylobacter* spp. from commercial hatchery trayliners (7). The researchers incorporated a pre-enrichment step and isolated

Campylobacter spp. from three different commercial hatcheries out of the eight hatcheries sampled. Trayliners (n=2,000) were evaluated which represented a screening of approximately 200,000 broiler chicks. Breeder lots were positive for *Campylobacter* spp. in 2 of the 3 hatcheries. The third hatchery's breeders were not sampled. In the five hatcheries that were negative for *Campylobacter* spp., the breeder lots sampled were also negative. *Campylobacter* spp. were recovered from 0.75% (15/2000) of the trayliners sampled. Taking into account only samples in which the breeder lots were positive, 1.6% (15/920) of the trayliners were positive. How, when, and to what extent *Campylobacter* spp. are transferred between broiler breeder flocks and its progeny (the broiler) is still not well understood or defined.

In the present study, only market age broilers were tested and further studies are needed to determine whether the presence of *Campylobacter* spp. in the blood of broilers can be found throughout the production cycle and the significance of the invasiveness of those organisms. Molecular techniques other than *flaA* SVR typing may provide for increased discriminatory power to evaluate the different invasiveness characteristics. The presence of *Campylobacter* spp. in broiler breeders throughout their production cycle also needs to be determined. Avian blood characteristically differs from mammalian blood (27). The blood glucose level in avian blood is approximately double that in mammals and plasma protein content is substantially lower (51). The interaction of *Campylobacter* spp. with the components within avian blood needs further study. The immunological

response within birds, the ability to survive in the blood over time, and transient time in blood of *Campylobacter* spp. needs to be elucidated.

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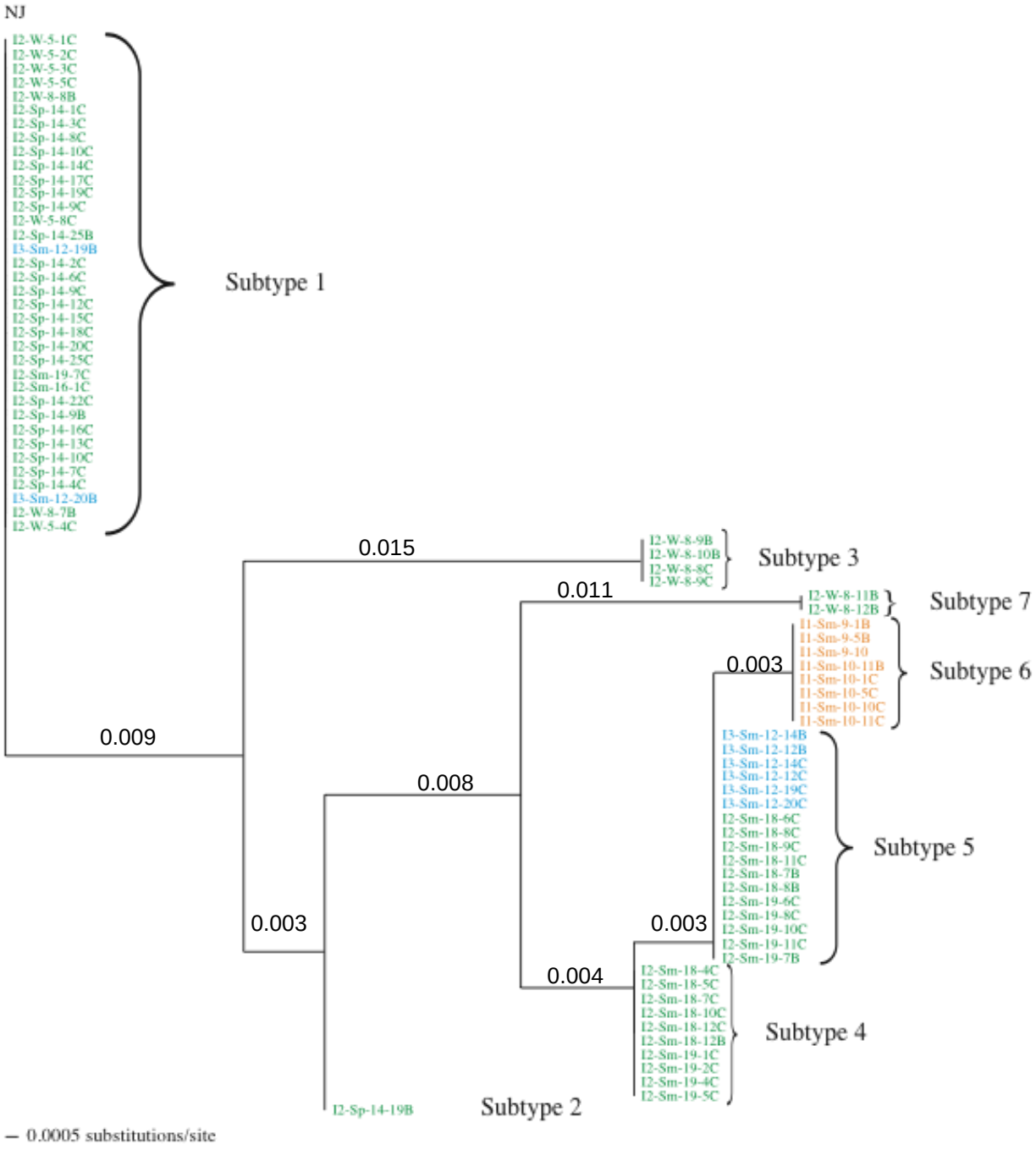
NJ
I2-W-5-1C
I2-W-5-2C
I2-W-5-3C
I2-W-5-5C
I2-W-8-8B
I2-Sp-14-1C
I2-Sp-14-3C
I2-Sp-14-8C
I2-Sp-14-10C
I2-Sp-14-14C
I2-Sp-14-17C
I2-Sp-14-19C
I2-Sp-14-9C
I2-W-5-8C
I2-Sp-14-25B
I3-Sm-12-19B
I2-Sp-14-2C
I2-Sp-14-6C
I2-Sp-14-9C
I2-Sp-14-12C
I2-Sp-14-15C
I2-Sp-14-18C
I2-Sp-14-20C
I2-Sp-14-25C
I2-Sm-19-7C
I2-Sm-16-1C
I2-Sp-14-22C
I2-Sp-14-9B
I2-Sp-14-16C
I2-Sp-14-13C
I2-Sp-14-10C
I2-Sp-14-7C
I2-Sp-14-4C
I3-Sm-12-20B
I2-W-8-7B
I2-W-5-4C
Subtype 1
0.015
I2-W-8-9B
I2-W-8-10B
I2-W-8-8C
I2-W-8-9C
Subtype 3
0.011
I2-W-8-11B
I2-W-8-12B
Subtype 7
I1-Sm-9-1B
I1-Sm-9-5B
I1-Sm-9-10
I1-Sm-10-11B
I1-Sm-10-1C
I1-Sm-10-5C
I1-Sm-10-10C
I1-Sm-10-11C
0.003
Subtype 6
0.009
I3-Sm-12-14B
I3-Sm-12-12B
I3-Sm-12-14C
I3-Sm-12-12C
I3-Sm-12-19C
I3-Sm-12-20C
I2-Sm-18-6C
I2-Sm-18-8C
I2-Sm-18-9C
I2-Sm-18-11C
I2-Sm-18-7B
I2-Sm-18-8B
I2-Sm-19-6C
I2-Sm-19-8C
I2-Sm-19-10C
I2-Sm-19-11C
I2-Sm-19-7B
Subtype 5
0.008
0.003
0.003
0.004
I2-Sm-18-4C
I2-Sm-18-5C
I2-Sm-18-7C
I2-Sm-18-10C
I2-Sm-18-12C
I2-Sm-18-12B
I2-Sm-19-1C
I2-Sm-19-2C
I2-Sm-19-4C
I2-Sm-19-5C
Subtype 4
I2-Sp-14-19B
Subtype 2
0.0005 substitutions/site

Figure 3.1. Relationships derived from comparison of the short variable region (SVR) of the *flaA* gene DNA sequences from *Campylobacter* spp. isolates obtained from the circulating blood and ceca of commercial broilers. The dendrogram was generated using the Neighbot-Joing algorithm with HKY85 distance measurements in PAUP*4.0b (Phylogenetic analysis utilizing Parsimony) following alignment of the sequences in ClustalX. Isolates are labeled as follows: the first letter set (I) and number set (1-3) indicate the integrator in which the isolate were recovered from the bird. The second letter set (F, W, Sp, S) indicate the season from which the isolates were recovered. The second number set (1-19) indicates the flock from which the sample was isolated. The third number set indicates the bird from which the isolate was recovered and the third letter set indicates whether the isolated came from the blood or ceca sample. A distance of 1% was arbitrary chosen for distinction of subtypes.

Table 3.1. Recovery of *Campylobacter* spp. from the blood of commercial broilers after disinfecting the skin surface and vena-puncture of the unexposed brachial vein, along with recovery from the ceca.

Flock number	Recovery[1]	
	Blood	Ceca
1	0/10	0/10
2	0/10	0/10
3	0/10	0/10
4	0/10	0/10
5	1/10	7/10
6	0/10	0/10
7	0/10	0/10
8	6/10	3/10
9	3/10	9/10
10	1/10	9/10
11	0/10	10/10
12	4/15	15/15
Total	15/125[b]	53/125[a]

[1] Recovery is expressed as number positive over number sampled
[a-b] Significance between total recovery for isolates from blood and ceca set at P>0.05

Table 3.2. Recovery of *Campylobacter* spp. from the blood of commercial broilers after disinfection of the skin and vena-puncture of the exposed brachial vein, along with recovery from the ceca.

Flock number	Recovery[1]	
	Blood	Ceca
1	0/25	0/25
2	4/25	25/25
3	0/25	0/25
4	4/12	12/12
5	0/12	1/12
6	3/12	12/12
7	3/12	12/12
Total	14/123[b]	62/123[a]

[1]Recovery is expressed as number positive over number sampled
[a-b] Significance between total recovery for isolates from blood and ceca set at P>0.05

Table 3.3. Incidence of *Campylobacter* spp. in the blood and ceca of commercial market age broilers evaluated by poultry integrator

Integrator	Season Sampled		Flock Sampled		Incidence[1]	
Sampled	E[2] 1	E 2	E 1	E 2	Blood	Ceca
1	F[3], Sm[4]	ns[5]	2, 9, 10	ns	4/30[b]	18/30[a]
2	W[6]	Sp[7], Sm	5-8	1-7	21/163[b]	72/163[a]
3	W, Sm	ns	1,3-4,11,12	ns	4/55[b]	25/55[a]
Total					29/248[b]	115/248[a]

[1] Recovery is expressed as number positive over number sampled
[2] Experiment
[3] Fall
[4] Summer
[5] No sample was tested
[6] Winter
[7] Spring
[ab] Significance between incidence for each integrator and total samples set at P>0.05

Table 3.4. Comparison of *Campylobacter* spp. recovery from the blood and ceca of commercial market age broilers by season.

Sample Site	Recovery by Season[1]			
	Fall	Winter	Spring	Summer
Blood	$^{z}0/20^{d}$	$^{z}7/85^{d}$	$^{z}4/50^{d}$	$^{y}18/93^{c}$
Ceca	$^{z}0/20^{d}$	$^{z}10/85^{d}$	$^{x}25/50^{b}$	$^{w}80/93^{a}$

[1] Recovery is expressed as number positive over number sampled
[a-d] Significance between samples within rows set at P>0.05
[w-z] Significance between samples within columns set at P>0.05

Table 3.5. Breakdown of isolates utilized for the *flaA* SVR DNA sequence of the *Campylobacter* isolates obtained from the blood and ceca of commercial broilers

Flock[1]	Isolates for *flaA* SVR DNA sequence			
	Total Isolates	Sequenced	Ceca	Blood
5	8	6	6	0
8	9	8	2	6
9	12	3	0	3
10	10	5	4	1
12	19	8	4	4
14	29	24	22	2
16	15	1	1	0
18	15	11	8	3
19	15	10	9	1
Total	132	76	56	20

[1] Isolates from one flock in experiment 1 and one flock in experiment 2 were not sampled. Flocks 5, 8, 9, 10, 12 are from experiment 1 and flocks 14, 16, 18, 19 are from experiment 2. Flock 14=flock 2; Flock 16=flock 4; flock 18=flock 6 and flock 19=flock 7 from experiment 2.

Table 3.6. The average percent of *Campylobacter* cells that adhered and invaded Caco-2 cells using a gentamicin resistant assay.

Strain[1]	% Adhered[2]	% Invaded[3]
C. jejuni 81-176[4]	0.958 +/- 0.026	0.951 +/- 0.000
E. coli DH5α[5]	0.064 +/- 0.014	0.000 +/- 0.000
I2-W-5-3B	0.035 +/- 0.003	0.039 +/- 0.006
I2-W-8-7B	0.369 +/- 0.363	0.012 +/- 0.011
I2-W-8-8B	0.035 +/- 0.015	0.018 +/- 0.013
I2-W-8-9B	**3.220 +/- 1.053**	**1.255 +/- 0.115**
I2-W-8-9C	**0.137 +/- 0.106**	**0.027 +/- 0.018**
I2-W-8-10B	0.003 +/- 0.000	0.003 +/- 0.001
I2-W-8-12B	0.094 +/- 0.081	0.030 +/- 0.029
I1-Sm-9-1B	0.240 +/- 0.010	0.003 +/- 0.003
I1-Sm-9-5B	**0.103 +/- 0.098**	**0.002 +/- 0.001**
I1-Sm-9-5C	**0.001 +/- 0.000**	**0.000 +/- 0.000**
I1-Sm-9-10B	0.125 +/- 0.116	0.008 +/- 0.008
I1-Sm-9-11B	1.850 +/- 0.150	0.008 +/- 0.000
I3-Sm-12-12B	0.525 +/- 0.527	0.050 +/- 0.050
I3-Sm-12-14B	0.088 +/- 0.072	0.019 +/- 0.009
I3-Sm-12-19B	0.004 +/- 0.002	0.000 +/- 0.000
I3-Sm-12-20B	1.243 +/- 1.231	0.010 +/- 0.003
I2-Sp-14-9B	1.685 +/- 0.105	0.029 +/- 0.021
I2-Sp-14-25B	**1.025 +/-0.276**	**0.960 +/- 0.261**
I2-Sp-14-25C	**0.74 +/- 0.366**	**0.048 +/- 0.029**

I2-Sm-16-1B	0.011 +/- 0.009	0.000 +/- 0.000
I2-Sm-16-2B	**0.084 +/- 0.072**	**0.105 +/- 0.045**
I2-Sm-16-2C	**0.233 +/- 0.175**	**0.063 +/- 0.031**
I2-Sm-16-4B	0.736 +/- 0.506	0.002 +/- 0.002
I2-Sm-16-8B	0.769 +/- 0.763	0.053 +/-0.046
I2-Sm-18-7B	0.005 +/- 0.004	0.001 +/- 0.000
I2-Sm-18-8B	0.355 +/- 0.216	0.000 +/- 0.000
I2-Sm-18-12B	0.065 +/- 0.035	0.000 +/- 0.000
I2-Sm-19-9B	0.022 +/- 0.016	0.004 +/- 0.004
I2-Sm-19-12B	0.001 +/- 0.000	0.000 +/- 0.000

[1] Isolates are labeled as follows: the first letter set (I) and number set (1-3) indicate the integrator, second letter set (F, W, Sp, Sm) indicate the season, second number set (1-19) indicates the flock; third number set indicates the bird from which the isolate was recovered, the third letter set indicates whether the isolated came from the blood (B) or ceca (C).
[2] Average % adherence of two replications performed per isolate and +/- standard error.
[3] Average % invasion of two replications performed per isolate and +/- standard error.
[4] Positive Control
[5] Negative Control

Chapter 4

Evaluation of TECRA® broth, Bolton broth and direct plating for recovery of *Campylobacter* spp. from broiler carcass rinses from several commercial processing plants[1]

[1] L. J. Richardson, J.S. Bailey, N. A. Cox, M. E. Berrang, J. E. Cox, P. Fedorka-Cray, and M. A. Harrison. To be submitted to the J. Food Protection. 2008.

Abstract

The purpose of this study was to compare a conventional culture method (Bolton enrichment broth containing lysed horse blood) to a newly developed proprietary TECRA® *Campylobacter* enrichment broth for the recovery of *Campylobacter* spp. from chicken carcass rinses. Whole carcass rinses were taken from 140 carcasses at rehang (immediately after defeathering but before evisceration) and 140 carcasses at post-chill from 8 different processing lines in the United States. The samples were ice packed and shipped overnight to the laboratory. An aliquot of the rinse from each sample was transferred into Bolton and TECRA® enrichment broths. In addition, direct plating was performed. Standard laboratory procedures using Campy-cefex plates were followed for the recovery of *Campylobacter* spp. For carcass rinse samples obtained at rehang, 86% of samples were positive for *Campylobacter* spp. by TECRA® enrichment broth and 74% by Bolton enrichment broth. For carcass rinse samples obtained post-chill, 74% of samples were positive for *Campylobacter* spp. by TECRA® enrichment broth and 71% by Bolton enrichment broth. Evaluation of non-*Campylobacter* colonies on Campy-cefex plates revealed that TECRA® enrichment broth significantly suppressed the competing organisms when compared to Bolton enrichment broth. Overall, TECRA® enrichment broth yielded a 7.5% increase in the total number of positive samples compared to Bolton enrichment broth. *Campylobacter* spp. detection in post-chill samples was significantly greater employing enrichment than direct plating. Direct plating only detected 19% of the samples positive. Analysis of *Campylobacter*-positive samples by both

enrichment procedures revealed that 90% of the carcass rinses obtained at rehang and 87% at post-chill contained *Campylobacter* spp.

The genus *Campylobacter* has had increased focus as a threat to food safety, due to the rise in enteritis in humans caused by consumption or handling of foods contaminated with the organism. The infectious dose for campylobacters can be as few hundred cells (*9, 15*). *Campylobacter* spp. can be sensitive to environmental conditions outside of an animal's intestinal tract (*28*). *Campylobacter* spp. are sensitive to drying, atmospheric concentration, and low pH (less than or equal to 4.7) but are still a significant cause of bacterial gastroenteritis in humans. In the U.S., approximately 2.5 million people are infected each year due to consumption of foods containing campylobacters (*27*). Four species (*C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*) are known as "thermophilic campylobacters" and are clinically significant due to their association as dominant causative agents of human campylobacteriosis (*10, 20, 23*). *C. jejuni* is the predominant species causing bacterial gastroenteritis in the U.S. and in many other developed countries with *C. coli* being second (*25*). In the U.S., campylobacteriosis and salmonellosis alternate as the leading bacterial foodborne illness (*1, 27*).

Cross-contamination of food products is a major factor that contributes to human illness. Outbreaks of human campylobacteriosis have been associated with raw milk, untreated water, and poultry meat. Poultry meat is frequently contaminated

with the organism and may be responsible for approximately 70% of sporadic campylobacteriosis cases (*14, 40*). Contamination is thought to originate from the intestinal tract of poultry and spread to the meat during transport and processing. Broiler crops, particularly after a feed withdrawal prior to transport to the processing facility, harbor large numbers of *Campylobacter* (*7, 18, 38*). *Campylobacter* levels in the intestinal tract of birds entering the plant can be 10^7 CFU/g cecal contents and when whole carcasses with feathers are rinsed 10^6 CFU/ ml of rinse can be recovered (*5, 29*).

Campylobacter spp. are fastidious organisms that require special growth temperature, gaseous environments and nutrient rich basal medium. Current conventional methods for detecting *Campylobacter* spp. in samples involve selective enrichment followed by plating or direct plating onto selective media, microscopic observation, and biochemical confirmation or confirmation through latex agglutination. There is no standard medium or procedure for *Campylobacter* spp. recovery. Numerous growth media (i.e., Skirrow formulation, Preston, semisolid blood-free selective medium, charcoal cefoperazone deoxycholate agar (CCDA); Campy-Brucella agar (CBA), Abeyta-Hunt-Bark agar, Campy-Line, and Campy-cefex) for *Campylobacter* spp. have been developed (*13, 16, 26, 35, 37*). Membrane filtration techniques and semisolid enrichment media have also been developed for recovery of *Campylobacter* spp. (*13, 21, 36, 39*).

Selective enrichment broths are used to improve the recovery of *Campylobacter* cells within a sample that may be in low numbers, injured or stressed (19, 39). Most enrichment broths differ in their nutrient composition, oxygen-quenching agent, incubation time, temperature, environment and the antibiotics used to suppress growth of competing organisms. Examples of selective enrichment broths that have been evaluated are Preston broth, Bolton broth, *Campylobacter* enrichment broth, blood-free enrichment broth, buffered peptone water, and Hunt enrichment broth (*3, 8, 30*). Using a selective enrichment broth for recovery of *Campylobacter* spp. has increased sensitivity compared to direct plating (*32*). An investigation of the efficacy of enrichment for isolation of *C. jejuni* from water and food found that the number of heat or freeze damaged cells recovered was increased by enrichment in basal or selective media (*19*). A newly developed *Campylobacter* enrichment broth (TECRA® broth) may allow for improved recovery of *Campylobacter* spp. than previous used enrichment broths (*2*).

The TECRA® CAMVIA protocol; enrichment in a proprietary TECRA® *Campylobacter* enrichment broth followed by an ELISA, was compared to a conventional cultural method, with enrichment in Bolton medium (containing lysed horse blood), followed by plating to Campy-cefex agar. Significantly more samples were positive for *Campylobacter* spp. by the ELISA assay than the conventional cultural method. The increased recovery was thought to be because of the differences in the enrichment broths.

The objectives of the current study were threefold: i) compare TECRA® broth to Bolton broth for the recovery of natural *Campylobacter* spp. from carcass rinse samples obtained at rehang and post-chill from commercial processing facilities; (ii) determine the efficiency of TECRA® broth to reduce background microflora present by evaluating contamination levels on the plating media (iii) compare enrichment to direct plating for recovery of *Campylobacter* spp. from carcass rinse samples.

Materials and Methods

Experimental design. The efficacy of TECRA® enrichment broth for recovery of *Campylobacter* spp. from broiler carcass rinses was compared to Bolton enrichment broth containing lysed horse blood and to direct plating. The plating medium used for all procedures was Campy-cefex agar. Samples were collected from 8 different processing facilities throughout the United States. A total of 140 carcasses were obtained from rehang and 140 post-chill. From each of 3 processing facilities, 10 carcasses were obtained from rehang (after defeathering, but before evisceration) and 10 carcasses post-chill. Twenty carcasses from the two sampling sites were obtained during visits to 4 processing facilities and 30 carcasses during a visit to one processing facility. Each carcass was placed in a sterile plastic bag and 100 ml of sterile water added prior to rinsing (*12*). Carcass rinses were then poured into sterile specimen cups, placed on ice and transported overnight to the laboratory. The next day sample analysis was performed.

Enrichment broth procedures. From each of the rinse samples obtained at rehang and post-chill, 5 ml of rinsate was transferred into sterile sampling bags containing 45 ml of Bolton enrichment broth (Acumedia Manufacturers, Inc., Baltimore, MD) containing lysed horse blood (Lampire Biological, Everett, PA) and antibiotic supplement (Dalynn Biologicals, Calgary, AB, Canada). Five ml were also dropped into bags of TECRA® enrichment broth (TECRA® Int. Pty. Ltd., Frenchs Forest, NSW, Australia). The Bolton enriched samples were incubated at 42ºC for 48 h in a microareophilic environment containing 5% O_2, 10% CO_2 and 85% N_2. The TECRA® enriched samples were incubated for 48 h at 42°C in an aerobic environment as specified in the TECRA® CAMVIA protocol. After incubation, 0.1ml aliquot from samples enriched in Bolton and TECRA® broth was streaked onto Campy-cefex agar plates (Acumedia). The plates were then incubated in a microareophilic environment for 48 h at 42ºC. Presence of *Campylobacter* colony forming units on the plates from all samples was confirmed using phase-contrast microscopy and observing the characteristic rapid, darting motility under wet mount. For further confirmation, latex slide agglutination (Microgen Bioproducts Limited, Camberley, Surrey, U.K.) was performed.

Direct plating procedure. A 0.25 ml aliquot of the undiluted sample was spread plated onto each of 4 Campy-cefex plates in order to eliminate dilution effect and increase the minimum detection level. Serial dilutions of each type of rinse sample were prepared using phosphate-buffered saline. A 0.1 ml aliquot from

the dilutions was plated in duplicate onto Campy-cefex agar plates. The plates were then incubated in a microaerophilic environment at 42ºC for 48 h. After incubation, the plates were observed for presumptive *Campylobacter* colonies and confirmation was performed as described for the enrichment samples.

Background microflora evaluation. To roughly evaluate the effects of both broths to suppress non-*Campylobacter* spp. present in the initial rinses, a four quadrant streak method was performed from samples obtained from 5 of the processing facilities (Figure 4.1). The thinking was that if greater non-*Campylobacter* microflora was present within the broths, a greater number of the quadrants would contain non-*Campylobacter* colonies. The identity of recurring non-*Campylobacter* colony types was determined. Each isolate was transferred onto total plate count agar and incubated for 24 h at 37ºC. An isolated colony was obtained from the plate and identity determined by biochemical testing using the Vitex 2 system (biomerieux, Inc., Durham, NC).

Statistical analysis. The Chi-Square test for independence was used to evaluate differences in the total number of carcasses determined to be *Campylobacter* positive from TECRA® broth, Bolton broth or by direct plating. The Kruskal-Wallis test along with the Dunn's multiple comparisons test was used to evaluate the differences in suppression of non-*Campylobacter* colonies in the enrichment broth. For all tests, significance was set at a p-value less than

0.05. All statistical analyses were conducted using InStat 3.0 (GrahPad Software, Inc., San Diego, CA).

Results

The incidence of *Campylobacter* spp. by processing plant determined by use of direct plating and enrichment in TECRA® and Bolton broth is shown in Tables 4.1 and 4.2. The overall incidence of *Campylobacter* spp. from the carcass rinses taken at rehang were not significantly different by the methods of direct plating or enrichment in TECRA® broth (Table 4.1). Significantly fewer carcasses overall were determined to be *Campylobacter* spp. positive by enrichment in Bolton broth. The overall incidence of *Campylobacter* spp. from the carcass rinses taken at post-chill was not significantly different between the two enrichment methods (Table 4.2). Significantly fewer carcasses overall were determined to be *Campylobacter* spp. positive by direct plating compared to enrichment of the post-chill samples.

The average number of quadrants on Campy-cefex agar plates containing non-*Campylobacter* colonies after streaking from the two enrichment broths from 5 of the processing plants is shown in Table 4.3. Overall, significantly fewer quadrants contained non-*Campylobacter* colonies from the sample that had been streaked from the TECRA® enrichment broth. In addition, significantly fewer quadrants contained non-*Campylobacter* colonies on Campy-cefex agar plates regardless of enrichment broth from post-chill compared to rehang samples. The

non-*Campylobacter* colonies most often observed on the Campy-cefex agar plates from aliquots originating from the TECRA® broth was *Candida albicans* and *E. coli*. The non-*Campylobacter* colonies most often observed on the Campy-cefex agar plates from aliquots originating from the Bolton broth were determined to be *Aeromonas sobria*, *Acinetobacter baumannii*, and *Pseudomonas flourescens*. The percent of samples where *Campylobacter* spp. were recovered by direct plating compared to evaluation of both enrichment procedures (two-way analysis) is shown in Figure 4.2. No significant difference in recovery of *Campylobacter* positive carcasses was observed at rehang when comparing direct plating to the two-way analysis of enrichment procedures. Significantly more carcasses where determined to be positive post-chill using the two enrichment broths and two-way analysis (positives obtained from both broths). From all samples analyzed: 148, 204, and 225 were determined to be *Campylobacter* positive by direct plating, Bolton enrichment and TECRA® enrichment, respectively.

Discussion

In a poultry processing plant, samples taken at the rehang station usually contain larger populations of *Campylobacter* spp. and other organisms than samples taken down the processing line where interventions have occurred such as exposure to chlorine in the chill tank (*6*). External contamination often increases during transport from grow-out houses to the processing plant. *Campylobacter* counts decrease in the scalding tank and increase during removal of feathers

(picking) (*4*). In most commercial processing facilities in the U.S., carcasses are chilled by immersion in air agitated ice water in a series of tanks. Examining carcass rinses taken from rehang and post-chill allowed for TECRA® broth to be evaluated for recovery of *Campylobacter* cells in samples where an increased number of competitive organisms are present or where injured or stressed cells are present. Bolton broth was employed as the standard broth for comparison because this broth has been used in numerous poultry research studies for recovery of *Campylobacter* spp. (*30, 35*). Results were compared to direct plating because of the possibility of competitive organisms being high in samples (*26*).

The method employed in the study to evaluate presence of non-*Campylobacter* organisms allowed for the quick estimation of the enrichment broths effectiveness in suppressing or eliminating these organisms. The organisms found in the study contaminating the media plates have been isolated in other studies (*11, 22*). Competitive organisms in TECRA® broth were found to be suppressed more than competitive organisms in Bolton broth. This might explain why fewer carcasses were determined to be *Campylobacter* spp. positive by Bolton broth from rehang samples due to the inability to suppress the competitive organisms. The difference in the suppression of competitive organisms and the type of organisms most often associated with the two broths could have been due to the antimicrobials used in the two broths. The supplement used in Bolton broth contained cefoperzaone, vancomycin, trimethoprim, and cycloheximide.

The supplement for TECRA® broth contained trimethoprim, rifampicin, and polymyxin B. The differences in selective supplements may have contributed to the differences in competitive organisms that were observed on the Campy-cefex plates. The incubation environment may have also contributed to the differences. In the TECRA® protocol, a microaerophilic environment was not used but the Bolton broth protocol used a microaerophilic environment.

TECRA® broth was efficient in suppressing competitive organisms and for recovering *Campylobacter* spp. from rehang samples compared to direct plating onto Campy-cefex agar plates. Direct plating was insufficient in evaluating the prevalence of *Campylobacter* spp. post-chill in the present study as seen in the incidence rate of only 19%. The low prevalence rate of *Campylobacter* spp. on carcasses determined by direct plating has been documented in other studies (*6, 41*). The chilling process which includes application of antimicrobials has a tendency to injure or kill *Campylobacter* spp., especially on those carcasses that are highly contaminated. This was evident in the study because the minimum detectable level by direct plating was presumed to be 1 CFU/ml, but the prevalence rate from post-chill carcasses was significantly higher after enrichment than direct plating. In the present study, the large numbers of carcasses after post-chill may have contained sub-lethally injured cells. The observation that 87% of the samples were positive by evaluation of both enrichment broths after chilling may suggest that more than one enrichment broth should be employed when analyzing samples believed to harbor low

numbers of injured or stressed cells. The impact of each component in an enrichment medium on recovery of *Campylobacter* spp. is not fully elucidated and might explain the variability of recovery between the two enrichments. In general, enrichment and plating media contain ingredients intended to protect *Campylobacter* spp. This includes but not limited to blood, ferrous suphate, sodium metabisulphite, sodium pyruvate, haematin and charcoal (*13*). One problem observed with media and enrichment broths is that *Campylobacter* spp. can be overgrown by other organism such as coliform bacteria, yeast and molds, and *Proteus* spp. making it difficult to evaluate whether *Campylobacter* spp. are present in the sample (*13, 26*). To combat the overgrowth in or on media and in enrichment broths, varying antibiotics such as vancomycin, rifampicin, polymyxin, trimethoprim, amphotericin B, cephalosporins and cycloheximide are added to the media and broths (*13*). Most are not efficient in reducing the level of competitive organisms when the levels of these organisms present in samples are in high numbers.

Certain media are better at recovery of *Campylobacter* spp. and exclusion of background microflora than others (*16, 26, 31, 36*). The media used can also affect the number of *Campylobacter* colonies that grow on the medium (*26*). The most popular medium believed to be used worldwide is charcoal cefoperazone deoxycholate agar (CCDA) or modified versions (*36*). Numerous other media are also used including Campy-cefex or modified versions (*31*). Evaluation of Campy-cefex, modified Campy-cefex, modified CCDA, Karmali, CAMPY, and

Campy-Line agars for recovery of *Campylobacter* spp. from poultry carcass rinses determined that Campy-cefex and its modification had the best recovery rates (*31*). As with plating media, certain enrichment broths are better at recovery as well (3, 30, 36). Comparing Bolton broth, Preston Broth and *Campylobacter* selective broth (CEB) it was determined that CEB and Bolton broth resulted in more *Campylobacter* spp. growth than Preston broth from foods (3). Bolton broth has also been found to be more sensitive than Preston broth in another study (36). A modified version of buffered peptone water is as efficient as Bolton broth for recovery of *Campylobacter* spp. (30).

The prevalence rate was 71, 74 or 87% using Bolton broth, TECRA® broth or positives from both broths, respectively, but only 19% by direct plating from post-chill samples. Prevalence and state of *Campylobacter* spp. on carcasses can vary greatly depending on the sensitivity of the cultural procedures used and by the point along the process chain where sampling is being conducted. The type of methodology employed significantly affects prevalence rates of *Campylobacter* spp. from carcasses at the final stages of processing. Several studies have been conducted in order to obtain a basic knowledge of survival mechanisms of *Campylobacter* spp. (*17, 24, 28, 34*). The ability of injured or stressed *Campylobacter* cells to recover in environments outside of the animal host or the significance to food safety is not fully understood (*33*). Even stressed or sub-lethal injured cells present on a carcass must be considered in prevalence studies. The differences in methodology procedures employed in the present

study further confirm the variability in procedures to recover *Campylobacter* spp. It is critical to consider the cultural procedures used and the impact those choices have on sensitivity to recover or detect the organism. Direct plating allows for the evaluation of a processing step on reducing the level of contamination on a carcass whereas this is not achievable by using enrichment (*6*). The method did not accurately assess the incidence rate of *Campylobacter* spp. on carcasses after the chilling process in the present study. In summary, the sensitivity of TECRA® broth was better than Bolton broth for recovery of *Campylobacter* spp. from carcass rinses but the use of both enrichment procedures on each sample allowed for the best sensitivity.

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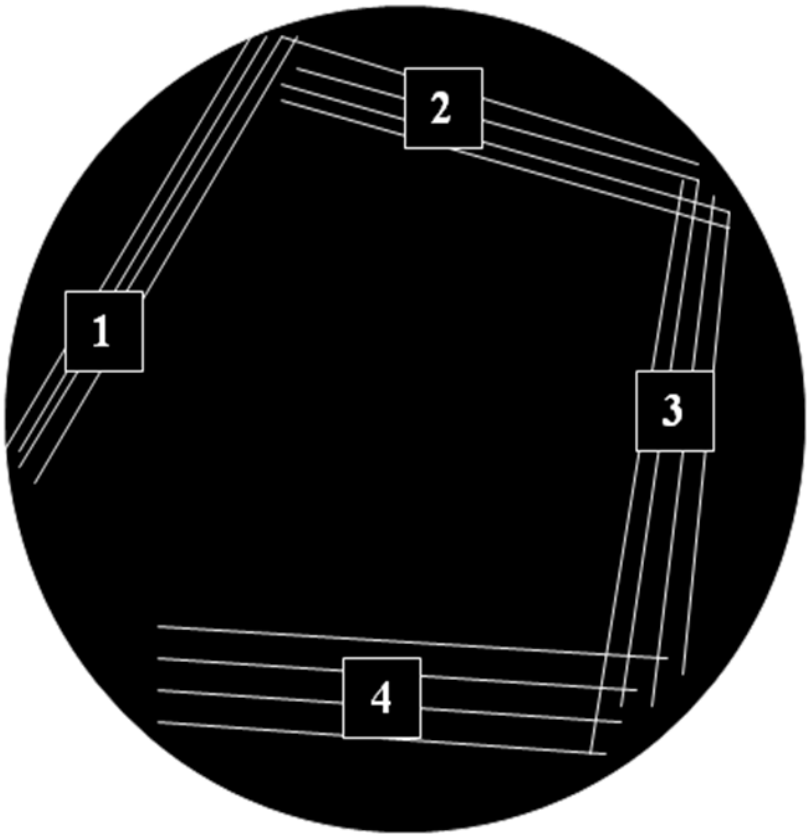

Figure 4.1. Illustration of the estimation method used to determine the efficacy level of the enrichment broths to suppress contaminants during enrichment. (contamination number = mean of highest quadrant)

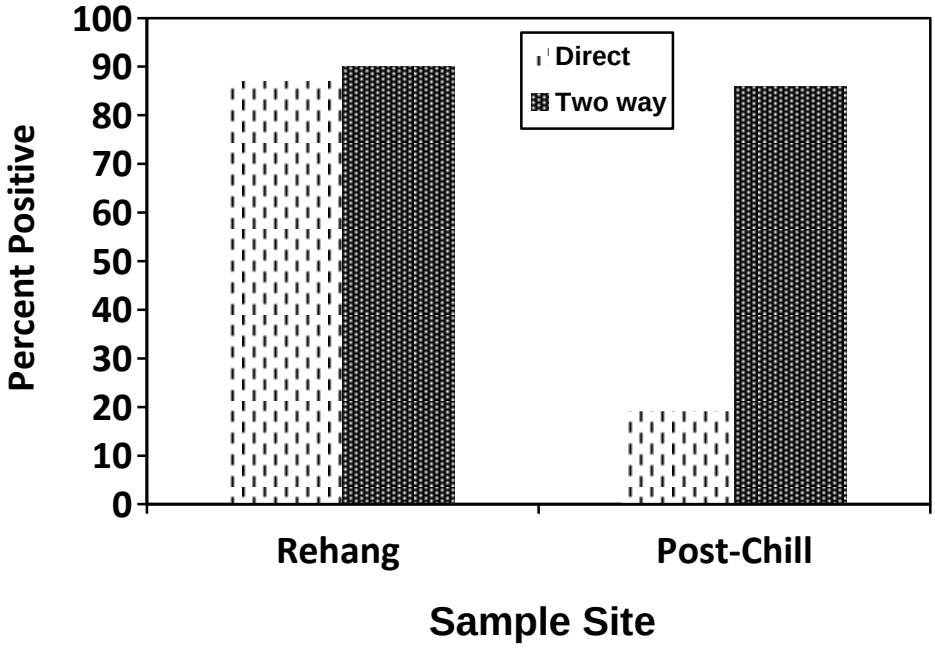

Figure 4.2. The overall percentage of *Campylobacter* spp. positive poultry carcasses obtained from rehang and after chilling from the eight processing facilities evaluated by direct plating and analysis of both enrichment broths (Two-way analysis).

Table 4.1. *Campylobacter* spp. incidence on carcass rinse samples obtained at rehang from 8 poultry processing facilities evaluated by TECRA® broth, Bolton broth, and direct plating

Plant no.	Methodology[1]		
	TECRA®[2]	Bolton	Direct Plating
1	10/10[a]	1/10[b]	0/10[b]
2	10/10[a]	10/10[a]	10/10[a]
3	9/10[a]	10/10[a]	10/10[a]
4	20/20[a]	15/20[a]	20/20[a]
5	26/30[a]	21/30[b]	30/30[a]
6	16/20[a]	16/20[a]	18/20[a]
7	20/20[a]	20/20[a]	20/20[a]
8	20/20[a]	10/20[b]	4/20[c]
Total	131/140[a]	103/140[b]	112/140[a]

[1] Values are no. of positive carcasses over the number of carcasses tested
[2]Within each row, mean prevalence's with different letters (a, b, or c) are significantly different ($P<0.05$, chi-square test for independence)

Table 4.2. *Campylobacter* spp. incidence on carcass rinse samples obtained post-chill from 8 poultry processing facilities evaluated by TECRA® broth, Bolton broth and direct plating

Plant no.	Methodology[1]		
	TECRA®[2]	Bolton	Direct Plating
1	9/10[a]	6/10[a]	0/10[b]
2	6/10[a]	9/10[a]	0/10[b]
3	10/10[a]	10/10[a]	0/10[b]
4	20/20[a]	18/20[a]	16/20[a]
5	24/30[a]	19/30[a]	4/30[b]
6	0/20[b]	6/20[b]	6/20[b]
7	16/20[a]	16/20[a]	0/20[b]
8	19/20[a]	16/20[a]	0/20[b]
Total	104/140[a]	100/140[a]	26/140[b]

[1] Values are no. of positive carcasses over the number of carcasses tested
[2]Within each row, mean prevalence's with different letters (a or b) are significantly different ($P<0.05$, chi-square test for independence).

Table 4.3. Mean number of quadrants where non-*Campylobacter* colonies were present on Campy-cefex agar plates after streaking from TECRA® and Bolton broth from rehang and post-chill samples from each of 5 poultry processing facilities

Plant	Competitors rehang		Competitors post-chill	
	Bolton	TECRA®	Bolton	TECRA®
1	4.0	2.7	3.6	0.9
2	3.0	0.0	2.7	0.7
3	2.7	1.0	0.7	0.2
5	3.8	2.0	1.0	0.3
7	3.5	2.4	1.0	0.2
Mean[12]	3.47+/-0.11[a]	1.09+/-0.12[b]	1.49+/-0.16[bc]	0.39+/-0.07[d]

[1]Within the means row prevalence with different letters (a, b, c or d) are significantly different (P<0.05, Kruskal-Wallis test).
[2]Values are mean +/- standard error

Chapter 5

Sensitivity of eleven enrichment procedures employing two enrichment broths and direct plating for *Campylobacter* spp. recovery from commercial poultry carcass rinses[1]

[1] L. J. Richardson, J. S. Bailey, N. A. Cox, M. E. Berrang, and M. A. Harrison. To be submitted to the International Journal of Food Microbiology. 2008.

Abstract

Improvements in cultural recovery methods for *Campylobacter* spp. are essential to accurately assess the epidemiology and ecology of this organism in poultry. The objectives of this study were to evaluate delayed addition of antibiotics, incubation temperature, modified atmosphere, and combinations of the three for recovery of *Campylobacter* from poultry carcass rinses using TECRA® and Bolton enrichment broths. In study 1, post-pick and post-chill carcass rinses (n=198) were obtained from 20 processing plants over a two month period. Five different Bolton enrichment procedures and eight different TECRA® enrichment procedures were used to recover *Campylobacter* spp. In study 2, post-chill carcass rinses (n=150) were obtained from a single commercial processing facility over three consecutive days. Six TECRA® enrichment procedures and two Bolton enrichment procedures were used to recover *Campylobacter* spp. From each carcass rinse sample in both studies, an aliquot (2 ml) was placed into 18 ml of enrichment broth for each enrichment procedure. Direct plating was also performed. After incubation, an aliquot was streaked from the different enrichments onto Campy-cefex plates and standard laboratory procedures performed. The level of non-*Campylobacter* spp. on the plating media was also determined. In study 1, from direct plating, 50% post-pick and 4% post-chill rinse samples were positive for *Campylobacter* spp. The delayed addition of antibiotics combined with reduced incubation temperature for 5 h in Campy-gas all contributed to increased recovery of *Campylobacter* from TECRA® enrichment broth. The best TECRA® enrichment procedure recovered

80% post-pick and 39% post-chill positive samples. The best Bolton enrichment procedure recovered 72% post-pick and 38% post-chill positive samples. In study 2, 16% of the samples from direct plating and 57% from enrichment samples (taking into account all enrichment procedures) were positive for *Campylobacter* spp. The best TECRA® and Bolton enrichment procedures determined that 13 and 2, respectively, of the 86 confirmed positive post-chill rinse samples were positive after being stored at 4ºC for 7 days . Overall, TECRA® broth with delayed addition of antibiotics and reduced temperature for 5 h with incorporation of Campy-gas resulted in the greatest number of positive samples in both experiments. The second best TECRA® broth procedure used Campy-gas with no delayed addition of antibiotics. The best Bolton broth procedure was a standard procedure incorporating lysed horse blood with Campy-gas. Overall, direct plating was not a reliable method to determine qualitatively the number of samples positive for *Campylobacter* spp. The level of non-*Campylobacter* colonies present on the plating media differed between the enrichment procedures used.

Keywords: *Campylobacter*, poultry, enrichment broth, enrichment procedures, carcass rinses

Introduction

Thermophilic *Campylobacter* spp. are one of the leading causes of bacterial gastroenteritis in the U.S. and poultry products implicated as a significant source of human infection (Anonymous, 2006; Jacobs-Reitsma, 2000; Keener *et al*., 2004; Lastovica, 2006). Four species (*C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*) are known as "thermophilic campylobacters" and are clinically significant due to their association as dominant causative agents of human campylobacteriosis (Blaser *et al.*, 1982; Jacobs-Reitsma, 2000; Keener *et al.*, 2004). In the U.S., approximately 2.5 million people are infected each year due to consumption of foods containing campylobacters (Mead *et al*., 1999). *C. jejuni* is the predominant species that causes bacterial gastroenteritis in the U.S. and in many other developed countries, with *C. coli* being second (Lastovia, 2006). Thus, it is pivotal that research be conducted to develop more standardized methodologies and focus on studies that further elucidate the life cycle of this organism in poultry. Recovery of injured, stressed or low numbers of *Campylobacter* cells from poultry samples continues to be a challenge.

Campylobacter spp. are fastidious organisms which require specific growth temperatures, gaseous environment and nutrient rich basal medium. Most of the media contains ingredients intended to protect *Campylobacter* spp. from the toxic effects of substances that form in the presence of oxygen and light. This includes, but is not limited to blood, ferrous suphate, sodium metabisulphite, sodium pyruvate, haematin and charcoal (Corry *et al*., 1995). To combat overgrowth in or on media, antibiotics such as vancomycin, rifampicin, polymyxin,

trimethoprim, amphotericin B, cephalosporins and cycloheximide have been incorporated (Correy *et al*., 1995).

Most enrichment broths differ in their nutrient composition, oxygen-quenching agent, incubation time, temperature, environment and the antibiotics used to suppress growth of competing organisms. Preston broth, Bolton broth, *Campylobacter* enrichment broth, blood-free enrichment broth, buffered peptone water, and Hunt enrichment broth are examples of selective enrichment broths that have been used (Baylis *et al*., 2000; Beuchat, 1985; Oyzarabal *et al*., 2007). Use of selective enrichment broths for recovery of *Campylobacter* spp. increases sensitivity compared to direct plating (Park, 1983). An investigation of the efficacy of enrichment for isolation of *C. jejuni* from water and food found that heat and freeze damaged cells could be recovered by enrichment in basal or selective media (Humphrey, 1989). Under certain conditions selective enrichments and incubation temperature can have a negative effect on recovery of very low numbers of cells or injured cells (Humphrey and Cruickshank, 1985; Ray and Johnson, 1984). Enrichments containing no selective supplements or enrichments where selective supplements are added after a period of incubation have been suggested to recover injured cells or to detect *Campylobacter* in samples containing very low numbers of the organism (Byrd *et al*., 2007; Humphrey, 1986, 1989). The presence of *Campylobacter* spp. from chick trayliners was only demonstrated after using a pre-enrichment step followed by enrichment in Bolton broth (Byrd *et al*., 2007).

The culture methods used for analysis of *Campylobacter* spp. from poultry samples have improved over the years. Further improvements in the enrichment broths and cultural procedures are key elements in order to evaluate the ecology of *Campylobacter* spp. in poultry. TECRA® broth was found to be a better enrichment media overall compared to Bolton broth (Richardson *et al*., 2008). In that study, significantly more broiler carcass rinse samples were determined to be *Campylobacter* spp. positive using TECRA® broth than Bolton broth.

In a poultry processing plant, samples taken before chilling usually contain larger populations of *Campylobacter* spp. and other organisms than samples taken after chilling (Berrang *et al*., 2007). This allows for samples to be taken before and after interventions where levels of competitive organisms differ and possible injured or stressed *Campylobacter* cells are present. The objectives of this study were threefold: 1) compare direct plating, TECRA® enrichment, and Bolton enrichment broth for recovery of *Campylobacter* from poultry carcass rinses 2) compare the effects of delayed addition of antibiotics, incubation temperature, modified atmosphere, and combinations of the three in increasing the sensitivity for recovery of *Campylobacter* spp. from different sets of poultry carcass rinses and 3) evaluate the ability of the enhanced enrichment procedures to recover *Campylobacter* cells from confirmed positive carcass rinse samples after storage at 4°C for 1 week.

Materials and Methods

Experimental Design

In study 1, 198 carcass rinse samples were obtained from 20 different processing plants across the U.S. over a two month period during the fall season. From each plant, 5 carcasses were selected post-pick and 5 post-chill. All carcasses from each plant came from a single flock. For analysis of each post-pick sample, two Bolton and three TECRA® enrichment procedures were used (Table 5.1). For analysis of each post-chill sample, three Bolton and five TECRA® enrichment procedures were used (Table 5.2). Direct plating was performed on each of the post-pick and post-chill samples.

In study 2, to eliminate plant-to-plant variation and better evaluate the efficacy of certain enrichment procedures, post-chill carcass rinses (n=150) were obtained from a commercial processing facility over a three day sampling period. Flocks from 4 different farms were sampled over the three days. Fifty carcasses were pulled from the processing line over two, eight hour processing shifts on each of three consecutive days. Using results from study 1, for analysis of each post-chill sample, two Bolton and six TECRA® enrichment procedures were employed (Table 5.3).

Sampling and Enrichment Laboratory Procedures

For both study 1 and 2, at each sampling the carcasses were rinsed with 100 ml of 1% buffered peptone, and the rinse was placed in sterile sampling cups, packed on ice and transported overnight to the laboratory. From each carcass rinse sample, an aliquot (2 ml) was placed into 18 ml of enrichment

broth. After incubation by the designated procedure, 0.1 ml aliquot from each of the enrichment samples was streaked onto Campy-cefex agar plates. The plates were then incubated in a microareophilic environment for 48 h at 42ºC. *Campylobacter* colony forming units present on the plates from all samples were presumptively classified using phase-contrast microscopy and observing the characteristic rapid, darting motility under wet mount and latex agglutination used for confirmation.

Direct plating procedure

A 0.25 ml aliquot from the original carcass rinses was spread plated onto each of 4 Campy-cefex plates in order to eliminate dilution effect and increase the minimum detection level. Serial dilutions of each type of rinse sample were prepared using phosphate-buffered saline. A 0.1 ml aliquot from the dilutions was plated in duplicate onto Campy-cefex agar plates. The plates were then incubated in a microaerophilic environment at 42ºC for 48 h. After incubation, the *Campylobacter* colonies were counted and confirmed described for the enrichment samples.

Background microflora evaluation

To get a rough evaluation on the effects of the broths to suppress non-*Campylobacter* spp. present in the initial rinses, a four quadrant streak method was used in study 1 (Richardson *et al*., 2008). Briefly, the theory of the evaluation was that by streaking the sample into quadrants, if greater non-*Campylobacter* microflora was present within the broths, a greater number of the quadrants would contain these types of microflora. This would allow for the

evaluation of the enrichment procedures to suppress the non-*Campylobacter* organisms. Each quadrant was assigned a number (1-4). The first quadrant correlated to the least number of non-*Campylobacter* colonies present and was designated with a numerical value of 1. The subsequent quadrants were designated with the numerical values 2, 3, and 4. The greater the number of positive quadrants, the greater the level of non-*Campylobacter* colonies present.

Analysis of 7 day cold stored samples

In study 2, after sampling, all rinses were immediately placed at 4ºC and held for 7 days. The samples determined to be positive during the initial sampling were removed after 7 days and re-analyzed for presence of *Campylobacter* spp. The TECRA® and Bolton procedure that resulted in the greatest number of *Campylobacter* spp. positive samples in the initial evaluation were used in the 7 day analysis. From each carcass rinse sample determined to be positive at initial sampling, an aliquot (2 ml) was placed into 18 ml of each of the enrichment broths. After incubation, positive samples were confirmed as described previously.

Statistical analysis

The Chi-square test for independence was utilized to evaluate differences in the total number of carcasses determined to be *Campylobacter* positive by the multiple enrichment procedures used. The Kruskal-Wallis test along with the Dunn's multiple comparisons test was performed to evaluate the differences in suppression of non-*Campylobacter* colonies from the enrichment procedure

used. Significance was set at a p-value less than 0.05. All statistical analyses were conducted using InStat 3.0 (GrahPad Software, Inc., San Diego, CA).

Results

Study 1

Only samples from 1 plant were negative for *Campylobacter* spp. by all methods. Nineteen out of twenty plants were positive by at least one method from evaluation of post-pick samples. The efficacy of 5 different enrichment procedures for recovery of *Campylobacter* spp. from post-chill carcass rinses is shown in Figure 5.1. The best Bolton enrichment procedure (A) determined *Campylobacter* spp. were present in 72/100 post-pick carcass rinses. Delayed addition of antibiotics was not an effective method for recovery of *Campylobacter* spp. from post-pick samples. The best TECRA® enrichment procedure (D) determined *Campylobacter* spp. were present in 80/100 post-pick rinses. The delayed addition of antibiotics using TECRA® broth did improve recovery. Variation from plant to plant was noted in post-pick samples and in some plants the Bolton procedures recovered *Campylobacter* spp. where they were not detected by any TECRA® procedure and vice versa. The best TECRA® method had an 8% increase in recovery of *Campylobacter* spp. but was not significantly better than the best Bolton method.

Analysis of post-chill rinse samples, determined that only 12/20 of the plants were positive by at least one of the methods. The efficacy of 8 different enrichment procedures for recovery of *Campylobacter* spp. from post-chill carcass rinse samples is shown in Figure 5.2. No significant difference was

found between the most efficient Bolton and TECRA® enrichment method. The best Bolton enrichment procedure (F) determined that 37/98 post-chill rinses were positive for *Campylobacter* spp. The best TECRA® enrichment procedure (L) recovered *Campylobacter* spp. from 38/98 of the carcass rinse samples. Delayed addition of antibiotics and reduced temperature for 5 h had a negative effect on recovery of *Campylobacter* spp. using Bolton enrichment broth, but a positive effect on recovery when using TECRA® enrichment broth. Variation in efficacy of enrichment procedures from plant-to-plant was observed in post-chill samples.

The incidence of *Campylobacter* spp. in post-pick and post-chill carcass rinses evaluated by direct plating, and multiple analyses of all enrichment procedures is shown in Figure 5.3. The combination of all enrichment methods increased the overall incidence of *Campylobacter* spp. from post-pick and post-chill carcass rinse samples. Direct plating significantly underestimated the incidence of *Campylobacter* spp. in post-pick and post-chill carcass rinse samples. The overall level of *Campylobacter* spp. in the positive post-pick carcass rinses was $\log_{10}$ 1.57 CFU/ml of rinse (SE +/- 0.18) and from post-chill samples, the level was $\log_{10}$ 0.81 CFU/ml of rinse (SE +/- 0.02). Evaluation of enrichment broths to suppress non-*Campylobacter* spp. in the broth determined by evaluation of Campy-cefex plates is shown in Figures 5.4 and 5.5. Overall, lower non-*Campylobacter* spp. on Campy-cefex plates after streaking from the different enrichment procedures were found with procedures using TECRA® broth.

Study 2

The incidence of *Campylobacter* spp. by day in post-chill carcass rinses obtained from a single processing facility over 3 days is shown in Figure 5.6. Variation in the incidence was observed from day-to-day. The efficacy of the 8 enrichment procedures for recovery of *Campylobacter* spp. from the post-chill carcass rinses is shown in Figure 5.7. A significant difference in the incidence of *Campylobacter* spp. in post-chill carcass rinses was observed by the best Bolton and TECRA® enrichment method. A greater number of *Campylobacter* spp. positive rinse samples were determined by the best TECRA® enrichment procedure than the best Bolton enrichment procedure. The combination of delayed addition of antibiotics, reduced temperature for 5 h and incorporating gas was the best overall method for increasing TECRA® sensitivity. Incorporating gas had the most significant impact on Bolton and TECRA® broth sensitivity.

The incidence of *Campylobacter* spp. in post-chill carcass rinse samples by direct plating and multiple analyses of enrichment procedures over the 3 day sampling period are shown in Figure 5.8. Direct plating significantly underestimated the incidence of *Campylobacter* spp. in post-chill carcass rinse samples. The overall level of *Campylobacter* spp. in the positive post-chill carcass rinse samples was $\log_{10}$ 0.31 CFU/ml of carcass rinse (SE +/- 0.05). The best TECRA® (P) and Bolton (N) enrichment procedures were able to recover *Campylobacter* spp. from 15% (13/86) and 2% (2/86), respectively, from the confirmed positive rinse samples which were stored at 4ºC for 7 days.

Discussion

Numerous approaches have been developed with success to control/prevent *Salmonella* serovars from entering and colonizing poultry flocks, therefore reducing the number of *Salmonella*-positive flocks entering the processing plant. The measures put into place for *Salmonella* serovars have had some success in controlling *Campylobacter* spp. colonization of poultry flocks and in the processing plant but a high incidence still remains. This has been postulated to be due to the differences in the ecology, physiology, and epidemiology of these two human pathogens (Newell and Fearnley, 2003). Evaluation of numerous studies determined that the average *Campylobacter* spp. prevalence rate on chicken at retail is 57% with a range of 23% to 100% (Humphrey *et al.*, 2007). *Campylobacter* spp. were isolated from 82%, 82%, and 71% of whole chickens, breast with skin attached, and pieces with skin, respectively from supermarkets (Harrison *et al.*, 2001). A significantly high prevalence rate of *Campylobacter* spp. contamination can be found in retail poultry and poultry products and is often directly related to the prevalence rate at the farm (Skovgaard, 2007). Evaluation of numerous studies has put the average prevalence rate of infected flocks at 44 to 59% with a range of 3 to 100% (Humphrey *et al.*, 2007; Nauta and Havelaar, 2008). The number of contaminated broilers accounts for the high incidence of *Campylobacter* spp. in poultry processing plants and on processed birds (Allen *et al.*, 2007; Jacobs-Reitsma, 2000; Lindqvist and Lindblad, 2008).

Evaluation of 20 processing plants in the first study found the incidence of *Campylobacter* spp. positive carcasses post-pick ranged from 24 to 80% and 5 to 39% post-chill depending on the recovery method employed. The variation in enrichment procedure performance may have been due to the variations in each plant's intervention procedures. To overcome plant-to-plant variation and further evaluate enrichment procedures for recovery of low numbers, injured or stressed *Campylobacter* spp., a single processing plant was used where intervention procedures were constant and an increased number of samples were taken. The overall incidence of *Campylobacter* spp. positive carcasses post-chill ranged from 8 to 52% depending on the recovery method employed.

Studies have been conducted to develop an understanding of the effects of different methodology procedures on recovery of *Campylobacter* spp. (Bailey *et al*., 2008; Baylis *et al*., 2000; Kelana and Griffiths, 2003; Khanna *et al*., 2006; Line, 2006; Madden *et al*., 2000; Ng *et al*., 1985; Oyzarabal *et al*., 2007; Paulsen *et al*., 2005; Richardson *et al*., 2008; Sandberg *et al*., 2006; Siogren *et al*., 1987; Solow *et al*., 2003; Tran, 1998). The use of delayed addition of antibiotics and reduced temperature for 5 h with incubation under Campy-gas had the greatest effect on improved recovery of *Campylobacter* cells from rinse samples using TECRA®. The increase in sensitivity using delayed addition of antibiotics or pre-enrichment has also been observed in other studies (Byrd *et al*., 2007; Humphrey, 1989; Jones *et al*., 1999). Delayed addition of antibiotics and reduced temperature had a negative effect on recovery from Bolton broth. This was due to the differences in the antibiotics used in the two broths. The

antibiotics in TECRA® enrichment broth were able to more effectively reduce the level of non-*Campylobacter* spp. than the antibiotics used in the Bolton enrichment broth. This may have negatively affected the ability of the TECRA® broth to recover injured or stressed cells in certain samples. This could explain why *Campylobacter* spp. were recovered in two plants post-chill by Bolton enrichment broth but not with any of the TECRA® enrichment broth procedures. The supplement used in Bolton broth contained cefoperazone, vancomycin, trimethoprim, and cycloheximide. The supplement for TECRA® broth contained trimethoprim, rifampicin, and polymyxin B. The negative effect of some of these antibiotics to recover certain strains of *Campylobacter* or injured/stressed cells has been observed in other studies (Humphrey and Cruikshank, 1985; Humphrey, 1990; Nachamkin *et al*., 2000; Ng, 1985). Rifampicin can be inhibitory to *Campylobacter* cells which are stressed or injured (Humphrey, 1990). Polymyxin in high levels has also shown some inhibitory effects (Lovett *et al*., 1983).

Incubation temperature has been shown to effect the sensitivity of enrichment broth to recover injured or stressed cells, and chemotactic behavior (Baylis *et al*., 2000; Humphrey 1989; Jasson *et al*., 2007; Khanna *et al*., 2006; Sandberg *et al*., 2006; Scotter *et al*., 1993; Solow *et al*., 2003). In the present study, incubation at 37ºC for 5 h resulted in recovery of *Campylobacter* spp. from a greater number of post-chill carcass rinses than did incubation at 42ºC for 5 h when utilizing TECRA® broth. The reduced temperature was not evaluated in post-pick samples due to the observations that these samples usually contain

larger numbers of non-injured *Campylobacter* cells and presence of non-*Campylobacter* spp. (Berrang *et al*., 2007; Richardson *et al*., 2008). Incubation at reduced temperatures with samples containing high levels of non-*Campylobacter* spp. has been shown to be a less sensitive method mainly due to overgrowth by competitors (Aquino *et al*., 1996; Musgrove *et al*., 2001; Sandberg *et al.*, 2006). The ability of *C. jejuni* to grow at 43ºC after exposure to heating or freezing is reduced, but reducing the temperature to 37ºC during the first 4 h of incubation can increase the isolation rate (Humphrey, 1989). Growth and chemotaxis is also greater at a temperature of 37ºC than 42ºC (Khanna *et al*., 2006). It has also been suggested that incubation at 37ºC and 42ºC may give the widest range in recoverability due to *Campylobacter* strain-to-strain differences (Scates *et al*., 2003). This was observed in the present study analyzing the multiple enrichment procedures used.

Both of the standard broth procedures do not require the addition of microaerophilic incubation due to the oxygen scavengers present. The oxygen scavengers are lysed horse blood and ferrous sulfate in Bolton and TECRA® broths, respectively. Enrichment in a microareophilic environment significantly influenced the incidence of *Campylobacter* spp. on post-chill carcasses in the present study. The significance of incorporating gas during incubation has been observed in other studies (Heisick *et al*., 1984; Solow *et al*., 2003; Bovill and Mackey, 1997). Bovill and Mackey (1997) found that when aged *Campylobacter* cultures are no longer culturable, allowing a microareophilic gas solution to be bubbled through the broth allowed the cells to become viable. A rise in the

number of cells of vibrioid morphology and fewer cells in coccoid morphology were observed in the aged cultures.

Direct plating was employed in the present study because it has been suggested that where the possibility of competitive organisms present are high, direct plating should be performed instead of enrichment to determine the level of *Campylobacter* spp. present on carcasses (Aquino et al., 1996; Berrang, 2007; Line, 2001; Stern 2006). Direct plating was not a reliable method to determine qualitatively the number of samples positive for *Campylobacter* spp. in the present study and another study (Richardson *et al*., 2008) and due to the level of *Campylobacter* spp. found in the positive carcass rinse samples. In a separate study, the level of *Campylobacter* spp. was 2.66 log CFU/ml of carcass rinse at rehang and 0.43 log CFU/ml of carcass rinse at post-chill (Berrang *et al*., 2007). The antibiotics in plating media can inhibit the growth of injured cells (Nachamkin *et al*., 2000). The efficiency of certain plating media on the culturability of injured cells can be strain and origin dependant (Cools *et al*., 2003).

In summary, differences were observed in the sensitivity of the enrichment procedures evaluated to recover *Campylobacter* spp. from carcass rinse samples. The efficacy of enrichment procedures differed from plant-to-plant. Analyses of incidence by multiple enrichment procedures comparison may indicate the need for more than one enrichment broth method when evaluating samples believed to harbor injured or stressed cells. Using the best TECRA® and Bolton procedure together will likely increase overall sensitivity and compensate for variations that were observed from plant to plant. The

incorporation of Campy-gas into the enrichment procedures had the greatest positive effect on recovery of *Campylobacter* spp. Delayed addition of antibiotics and reduced temperature enhanced recovery rate of *Campylobacter* from TECRA® broth. The value of a more sensitive enrichment procedure was best observed when evaluating (naturally occurring) cold stressed *Campylobacter* spp. from the post-chill carcass rinse samples. As studies are conducted to further evaluate the ability of *Campylobacter* spp. to survive in certain environments and the resuscitation mechanisms used by injured or stressed cells, this may lead to more sensitive enrichment broths, methodology procedures and better understanding of the ecology of the organism in poultry.

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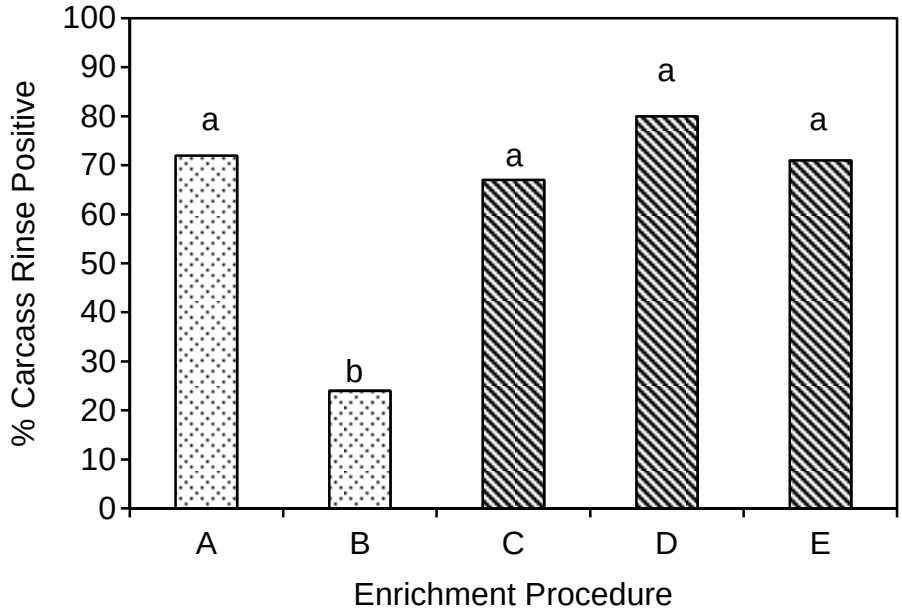

Figure 5.1. The total incidence in study 1 of *Campylobacter* spp. positive carcass rinses post-pick (n=100) obtained from 20 processing plants evaluated by 5 different enrichment procedures in study 1. Enrichment procedures A and B used Bolton enrichment broth (Table 5.1). Enrichment procedures C-E used TECRA® enrichment broth (Table 5.1). Prevalence's between bars with different symbols (a, b) are significantly different ($P<0.05$, Chi-square test for independence). Values are presented as average of the samples taken over the 20 plants.

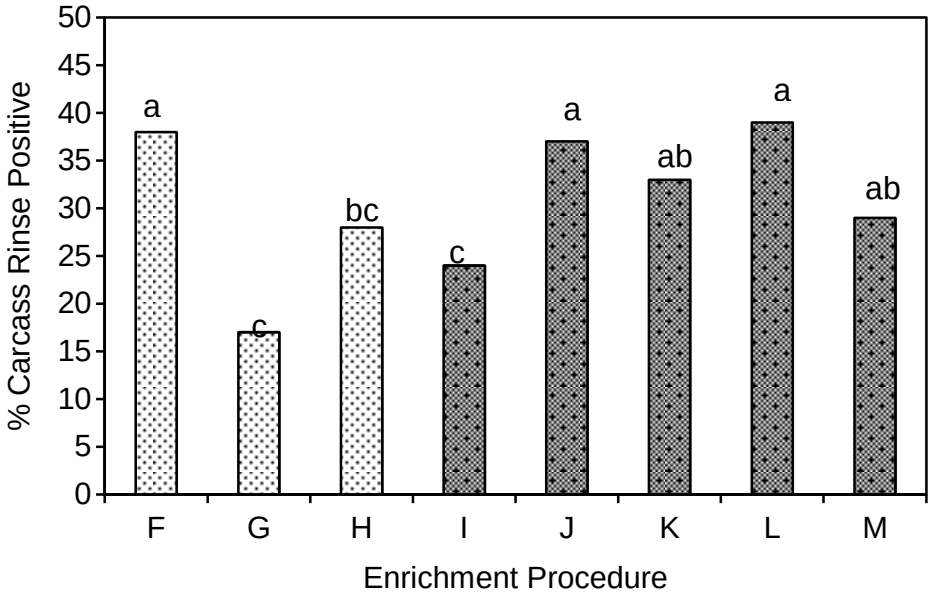

Figure 5.2. The overall incidence in study 1 of *Campylobacter* spp. positive post-chill carcass rinses (n=98) obtained from 20 processing plants evaluated by 8 different enrichment procedures. Enrichment procedures F-H used Bolton enrichment broth (Table 5.2). Enrichment procedures I-M used TECRA® enrichment broth (Table 5.2). Prevalence's between bars with different symbols (a, b, c) are significantly different ($P<0.05$, Chi-square test for independence). Values are presented as average of the samples taken over the 20 plants.

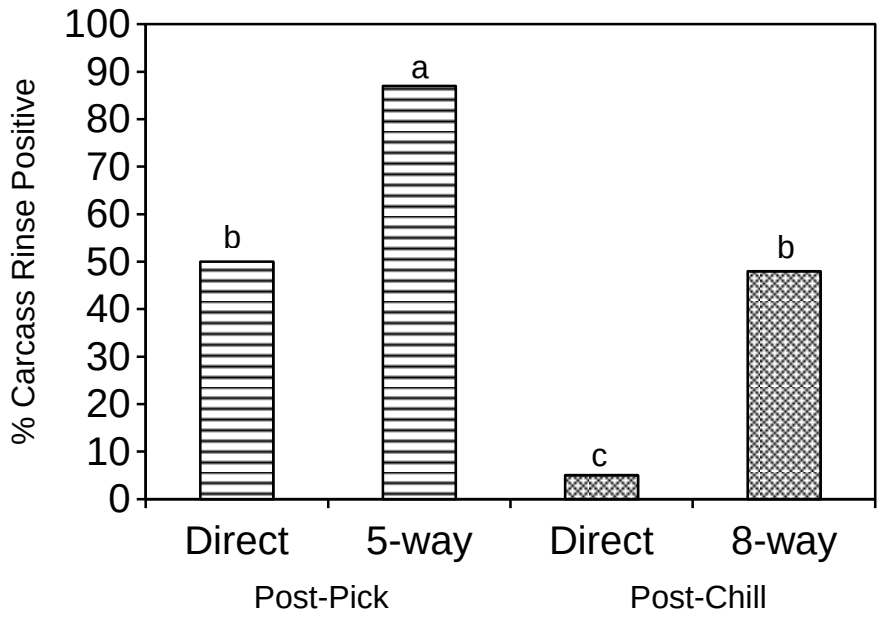

Figure 5.3. Comparison of direct plating and enrichment on the overall incidence of *Campylobacter* spp. positive carcass rinse samples post-pick (n=100) and post-chill (n=98) obtained from 20 processing plants in study 1. Post-pick incidence is evaluated by direct plating (minimum detection level 1 CFU/ml of carcass rinse) and the 5-way analysis (positives from evaluation of all enrichment procedures used in Table 5.1). Post-chill incidence was evaluated by direct plating and the 8-way analysis (positives from evaluation of all enrichment procedures used in Table 5.2). Prevalence's between bars with different symbols (a, b, c) are significantly different ($P<0.05$, Chi-square test for independence). Values are presented as average of the samples taken over the 20 plants.

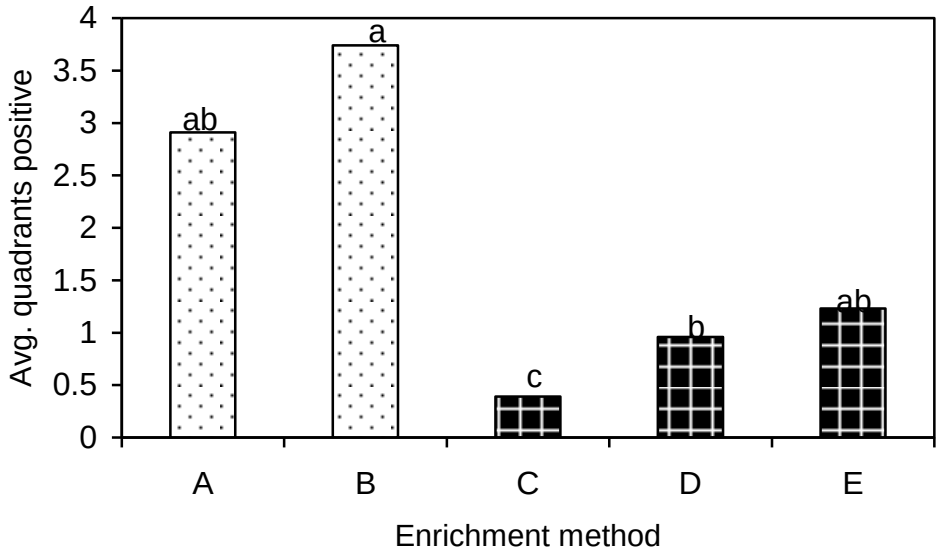

Figure 5.4. Evaluation of the ability of enrichment broth methods to suppress the level of non-*Campylobacter* colonies present on Campy-cefex agar plates from post-pick carcass rinses. The evaluation was determined by the four quadrant streak method. Methods A and B used Bolton enrichment broth and methods C-E used TECRA® enrichment broth (Table 5.1). Mean prevalence's between bars with different symbols (a, b, c) are significantly different ($P<0.05$, Kruskal-Wallis test using Dunn's multiple comparisons). Values are mean of the 20 plants.

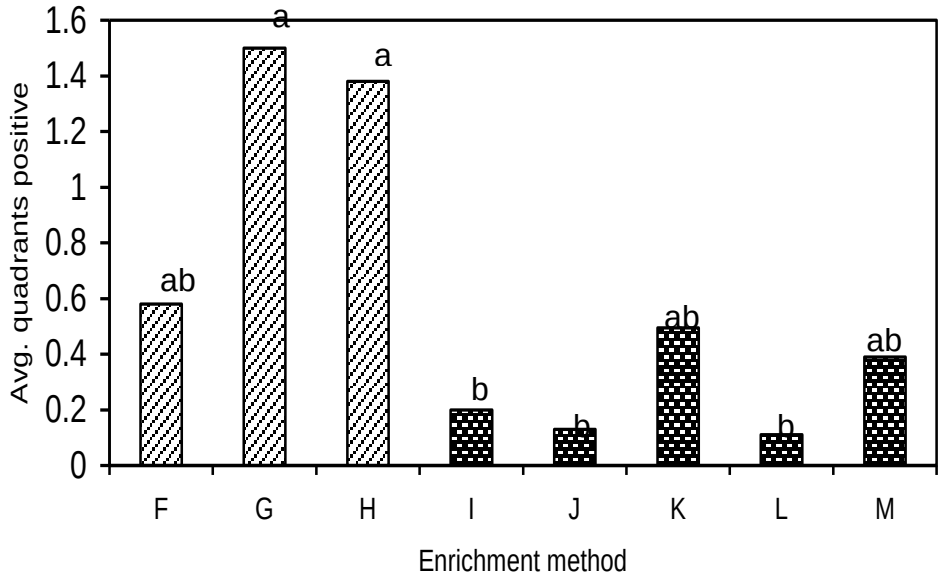

Figure 5.5. Evaluation of the ability of enrichment broth methods to suppress the level of non-*Campylobacter* colonies present on Campy-cefex agar plates from post-chill carcass rinses. The evaluation was determined by the four quadrant streak method. Methods F-H used Bolton enrichment broth and methods I-M used TECRA® enrichment broth (Table 5.2). Mean prevalence's between bars with different symbols (a, b) are significantly different ($P<0.05$, Kruskal-Wallis test using Dunn's multiple comparisons). Values are mean of the 20 plants.

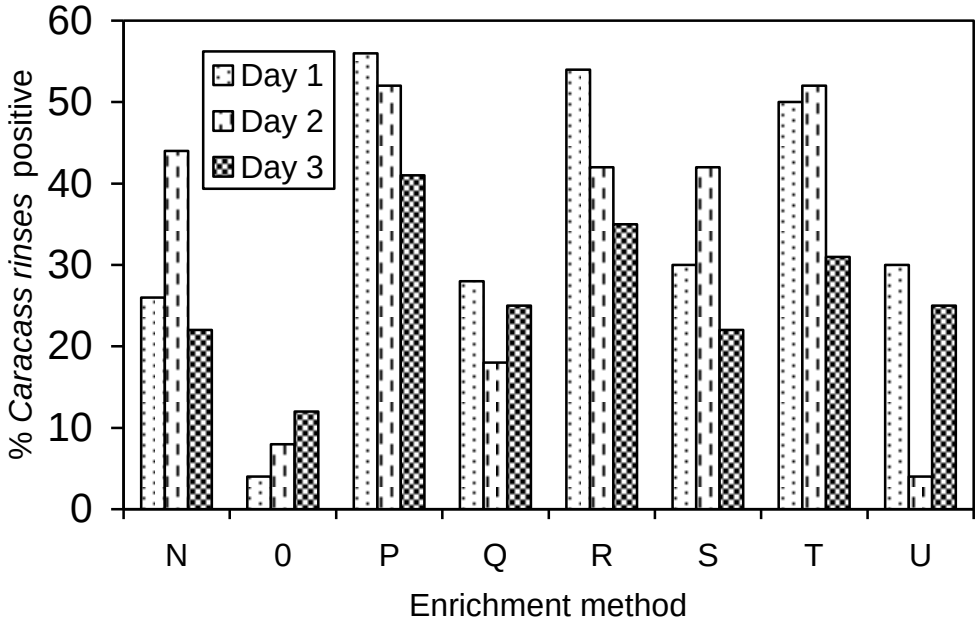

Figure 5.6. Evaluation of *Campylobacter* spp. positive rinse samples (n=50/day) obtained from a single processing plant over three consecutive days by eight different enrichment procedures in study 2. Enrichment procedures N and O used Bolton enrichment broth (Table 5.3). Enrichment procedures P-U used TECRA® enrichment broth (Table 5.3). Values are presented as average of the samples taken over the day.

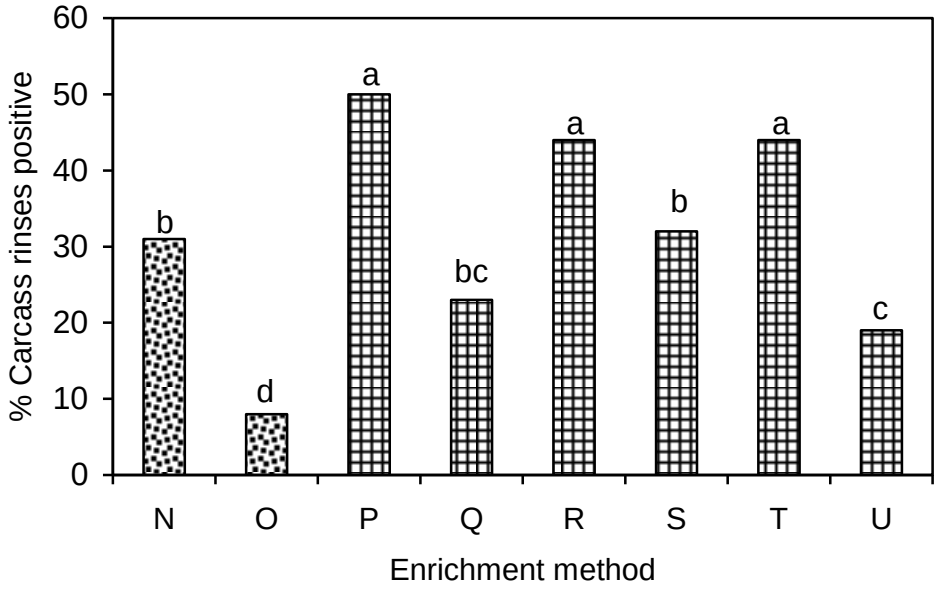

Figure 5.7. The overall incidence in study 2 of *Campylobacter* spp. positive carcass rinses (n=150) obtained from a single processing plant over three consecutive days. Enrichment procedures N and O used Bolton enrichment broth (Table 5.3). Enrichment procedures P-U used TECRA® enrichment broth (Table 5.3). Prevalence's between bars with different symbols (a, b, c, d) are significantly different ($P<0.05$, Chi-square test for independence). Values are presented as average of the samples taken over the 3 days.

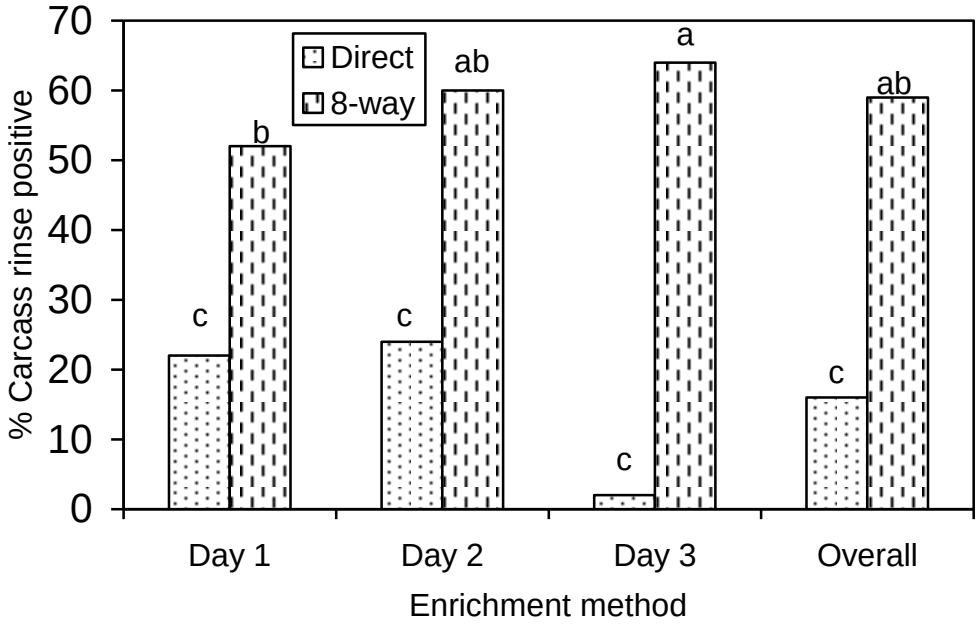

Figure 5.8. Comparison of direct plating and enrichment on the overall incidence of *Campylobacter* spp. positive carcass rinse samples post-pick (n=50/day) over three consecutive days in a single processing plants in study 1. Post-chill incidence is evaluated by direct plating (minimum detection level 1 CFU/ml of carcass rinse) and the analysis of positive samples (8-way analysis) from all enrichment procedures (Table 5.3) used. Prevalence's between bars with different symbols (a, b, c) are significantly different ($P<0.05$, Chi-square test for independence). Values are presented as average of the positive samples each day from the processing plant.

Table 5.1. Enrichment procedures evaluated for the recovery of *Campylobacter* spp. from post-pick carcass rinse samples obtained from 20 processing plants in (Study 1).

Method	Bolton enrichment procedures post-pick
A	Standard Bolton broth (5% lysed horse blood incorporated) method incubated at 42°C in a microareophilic environment (5% O_2, 10% CO_2 and 85% N_2)
B	Standard Bolton broth without antibiotics for 5 h, incubated at 42°C with gas, then addition of antibiotics and incubated at 42°C for an additional 43 h with gas

Method	TECRA® enrichment procedures post-pick
C	Standard TECRA® broth method (incubated at 42°C for 48 h without gas)
D	TECRA® broth without antibiotics for 5 h, incubated at 42°C with gas, then addition of antibiotics and incubated at 42°C for additional 43 h with gas
E	TECRA® broth without antibiotics for 5 h, incubated at 42°C without gas, then addition of antibiotics and incubated at 42°C for additional 43 h without gas

Table 5.2. Enrichment procedures evaluated for the recovery of *Campylobacter* spp. from post-chill carcass rinse samples obtained from 20 processing plants (Study 1).

Method	Bolton enrichment procedures post-chill
F	Same as method A in Table 5.1
G	Same as Method B in Table 5.1
H	Bolton broth without antibiotics for 5 h, incubated at 37°C with gas, then addition of antibiotics and incubated at 42°C for an additional 43 h with gas

Method	TECRA® enrichment procedures post-chill
I	Same as method C in Table 5.1
J	Same as Method D in Table 5.1
K	Same as method E in Table 5.1
L	TECRA® broth without antibiotics for 5 h, incubated at 37°C with gas, then addition of antibiotics and incubated at 42°C for additional 43 h with gas
M	TECRA® broth without antibiotics for 5 h, incubated at 37°C without gas, then addition of antibiotics and incubated at 42°C for additional 43 h without gas

Table 5.3. Enrichment procedures evaluated for the recovery of *Campylobacter* spp. from post-chill carcass rinse samples from a processing plant over three consecutive days (Study 2).

Method	Bolton enrichment procedures post-chill
N	Same as method A in Table 5.1
O	Standard Bolton broth method (Incubated at 42°C without gas)
Method	**TECRA® enrichment procedures post-chill**
P	TECRA® broth without antibiotics for 5 h, incubated at 37°C with gas, then addition of antibiotics and incubated at 42°C for additional 43 h with gas
Q	TECRA® broth without antibiotics for 5 h, incubated at 37°C without gas, then addition of antibiotics and incubated at 42°C for additional 43 h without gas
R	TECRA® broth without antibiotics for 5 h, incubated at 42°C with gas, then addition of antibiotics and incubated at 42°C for additional 43 h with gas
S	TECRA® broth without antibiotics for 5 h, incubated at 42°C without gas, then addition of antibiotics and incubated at 42°C for additional 43 h without gas
T	TECRA® broth method: incubated at 42°C with gas
U	Standard TECRA® broth method: incubated at 42°C without gas

Chapter 6

Efficacy of several enrichment broth procedures for recovery of dry-atmospheric-temperature stressed *C. jejuni* and *C. coli* and determining the viable state of the non-culturable isolates using a chick bioassay[1]

[1] L. J. Richardson, N. A. Cox, R. J. Buhr, J. S. Bailey and M. A. Harrison. To be submitted to Letters in Applied Microbiology

Abstract

The aims of the study were to evaluate the efficacy of 5 enrichment procedures for recovery of dry-atmospheric-temperature stressed *C. jejuni* and *C. coli* and determine the viable status of the non-culturable strains using a chick bioassay. Sterile chick paper pads (PP) and filter papers (FP) were inoculated at a low (L) and high (H) inoculum level. Inoculated samples were left at room temperature, exposed to atmospheric conditions for up to 24 h with sampling performed periodically. Recovery rate decreased gradually from 0 to 2 h and then sharply from 2 to 4 h. For the chick bioassay, negative dry-stressed samples at 6 and 24 h post-inoculation were administered orally and intracloacally to day-of-age chicks. Seven days post-inoculation, the chick's ceca were analyzed. *C. jejuni* strain on the L-FP, H-FP, and H-PP were recovered from the chicks at a rate of 11%, 3%, and 6% respectively from 6 h samples but not recovered from 24 h old samples. *C. coli* strain on the L-FP, H-FP, L-PP, and H-PP were recovered from the chicks at a rate of 2%, 2%, 2% and 4% respectively from 6 h samples and 4% of the H-PP 24 h samples. This is the first study to report on the VBNC state after subjecting the strains to dry-atmospheric-temperature stress.

Keywords: *C. jejuni, C. coli*, enrichment, dry-stress, atmospheric-stress, temperature-stress, VBNC, chick bioassay

Introduction

Campylobacter spp. are one of the leading bacterial etiological agents of acute gastroenteritis in the human population (Mead, 1999; Anonymous, 2006). Contaminated poultry meat is considered to be an important vehicle of human infection (Bryan and Doyle, 1995; On *et al.*, 1998; Altekruse *et al*., 1999). Due to the difficulties in controlling the spread of these bacteria in the kitchen (Cogan *et al*., 1999) and abattoir (Jones *et al*., 1991), control on the farm may be a more effective way of reducing the incidence of this organism in processing plants. Before more effective intervention strategies can be implemented and achieved a better understanding of the epidemiology and ecology of this organism in poultry confinement rearing facilities needs to be further elucidated.

C. jejuni and *C. coli* can enter into a viable, but non-culturable state when environments become unfavorable for growth. *Campylobacter* cells transition from a curved, spiral morphology to a coccoid morphology when subjected to conditions that are not optimum for growth (Ng *et al.*, 1985; Giffiths, 1999; Tholozan *et al*., 1999; Thomas *et al*., 1999). The cells are metabolically active and show signs of respiratory activity, but are unable to be cultured through conventional methodology procedures. The viable, but nonculturable (VBNC) stage was first described by Rollins and Colwell (1986) who postulated they could play a role in human infection and illness. The VBNC state arises from exposure to sublethal adverse environmental conditions and recovery occurs by passage of the organism through a susceptible host.

Several studies have explored the recovery of VBNC forms of *Campylobacter* cells (Jones *et al.*, 1991; Saha *et al.*, 1991; Bovill and Mackey, 1997; Cappelier *et al*., 1997; Talibart *et al*., 2000; Chaveerach *et al.*, 2003). Non-culturable *C. jejuni* and C. *coli* after subjection to acid stress was shown to be viable by injecting the cultures into the amniotic fluid and yolk sac of fertilized eggs (Chaveerach *et al.*, 2003). *C. jejuni* and *C. coli* strains which were in an aqueous microcosm and became non-culturable after 30 days were determined viable after injection into 9-day fertilized eggs (Talibart *et al*., 2000). Freeze-thaw-injured *C. jejuni* cells that were non-culturable were converted back to culturable after passage through the rat gut (Saha *et al.*, 1991). Stern *et al*. (1994) found that non-culturable *Campylobacter* cells stored in water at 4°C for eight weeks were viable using a chick bioassay.

Studies have produced contradictory results on the ability of *Campylobacter* spp. to become VBNC and could be strain dependant (Beumer *et al*., 1992; Medema *et al*., 1992; van de Giessen *et al*., 1996). The significance of the VBNC state remains unclear and controversial, but as the understanding of this phenomenon unfolds, this could provide answers to better understand the epidemiology and ecology of *Campylobacter* spp. transmission within poultry.

Enrichment broths have been evaluated for their sensitivity for recovery of *Campylobacter* spp. from numerous types of samples and certain procedures are more sensitive than others (Byrd *et al*., 2007; Oyzarabal *et al*., 2007; Richardson *et al*., 2008). Few studies have been able to isolate *Campylobacter* spp. from samples were the water activity is very low and the ability of these organisms to

survive desiccation is thought to be unlikely. *Campylobacter* spp. have been shown to be present on chick trayliners after a 24 h pre-enrichment in buffered peptone before transferring the samples to Bolton's enrichment broth (Bryd *et al*., 2007). The inability to recover *Campylobacter* spp. from these types of environments may be due to the inadequacies in the enrichment broths and procedures (Cox *et al*., 2001). VBNC state of *Campylobacter* spp. when subjected to acid or cold stress has been studied. Studies have not been conducted evaluating the ability of *Campylobacter* spp. to be non-culturable but viable when subjected to dry, atmospheric, temperature stress. This is a more realistic form of stress in the poultry environment due to the fact that these bacteria are subjected to the above mentioned stress in commercial hatcheries and grow-out facilities. The objectives of this study were to: 1) evaluate several enrichment procedures for recovery of a dry-atmospheric-temperature stressed *C. jejuni* and *C. coli* and 2) determine whether dry-atmospheric-temperature stressed *C. jejuni* or *C. coli* strain which has become non-culturable could be determined to be viable using a chick bioassay.

Materials and Methods

Experimental design: To evaluate the recovery of dry-stressed *C. jejuni* and *C. coli* several enrichment procedures were used (Table 6.1). For experiment 1, sterile chick paper pads (n=640) and sterile Whatman's #6 filter papers (n=640) 2.54 cm^2 were used. Paper pads (n=140/repetition and inoculum level) and Whatman's #6 filter papers (n=70/repetition and inoculum level) were inoculated

with a 10^3 (L) CFU/ml or a 10^6 (H) CFU/ml of a characterized field strain of *C. jejuni*. A subset (n=20 at 6 h and 24 h post-inoculation) of paper pads and filter papers from each inoculum level were used for the chick bioassay. The two types of papers were left at room temperature exposed to atmospheric conditions and allowed to dry for up to 24 h. At 0, 30 min, 1, 2, 4, 6, and 24 h post-inoculation, five different enrichment methodology procedures for cultivability of the inoculated strain were used (Table 6.1). In experiment 2, the same procedures as above were used and a gentamicin resistant strain of *C. coli* (Cox *et al*., 2008) was used. The water activity was measured using a A2101 AwQuick water activity meter (Rotronic Instrument Corp., Huntington, NY) of a subset of the paper pads (n=5) and filter pads (n=5) were recorded at each sampling time.

Laboratory procedures: At each sampling period, 20 paper pads and 20 filter papers were collected and randomly divided into 5 sets of 4 (each set representing a particular enrichment procedure). For each enrichment procedure, 4 individual paper pads and 4 individual filter papers were placed into individual sterile sampling bags, 10 ml of the enrichment added, and the appropriate enrichment procedure conducted. After incubation, from each of the enrichment samples a 0.1 ml aliquot was streaked onto Campy-cefex agar plates. The plates were then incubated for 48 h at 42ºC in a microareophilic environment containing 5% O_2, 10% CO_2 and 85% N_2. *Campylobacter* colony forming units present on the plates from all samples were confirmed using phase-contrast

microscopy and observing the characteristic rapid, darting motility under wet mount.

Chick bioassay: A subset of the 6 and 24 h dry-stressed papers (paper pads and filter papers) were used. For the high and low inoculated papers, twenty of each type of sample were collected, placed into two sterile individual sampling bags and 40 ml of physiological saline added. The samples were then homogenized and transported to the research farm (homogenization and drive time to farm was approximately 1 h). For each type of sample, 0.2 ml of the homogenate was administered both orally and intracloacally to day-of-age chicks. The chicks within each group (H-PP, L-PP, H-FP, and L-FP) were placed into sterile isolation units and provided feed and water *ad libitum*. Seven days post-inoculation, the chicks were euthanized and the ceca aseptically removed, placed on ice and transported back to the laboratory. The ceca were individually weighed, macerated with a rubber mallet and 3 times the weight-to-volume of Bolton broth containing lysed horse blood and supplements were added. The samples were stomached for 1 min. For the *C. jejuni* samples, a 0.1 ml aliquot from the ceca sample was direct plated onto Campy-cefex agar and the enrichment and direct plates incubated for 48 h at 42°C in microareophilic conditions. After incubation, the previous laboratory methods were conducted for isolation and confirmation. For the *C. coli* sample groups, a 0.1 ml aliquot from the ceca sample was direct plated onto Campy-cefex agar plates (containing 200 ppm gentamicin) and the enrichment and direct plates incubated for 48 h at 42°C

in microareophilic conditions. After incubation, the above laboratory methods were conducted for isolation and confirmation except that Campy-cefex agar plates containing 200 ppm of gentamicin were used.

Strain confirmation from chick bioassay: FlaA SVR DNA sequencing was used to confirm that the *C. jejuni* recovered from the chick bioassay was the same as the one inoculated. A *Campylobacter* isolate from each of the positive chicks (n=7) of the *C. jejuni* bioassay's was compared to the marker *C. jejuni* originally inoculated onto the papers. Isolated colonies were resuspended in 300 µL of sterile H_2O and placed at 100°C for 10 min. For each boiled cell suspension, 10 µL was used as template for *flaA* SVR PCR with the following primers: FLA242FU: $^{5'}$CTA TGG ATG AGC AAT TWA AAA T$^{3'}$ and FLA625RU: $^{5'}$CAA GWC CTG TTC CWA CTG AAG$^{3'}$ (Meinersmann *et al*., 1997). A 35-cycle reaction was used with 1 min. denaturing at 96°C, annealing at 52°C, and extension at 72°C. This resulted in a product of approximately 425 bp. Sequence data was generated using either the FLA242FU primer or the FLA625RU primer with the Big-Dye Dye-Terminator Cycle Sequencing Kit (ABI-PE, Foster City, CA). Data was assembled with Sequencher 4.2 (GeneCodes Corp., Ann Arbor, MI) and aligned using Clustal X (Thompson *et al*., 1994). The aligned sequences were compared and dendograms were generated using the Neighbor-Joining algorithm with HKY85 distance measurements in PAUP*4.0 (Phylogenetic Analysis Using Parsimony) (Swofford, 1988). Since a gentamicin resistant

marker strain of *C. coli* was used, the *flaA* SVR PCR was not performed on isolates obtained from *C. coli* chick bioassay's performed.

Statistical analysis: The Chi-square test for independence was used to evaluate differences in the total number of inoculated samples determined to be positive for the inoculated strains. The paired T-test was used to evaluate differences between the water activities of the two papers at each sampling time. In addition, linear regression was calculated to determine whether the decrease in water activity followed a linear or non-linear trend over the 24 h sampling period. For all tests performed, significance was set at a p-value less than 0.05. All statistical analyses were conducted using InStat 3.0 (GrahPad Software, Inc., San Diego, CA). Incidence data is represented as total number of positive samples over the total number of samples tested or as a percent positive of the samples taken.

Results

For experiment 1, the rate of recovery of *C. jejuni* from the different sample types and enrichment procedure from 0 to 24 h is shown in Figures 6.1, 6.2, 6.3, and 6.4. In one repetition using *C. jejuni*, the strain was recovered at the 6 h sampling time by enrichment procedure E. This group was not used for the chick bioassay. For experiment 2, the rate of recovery of *C. coli* from the different sample types and enrichment procedures from 0 to 24 h is shown in Figures 6.5, 6.6, 6.7, and 6.8. In the *C. coli* groups, the strains were not

recovered after 4 h by any method. The most recovered samples from both experiments taken at 0 to 24 h were from enrichment procedure E which used TECRA® enrichment broth and a procedure incorporating a reduced temperature and delayed addition of antibiotics (37°C for 5 h) with incubation in microareophilic environment (Table 6.2 and 6.3). No significant differences ($P>0.05$) between enrichment procedure E and procedures B, C, and D were determined. The least sensitive enrichment procedure (A) used buffered peptone incubated at 42°C for 48 h in microareophilic environment and was significantly different ($P<0.05$) compared to the other enrichment procedures (Table 6.2 and 6.3).

In experiment 1, for the chick bioassay using 6 h samples, the non-culturable *C jejuni* strain on the L-FP, H-FP, and H-PP were recovered from the chicks at a rate of 11% (4/35), 3% (1/35), and 6% (2/35), respectively. The *C. jejuni* strain was not recovered using the 6 h samples from the low paper pad groups. The *C. jejuni* strain was also not recovered from any of the chick bioassay's using the 24 h samples. The *C. jejuni* isolates obtained from the ceca of the chicks were not distinct from the *C. jejuni* strain inoculated onto the papers using the *flaA* SVR PCR and the strains recovered from the chicks were the same strain inoculated onto the paper pads.

In experiment 2, for the chick bioassay using 6 h samples, the non-culturable marker *C. coli* strain on the L-FP, H-FP, L-PP and H-PP were recovered from the chicks at a rate of 2% (1/55), 2% (1/55), 2% (1/55) and 4%

(2/55), respectively. For the 24 h H-PP samples, 4% (2/55) of the chicks contained the marker *C. coli* strain.

The water activity from the filter papers and paper pads are shown in Figure 6.9. A continuous decrease in the water activity was observed from 0 to 24 h post-inoculation for both sample types. The decrease in water activity followed a non-linear trend. No significant difference ($P>0.05$) in the water activity was observed between the two paper types. The water activity of the two paper types at each sampling time were also found to be highly correlated ($r=0.99$).

Discussion

In the present study, using different enrichment procedures did have an effect on the recovery of the two *Campylobacter* strains which were subjected to dry-atmospheric-temperature stress. The ability to recover the strains only for a few hours after inoculation may suggest that different basal media or enrichment procedures should be investigated. In a study by Cox *et al*. (2001), the inefficiency of *Campylobacter* enrichment broths were noted when evaluating the recovery of *Campylobacter* spp. from dry samples. The ability to recover the two *Campylobacter* strains by using a chick bioassay further confirms the inadequacies of enrichment broths and procedures used for recovery of *Campylobacter* spp. that have been subjected to these types of stresses. Recovery of *Campylobacter* strains when they have become non-culturable using an animal model has been observed in other studies (Saha *et al*., 1991; Stern *et*

al., 1994; Hald *et al*., 2001; Baffone *et al*., 2006). All of these studies have evaluated the VBNC state of *Campylobacter* strains that became non-culturable in aqueous environments over extended time frames and/or have been subjected to cold stress but not dry-atmospheric-temperature stress. The stresses *Campylobacter* strains were subjected to in the present study are similar to conditions found in commercial hatcheries and the poultry environments.

Previous studies administered the VBNC sample orally, but in the present study the VBNC sample was administered both orally and intracloacally. By administering the VBNC samples intracloacally, the sample bypassed the low pH of the gizzard. The route of inoculation has been shown to affect the level of cells needed for colonization to occur (Cox *et al.*, 1990). In that study, the oral route of inoculation needed substantially greater cells to colonize chicks than the intracloacal route of inoculation. The intracloacal route of inoculation used in the present study may have improved the ability of non-culturable cells to be shown viable by the chick bioassay. Studies have demonstrated that the VBNC state of *Campylobacter* can be strain dependent (Beumer *et al*., 1992; Medema *et al*., 1992; van de Giessen *et al*., 1996). Further studies evaluating a broader number of *Campylobacter* strains may give a better indication on the frequency of the VBNC state and significance in relation to different poultry environments.

Most broths contain ingredients to protect *Campylobacter* spp. from toxic effects formed in the presence of oxygen and light. This includes, but not limited to blood, ferrous suphate, sodium metabisulphite, sodium pyruvate, haematin and charcoal (Corry *et al*., 1995). In certain conditions, selective enrichments

and incubation temperature can have a negative effect on recovery of very low cell numbers or injured/stressed cells (Ray and Johnson, 1984; Humphrey and Cruickshank, 1985). Enrichments containing no selective supplements or enrichments where selective supplements are added after a period of incubation have been suggested (Humphrey, 1989). Using a pre-enrichment step in a study increased the ability of the researchers to recover *Campylobacter* cells from samples where dry, atmospheric and temperature stress could have occurred (Byrd *et al*., 2007). In that study, the presence of *Campylobacter* spp. from chick trayliners was evaluated, a pre-enrichment step using buffered peptone followed by enrichment in Bolton broth allowed for the recovery of *Campylobacter* spp. chick trayliners (Byrd *et al*., 2007). Buffered peptone used in one of the enrichment procedures in the present study was the least sensitive of all methods employed. The sensitivity of *Campylobacter* spp. to desiccation is known, but the VBNC state aids certain *Campylobacter* strains in survival in unfavorable conditions (Cappelier *et al*., 1999). The ability of certain *Campylobacter* strains to enter into a VBNC state and the significance of this for survival during exposure to desiccation needs to be further studied along with the sensitivity of current enrichment broths and methodology procedures.

Thermophilic *Campylobacter* spp. colonize the gastrointestinal tract of avian species. Once out of the hosts digestive tract and exposed to environmental conditions *Campylobacter* spp. have to overcome numerous stresses to survive. Growth and survival of *Campylobacter* spp. is depressed once exposed to temperatures outside their growth range, oxygen and

desiccation (Park, 2002). Even though evidence supports the conclusion that *Campylobacter* spp. are fragile organisms, they are still one of the most prominent etiological agents responsible for bacterial human foodborne illness (Murphy *et al*., 2006). *Campylobacter* spp. are able to adapt to certain limits of heat stress, acid stress, and cold stress (Lazaro *et al*., 1999; Moore and Madden, 2000; Kelana and Griffiths, 2003; Murphy *et al*., 2003; Moen *et al*., 2005). Whether *Campylobacter* spp. have the ability to adapt to certain levels of desiccation is not well defined. Certain strains of *Campylobacter* spp. have an inability to survive or be recovered after increasing levels of desiccation (Luechtefeld and Wang, 1981; Oosterom *et al*., 1983; Cox *et al*., 2001).

The ability of a *Campylobacter* spp. to enter into the VBNC state as mentioned previously seems to be strain dependant (Medema *et al*., 1992; Stern *et al*., 1994; Tholozan *et al*., 1999; Baffone *et al*., 2006). The majority of investigations into the VBNC state of *Campylobacter* spp. have also been performed using *C. jejuni* strains (Bovill and Mackey, 1997; Tholozan *et al*., 1999; Thomas *et al*., 1999). A few studies have looked at other *Campylobacter* species (Stern *et al*., 1994; Talibart *et al*., 2000). The two *Campylobacter* strains in the present study both originated from the poultry environment. In our study, the *C. jejuni* strain stayed culturable longer and more frequently than the *C. coli* strain. This would indicate that the *C. coli* strain was more susceptible to the stresses than the *C. jejuni* strain. In the chick bioassays, *C. coli* was recovered from more sample types than *C. jejuni*. The *C. coli* strain was shown to be viable after

subjected to 24 h of dry-atmsopheric-temperature stress. This further confirms the strain-to-strain variability observed in other studies.

In order for organisms to adapt to unfavorable conditions usually a series of genetic responses occur. The capacity of *Campylobacter* strains to regulate gene expression in response to environmental stresses is thought to be limited compared to other bacteria (Park, 2002). Some of the defensive responses to environmental stresses found in other organisms have not been observed in *Campylobacter* spp. (Gaynor *et al*., 2005; Murphey *et al*., 2006). *Campylobacter* spp. posses a two-component regulator defense system which regulates sets of genes in response to environmental stresses (Parkhill *et al*., 2000; Stock *et al*., 2000). Genome plasticity and adaptive tolerance of *Campylobacter* strains may also play a role in survival (Park, 2002; Murphy *et al*., 2003). As the understanding evolves on the role certain genes play in the survival of *Campylobacter* spp., this may provide answers to how *Campylobacter* spp. readily colonize and disseminate within poultry and the survival mechanisms used outside of the avian host where the organism is constantly subjected to stress factors.

In summary, this is believed to be the first report of the VBNC state after subjecting *Campylobacter* strains to dry-atmospheric-temperature stress. This is the first report performing intracloacal inoculation of the VBNC samples. Better resuscitation broths and methods are vital in order to accurately access the ecology of *Campylobacter* spp. in poultry. Even after using 5 different enrichment procedures the *C. jejuni* and *C. coli* strains could not be recovered

frequently after 2 h from any treatment group. Studies evaluating the environment within a bird and correlation with the survival of *Campylobacter* spp. may allow for better interpellation on the epidemiology and ecology of this organism in poultry.

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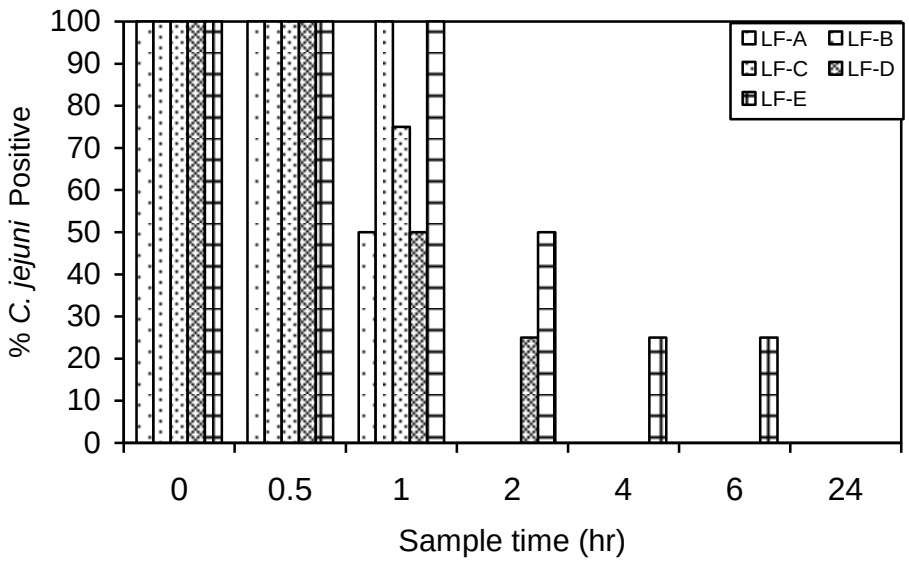

Figure 6.1. Average recovery over time of approximately 10^3 CFU's of *C. jejuni* inoculated on Whatman's filter paper (LF) and left at room temperature exposed to the laboratory environment for 24 h. Letters (A-E) represent the recovery rate determined by the 5 different enrichment procedures (Table 6.1).

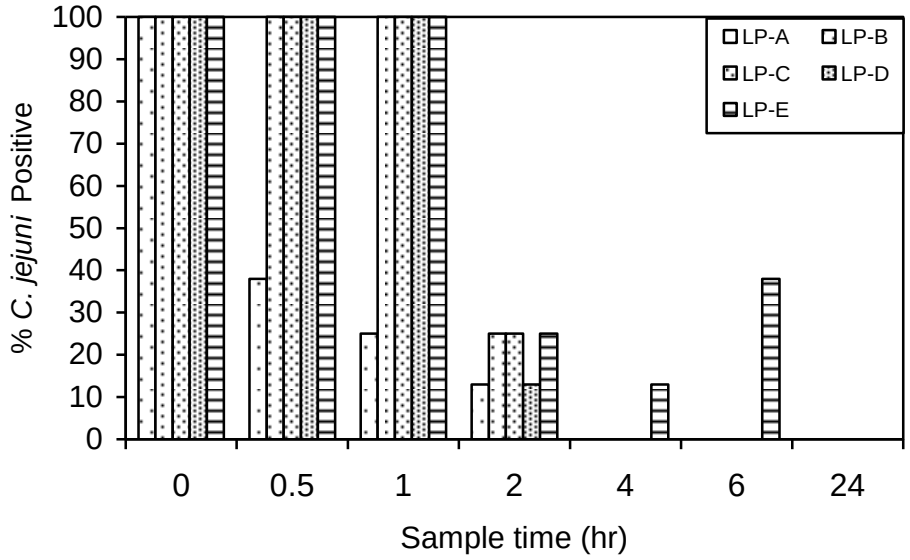

Figure 6.2. Average recovery over time of approximately 10^3 CFU's of *C. jejuni* inoculated on chick paper pads (LP) left at room temperature exposed to the laboratory environment for 24 h. Letters (A-E) represent the recovery rate determined by the 5 different enrichment procedures (Table 6.1).

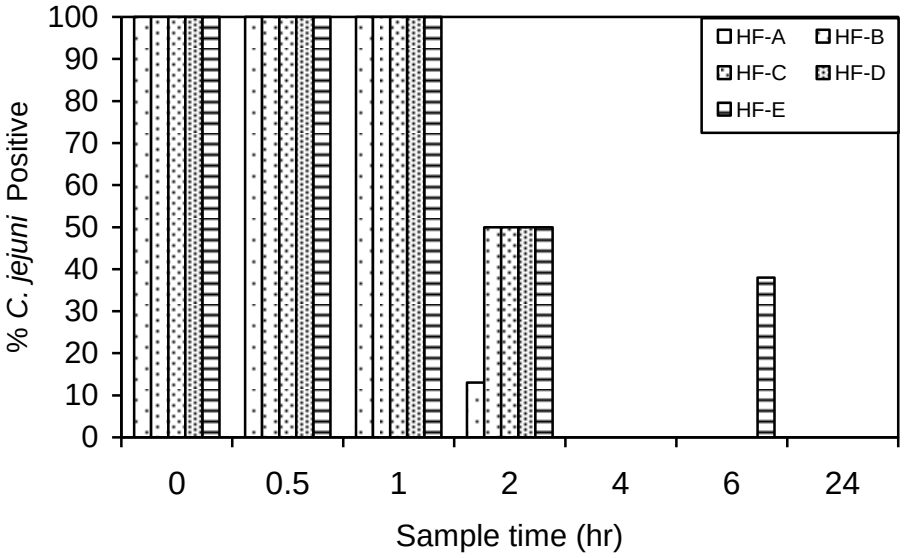

Figure 6.3. Average recovery over time of approximately 10^6 CFU's of *C. jejuni* inoculated on Whatman's filter paper (HF) left at room temperature exposed to the laboratory environment for 24 h. Letters (A-E) represent the recovery rate determined by the 5 different enrichment procedures (Table 6.1).

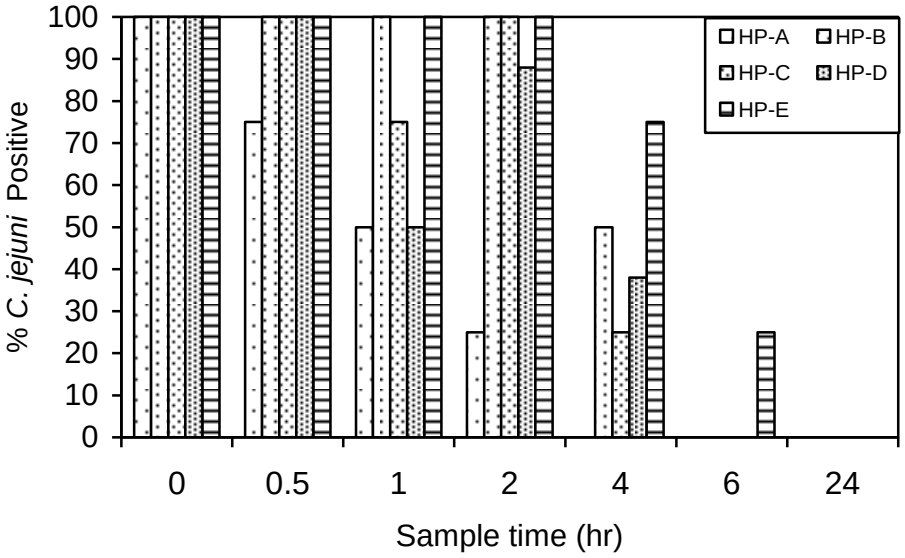

Figure 6.4. Average recovery over time of approximately 10^6 CFU's of *C. jejuni* inoculated on chick paper pads (HP) left at room temperature exposed to the laboratory environment for 24 h. Letters (A-E) represent the recovery rate determined by the 5 different enrichment procedures (Table 6.1).

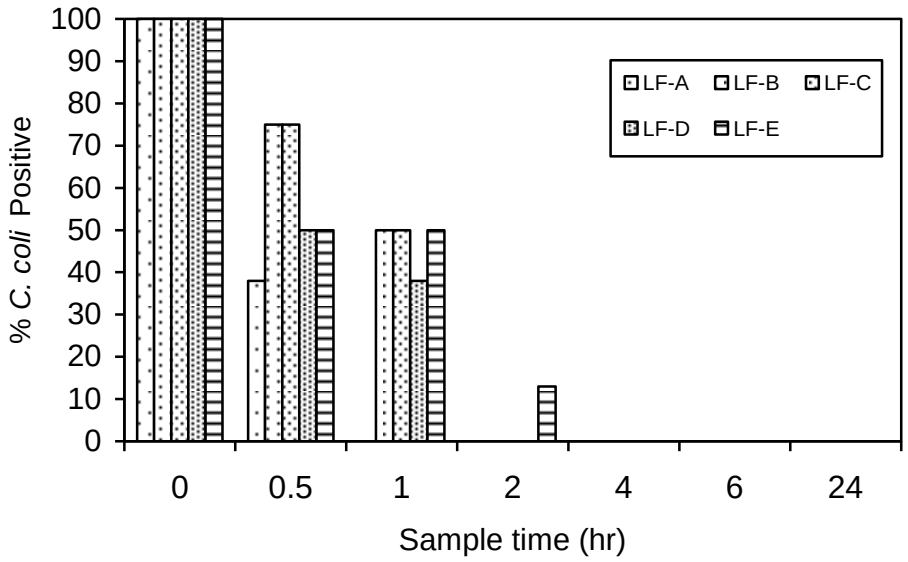

Figure 6.5. Average recovery over time of approximately 10^3 CFU's of *C. coli* inoculated on Whatman's filter paper (LF) left at room temperature exposed to the laboratory environment for 24 h. Letters (A-E) represent the recovery rate determined by the 5 different enrichment procedures (Table 6.1).

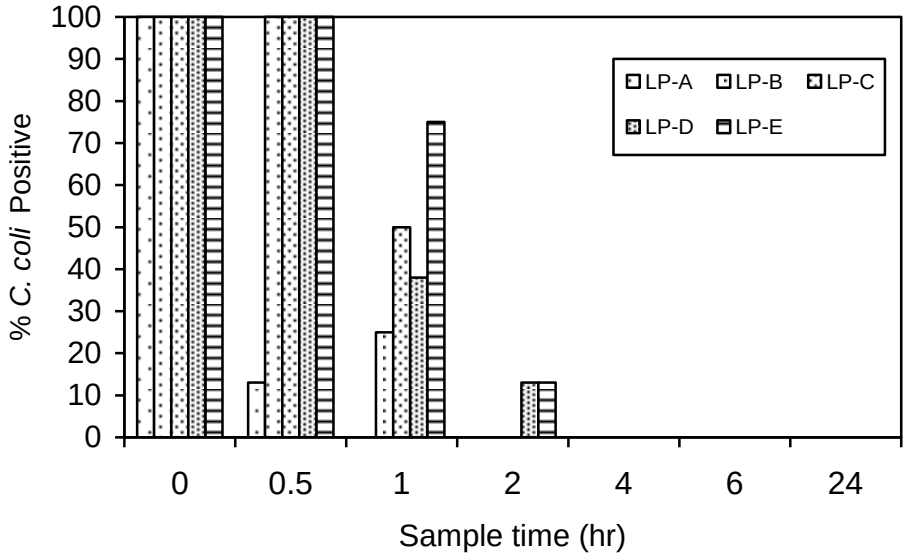

Figure 6.6. Average recovery over time of approximately 10^3 CFU's of *C. coli* inoculated on chick paper pads (LP) left at room temperature exposed to the laboratory environment for 24 h. Letters (A-E) represent the recovery rate determined by the 5 different enrichment procedures (Table 6.1).

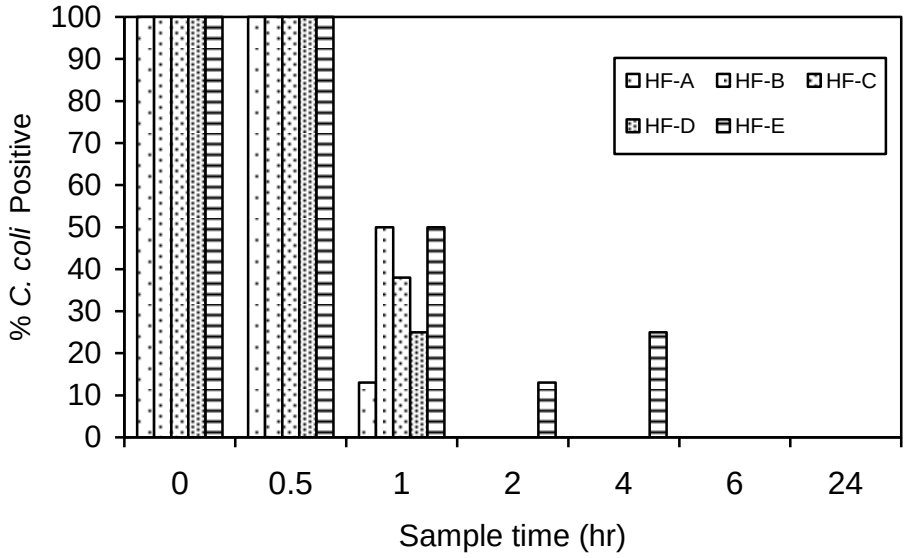

Figure 6.7. Average recovery over time of approximately 10^6 CFU's of *C. coli* inoculated on Whatman's filter paper (HF) left at room temperature exposed to the laboratory environment for 24 h. Letters (A-E) represent the recovery rate determined by 5 different enrichment procedures (Table 6.1).

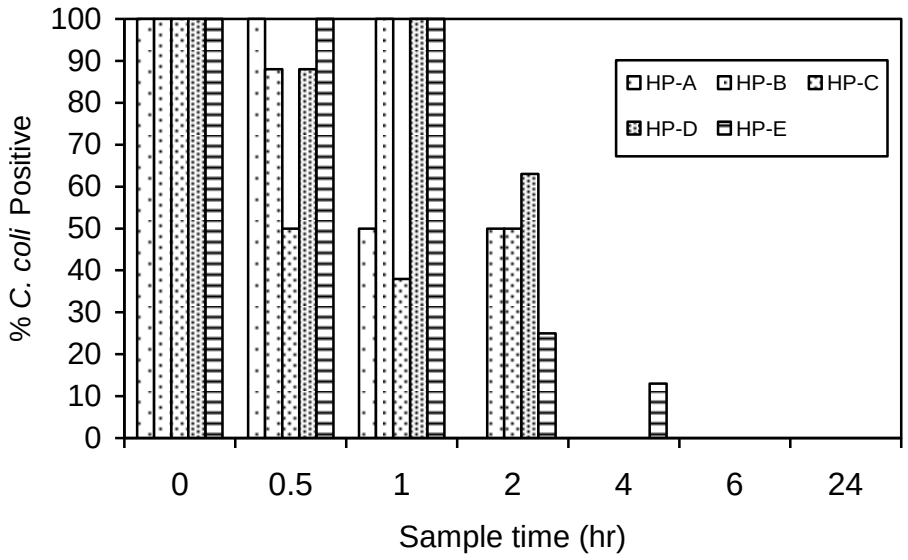

Figure 6.8. Average recovery over time of approximately 10^6 CFU's of *C. coli* inoculated on chick paper pads (HP) left at room temperature exposed to the laboratory environment for 24 h. Letters (A-E) represent the recovery rate determined by 5 different enrichment procedures (Table 6.1).

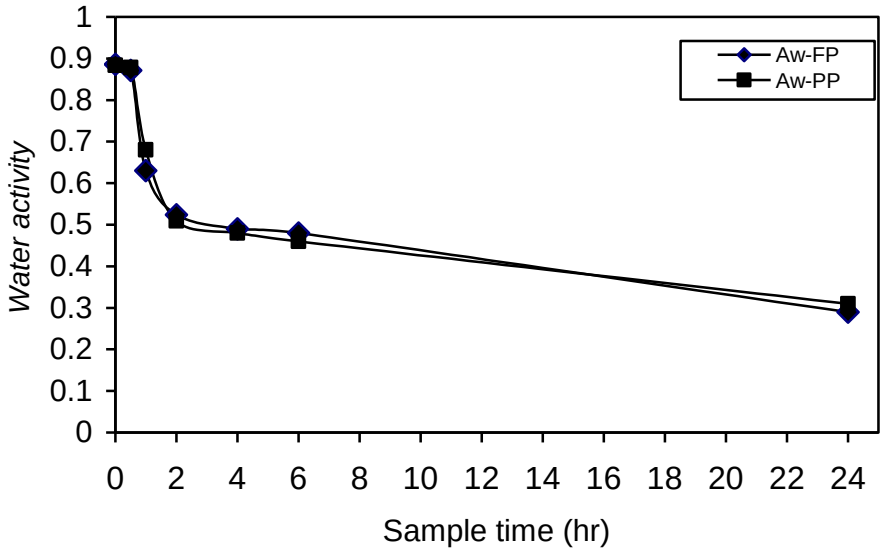

Figure 6.9. The average water activity (Aw) of the Whatman's filter paper (FP) and the chick paper pads (PP) over the course of the sampling times.

Table 6.1. Enrichment procedures used for the recovery of *C. jejuni* and *C. coli* inoculated strains onto Whatman's filter paper and chick paper pads at a low and high concentration of cells.

Method	Enrichment Procedure
A	2% Buffered peptone incubated at 42°C for 48 h in Campy-gas (a microareophilic condition containing 5% O_2, 10% CO_2 and 85% N_2)
B	Bolton broth with 5% lysed horse blood without incorporation of supplements incubated at 42°C for 48 h in Campy-gas
C	TECRA® broth without incorporation of supplements incubated at 42°C for 48 h in Campy-gas
D	Bolton broth with 5% lysed horse blood with supplements incubated at 42°C for 48 h in Campy-gas
E	TECRA® broth without incorporation of supplements incubated at 37°C for 5 h then supplement added and incubated at 42°C in Campy-gas.

Table 6.2. Assessment of the sensitivity of the 5 individual enrichment procedures for recovering artificially dry-oxidative-temperature stressed *C. jejuni* using all sample times.

Sample[1]	Enrichment Procedure[2]				
	A	B	C	D	E
LF[3]	20/56	24/56	22/56	22/56	32/56
LP[4]	14/56	26/56	27/56	25/56	30/56
HF[5]	25/56	28/56	28/56	28/56	31/56
HP[6]	20/56	36/56	32/56	30/56	40/56
Total[7]	79/224^{c}	114/224^{a}	109/224^{ab}	105/224^{ab}	133/224^{a}

[1] Overall incidence from each sample evaluated from 0 to 24 h
[2] Data represented as the number of positive samples / number of samples taken
[3] Whatman's filter paper inoculated with 10^3 CFU of *C. jejuni*
[4] Chick paper pad inoculated with 10^3 CFU of *C. jejuni*
[5] Whatman's filter paper inoculated with 10^6 CFU of *C. jejuni*
[6] Chick paper pad inoculated with 10^6 CFU of *C. jejuni*
[7] Total incidence within row with different symbols (a, b, c) are significantly different ($P<0.05$, chi-square test for independence)

Table 6.3. Assessment of the sensitivity of the 5 individual enrichment procedures for recovering artificially dry-oxidative-temperature stressed *C. coli* using all sample times.

Sample[1]	Enrichment Procedure[2]				
	A	B	C	D	E
LF[3]	11/56	20/56	18/56	15/56	17/56
LP[4]	16/56	18/56	20/56	20/56	23/56
HF[5]	17/56	20/56	19/56	18/56	23/56
HP[6]	20/56	25/56	19/56	28/56	27/56
Total[7]	64/224[b]	83/224[a]	76/224[a]	81/224[a]	90/224[a]

[1] Overall incidence from each sample evaluated from 0 to 24 h
[2] Data represented as the number of positive samples / number of samples taken
[3] Whatman's filter paper inoculated with 10^3 CFU of *C. coli*
[4] Chick paper pad inoculated with 10^3 CFU of *C. coli*
[5] Whatman's filter paper inoculated with 10^6 CFU of *C. coli*
[6] Chick paper pad inoculated with 10^6 CFU of *C. coli*
[7] Total incidence within row with different symbols (a and b) are significantly different ($P<0.05$, chi-square test for independence)

CHAPTER 7

SUMMARY AND CONCLUSION

This was the first report of *Campylobacter* spp. being isolated from the circulating blood of commercial broilers. *Campylobacter* enrichment broths may not be sensitive enough to recover low levels or injured/stressed cells due to the negative effects of the antibiotics in the enrichment and is why antibiotics were not used in the enrichment broth for blood samples. The mode into the circulating blood was not fully elucidated but if *Campylobacter* spp. are invading into the epithelial cells then this could be one route in which the organisms are entering into the circulating blood. The presence of *Campylobacter* spp. in the circulating blood may explain why the organisms can be recovered from tissues and organs within birds. The significance of *Campylobacter* spp. in the circulating blood and the ability of these organisms to invade chicken epithelial cells are still unknown. Vertical transmission of *Campylobacter* spp. from the broiler breeder hen to its progeny the broiler through the ovarian follicles could be occurring.

Improvements in cultural recovery methods for *Campylobacter* spp. are essential to accurately assess the epidemiology and ecology of this organism in poultry. Differences existed in the sensitivity of the enrichment procedures

evaluated to recover *Campylobacter* spp. from carcass rinse samples. The efficacy of enrichment procedures varied from plant-to-plant. In addition the four-quadrant streak method developed allowed for the level of non-*Campylobacter* colonies present on the plating media to be determined. The level of non-*Campylobacter* colonies varied between the enrichment procedures that were used and different procedures performed better at suppressing non-*Campylobacter* microflora in the broths than other procedures. Overall, the antibiotics in TECRA® broth significantly suppressed background organisms compared to the antibiotics in Bolton broth. Analyses of incidence by multiple comparisons of enrichment procedures indicated that more than one enrichment broth method is needed when evaluating samples believed to harbor injured or stressed cells. Using the best TECRA® and Bolton enrichment broth procedure together increases overall sensitivity and compensates for variations observed plant-to-plant. The incorporation of Campy-gas into the enrichment procedures had the greatest positive effect on recovery of *Campylobacter* spp. Delayed addition of antibiotics and reduced temperature enhanced recovery rate of *Campylobacter* from TECRA® broth. The value of a more sensitive enrichment procedure was best observed when evaluating (naturally occurring) cold stressed *Campylobacter* spp. from the post-chill carcass rinse samples.

Better resuscitation broths and methods are vital in order to accurately access the ecology of *Campylobacter* spp. in poultry. Even after using 5 different enrichment procedures, *C. jejuni* and *C. coli* strains subjected to dry-atmospheric-temperature stress could not be recovered frequently after 2 h from

any treatment group but were determined to be viable using the chick bioassay. This is the first report on the VBNC state after subjecting *Campylobacter* strains to dry-atmospheric-temperature stress using intracloacal inoculation as a route for recovery of VBNC cells using the chick bioassay.

Additional studies evaluating the environment within a bird and correlation with the survival of *Campylobacter* spp. needs to be further researched. As studies are conducted to further assess the ability of *Campylobacter* spp. to survive in certain environments and the resuscitation mechanisms used by injured or stressed cells, this may lead to better enrichment broths, methodology procedures and a clearer understanding of the ecology of the organism in poultry. As the understanding of *Campylobacter* spp. evolves, this may allow for development of interventions or intervention strategies to be implemented at critical points in the production chain that have been previously overlooked. Reducing *Campylobacter* spp. in poultry pre- and post-harvest is critical in order to decrease or eliminate the number of contaminated products leaving the processing plant. This should reduce the probability that one of these contaminated products with this organism will eventually windup in a consumer's kitchen.

APPENDICES

The appendices are intended to provide readers with additional data that was not extensively discussed on two preliminary studies that were conducted without an extensive literature review, statistical analysis or discussion of the results obtained. Appendix A presents materials and methods along with results on a method that was developed to aseptically sample the outside of the spleen and the internal contents of the spleen after birds have been inoculated with *C. jejuni*. Appendix B presents materials and methods along with data on recovery of inoculate *C. jejuni* in the circulating blood of broilers.

APPENDIX A. ETHANOL IMMERSION METHOD FOR THE COLLECTION OF SEPARATE EXTERNAL AND INTERNAL MICROBIOLOGICAL SPLEEN SAMPLES OF BABY BROILER CHICKS

Materials and Methods

Experimental design

Four experiments were conducted and for each experiment, day-of-age broiler chicks were obtained and inoculated by different routes with a 10^5 CFU/ml inoculum of *C. jejuni*. For experiment 1, broiler chicks were inoculated both oral and intracloacal on three separate occasions. For experiment 2, broiler chicks were inoculated orally on two separate occasions. For experiment 3, broiler chicks were inoculated intracloacal on two separate occasions. For experiment 4, broiler chicks were inoculated ocularly on two separate occasions.

Two days post-inoculation for each experiment, necropsy was performed and the spleen and ceca were aseptically removed from each bird and individually placed into sterile sampling bags, packed on ice and transported back to the laboratory for evaluation. For external (ES) spleen sampling, 3 ml of Bolton enrichment broth was added to each spleen sample and shaken for 30 seconds. The spleen was then aseptically removed from the bag. For internal (IS) spleen sampling, the spleen was submerged into a 70% ethanol solution for 10 seconds, then removed and submerged into a saline solution to detect sterilization ability of the immersion technique. The spleen was then placed into a sterile sampling bag,

macerated and 3 ml of Bolton enrichment broth was added and the sample was stomached for 30 seconds.

Lab Procedure

The samples were bagged, gassed (5% O_2, 10% CO_2, 85% N) to simulate a microaerophilic environment, and placed in the incubator at 42ºC for 48 h. A 0.1 ml of the enrichment broth from each sample was then plated onto Campy-*c*efex plates. The plates were also bagged and gassed and placed in the incubator at 42ºC for 48 h. The plates were then removed and presumptive *Campylobacter* positive colonies were isolated and confirmation was determined by observation of the characteristic darting motility using wet mounts under the microscope.

Results

Overall, *C. jejuni* was recovered from 75% (82/109) of the ES samples, 71% (77/109) of the IS samples and from 100% (109/109) of the ceca samples (Tables A.1, A.2, A.3, A.4). Furthermore, ethanol and saline samples were negative for *C. jejuni* suggesting that the ethanol immersion method was a good sterilization method of the external surface of the spleen.

Table A.1. Number of positive samples after both oral and intracloacal inoculation with *C. jejuni*

	Sample Site				
	Spleen				
Repetitions	Internal	External	Ceca	Ethanol	Saline
1	12/20	20/20	20/20	0/20	0/20
2	20/20	18/20	20/20	0/20	0/20
3	5/10	2/10	10/10	0/10	0/10
Total	37/50	40/50	50/50	0/50	0/50

Table A.2. Number of positive samples after oral inoculation with *C. jejuni*

	Sample Site				
	Spleen				
Repetitions	Internal	External	Ceca	Ethanol	Saline
1	2/10	5/10	10/10	0/10	0/10
2	9/9	9/9	9/9	0/9	0/9
Total	11/19	14/19	19/19	0/19	0/19

Table A.3. Number of positive samples after intracloacal inoculation with *C. jejuni*

	Sample Site				
	Spleen				
Repetitions	Internal	External	Ceca	Ethanol	Saline
1	4/10	2/10	10/10	0/10	0/10
2	10/10	10/10	10/10	0/10	0/10
Total	14/20	12/20	20/20	0/20	0/20

Table A.4. Number of positive samples after inoculation ocularly with *C. jejuni*

	Sample Site				
	Spleen				
Repetitions	Internal	External	Ceca	Ethanol	Saline
1	4/10	2/10	10/10	0/10	0/10
2	10/10	10/10	10/10	0/10	0/10
Total	14/20	12/20	20/20	0/20	0/20

APPNEDIX B. ISOLATION OF INOCULATED *C. JEJUNI* FROM THE CIRCULATING BLOOD OF EIGHT AND TEN WEEK OLD BROILERS.

Materials and Methods

Experimental design

To evaluate whether inoculated *C. jejuni* could be recovered from the circulating blood, eight (repetition 1) and ten week (repetition 2) university raised broilers were used. The cecal droppings of the birds were screened prior to inoculation to ensure the birds were negative for *Campylobacter* spp. All cecal droppings were negative therefore the birds were presumed to be *Campylobacter* spp. negative. The eight week old broilers were inoculated with $\log_{10}$ 6.6 CFU/ml of *C. jejuni* orally and the ten week old birds were inoculated with $\log_{10}$ 6.2 CFU/ml of *C. jejuni* orally. The broilers were then housed in a confinement rearing facility with access to feed and water *ad libitum*. Seven days post-inoculation, the broilers were euthanized. For collection of blood, the feathers were removed from the skin overlying the brachial vein and surrounding area. Seventy percent ethanol was sprayed on the skin and allowed to sit for 30 sec. before vena-puncture (brachial vein) with a sterile needle. Using sterile 5 ml syringes and 20 gauge needles, 5 ml of circulating blood was collected from each broiler. The blood from each bird was then added into 50 ml sterile conjugal centrifuge tubes which contained 45 ml of modified Bolton enrichment broth (broth without the addition of lysed horse blood and supplements). The ceca from each bird was also aseptically removed and placed into individual sterile stomacher bags.

Blood and ceca samples were then placed on ice and transported back to the laboratory for microbiological analysis. The blood samples were bagged, gassed (5% O_2, 10% CO_2, 85% N) to simulate a microaerophilic environment, and placed in the incubator at 42ºC for 48 h. For the ceca samples, standard laboratory methods were utilized for the recovery of the inoculated *C. jejuni*. After incubation, 0.1 ml of the enrichment broth from each sample was then plated onto Campy-cefex plates. The plates were bagged, gassed and placed in the incubator at 42ºC for 48 h. The plates were then removed and presumptive *C. jejuni* positive colonies were isolated and confirmation was determined by observation of the characteristic darting motility using wet mounts under the microscope.

Results

The inoculated *C. jejuni* was recovered in the circulating blood of 8/20 of the eight week old broilers and from 2/20 of the ten week old broilers. *C. jejuni* was recovered from 19/20 of the ceca from the eight week old broilers and from 12/20 of the ten week old birds. All broilers that were positive for *C. jejuni* in their blood also had *C. jejuni* in their ceca.