CAMPYLOBACTER SPP. IN CONVENTIONAL AND ORGANIC POULTRY OPERATIONS

DISSERTATION

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By

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ABSTRACT

_Campylobacter_ spp. particularly _C. jejuni_ has been recognized as one of the most important causes of foodborne bacterial diarrhea in humans worldwide. Since poultry are considered the major reservoir of this foodborne pathogen, the prevalence along with the antimicrobial resistance of _Campylobacter_ spp. in conventional and organic poultry operations is a matter of concern. However, relatively little information on the impact of different food animal production practices on the prevalence of antibiotic-resistant _Campylobacter_ is available. Hence, a farm-based study was conducted to determine the prevalence and the antimicrobial resistance rates of _Campylobacter_ isolates from both conventionally-raised and organically-raised poultry. Although _Campylobacter_ was highly prevalent in both organic and conventional production systems, the antibiotic resistance rates were significantly higher in conventional operations than in organic operations. These findings clearly indicate the influence of different production practices on the antibiotic resistance rates of _Campylobacter_ spp. on poultry farms, which is likely due to the result of antimicrobial usage in conventional poultry production. However, antimicrobial usage alone may not solely be responsible for the increased antibiotic resistance in _Campylobacter_ isolates because even in the absence of antibiotic exposure, a high level of tetracycline resistance was also observed in _Campylobacter_ isolates from organic poultry farms. Since antibiotics including tetracycline have never been used on these organic poultry farms, a high
prevalence of tetracycline resistance observed among *Campylobacter* isolates from this operation type is interesting. Hence, another part of this study was conducted to investigate the epidemiology of tetracycline resistance of thermophilic *Campylobacter* on organic poultry farms. The results showed the changes of tetracycline resistance rates in *Campylobacter* isolates during the production cycle of one organic broiler flock with 0%, 100%, and 33.33% of tetracycline resistance rates detected at week 3, 6, and 10 of the production period, respectively. Although no tetracycline-resistant *Campylobacter* strains were isolated from unexposed environmental samples collected from these organic poultry farms except for one organic broiler farm, some of these samples were positive for tetracycline resistance gene [*tet*(O) gene]. In addition, the agreement between the disk diffusion method and the agar dilution method in identification of antimicrobial resistance of thermophilic *Campylobacter* was also investigated. The results indicated that the disk diffusion method can be used as a reliable alternative method for susceptibility testing of *Campylobacter* species to fluoroquinolone and aminoglycoside antibiotics; however, until the standard resistance breakpoints specific for *Campylobacter* is established and validated, the agar dilution method should be used to determine antimicrobial resistance of *Campylobacter* to other classes of antibiotics. Together, this study reveals the complex nature in the spread of antibiotic resistance and further highlights the need for prudent measures to prevent the occurrence and transmission of antibiotic-resistant *Campylobacter* in the poultry reservoir.
Dedicated to my parents, Songkram and Theraphan Luangtongkum

Without their love and support this dissertation would not have been possible
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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

*Campylobacter jejuni* has been recognized as an important cause of food-borne illness in humans since the late 1970s (Butzler, 2004; Skirrow, 1977). *Campylobacter* species are gram-negative, motile, nonspore-forming, curved-rod shaped bacteria that are approximately 0.2 to 0.5 µm wide and about 0.5 to 5 µm long (Doyle, 1990; Kaplan and Weissfeld, 1994; Mahon and Manuselis, 2000; Penner, 1988; Rowe and Madden, 2000; Shane and Montrose, 1985). Unlike other recognized food-borne pathogens, *Campylobacter* spp. particularly *C. jejuni* is strictly microaerophilic. This means they require low level of oxygen for growth (Doyle, 1990; Rowe and Madden, 2000). *C. jejuni* and related organisms grow best in an atmosphere containing approximately 3 to 8% O₂ and 5 to 15% CO₂ (Doyle, 1990; Nachamkin, 1999; Rowe and Madden, 2000). Although most *Campylobacter* spp. grow at 37 °C, *C. jejuni* and *C. coli* show optimal growth at 42 °C (Blaser, 2000; Doyle, 1990; Shane and Montrose, 1985). In order to successfully isolate *Campylobacter* spp., appropriate selective media and optimal incubation conditions should be used (Forbes et al., 1998). As mentioned earlier that most
Campylobacter species are strictly microaerophilic, the the ideal environment for optimal recovery of Campylobacter spp. is an atmosphere containing approximately 5% O2, 10% CO2, and 85% N2. Therefore, the selective agar plates should be incubated under this microaerophilic condition at 37 °C or even better at 42 °C for 24 to 48 hours. Currently, new techniques such as latex agglutination tests, enzyme immunoassays (EIA), enzyme-linked immunosorbent assay (ELISA), DNA hybridization assays, and polymerase chain reaction (PCR) have been developed for detection and identification of Campylobacter species (Sahin et al., 2003c); however, these methods should be used with the traditional culture method for the best isolation and identification results.

Most Campylobacter infections in humans usually occur as sporadic cases and consumption of undercooked poultry and/or other foods that are cross-contaminated with raw poultry meat during food preparation is considered to be a major risk factor of this food-borne campylobacteriosis (Allos, 2001; Altekruse et al., 1999; Altekruse and Tollefson, 2003; Blaser, 1997; Harris et al., 1986b; Kapperud et al., 1992; Norkrans and Svedhem, 1982; Pearson et al., 2000; Skirrow, 1990). Generally, clinical symptoms of C. jejuni infection including diarrhea, abdominal pain, high fever, and malaise commonly occur within 2 – 5 days after ingestion of contaminated foods or water (Blaser, 1997; Blaser, 2000; Butzler, 2004; Butzler and Skirrow, 1979; Matthews, 1999; Skirrow, 1990). Since the symptoms of Campylobacter gastroenteritis are indistinguishable from those caused by Salmonella, Shigella, or other enteric bacterial pathogens, a definitive diagnosis of food-borne Campylobacter infection requires isolating the organism from the stools of infected persons (Allos, 1998; Allos, 2001; Allos and Taylor, 1998; Blaser,
In addition to an important cause of bacterial gastroenteritis in humans, *C. jejuni* has also been associated with Guillane-Barré Syndrome (GBS), an acute immune-mediated demyelinating disorder of the peripheral nervous system (Allos, 1997; Allos, 1998; Allos, 2001; Allos and Taylor, 1998; Blaser, 1997; Butzler, 2004; Nachamkin et al., 1998; Nachamkin, 1999; Wassenaar and Blaser, 1999). Although most *Campylobacter* infections are mild, self-limiting, and usually resolve within a few days without any antibiotic treatment, severe or prolonged infections can also occur under certain circumstances, in which antimicrobial therapy is usually warranted (Allos, 1998; Allos, 2001; Allos and Blaser, 1999; Blaser, 1997; Blaser, 2000; Butzler, 2004; Skirrow, 1990). Nevertheless, supportive treatment consisting of fluid and electrolyte replacement seems to be the most important management of *Campylobacter* infections in humans (Allos, 1998; Allos, 2001; Blaser, 2000; Butzler, 2004; Hoeprich et al., 1994; Perez-Perez and Blaser, 1996). If antimicrobial therapy is indicated, erythromycin and fluoroquinolones such as ciprofloxacin are usually considered the drugs of choice for treatment of food-borne campylobacteriosis (Allos, 1998; Allos, 2001; Allos and Blaser, 1999; Allos and Taylor, 1998; Blaser, 1997; Blaser, 2000; Butzler, 2004; Butzler and Skirrow, 1979; Nachamkin, 1999). Since the major risk factor of *Campylobacter* infections in humans is consumption of undercooked poultry and/or other foods that are cross-contaminated with raw poultry meat during food preparation, proper handling and cooking of foods of animal origins particularly poultry meat as well as prevention of cross-contamination of cooked or ready-to-eat foods with raw poultry and other meats seem to be one of the most important actions that can help reduce the risk of food-borne
campylobacteriosis. Other actions that can also provide protection against the risk of *Campylobacter* infections include avoiding drinking untreated water and unpasteurized milk as well as handwashing after contact with animals (Allos, 1998; Allos, 2001; Blaser, 1986).

Although thermophilic *Campylobacter* is highly prevalent in poultry particularly broilers and turkeys, this organism is rarely detected in commercial broiler flocks under the age of 2 - 3 weeks old (Berndtson et al., 1996b; Engvall et al., 1986; Evans and Sayers, 2000; Jacobs-Reitsma, 1997; Jacobs-Reitsma et al., 1995; Newell and Fearnley, 2003; Pokamunski et al., 1986; Sahin et al., 2002; Shane, 1992; Shane, 2000; Stern, 1992). Interestingly, once *Campylobacter* organisms are isolated from the flock, which is probably around 3 weeks of age, most of the birds in that particular flock will become colonized and environment within or around the poultry house seems to be contaminated with *Campylobacter* spp. (Jacobs-Reitsma, 1997; Sahin et al., 2002; Saleha et al., 1998). Although many studies suggest that horizontal transmission from contaminated farm environment is the major mode of *Campylobacter* colonization in broiler flocks (Jacobs-Reitsma et al., 1995; Newell and Fearnley, 2003; Pearson et al., 1993; Stern, 1992), several findings indicate that vertical transmission from breeders may also play a role on *Campylobacter* infections in broilers (Chuma et al., 1994; Chuma et al., 1997b; Doyle, 1984; Pearson et al., 1996; Shane et al., 1986; Shanker et al., 1986). Even though the sources of poultry flock colonization are still unclear, it is unlikely that the vertical transmission will be the major source (Altekruse and Tollefson, 2003; Newell and Wagenaar, 2000). Since the horizontal transmission seems to be the primary route for
introduction of *Campylobacter* spp. to broiler flocks, the current intervention measures are mainly focused on strict biosecurity along with other supportive measures such as competitive exclusion or vaccination in order to control and prevent the introduction of *Campylobacter* species from contaminated farm environment to the poultry flock via the horizontal transmission (Altekruse and Tollefson, 2003; Newell and Davison, 2003; Newell and Wagenaar, 2000; Rowe and Madden, 1999).

Over the last decade, the emergence of antimicrobial resistance has increased dramatically and this problem is likely due to the widespread use of antimicrobial agents in both humans and animals (Khachatourians, 1998; Threlfall et al., 2000; White et al., 2002). In general, antimicrobial agents have been used in livestock and poultry for treatment, control, and prevention of the diseases as well as for improving growth and feed efficiency in animals for long period of time (Aarestrup and Wegener, 1999; Khachatourians, 1998; McEwen and Fedorka-Cray, 2002; Threlfall et al., 2000; Van den Bogaard and Stobberingh, 1999). Recently, a rapid increase in the proportion of *Campylobacter* strains resistant to antimicrobial agents particularly to fluoroquinolones has been reported from many countries around the world (Engberg et al., 2001; Nachamkin et al., 2000b; Smith et al., 2000). Because fluoroquinolones are effective against a wide range of enteric pathogens, this class of antibiotics is not only considered the drug of choice for treatment of food-borne campylobacteriosis, but it is also considered the drug of choice for treatment of gastroenteritis caused by other bacteria as well as for bacterial gastroenteritis that a cause of diarrheal illness has not yet been identified (Allos, 1998; Allos, 2001; Blaser, 2000; Hoeprich et al., 1994; Nachamkin,
Thus, a high prevalence of fluoroquinolone resistance among food-borne pathogens including *Campylobacter* spp. is undesirable. There is a direct evidence suggested that the emergence of fluoroquinolone resistance in *Campylobacter* spp. could occur very rapidly after the chickens were treated with fluoroquinolones (Griggs et al., 2005; Jacobs-Reitsma et al., 1994b; Luo et al., 2003; McDermott et al., 2002). In addition, several studies also revealed the association between the licensing of fluoroquinolones for use in food animals and the increasing numbers of fluoroquinolone resistance in *Campylobacter* isolates from both humans and animals (Endtz et al., 1991; Gaunt and Piddock, 1996; Sanchez et al., 1994; Smith et al., 1999; Velazquez et al., 1995). Since no antibiotics have been used in the organic poultry production system, the differences in antimicrobial susceptibility patterns of *Campylobacter* isolates from conventional and organic poultry operations should reveal the relationship between antimicrobial usage in animal agriculture and the development of antimicrobial resistance in food-borne bacterial pathogens.

Recently, organic poultry are becoming an increasingly important sector of the retail chicken market in many industrialized countries (El-Shibiny et al., 2005). In addition to the strict rules regarding the use of antimicrobial substances, the organic birds must be fed on organically-produced feed and they also need to have access to the outside environment (El-Shibiny et al., 2005). When compared to the conventional production system, the organic production system usually has significantly lower density of the birds in each flock but longer rearing cycle. The organic broiler chickens are generally raised on the farm for 8 to 12 weeks before they are sent to the processing plant, while the
conventional broiler chickens are commonly sent to the processing plant at 6 weeks of age. Interestingly, since there is no regulation regarding the source of the birds, all organic birds are usually obtained from conventional breeder flocks.

1.2 *Campylobacter*: The Organism

1.2.1 Historical Background

The pathogenic bacteria that are now known as *Campylobacter* were first recognized as a cause of infectious abortion in sheep during the early 1900s by McFadyean and Stockman (Butzler, 1984). At that time, these bacteria were classified as “*Vibrio fetus*” (*V. fetus*) because their morphology was similar to those of *Vibrio* species (Smith and Taylor, 1919). Several years later the first document indicated that a “vibrio” organism could also cause enteric infection and diarrhea was reported by Jones et al. (1931). They revealed that a “vibrio” called “*V. jejuni*” was associated with enteritis and dysentery in calves and cattle (Jones et al., 1931). Similarly during the mid-1940s, Doyle isolated another microaerophilic “vibrio”, which he called “*V. coli*”, from swine with diarrhea. He proposed that this organism was the cause of swine dysentery (Doyle, 1944). However, the association between microaerophilic “vibrio” and human infections was not reported until 1946 when Levy isolated the organisms that were similar to “*V. jejuni*” from blood cultures of patients suffered from acute diarrheal illness during a milk-borne outbreak in Illinois (Levy, 1946). In 1947, Vinzent et al. reported the first isolation of “*V. fetus*” from blood culture of a woman who had septic abortion (Butzler, 1984). During the late 1940s and 1950s, the clinical spectrum of infectious abortion due to “*V. fetus*” in sheep and
cattle was becoming better understood (Bryner et al., 1964; Butzler, 1984; Clark, 1971; Miller et al., 1959; Plastridge et al., 1947). As a result, two different varieties of “\textit{V. fetus}”, \textit{V. fetus} var. \textit{intestinalis} and \textit{V. fetus} var. \textit{venerealis}, were identified (Butzler, 1984).

One of the most important studies of \textit{Campylobacter} occurred in 1957 when Elizabeth King found that microaerophilic “vibrio” could be distinguished into two major groups based on their ability to grow at different temperatures (King, 1957). The first group, which had been known as “\textit{V. fetus}”, was the organisms that were able to grow at 25 and 37 °C but not at 42 °C, whereas the other group, which she called “related vibrios”, was the organisms that could grow at 37 °C and even better at 42 °C but not at 25 °C (King, 1957). In addition, Elizabeth King also described that these “related vibrios” were identical to “\textit{V. jejuni}” and “\textit{V. coli}” identified by Jones et al. in 1931 and Doyle in 1944, respectively. Since these “related vibrios” most of the time were isolated from bloodstream of the patients with diarrhea, she suggested that these “related vibrios” might be an important cause of acute diarrheal illness in humans even though these organisms could not be isolated from fecal samples during that time (King, 1957). In the early 1970s, a major progress in \textit{Campylobacter} studies occurred when the technique for culturing \textit{Campylobacter} from fecal specimens were available (Butzler et al., 1973; Dekeyser et al., 1972). During the same period of time, a major taxonomic study of \textit{Campylobacter} was also reported after Veron and Chatelain found that the biochemical characteristics of “\textit{V. fetus}” and “related vibrios” were different from those of the Vibrionaceae. As a result, a new genus, \textit{Campylobacter}, was proposed (Veron and
Chatelain, 1973). Under this scheme, King’s “related vibrios” (“V. jejuni” and “V. coli”) became *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*), while *V. fetus* var. *intestinalis* and *V. fetus* var. *venerealis* became *Campylobacter fetus* subsp. *fetus* (*C. fetus* subsp. *fetus*) and *Campylobacter fetus* subsp. *venerealis* (*C. fetus* subsp. *venerealis*), respectively (Veron and Chatelain, 1973). A significant development of *Campylobacter* studies occurred once again during the late 1970s when selective media for culturing *Campylobacter* was developed (Skirrow, 1977). The use of antibiotic-containing media in combination with simple methods for obtaining a microaerophilic environment not only made a routine diagnosis of *Campylobacter* infections possible, but also opened up the study of *Campylobacter* on a worldwide scale (Allos and Taylor, 1998).

### 1.2.2 General Characteristics

*Campylobacter* species belong to the family *Campylobacteraceae* (Vandamme, 2000). The genus name *Campylobacter* is derived from the Greek words “campylos”, which means “curved” and “baktron”, which means “rod”. The name *Campylobacter* or “curved rod” therefore describes the appearance of this organism under the microscope (Blaser, 1986). Currently, 14 *Campylobacter* species are classified in the genus *Campylobacter* including *C. jejuni* and *C. coli*, the major cause of food-borne *Campylobacter* infection in humans, *C. lari*, *C. fetus*, *C. hyointestinalis*, *C. upsaliensis*, *C. helveticus*, *C. mucosalis*, *C. concisus*, *C. curvus*, *C. rectus*, *C. showae*, *C. sputorum*, and *C. gracilis* (Vandamme, 2000). The main characteristic of this genus is the low level of the G + C content of the DNA ranging from 28 to 47 mol% (Parkhill et al., 2000;
Penner, 1988; Vandamme, 2000). In addition, *Campylobacter* species also have a small genome with approximately 1,600 – 1,700 kilobases (kb) in size, which is about one-third the size of the *E. coli* genome (Chang and Taylor, 1990; Nachamkin, 1997; Parkhill et al., 2000).

*Campylobacter* spp. are gram-negative, nonspore-forming, curved-rod shaped bacteria that are approximately 0.2 to 0.5 µm wide and about 0.5 to 5 µm long (Doyle, 1990; Kaplan and Weissfeld, 1994; Mahon and Manuselis, 2000; Penner, 1988; Rowe and Madden, 2000; Shane and Montrose, 1985). In addition to curved-rod shape, other forms of *Campylobacter* such as S shapes, seagull-wing shapes, spiral rods, commas, and coccoid shapes have also been reported (Blaser, 1986; Doyle, 1990; Kaplan and Weissfeld, 1994; Mahon and Manuselis, 2000). However, spherical or coccoid forms of *Campylobacter* seem to occur only when the culture is old or exposed to the air for a long period of time (Blaser, 1986; Doyle, 1990; Kaplan and Weissfeld, 1994; Mahon and Manuselis, 2000). Although most *Campylobacter* are motile with a distinctive corkscrew darting type motility using a single polar flagellum at one or both ends (Blaser, 1986; Penner, 1988; Rowe and Madden, 2000; Shane and Montrose, 1985), some *Campylobacter* species such as *C. gracilis* is not motile, while other species such as *C. showae* are motile by multiple flagella (Vandamme, 2000).

The colony morphology of thermophilic *Campylobacter* has been reported in two major types (Kaplan and Weissfeld, 1994; Shane and Montrose, 1985; Skirrow and Benjamin, 1980). The first type of *C. jejuni* and *C. coli* colonies is flat, mucoid, or wet appearing, and usually formed large islands of growth. Spreading along the streak line or
swarming on the agar is also commonly observed. The other type of these *C. jejuni* and *C. coli* colonies is round, convex, or raised, and has a discrete margin. Occasionally, the simultaneous occurrence of both types of *Campylobacter* colony in one culture on the agar plate has been reported (Skirrow and Benjamin, 1980). In addition, Skirrow and Benjamin (1980) also described that the swarming growth of *Campylobacter* colony seemed to be more characteristic of *C. jejuni* strain than *C. coli* strain. In general, the transparent colonies like droplets of water sprayed on the medium or the grayish translucent *Campylobacter* colonies are usually present on the agar plate after 18 to 24 hours of incubation (Shane and Montrose, 1985; Skirrow and Benjamin, 1980). If the incubation is continued for 24 to 48 hours, the colonies will be thickened and appear in gray or pinkish and yellowish gray or even tan or slightly pink and orange in color (Allos, 1998; Forbes et al., 1998; Kaplan and Weissfeld, 1994; Shane and Montrose, 1985; Skirrow and Benjamin, 1980). Interestingly, a “metallic” surface sheen is frequently observed in mature cultures of *C. jejuni* and *C. jejuni*-like strains, while this feature is absent or much less evident in *C. coli* and *C. coli*-like strains (Skirrow and Benjamin, 1980).

Although almost every strain of *Campylobacter* is oxidase- and catalase-positive, a wide range of oxidase and catalase activity can be noticed especially among *C. jejuni* and *C. coli* strains. On average, *C. jejuni*-like strains were reported to be less active than *C. coli*-like strains and on a few occasions these *C. jejuni*-like strains were described as catalase-negative *Campylobacter* spp. (Skirrow and Benjamin, 1980). Besides oxidase- and catalase-positive, thermophilic *Campylobacter* strains can also reduce fumarate to
succinate, reduce nitrate to nitrite as well as reduce selenite, but they cannot hydrolyze urea or grow in the presence of 3.5% NaCl and they are acetoin- and indole-negative (Forbes et al., 1998; Penner, 1988; Shane and Montrose, 1985; Skirrow and Benjamin, 1980; Vandamme, 2000). The ability to grow in 1% glycine is variable among species (Forbes et al., 1998). Although \textit{C. jejuni}, \textit{C. coli}, and \textit{C. lari} are closely related, only \textit{C. jejuni} can hydrolyze hippurate. The ability to hydrolyze hippurate helps distinguish \textit{C. jejuni} from other \textit{Campylobacter} species; however, hippurate-negative \textit{C. jejuni} can also occur (Blaser, 2000; Doyle, 1990; Kaplan and Weissfeld, 1994; Nachamkin, 1999). Since thermophilic \textit{Campylobacter} strains do not ferment or oxidize carbohydrates, e.g. glucose, their energy sources for growth are mainly obtained from either amino acids such as aspartate and glutamate or tricarboxylic acid (TCA) cycle intermediates (Blaser, 1986; Penner, 1988; Rowe and Madden, 2000; Shane and Montrose, 1985; Skirrow and Benjamin, 1980; Vandamme, 2000).

Unlike other recognized food-borne pathogens, \textit{Campylobacter} spp. particularly \textit{C. jejuni} is strictly microaerophilic. This means they require low level of oxygen for growth (Doyle, 1990; Rowe and Madden, 2000). \textit{C. jejuni} and related organisms grow best in an atmosphere containing approximately 3 to 8% O$_2$ and 5 to 15% CO$_2$ (Doyle, 1990; Nachamkin, 1999; Rowe and Madden, 2000). Although most \textit{Campylobacter} grow at 37 °C, \textit{C. jejuni} and \textit{C. coli} show optimal growth at 42 °C (Blaser, 2000; Doyle, 1990; Shane and Montrose, 1985). In contrast to the thermophilic \textit{Campylobacter}, \textit{C. fetus} grow
well at 25 °C (Skirrow and Benjamin, 1980). This characteristic helps differentiate *C. fetus* from other *Campylobacter* species (Shane and Montrose, 1985; Skirrow and Benjamin, 1980).

### 1.2.3 Isolation and Identification

**Isolation of *Campylobacter* spp.**

In order to isolate *Campylobacter* from the samples that may contain different varieties of bacteria such as fecal samples, the selective media should be used to inhibit the growth of more rapidly growing components of the enteric bacterial flora because *Campylobacter* species multiply much more slowly than other enteric bacteria (Allos, 1998; Blaser, 2000). Since the late 1970s a number of *Campylobacter* selective media have been described including blood-free media such as modified charcoal cefoperazone deoxycholate agar (mCCDA), charcoal-based selective medium (CSM), and Karmali agar; semi-solid blood-free selective motility medium; and blood-containing media such as Skirrow’s medium, Butzler’s medium, Blaser’s medium, Campy-BAP medium, Preston agar, and Campy CVA agar (Bolton and Robertson, 1982; Bolton et al., 1983; Bolton et al., 1984; Corry et al., 1995; Corry, 2000; Forbes et al., 1998; Goossens et al., 1989; Gun-Munro et al., 1987; Karmali et al., 1986; Kaplan and Weissfeld, 1994; Mahon and Manuselis, 2000; Nachamkin, 1999; Sahin et al., 2003c; Skirrow, 1977). These selective media usually contain combinations of antibiotics to which thermophilic *Campylobacter* are intrinsically resistant but other bacteria particularly enteric microbial flora are susceptible (Corry et al., 1995; Sahin et al., 2003c). Antimicrobial agents that
are usually used in *Campylobacter* selective media include a combination of cephalosporins such as cephalothin or cefoperazone and vancomycin or bacitracin. Although both cephalothin and cefoperazone have been used in *Campylobacter* selective media, cefoperazone is now more preferred because some strains of *C. jejuni* such as *C. jejuni* subsp. *doylei* and *C. coli* as well as *C. fetus* and *C. upsaliensis* are inhibited by cephalothin (Corry et al., 1995; Nachamkin, 1999; Ng et al., 1988). Since these antibiotics are mainly active against gram-positive bacteria, other antibiotics that active against gram-negative rod-shaped bacteria such as polymyxin B or closely related antibiotic, polymyxin E (colistin), are often added to the selective media (Corry et al., 1995; Corry, 2000). Although most gram-negative bacteria are inhibited by polymyxin, *Proteus* spp. seem to be resistant to this antibiotic. Because trimethoprim generally inhibits *Proteus* spp. as well as other gram-negative bacteria and because it does not have inhibitory effect on some strains of *Campylobacter* as polymyxin B or colistin, it has been used in several selective media (Corry et al., 1995; Corry, 2000). Rifampicin is another antibiotic that is used in *Campylobacter* selective media. This antibiotic is active against both gram-positive and particularly gram-negative bacteria (Corry, 2000). Other antimicrobials that are effective against yeasts and molds such as cycloheximide (actidione), amphotericin B, or nystatin are also present in many *Campylobacter* selective media (Corry et al., 1995; Corry, 2000). The detail formulation of each *Campylobacter* selective medium has been previously summarized by Corry et al. (1995). Since each selective medium contains different combination and amount of antibiotics, a combination of media should be used in order to increase the success rates of
Campylobacter isolation (Nachamkin, 1999). In addition, since Campylobacter spp. are sensitive to oxygen, most selective media for thermophilic Campylobacter usually contain substances that help protect Campylobacter from the toxic effect of oxygen derivatives (Corry et al., 1995; Corry, 2000; Sahin et al., 2003c). The most commonly used substances for neutralizing these toxic oxygen derivatives include whole, lysed, or defibrinated blood; charcoal; a combination of ferrous sulfate, sodium metabisulfite, and sodium pyruvate; and haemin or haematin (Corry et al., 1995; Corry, 2000; Sahin et al., 2003c).

Besides Campylobacter selective media, a number of enrichment broths and transport media have also been formulated to enhance the recovery rates of thermophilic Campylobacter. The most widely used enrichment broths for Campylobacter spp. include Preston broth, Doyle and Roman broth, Park and Sanders broth, Hunt and Radle broth, and Exeter broth (Bolton et al., 1982; Corry et al., 1995; Corry, 2000; Doyle and Roman, 1982). These enrichment broths seem to be very beneficial especially when low numbers of Campylobacter in the samples are expected. In addition, if the samples cannot be processed within 2 or 3 hours of collection, the transport media such as Cary-Blair or Campy-thioglycollate broth should be used to maintain the viability of thermophilic Campylobacter in the samples (Allos and Taylor, 1998; Forbes et al., 1998; Kaplan and Weissfeld, 1994; Martin et al., 1983; Rubin et al., 1983; Wang et al., 1983).

Campylobacter isolation can be obtained by a direct plating method or a selective enrichment method depending on the types of sample and the numbers of Campylobacter present in those samples (Sahin et al., 2003c). For example, feces or intestinal/cecal
contents from chickens usually contain high numbers of *Campylobacter* organisms. Therefore, these types of sample can be directly plated onto *Campylobacter* selective agar (Musgrove et al., 2001; Sahin et al., 2003c). On the other hand, *Campylobacter* species are unlikely to be present in high numbers in food or environmental samples such as water samples. Therefore, the selective enrichment method should be performed prior to plating these samples directly onto selective media (Sahin et al., 2003c). For the optimal recovery, samples should be enriched in the enrichment broth no longer than 24 hours because a prolonged enrichment can decrease the isolation rate to a level even below the recovery rate obtained from the direct plating method (Sahin et al., 2003c). Interestingly, the selective enrichment method may not always be superior for *Campylobacter* isolation especially when the samples containing high numbers of fast-growing background flora such as fecal samples are enriched. This is because the background flora may overgrow thermophilic *Campylobacter* during the enrichment step leading to the reduction of *Campylobacter* recovery rates (Sahin et al., 2003c). Because of their size and motility, *Campylobacter* species can be isolated by the filtration method as well (Blaser, 2000; Corry, 2000; Forbes et al., 1998). In fact, the filtration method in conjunction with a nonselective medium such as blood agar has been used to isolate *Campylobacter* species from fecal samples since 1970s (Kaplan and Weissfeld, 1994; Shane and Montrose, 1985). This method is based on the principle that *Campylobacter* can pass through the 0.45 µm or 0.65 µm pore-size membrane filter relatively easy during the short processing time (about 30 to 60 minutes), while other enteric bacteria cannot (Allos and Taylor, 1998; Blaser, 2000; Corry, 2000; Forbes et al., 1998; Nachamkin,
However, the filtration method can recover *Campylobacter* spp. from the samples only when those samples contain at least $10^5$ CFU of *Campylobacter* microorganisms (Corry, 2000; Nachamkin, 1999). Although this method is not quite as sensitive as primary culturing with selective media, it is very useful especially for recovering *Campylobacter* spp. such as *C. upsaliensis* that are susceptible to antibiotics used in the selective media (Corry, 2000; Forbes et al., 1998; Kaplan and Weissfeld, 1994; Nachamkin, 1999). Therefore, the filtration method should be used only to complement the direct plating or the selective enrichment methods but not as a replacement (Corry, 2000; Nachamkin, 1999). A combination of the filtration method and the selective enrichment method has been used successfully for isolation of thermophilic *Campylobacter* from water samples (Blaser and Cody, 1986; Pearson et al., 1993; Sahin et al., 2003c).

To successfully isolate *Campylobacter* spp. from samples, selective media and optimal incubation conditions are very crucial (Forbes et al., 1998). As mentioned earlier that most *Campylobacter* species are strictly microaerophilic, the ideal environment for optimal recovery of *Campylobacter* is the atmosphere containing approximately 5% O$_2$, 10% CO$_2$, and 85% N$_2$. However, some *Campylobacter* species such as *C. hyointestinalis*, *C. mucosalis*, *C. concisus*, *C. curvus*, *C. rectus*, and *C. sputorum* seem to require higher level of hydrogen (about 6% H$_2$) for growth and for primary isolation, so these *Campylobacter* species may not be recovered under the conventional microaerophilic conditions (Mahon and Manuselis, 2000; Nachamkin, 1999). The methods used to create the suitable microaerophilic environment for *Campylobacter* spp.
isolation have been summarized by Kaplan and Weissfeld (1994) and by Mahon and Manuselis (2000). Since different *Campylobacter* species seem to have different optimal temperatures for growth, the choice of incubation temperature for isolating *Campylobacter* species from samples is critical (Nachamkin, 1999). In general, most laboratories use 42 °C as the primary incubation temperature for *Campylobacter* isolation (Nachamkin, 1999). This temperature is the optimal growth temperature of thermophilic *Campylobacter* including *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*; however, it may not be suitable for the growth of several *Campylobacter* species (Corry, 2000; Nachamkin, 1999). For example, *C. fetus*, an opportunistic pathogen that is commonly involved in extraintestinal *Campylobacter* infections in humans, is unlikely to grow and most of the time is not recovered at 42 °C (Nachamkin, 1999; Shane and Montrose, 1985; Skirrow and Benjamin, 1980). Since *C. fetus* grows well at 25 °C, this temperature should be used for isolating this organism (Joklik et al., 1992; Nachamkin, 1999; Shane and Montrose, 1985; Skirrow and Benjamin, 1980). Besides the optimal incubation conditions that directly affect the recovery rates of *Campylobacter*, another thing that is also important when trying to isolate *Campylobacter* spp. from samples is the susceptibility of *Campylobacter* strains to antibiotics used in the selective media. Since several *Campylobacter* strains may be inhibited by cephalothin, rifampin, colistin, and polymyxin B, the isolation of *Campylobacter* spp. on the selective agar containing these antimicrobial agents should be performed carefully (Allos and Taylor, 1998; Forbes et al., 1998; Joklik et al., 1992; Nachamkin, 1999). In addition, because *C. upsaliensis* and *C. fetus* are generally sensitive to cephalothin as well as other cephalosporin antibiotics,
these organisms should not be isolated on the selective media containing these types of antimicrobial agents (Forbes et al., 1998; Joklik et al., 1992; Nachamkin, 1999). Finally, in order to isolate thermophilic *Campylobacter*, the selective agar plates should be incubated at 37 °C or even better at 42 °C for 24 to 48 hours under a microaerophilic condition. If no suspected *Campylobacter* colonies are present on the plates, the incubation should be extended to 72 to 96 hours before being reported as negative; however, *Campylobacter* strains that cause septicemia or other extraintestinal infections such as *C. fetus* may require as long as 2 weeks before their growth can be detected (Forbes et al., 1998; Nachamkin, 1999).

**Identification of *Campylobacter* spp.**

As mentioned earlier, *Campylobacter* colonies may appear differently depending on the media used; however, colonies that appear gray to pinkish gray in color, nonhemolytic, flat, and slightly mucoid should be suspected of being *Campylobacter* species (Forbes et al., 1998; Kaplan and Weissfeld, 1994; Nachamkin, 1999; Sahin et al., 2003c). When *Campylobacter* spp. are subcultured onto freshly prepared moist media, spreading of the colonies along the streak line or swarming of the colonies on the agar plate is commonly noticed. As the moisture content decreases, *Campylobacter* colonies may become round, convex, and glistening with little spreading (Nachamkin, 1999; Sahin et al., 2003c; Shane and Montrose, 1985). Besides colony morphology, microscopic morphology can help identify *Campylobacter* species as well. *Campylobacter* spp. are gram-negative, curved-rod shaped bacteria that are approximately 0.2 to 0.5 µm wide and
about 0.5 to 5 µm long (Doyle, 1990; Kaplan and Weissfeld, 1994; Mahon and Manuselis, 2000; Penner, 1988; Rowe and Madden, 2000; Shane and Montrose, 1985). Since *Campylobacter* species are not stained very well and are not easily visualized with the safranin counterstain as commonly used in the standard Gram stain procedure, carbolfuchsin or 0.1% – 1% aqueous basic fuchsin should be used as the counterstain instead. However, if safranin is used, counterstaining should be extended to 2 to 3 minutes (Allos and Taylor, 1998; Kaplan and Weissfeld, 1994; Mahon and Manuselis, 2000; Nachamkin, 1999). In general, when the smears are prepared from fresh, young cultures, *Campylobacter* spp. may appear in different shapes such as S shapes, seagull-wing shapes, long spirals, short curves, or comma shapes. However, these organisms can also appear as coccoid or coccobacilli especially when the smears are prepared from older cultures (Blaser, 1986; Doyle, 1990; Kaplan and Weissfeld, 1994; Mahon and Manuselis, 2000).

Although the presumptive identification of *Campylobacter* spp. can be made by typical colonial and typical microscopic morphology as well as the characteristic rapid darting motility as observed under phase-contrast or darkfield microscope, other phenotypic tests especially oxidase and catalase tests should also be performed to confirm the identification (Kaplan and Weissfeld, 1994; Mahon and Manuselis, 2000; Nachamkin, 1999; Sahin et al., 2003c). For oxidase test, a heavy inoculum sometimes is required to conduct this phenotypic test (Kaplan and Weissfeld, 1994). Several biochemical tests have been described for identifying *Campylobacter* spp.; however, the most routinely useful tests for initial identification of thermophilic *Campylobacter*
include ability to grow at 37 or 42 °C but not at 25 °C, oxidase test, catalase test, hippurate hydrolysis test, production of hydrogen sulfide (H₂S) in triple sugar iron agar butts, nitrate reduction, urease production, indoxyl acetate hydrolysis test, cephalothin sensitivity test, and nalidixic acid susceptibility test (Doyle, 1990; Forbes et al., 1998; Kaplan and Weissfeld, 1994; Nachamkin, 1999; Sahin et al., 2003c; Shane and Montrose, 1985; Skirrow and Benjamin, 1980).

As mentioned earlier, hydrolysis of sodium hippurate is the major biochemical criterion that is commonly used to differentiate C. jejuni from other thermophilic Campylobacter species because only C. jejuni can hydrolyze sodium hippurate and give a positive result to this test. However, some strains of C. jejuni may occasionally be hippurate-negative (Blaser, 2000; Doyle, 1990; Kaplan and Weissfeld, 1994; Nachamkin, 1999; Sahin et al., 2003c). Susceptibility to cephalothin and nalidixic acid is also another important criterion for differentiation among thermophilic Campylobacter species. In general, both C. jejuni and C. coli are resistant to cephalothin, but they both are susceptible to nalidixic acid. On the other hand, C. lari is resistant to both cephalothin and nalidixic acid, while C. upsaliensis is susceptible to both of these antimicrobial agents (Kaplan and Weissfeld, 1994; Nachamkin, 1999). Over the last decade, the emergence of fluoroquinolone resistance among C. jejuni and C. coli isolates has increased drastically (Endtz et al., 1991; Nachamkin et al., 2000). Since fluoroquinolone-resistant C. jejuni and C. coli strains are also cross-resistant to nalidixic acid, the occurrence of nalidixic acid resistance among C. jejuni and C. coli strains can directly lead to problems with identification of thermophilic Campylobacter to the species level.
(Endtz et al., 1991; Nachamkin, 1999; Nachamkin et al., 2000). Therefore, if the susceptibility to nalidixic acid is going to be used for identification of *Campylobacter* species, the interpretation of this test should be conducted carefully. Recently, the phenotypic identification of thermophilic *Campylobacter* also can be performed with commercial kits such as API Campy (Huysmans et al., 1995; Sahin et al., 2003c). The biochemical characteristics of each *Campylobacter* species have been summarized by Kaplan and Weissfeld (1994).

At present, new techniques have been developed for detection and identification of *Campylobacter* species. These techniques are mainly based on antigen-antibody interactions or nucleic acid (DNA) detection (Sahin et al., 2003c). The methods for rapid detection and identification of *Campylobacter* species include latex agglutination tests, enzyme immunoassays (EIA), enzyme-linked immunosorbent assay (ELISA), immunoblotting technique, colony blotting technique, colony lift immunoassay (CLI), immunomagnetic separation (IMS), DNA hybridization assays, and polymerase chain reaction (PCR) (Sahin et al., 2003c).

Latex agglutination tests are mainly used for rapid identification and confirmation of *Campylobacter* colonies (Hazeleger and Beumer, 2000; Hazeleger et al., 1992; Hodinka and Gilligan, 1988; Kaplan and Weissfeld, 1994; Mahon and Manouselis, 2000; Nachamkin and Barbagallo, 1990; Sahin et al., 2003c; Sutcliffe et al., 1991). These tests help identify whether the suspected colonies are *Campylobacter* or not, but they cannot differentiate between *Campylobacter* species; i.e., the latex agglutination tests can identify *Campylobacter* spp. at the genus level not at the species level (Hazeleger and
Latex agglutination tests are usually performed after the primary isolation of thermophilic Campylobacter on selective agar plates and these tests are not intended to use for direct detection of Campylobacter spp. from field samples (Hazeleger and Beumer, 2000; Hazeleger et al., 1992; Mahon and Manuselis, 2000; Sahin et al., 2003c). Examples of the commercially available latex agglutination tests include Campyslide (BBL Microbiology Systems, Cockeysville, MD), Meritec Campy [jcl] (Meridian Diagnostics, Cincinnati, OH), ID Campy (Integrated Diagnostics, Baltimore, MD), INDX-Campy [jcl] (PanBio INDX, Inc., Baltimore, MD), and Microscreen Campylobacter (Mercia Diagnostics, Guildford, UK) (Hazeleger and Beumer, 2000; Hazeleger et al., 1992; Hodinka and Gilligan, 1988; Kaplan and Weissfeld, 1994; Mahon and Manuselis, 2000; Nachamkin, 1999; Nachamkin and Barbagallo, 1990; Sahin et al., 2003c). Most of these tests can detect C. jejuni, C. coli, and C. lari (Hazeleger and Beumer, 2000; Hazeleger et al., 1992; Hodinka and Gilligan, 1988; Kaplan and Weissfeld, 1994; Mahon and Manuselis, 2000; Nachamkin, 1999; Nachamkin and Barbagallo, 1990; Sutcliffe et al., 1991), while some of these tests can also detect C. fetus and C. upsaliensis (Hazeleger and Beumer, 2000; Kaplan and Weissfeld, 1994; Mahon and Manuselis, 2000; Nachamkin and Barbagallo, 1990; Sutcliffe et al., 1991). More details on latex agglutination tests can be obtained from the previously published articles by Hazeleger and Beumer (2000) and On (1996). When compared to the latex agglutination tests, the enzyme immunoassays (EIA) seem to have better sensitivity and specificity and they can also be used for direct detection of
Campylobacter spp. in field samples (Hindiyeh et al., 2000; Sahin et al., 2003c). Although these methods provide rapid detection and identification of Campylobacter species, they are not as sensitive as the traditional culture methods; hence, they should not be used for detection of Campylobacter spp. from samples in which the organisms are suspected to be low in numbers (Sahin et al., 2003c). Examples of the commercially available EIA kits include VIDAS Campylobacter kit (bioMerieux, France), EIA-Foss Campylobacter enzyme-linked immunosorbent assay kit (Foss Electric, Denmark), and ProSpecT Campylobacter microplate assay (Alexon-Trend, Inc., Ramsey, MN) (Hindiyeh et al., 2000; Hoorfar et al., 1999; Sahin et al., 2003c). In addition, a commercial enzyme-linked immunosorbent assay (Alexon-Trend, Inc., Minneapolis, MN) for direct detection of Campylobacter antigen in field samples; e.g., fecal samples was also recently introduced (Nachamkin et al., 2000).

The use of nucleic acid or DNA probes for detection and identification of Campylobacter species has increased in recent years (On, 1996). These DNA probes may be genus or species specific (On, 1996). In general, the majority of DNA probes used for hybridization assays of Campylobacter species are designed from 16 S rRNA genes of these organisms (On, 1996; Sahin et al., 2003c). The major advantage of DNA probes over conventional methods for identification of Campylobacter spp. is that Campylobacter colonies can be identified relatively quickly and efficiently. Moreover, certain probes can also be used for direct detection of thermophilic Campylobacter from field samples (On, 1996; Sahin et al., 2003c). However, the sensitivity and specificity of DNA probes seem to be lower than those of traditional culture methods especially when
the target gene sequence is mutated or absent. In addition, these methods also are costly and require specialized reagents and equipment (Olsen et al., 1995; On, 1996; Sahin et al., 2003c; Stucki et al., 1995). Generally, DNA probes are often used for culture confirmation and usually combined with culture methods to increase detection efficiency (Nachamkin, 1999; Sahin et al., 2003c). Examples of the commercially available DNA probes include SNAP (Syngene, San Diego, CA), DNA Probe System (Enzo Biochemicals, New York, NY), and AccuProbe (Gen-Probe, Inc., San Diego, CA). These DNA probes are commonly used for identification of thermophilic *Campylobacter* including *C. jejuni*, *C. coli*, and *C. lari* (Kaplan and Weissfeld, 1994; Nachamkin, 1999; On, 1996; Sahin et al., 2003c).

Recently, polymerase chain reaction (PCR)-based methods have been developed for rapid detection, identification, and confirmation of *Campylobacter* species as well as for typing of *Campylobacter* strains (Blaser, 2000; Englen et al., 2003; Sahin et al., 2003c). When compared to DNA hybridization assays, PCR-based methods are more commonly used than DNA probes not only for detection and identification of *Campylobacter* spp. but also for detection and identification of other food-borne pathogens (Sahin et al., 2003c). One of the most important components of PCR-based method is oligonucleotide primers (PCR primers), which can be designed from either variable or conserved gene sequences of *Campylobacter* species. Depending on the primers used, PCR methods can be used for detection and identification of *Campylobacter* genus as well as for differentiation between *Campylobacter* species. In general, PCR primers designed from conserved regions; for example, 16S rRNA genes or
23S rRNA genes are commonly used for general or genus detection, while primers designed from variable regions such as \textit{hipO} gene, \textit{flaA} gene, \textit{mapA} gene, \textit{ceuE} gene, \textit{glyA} gene, \textit{cadF} gene, or \textit{lpxA} gene are normally used for differentiation of species or strains (Al Rashid et al., 2000; Bang et al., 2002; Cloak and Fratamico, 2002; Englen et al., 2003; Eyers et al., 1993; Fermer and Engvall, 1999; Giesendorf et al., 1992; Gonzalez et al., 1997; Hiett et al., 2002a; Houng et al., 2001; Itoh et al., 1995; Kirk and Rowe, 1994; Klena et al., 2004; Konkel et al., 1999; Kulkarni et al., 2002; Linton et al., 1997; Logan et al., 2001; Lubeck et al., 2003; Metherell et al., 1999; O’Sullivan et al., 2000; Oyofo et al., 1992; Perelle et al., 2004; Rasmussen et al., 1996; Sahin et al., 2003c; Sticki et al., 1995; Waage et al., 1999; Waegel and Nachamkin, 1996; Wang et al., 2002). Over the last several years, PCR methods targeting genus-specific sequences or species-specific sequences have been developed and used for detection and identification of \textit{Campylobacter} spp. in different types of samples (Bang et al., 2002; Bolton et al., 2002; Chuma et al., 1997b; Collins et al., 2001; Denis et al., 2001; Docherty et al., 1996; Engvall et al., 2002; Fermer and Engvall, 1999; Giesendorf et al., 1992; Hernandez et al., 1995; Hiett et al., 2002a; Inglis and Kalischuk, 2003; Itoh et al., 1995; Josefsen et al., 2004; Kirk and Rowe, 1994; Lawson et al., 1998; Linton et al., 1997; Lund et al., 2003; Magistrado et al., 2001; Metherell et al., 1999; O’Sullivan et al., 2000; Oyofo et al., 1992; Padungtod et al., 2002; Rasmussen et al., 1996; Sails et al., 2002; Sails et al., 2003; Sahin et al., 2003c; Steinbrueckner et al., 1999; Studer et al., 1999; Vanniasinkam et al., 1999; Waage et al., 1999; Waegel and Nachamkin, 1996; Waino et al., 2003; Waller and Ogata, 2000; Wang et al., 2002; Winters and Slavik, 2000).
Since PCR methods can amplify targeted or specific genetic sequences over a billion fold within a short period of time, these methods definitely improve detection and identification of *Campylobacter* spp. from samples containing low numbers of *Campylobacter* species (Englen et al., 2003; Sahin et al., 2003c). In addition, when species-specific primer sets are used, PCR can efficiently distinguish *C. jejuni* from *C. coli*. Therefore, it is very useful for differentiating between hippurate-negative *C. jejuni* and *C. coli*, which cannot be differentiated by phenotypic methods (Nachamkin et al., 2000; Rautelin et al., 1999). Several specific genes such as *hipO* gene, *flaA* gene, *mapA* gene, *ceuE* gene, and *glyA* gene have been reliably used to distinguish between *C. jejuni* and *C. coli* by PCR methods over the last several years (Al Rashid et al., 2000; Bang et al., 2002; Cloak and Fratamico, 2002; Englen et al., 2003; Fermer and Engvall, 1999; Gonzalez et al., 1997; Hiett et al., 2002; Houng et al., 2001; Itoh et al., 1995; Kirk and Rowe, 1994; Linton et al., 1997; Nachamkin et al., 2000; Oyofo et al., 1992; Rasmussen et al., 1996; Rautelin et al., 1999; Stucki et al., 1995; Waage et al., 1999; Waegel and Nachamkin, 1996; Wang et al., 2002). Although PCR method provides rapid detection as well as high sensitivity and specificity in identification of *Campylobacter* species, it usually works well with bacterial isolates and its performance on direct testing of field samples seems to be reduced drastically due to PCR inhibitors that may be present in some types of field samples such as in fecal samples (Sahin et al., 2003c). Another drawback of PCR method is that it is unable to discriminate between viable and non-viable *Campylobacter* cells, which may be essential in some epidemiological studies (Sahin et al., 2003c).
A variety of PCR assays such as multiplex PCR (Cloak and Fratamico, 2002; Denis et al., 1999; Harmon et al., 1997; Houng et al., 2001; Klena et al., 2004; Korolik et al., 2001; Wang et al., 2002; Winters and Slavik, 2000), real-time PCR (Logan et al., 2001; Lund et al., 2004; Nogva et al., 2000; Perelle et al., 2004; Rudi et al., 2004; Sails et al., 2003), reverse transcriptase-PCR (RT-PCR) (Sails et al., 1998) have been designed and used in order to improve detection and identification of *Campylobacter* species (Sahin et al., 2003c). For multiplex PCR, multiples sets of primers such as genus-specific and species-specific primers or several species-specific primers are included in one PCR reaction so that detection and identification can be simultaneously accomplished in a single test (Sahin et al., 2003c). In terms of real-time PCR, although this method can accurately measure the quantity of template DNA and consequently provide approximate numbers of organisms present in the sample, it requires special equipment and reagents that usually are more expensive than conventional PCR method (Sahin et al., 2003c). Reverse transcriptase-PCR (RT-PCR) has been used to differentiate between viable and non-viable *Campylobacter* cells by amplifying mRNA, which is regarded as a marker for cell viability because it is much less stable than DNA and usually disappears quickly after cells die (Sahin et al., 2003c; Sails et al., 1998). However, this method requires pure RNA preparations because DNA contamination in the reaction can lead to unreliable results (Sahin et al., 2003c; Sails et al., 1998). Besides different PCR assays previously mentioned, PCR can also be combined with other identification or typing techniques such as ELISA (Bolton et al., 2002; Kulkarni et al., 2002; Lawson et al., 1999; Metherell et al., 1999; Sails et al., 2001; Sails et al., 2002; Waller and Ogata, 2000), hybridization assays;
e.g., Southern blot (Al Rashid et al., 2000; Collins et al., 2001; Geisendorf et al., 1993; Konkel et al., 1999; Lawson et al., 1998; O’Sullivan et al., 2000), or reverse hybridization assay (O’Sullivan et al., 2000; van Doorn et al., 1999), restriction fragment length polymorphism (RFLP) (Fermer and Engvall, 1999; Nachamkin et al., 2000; Steinhauserova et al., 2001; Steinhauserova et al., 2002), and restriction enzyme assay (REA) (Comi et al., 1996; Engvall et al., 2002) to enhance detection efficiency (Sahin et al., 2003c). Other molecular detection and identification methods for *Campylobacter* species have been reviewed by On (1996) and Sahin et al. (2003c).

As mentioned earlier, nucleic acids (DNA)-based methods such as PCR are not only useful for rapid detection and identification of *Campylobacter* species, but they are also useful for epidemiological studies of *Campylobacter* infections in both humans and animals (Sahin et al., 2003c; Sails et al., 1998). Several typing systems have been developed to study the epidemiology of *Campylobacter* infections (Nachamkin, 1999; Nachamkin et al., 2000). These typing systems can be divided into two major groups, which are phenotypic typing methods and genotypic typing methods (Farber et al., 2001). These methods seem to vary in complexity and ability to discriminate between *Campylobacter* strains (Nachamkin, 1999; Nachamkin et al., 2000). The methods for epidemiological typing of *Campylobacter* species include serotyping, biotyping, phage typing, auxotyping, lectin binding, bacteriocin sensitivity testing, detection of preformed enzymes, multilocus enzyme electrophoresis (MEE) and molecular typing methods such as pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), PCR-restriction fragment
length polymorphism (PCR-RFLP), restriction endonuclease enzyme analysis (REA), ribotyping, plasmid typing, and flagellin typing (*fla* typing) (Farber et al., 2001; Kaplan and Weissfeld, 1994; Nachamkin, 1999; Nachamkin et al., 2000; Newell et al., 2000; Sahin et al., 2003c; Wassenaar and Newell, 2000). Among the phenotypic typing methods, serotyping is the most frequently used method (Nachamkin, 1999). The two most widely used serotyping systems for *Campylobacter* species particularly for *C. jejuni* and *C. coli* are the Penner system and the Lior system (Allos and Taylor, 1998; Nachamkin et al., 2000). The Penner system identifies soluble heat-stable (somatic, O) antigens; e.g. lipopolysaccharide, by passive or indirect hemagglutination technique, whereas the Lior system measures heat-labile (flagellar, H) antigens by slide agglutination technique (Allos and Taylor, 1998; Joklik et al., 1992 Nachamkin et al., 2000). Recently, there are more than 90 reference serotypes defined by the Penner system and 112 serotypes defined by the Lior system (Allos and Taylor, 1998). Both systems identify more than 90% of *Campylobacter* species isolated from humans and nonhuman sources (Allos and Taylor, 1998; Jones et al., 1985; Patton et al., 1985). Although serotyping method is a useful tool for epidemiological studies of *Campylobacter* infections in both humans and animals, it is performed only in a few reference laboratories because of the time and expense needed to maintain quality antisera (Nachamkin, 1999). In terms of genotypic typing methods, several methods have been used and each method seems to have its own advantages and disadvantages. More details
of molecular typing methods of *Campylobacter* species can be obtained from comprehensive reviews previously published by Newell et al. (2000), and Wassenaar and Newell (2000).

1.3 *Campylobacter* spp. in Humans

1.3.1 Sources and Modes of Transmission

*Campylobacter* species particularly *C. jejuni* and *C. coli* have been recognized as important causes of bacterial gastrointestinal infections in humans since the late 1970s (Butzler, 2004). Human campylobacteriosis is a zoonosis and is mainly a food-borne infection in which foods of animal origin particularly poultry seem to play an important role (Allos, 2001; Altekruse et al., 1999; Altekruse and Tollefson, 2003; Butzler, 2004; Butzler and Skirrow, 1979; Skirrow, 1982). Most *Campylobacter* infections in humans usually occur as sporadic cases and consumption of undercooked poultry and/or other foods that are cross-contaminated with raw poultry meat during food preparation is considered to be a major risk factor of this food-borne campylobacteriosis (Allos, 2001; Altekruse et al., 1999; Altekruse and Tollefson, 2003; Blaser, 1997; Harris et al., 1986b; Kapperud et al., 1992; Norkrans and Svedhem, 1982; Pearson et al., 2000; Skirrow, 1990). Since *Campylobacter* species particularly *C. jejuni* are found as commensals in the intestinal tracts of warm-blooded animals especially poultry, it is not surprising that the majority of retail chicken meats will be contaminated with these organisms (Butzler and Skirrow, 1979). Several survey studies revealed that at least 70% of retail chickens sold in the United States were contaminated with *Campylobacter* spp., while less than 2% of
pork and beef were contaminated with these organisms (Smith et al., 1999; Zhao et al., 2001). Likewise, about 40% of poultry meats sold in Europe and Asia were contaminated with *Campylobacter* spp., which was 10 times higher than that of pork meats sold in the same area (Ono and Yamamoto, 1999; Zanetti et al., 1996). Although other risk factors such as eating barbequed pork or sausage, drinking raw milk or untreated water, and traveling abroad have been reported to be associated with sporadic *Campylobacter* infections (Altekruse et al., 1999; Hopkins et al., 1984; Kapperud et al., 1992; Norkrans and Svedhem, 1982; Schmid et al., 1987), a majority (50% - 70%) of *Campylobacter* infections in humans in many parts of the United States, Europe, and Australia have been attributed to consumption of chickens (Allos, 2001).

Besides poultry meats, raw or unpasteurized milk is also recognized as another important source of *Campylobacter* infections in humans (Skirrow, 1982). Unlike sporadic infections, most outbreaks of food-borne campylobacteriosis are associated with consumption of raw or improperly pasteurized milk (Allos, 2001; Friedman et al., 2000; Robinson and Jones, 1981; Skirrow, 1982). Since *Campylobacter* spp. are commonly found in cow feces, it is possible that these organisms may probably be introduced into the milk by fecal contamination at the time of milking (Butzler and Skirrow, 1979; Skirrow, 1990). Occasionally, *Campylobacter* spp. may be excreted into the milk from cows that have *Campylobacter* mastitis as well (Skirrow, 1990).

Another important source of outbreaks of *Campylobacter* gastroenteritis is improperly treated or untreated water (Friedman et al., 2000; Skirrow, 1982). Large waterborne outbreaks have occurred in municipal water system as a result of a break in
chlorination or when a non-chlorinated ground water supply becomes contaminated (Friedman et al., 2000). In addition, consumption of untreated natural water from lakes, rivers, streams, or even the sea that may be contaminated with feces from wild birds or waterfowl has also been described in several Campylobacter gastroenteritis cases (Butzler and Skirrow, 1979; Skirrow, 1982; Skirrow, 1990).

The transmission of Campylobacter spp. from food producing animals to humans usually occurs as indirect transmission via contaminated meats and meat products (Skirrow, 1982; Skirrow, 1990). Since food producing animals such as cattle, sheep, and pigs commonly carry Campylobacter spp. in their intestinal tracts, it is not unusual that carcasses of these animals will regularly become contaminated with Campylobacter spp. at slaughter (Skirrow, 1982; Skirrow, 1990). Fortunately, the process of carcass dressing particularly at air chilling step greatly helps reduce numbers of Campylobacter spp. on the carcass by means of surface drying (Skirrow, 1990). Thus, contamination rates of red meats at retail outlets are usually low as previously mentioned (Ono and Yamamoto, 1999; Skirrow, 1982; Skirrow, 1990; Zanetti et al., 1996; Zhao et al., 2001).

In general, people can become infected with Campylobacter spp. from contaminated meats by three different ways: (i) by handling raw meats and meat products, which is commonly reported in persons preparing the food or in inexperienced workers in processing plants; (ii) by consumption of raw or undercooked beef, hamburgers, and sausages; salted, smoked, or slightly cooked pork; as well as raw fish and shellfish; e.g. clams; (iii) by consumption of foods that are usually eaten raw or without further cooking such as salads and breads, which may become cross-
contaminated with raw meats in the kitchen (Skirrow, 1982; Skirrow, 1990). Since the infectious dose of *Campylobacter* species can be as low as a few hundred bacteria such as 500 microorganisms, the cross-contamination of other foods in the kitchen, which is probably one of the most important routes of food-borne campylobacteriosis in humans, should be concerned (Robinson and Jones, 1981; Skirrow, 1982; Skirrow, 1990). In addition, it has been reported that cooking methods also have an effect on the risk of *Campylobacter* infections in humans. Generally, the methods that require shorter cooking times such as fondue or barbecue seem to carry an increased risk of infections and seem to be more often associated with *Campylobacter* infections than the methods that require longer cooking times such as roasting, baking, or boiling (Skirrow, 1982; Skirrow, 1990).

Besides the foods mentioned earlier, non-meat products including mushrooms and other vegetables can be the sources of *Campylobacter* infections in humans as well. These products may be contaminated with *Campylobacter* spp. by several routes such as the application of fertilizers or contaminated surface waters (Allos and Taylor, 1998). Although there have been some reported outbreaks due to sources of raw foods such as lettuce or salad, it has been suggested that these foods were not the original source but rather the vehicle, which became contaminated during preparation (Jacobs-Reitsma, 2000). Unlike other food-borne pathogens including *Salmonella* and *Staphylococcus*, *Campylobacter* species do not multiply in foods. This may be the reason why outbreaks of food-borne campylobacteriosis do not occur frequently (Skirrow, 1990; Skirrow, 1997).
In addition to the indirect transmission, *Campylobacter* infections in humans can also be acquired through direct contact with infected animals (Skirrow, 1982). Direct transmission is mainly occupational and individuals who are regularly exposed to infected animals seem to develop immunity to *Campylobacter* species (Altekruse and Tollefson, 2003; Butzler, 2004; Skirrow, 1982; Skirrow, 1990). Veterinarians, farmers, abattoir workers, and those engaged in meat processing are likely to be at an increased risk of exposure to *Campylobacter* species (Altekruse and Tollefson, 2003; Butzler, 2004; Skirrow, 1982; Skirrow, 1990). In addition, contact with household pets, especially the ones with diarrhea or puppies and kittens as well as pet chickens has also been identified as risk factor for human campylobacteriosis, accounting for perhaps 5% of *Campylobacter* infections in humans (Altekruse et al., 1999; Altekruse and Tollefson, 2003; Butzler, 2004; Hopkins et al., 1984; Kapperud et al., 1992; Norkrans and Svedhem, 1982; Skirrow, 1981; Skirrow, 1982; Skirrow, 1990). Since *Campylobacter* spp. has been found in sand from bathing beaches, direct contact with this type of environment may be considered as a risk for *Campylobacter* infections in humans as well although it is probably infrequent. Shedding of *Campylobacter* spp. in the feces of wild animals, particularly wild birds seem to be an important source of such environmental contamination (Allos and Taylor, 1998).

Like other enteric pathogens, person-to-person transmission of *Campylobacter* spp. can occur although it is unlikely to happen (Altekruse et al., 1999). In addition, although transmission from infected food handlers who are symptomatic or asymptomatic is uncommon, an outbreak resulted from eating food contaminated with *Campylobacter*
spp. by a food handler may occur (Allos and Taylor, 1998). However, if person-to-person spread does occur, it is mainly observed among young children (Butzler, 2004; Butzler and Skirrow, 1979; Skirrow, 1991). The transmission of *Campylobacter* species from small children with diarrhea to their siblings or their parents taking care of them has been documented (Butzler and Skirrow, 1979). There have been several reports of vertical transmission of *Campylobacter* species from mothers who have *Campylobacter* enteritis to their neonates (Butzler and Skirrow, 1979). Perinatal transmission from a mother, who is not necessarily symptomatic, may occur following exposure *in utero*, during passage through the birth canal, or during the first days of life (Butzler, 2004).

### 1.3.2 Infection and Clinical Manifestation

Infection with *Campylobacter* spp. does not always produce clinical symptoms. Clinical manifestation due to *Campylobacter* infections can vary from asymptomatic to severe illnesses (Allos, 1998; Allos and Taylor, 1998; Blaser, 2000; Butzler and Skirrow, 1979). Although the factors responsible for this phenomenon are unclear, it appears that the numbers of *Campylobacter* spp. reaching the small intestinal tract and the host’s immunity seem to play important roles (Blaser, 2000). Some experimental studies have shown that ingestion of as low as 500 organisms can cause illness in some persons, while in other studies, higher numbers, e.g. $10^6$ organisms, are required to cause clinical symptoms. This information suggests that host susceptibility also dictates infectious dose to some degree (Allos and Taylor, 1998; Black et al., 1988; Blaser, 2000; Matthews, 1999). In general, a dose of less than $10^4$ organisms does not seem to cause illness very
frequently (Black et al., 1988; Blaser, 2000). Although the exact number of *Campylobacter* cells needed to cause the disease in humans is unknown, a high dose is probably required because *Campylobacter* spp. are susceptible and likely to be destroyed by acid in the stomach (Blaser, 2000). However, vehicles such as milk or fatty foods that favor passage through the gastric acid barrier may allow some infections to occur at relatively low doses (Blaser, 2000).

Infection with *Campylobacter* spp. particularly *C. jejuni* produces acute inflammatory enteritis affecting both small and large intestines (Allos, 1998). Generally, jejunum and ileum are the first sites to become colonized by *Campylobacter* organisms and then the infection extends distally to affect terminal ileum and usually colon and rectum (Blaser, 2000; Skirrow, 1997). The signs and symptoms of *Campylobacter* infections suggest an invasive mechanism of the disease. *Campylobacter* first penetrates and colonizes the intestinal mucous layer and then invades and/or translocates through the epithelial surface to the underlying tissue (Nachamkin, 1999). Penetration and colonization of the mucous lining of the intestinal tract is an important ability of *Campylobacter* to produce the disease. A polar flagellum at one or both ends in combination with a rapid motility gives *Campylobacter* a selective advantage in penetrating and colonizing the thick mucous layer covering the gut surface (Rowe and Madden, 2000). The pathologic lesion of *Campylobacter* enteritis is a mucosal invasion characterized by ulceration of the mucosal epithelium and destruction of epithelial glands with crypt abscess formation leading to degeneration, atrophy, and loss of mucus as well as inflammatory infiltration of the lamina propria with acute and chronic inflammatory
cells including neutrophils, eosinophils, and mononuclear cells (Allos, 1998; Allos and Taylor, 1998; Blaser, 2000; Perez-Perez and Blaser, 1996; Skirrow, 1997). These changes; however, are not pathognomonic lesions and are indistinguishable from those seen in ulcerative colitis, Crohn’s disease, or Salmonella, Shigella, or Yersinia infections (Allos, 1998; Allos and Taylor, 1998; Blaser, 2000; Hoeprich et al., 1994; Skirrow, 1997). In addition to an invasive mechanism, some Campylobacter strains can also produce enterotoxin and cytotoxin. A heat-labile enterotoxin produced by some Campylobacter strains is similar to the toxin produced by E. coli or cholera toxin, which is commonly detected from the patients with watery diarrhea. This enterotoxin causes a secretory diarrhea by stimulating adenylate cyclase activity in the intestinal mucosa and disrupting the normal ion transport in the enterocytes. Cytotoxin, verotoxin, or Shiga-like toxin is also produced by some strains of Campylobacter species. This toxin is similar to the toxins produced by Shigella species and E. coli O157:H7. The roles of these toxins in the pathogenesis of Campylobacter infections are unclear (Allos and Taylor, 1998; Blaser, 2000; Hoeprich et al., 1994; Perez-Perez and Blaser, 1996).

The incubation period of Campylobacter infections is commonly 2 – 5 days after ingestion of contaminated foods or water, but the range can extend from 1 - 7 days and perhaps up to 10 days (Blaser, 1997; Blaser, 2000; Butzler, 2004; Butzler and Skirrow, 1979; Matthews, 1999; Skirrow, 1990). In general, the length of the incubation period seems to be reversely related to the dose ingested (Allos and Taylor, 1998; Blaser, 2000). The typical symptom of Campylobacter infection is characterized by an acute diarrheal illness, which is indistinguishable from gastroenteritis caused by Salmonella, Shigella, or
other enteric bacterial pathogens (Allos, 1998; Allos, 2001; Allos and Taylor, 1998; Blaser, 1997; Skirrow, 1990). Symptoms may range from mild gastrointestinal distress lasting 24 hours to severe relapsing colitis lasting several weeks (Allos, 1998; Allos and Taylor, 1998; Blaser, 1997). Although *Campylobacter* enteritis may persist from 1 day to a few weeks, most patients recover from illness within a week without any antibiotic treatment (Allos, 1998; Skirrow, 1990).

Clinical manifestation of human campylobacteriosis usually starts with abdominal pain; however, in some patients, an influenza-like prodrome consisting of fever, headache, myalgia, malaise, and dizziness may happen (Allos, 1998; Allos and Taylor, 1998; Butzler and Skirrow, 1979). Although the predromal symptoms commonly occur 12 to 24 hours before the onset of intestinal symptoms, these symptoms may coincide with the intestinal phase or, less often, may follow it (Blaser, 2000). The most common symptoms of *Campylobacter* enteritis include diarrhea, abdominal pain, fever, and malaise (Blaser, 2000). Nausea is also frequently reported among the patients with *Campylobacter* infections, while vomiting is uncommon (Allos, 1998).

Diarrhea due to *Campylobacter* infections may range in severity from loose stools to massive watery or even bloody stools with 8 or more bowel movements within one day (Allos, 1998; Blaser, 1997; Blaser, 2000). This acute diarrhea usually lasts for 2 to 3 days; however, it may persist for 1 week or longer particularly in patients with an acute colitis (Blaser, 2000; Butzler and Skirrow, 1979). Occasionally, acute abdominal pain may be the major and only symptom of *Campylobacter* infections. Since the abdominal pain is usually cramping in nature and most often occurs at the right lower quadrant of
the abdomen, severe abdominal pain may mimic acute appendicitis (Allos, 1998; Allos and Taylor, 1998; Blaser, 1997; Blaser, 2000; Butzler, 2004). Other common symptoms of Campylobacter enteritis include tenesmus and high fever (40 °C) (Blaser, 2000). Like acute abdominal pain, high and persistent fever may be the sole manifestation of Campylobacter infections (Blaser, 2000).

In general, gastrointestinal complications due to Campylobacter infections are rarely occur. However, Campylobacter spp. may infect the biliary tract and lead to cholecystitis, pancreatitis, or obstructive hepatitis (Allos, 1998; Allos and Taylor, 1998; Blaser, 2000; Butzler, 2004). Massive gastrointestinal hemorrhage and toxic megacolon may also occur in some severe cases (Allos, 1998; Blaser, 2000). In patients undergoing peritoneal dialysis, Campylobacter infections may cause peritonitis although it rarely occurs (Allos, 1998; Allos and Taylor, 1998; Butzler, 2004). Unlike gastrointestinal complications that seem to be the result of local invasion, extraintestinal complications such as bacteremia, meningitis, endocarditis, septic arthritis, and osteomyelitis are usually the result of systemic spread (Allos, 1998; Allos, 2001; Blaser, 2000; Butzler, 2004). Although bacteremia has been noted in less than 1% of patients with Campylobacter infections, it seems to be more common in immunocompromised or very young or very old persons (Allos, 1998; Allos, 2001; Blaser, 2000; Butzler, 2004). In addition, immunodeficient persons also often develop prolonged, severe, and recurrent Campylobacter infections, especially with bacteremia and other extraintestinal manifestations (Blaser, 2000). In terms of Campylobacter infections in pregnant women,
although the symptoms are likely to be mild and self-limited, neonatal sepsis and death can occur particularly if the woman is infected during her trimester (Allos, 1998; Allos and Taylor, 1998).

The most important postinfectious complication of *C. jejuni* infection is Guillane-Barré Syndrome (GBS), an acute immune-mediated demyelinating disorder of the peripheral nervous system (Allos, 1998; Allos, 2001; Allos and Taylor, 1998; Blaser, 1997; Butzler, 2004; Nachamkin et al., 1998; Nachamkin, 1999; Wassenaar and Blaser, 1999). Generally, about 20 – 40% of GBS patients are infected with *C. jejuni* (Allos and Taylor, 1998). Symptoms of GBS usually occur 1 - 3 weeks after the onset of *Campylobacter* enteritis (Allos, 1998; Allos and Taylor, 1998; Butzler, 2004). Although *C. jejuni* infection seems to be a common trigger of GBS, the risk of developing GBS after *C. jejuni* infection is actually quite small (less than 1 case of GBS per 1000 cases of *C. jejuni* infection) (Allos, 1997; Allos, 1998; Allos, 2001; Butzler, 2004; Nachamkin, 2002; Nachamkin et al., 2000a). In addition, the development of GBS after *C. jejuni* infections does not appear to be related to the severity of gastrointestinal symptoms. In fact, GBS can follow asymptomatic *C. jejuni* infections (Allos, 1998; Allos, 2001; Allos and Taylor, 1998). Although different *C. jejuni* strains may involve with GBS, 30 – 80% of *C. jejuni* isolates from GBS patients belong to Penner serotype O:19 (Allos, 1998; Allos, 2001; Allos and Taylor, 1998; Nachamkin, 1999). Early symptoms of GBS generally include burning sensation and numbness that can progress to flaccid paralysis (Allos, 1997; Nachamkin et al., 1998; Nachamkin, 2002; Nachamkin et al., 2000a). Interestingly, GBS that occurs after *C. jejuni* infection is usually more severe, associated
with an irreversible neurological damage, and may require intensive treatment with possible long-term disability (Allos, 2001; Butzler, 2004). Because the neurological symptoms of GBS that follow *C. jejuni* infection typically occur 1 – 3 weeks after the onset of diarrheal illness, humoral immunopathogenic mechanisms seem to play important roles in the pathogenesis of *Campylobacter*-induced GBS (Allos, 1998; Allos, 2001; Butzler, 2004; Nachamkin, 2002; Nachamkin et al., 2000a). It is possible that antibodies and/or T-cells are induced by the infection and are initially directed against *Campylobacter*, leading to eradication of the organism. However, because peripheral nerve glycolipids or myelin proteins (self-antigens) are similar to structures on the lipopolysaccharides of some *Campylobacter* strains (microbial antigens), the self-antigens; e.g. peripheral nerve cells or tissues, may be destroyed by antibodies and/or T-cells as well, leading to GBS (Allos, 1998; Allos, 2001; Butzler, 2004; Nachamkin, 2002; Nachamkin et al., 2000a). More details on Guillane-Barré Syndrome and *Campylobacter* infections have been reviewed by Allos (1997), Nachamkin et al. (1998), and Nachamkin et al. (2000a).

In addition to GBS, *C. jejuni* infection has been implicated as a potential cause of acute motor axon neuropathy (AMAN), which is clinically indistinguishable from GBS (Allos and Taylor, 1998) and Miller Fisher syndrome (MFS), which is another related neurological syndrome that can follow *C. jejuni* infection (Endtz et al., 2000; Takahashi et al., 2005). MFS is characterized by acute onset of ophthalmoplegia, ataxia, and areflexia. This complication is considered a variant of GBS because some patients who have MFS can progress to GBS (Endtz et al., 2000; Takahashi et al., 2005). Another
postinfectious complication of *C. jejuni* infection is reactive arthritis, which may occur up to several weeks after *Campylobacter* infections. Reactive arthritis most commonly affects large weight-bearing joints such as the knees and the lower back. Like GBS, reactive arthritis can follow symptomatic or asymptomatic *C. jejuni* infection and both are thought to be autoimmune responses triggered by the infection. However, reactive arthritis can occur after *Salmonella*, *Shigella*, and *Yersinia* infections, while GBS is associated only with *Campylobacter* infections (Allos, 1998; Allos, 2001; Blaser, 2000). When reactive arthritis occurs as a part of a triad of symptoms with inflammation of the urethra and conjunctiva, it is referred to as Reiter’s syndrome (Altekruse et al., 1998).

Other reported but rare postinfectious complications of *Campylobacter* infections include uveitis, hemolytic anemia, hemolytic uremic syndrome, IgA nephropathy, interstitial nephritis, hepatitis, and encephalopathy (Allos, 1998; Allos, 2001; Allos and Taylor, 1998; Blaser, 2000).

The clinical manifestations of infections due to other *Campylobacter* species overlap substantially with those of *C. jejuni* infections. In general, *C. coli* infections seem to produce more mild diseases than *C. jejuni* infections (Blaser, 2000; Nachamkin, 1999). Although *C. fetus* can cause intermittent diarrhea with or without nonspecific abdominal pain, it is primarily associated with bacteremia and extraintestinal infections, especially in patients with underlying diseases such as acquired immunodeficiency syndrome (AIDS) (Blaser, 2000; Nachamkin, 1999). Clinical aspects of *Campylobacter* infections by *C. jejuni* and other *Campylobacter* species have been summarized by Skirrow and Blaser (2000) and Lastovica and Skirrow (2000).
In general, specific humoral antibodies IgG, IgM, and IgA and specific intestinal antibody IgA are usually developed after *Campylobacter* infections (Allos, 1998; Black et al., 1988; Black et al., 1992; Blaser, 2000; Perez-Perez and Blaser, 1996; Skirrow, 1997). In addition, serum antibodies developed in response to infection with one *Campylobacter* strain are also cross-reactive with other *Campylobacter* strains (Allos and Taylor, 1998). Although the specific humoral antibodies particularly IgA and IgM antibodies commonly appear within 10 days and peak within 2 to 4 weeks after the infection, they decline rapidly (Allos, 1998; Skirrow, 1997). Immunoglobulin G (IgG), on the other hand, may not peak until 3 – 4 weeks after the onset of illness; however, it can persist for several weeks or months (Blaser, 2000; Skirrow, 1997). It has been reported that repeated infections may stimulate specific IgA production, but these seem to have little effect on systemic IgG production (Allos and Taylor, 1998). In addition, high exposure to *Campylobacter* can also lead to the development of solid gut immunity (Allos and Blaser, 1999). Currently, it is not known whether the antibody response helps eliminate the infection or helps protect against reinfection (Perez-Perez and Blaser, 1996). However, it has been shown that persons with elevated serum and intestinal antibody levels were likely to develop asymptomatic infection with a brief duration of pathogen excretion after they were challenged with *C. jejuni* (Allos and Taylor, 1998; Black et al., 1988; Nachamkin, 1997; Perez-Perez and Blaser, 1996). Likewise, persons who regularly drink raw milk seem to have specific antibody against *Campylobacter* species and seem to be protected from illness when exposed to contaminated raw milk that led to illness in other persons (Allos and Taylor, 1998; Jones et al., 1981; Blaser et
al., 1987; Nachamkin, 1997; Perez-Perez and Blaser, 1996; Skirrow, 1982). Based on the above information, it looks like specific serum and intestinal antibodies can help protect against illness but not necessarily against *Campylobacter* infection colonization (Black et al., 1988; Nachamkin, 1997). Although the role of the cellular immune response in the control of *Campylobacter* infections is unclear, the increased incidence, severity, and duration of *Campylobacter* infections in HIV-infected persons suggest that cell-mediated immunity seem to play an important role as well (Allos, 1998; Allos and Taylor, 1998; Allos and Blaser, 1999; Blaser, 2000).

1.3.3 Treatment and Control and Prevention

**Treatment**

The most important intervention in treatment of *Campylobacter* infections or any other diarrheal illnesses is maintenance of proper rehydration and electrolyte balance, which in most cases can be accomplished by encouraging oral intake of water and other fluids such as glucose and electrolyte solutions (Allos, 1998; Allos, 2001; Blaser, 2000; Butzler, 2004; Hoeprich et al., 1994; Perez-Perez and Blaser, 1996). Although oral rehydration solutions are the best method of maintaining fluid and electrolyte balance, intravenous fluids may be needed especially in the persons who are severely dehydrated or in the very young and very old persons (Allos, 1998; Allos and Blaser, 1999; Blaser, 2000). Since most clinical symptoms of *Campylobacter* infections are mild, self-limiting, and usually resolve within a few days without any antimicrobial therapy, treatment with antibiotics seems to be unnecessary (Allos, 2001; Allos and Blaser, 1999; Blaser, 1997;
Butzler, 2004). However, in some certain circumstances such as in severe infections (persistent high fever, bloody diarrhea, or more than eight bowel movements within a day), prolonged illness (symptoms last longer than 1 week), or systemic infections particularly in infants, elderly, or immunocompromised persons, antimicrobial treatment is usually warranted (Allos, 1998; Allos, 2001; Allos and Blaser, 1999; Blaser, 1997; Blaser, 2000; Butzler, 2004). If possible, antibiotic treatment should be initiated on the first day of illness. Several studies have shown shortened duration of symptoms and Campylobacter excretion in patients who received antibiotics at the onset of their illness, while other studies in which initiation of treatment was delayed for several days after the onset of symptoms did not show a therapeutic effect (Allos, 1998; Blaser, 2000).

In vitro, Campylobacter spp. particularly C. jejuni are susceptible to a wide variety of antimicrobial agents including macrolides (e.g. erythromycin), fluoroquinolones (e.g. ciprofloxacin), aminoglycosides (e.g. gentamicin), and chloramphenicol, clindamycin and imipenem. In contrast, they are generally resistant to cephalosporins (e.g. cephalothin), penicillin, vancomycin, bacitracin, lincomycin, and trimethoprim. Although a majority of Campylobacter isolates are susceptible to tetracycline, resistance to this antibiotic has been reported. For ampicillin and trimethoprim-sulfamethoxazole, susceptibility to these antimicrobials is variable (Allos, 1998; Allos, 2001; Allos and Blaser, 1999; Allos and Taylor, 1998; Blaser, 2000; Butzler and Skirrow, 1979; Nachamkin, 1999; Skirrow, 1997). When antimicrobial therapy is indicated, erythromycin is commonly recognized as the drug of choice for treatment of Campylobacter infections because of its efficacy, safety (low toxicity), ease of
administration, low cost, and relatively narrow spectrum of activity (less inhibitory effect
on fecal flora) (Allos, 1998; Allos, 2001; Allos and Blaser, 1999; Allos and Taylor, 1998;
Blaser, 1997; Blaser, 2000; Butzler, 2004; Butzler and Skirrow, 1979; Nachamkin, 1999).
Unlike other antibiotics such as fluoroquinolones or tetracyclines, erythromycin can be
used safely with children and pregnant women and the resistance rate of *Campylobacter*
spp. particularly *C. jejuni* to this antibiotic is still low when compared to other antibiotics
(Allos, 2001; Allos and Blaser, 1999; Nachamkin, 1999). In addition to its systemic
effects, erythromycin seems to have a local or a contact effect throughout the bowel as
well because some forms of erythromycin; e.g. erythromycin stearate, is quite stable and
incompletely absorbed (Allos, 2001; Allos and Blaser, 1999). The recommended dosage
of erythromycin for adults is 250 mg administered orally 4 times per day or 500 mg
administered orally twice a day for 5 to 7 days. For children, the recommended dosage is
30 – 50 mg/kg/day in divided doses for 5 days (Allos, 2001; Blaser, 2000; Butzler and
Skirrow, 1979). The new macrolides such as azithromycin and clarithromycin are also
effective in treatment of *Campylobacter* infections; however, they are more expensive
and provide little or no clinical advantage over erythromycin (Allos, 1998; Allos, 2001;
Allos and Blaser, 1999). Interestingly, *Campylobacter* strains that are resistant to
erthyromycin seem to be resistant to these new extended-spectrum macrolides as well
(Allos and Blaser, 1999).

In addition to erythromycin, fluoroquinolones such as ciprofloxacin is considered
the treatment of choice for *Campylobacter* enteritis as well (Allos, 1998; Allos, 2001;
Allos and Blaser, 1999; Allos and Taylor, 1998; Blaser, 1997, Blaser, 2000; Butzler,
2004; Hoeprich et al., 1994; Nachamkin, 1999). Unfortunately, the emergence of fluoroquinolones resistance that has been increased drastically over the last decade in many parts of the world has limited their effectiveness (Allos, 1998; Allos, 2001; Allos and Blaser, 1999; Allos and Taylor, 1998; Blaser, 2000; Butzler, 2004; Nachamkin, 1999). In addition, although the development of fluoroquinolone particularly ciprofloxacin resistance during therapy has been reported, fluoroquinolones have retained the advantage of being effective against a wide range of enteric pathogens (Allos, 1998; Hoeprich et al., 1994; Nachamkin, 1999). Because symptoms of *Campylobacter* enteritis are clinically indistinguishable from those of gastroenteritis caused by other enteric bacteria, fluoroquinolones may still be considered the drug of choice especially when bacterial gastroenteritis is suspected and a cause of diarrheal illness has not yet been identified (Allos, 1998; Allos, 2001; Blaser, 2000). The recommended dosage of ciprofloxacin is 500 mg administered orally twice daily for 5 to 7 days (Blaser, 2000; Hoeprich et al., 1994). Although new fluoroquinolones exhibit considerable activity against *Campylobacter* species, it is possible that these agents might not be effective against *Campylobacter* strains already resistant to ciprofloxacin (Allos and Blaser, 1999).

Other antimicrobial agents that can be used as alternative agents for treatment of *Campylobacter* infections include tetracycline, clindamycin, chloramphenicol, amoxicillin plus clavulanic acid, third-generation or fourth-generation cephalosporins (e.g. cefotaxime), imipenem, and gentamicin (Allos, 1998; Allos, 2001; Allos and Blaser, 1999; Allos and Taylor, 1998; Blaser, 1997; Blaser, 2000; Butzler and Skirrow, 1979). Since small to moderate numbers of *Campylobacter* isolates are resistant to tetracycline,
care should be taken before prescribing this antibiotic. In addition, because tetracycline is contraindicated in children under 9 years of age, clindamycin may be used in such patients (Allos and Blaser, 1999; Blaser, 2000; Butzler and Skirrow, 1979). It has been reported that the activity of clindamycin against *C. jejuni* is not different from that of erythromycin (Allos, 2001; Allos and Blaser, 1999). In special circumstances, as when *Campylobacter* strain has an unusual antimicrobial resistance pattern or when antibiotics need to be prescribed for a patient who is intolerant of many classes of antimicrobial agents, alternative agents such as chloramphenicol should be used; nearly all *Campylobacter* strains are susceptible to this antibiotic (Allos, 2001; Allos and Blaser, 1999; Butzler and Skirrow, 1979). Although *C. jejuni* and *C. coli* may produce enzyme β-lactamase causing penicillin and cephalosporin resistance in these *Campylobacter* isolates, this enzyme has been reported to be inhibited by clavulanic acid but not by sulbactam or tazobactam. This is why amoxicillin plus clavulanic acid (but not sulbactam or tazobactam) appears to be generally effective against *Campylobacter* infections (Blaser, 2000;Nachamkin, 1999). For bacteremia and other extraintestinal infections, gentamicin and imipenem are the most commonly prescribed antibiotics in these circumstances because less than 1% of *Campylobacter* isolates are resistant to these antimicrobial agents (Allos, 1998; Allos and Blaser, 1999; Allos and Taylor, 1998; Blaser, 2000; Butzler and Skirrow, 1979). However, because gentamicin is ineffective against *Campylobacter* infections in the gut, oral therapy with an effective and absorbable drug should be given as well (Allos, 1998; Allos and Blaser, 1999; Allos and
Taylor, 1998). Other alternative agents such as cefotaxime or chloramphenicol also have been indicated for treatment of bacteremia or other extraintestinal infections by *Campylobacter* species (Blaser, 2000).

Systemic *C. fetus* infections or other severe *Campylobacter* infections should be treated parenterally with erythromycin, gentamicin or other aminoglycosides, and chloramphenicol, depending upon the type of infection. In addition, ampicillin and third-generation cephalosporins may be listed as alternative agents as well (Blaser, 2000; Butzler and Skirrow, 1979; Nachamkin, 1999). Unlike in *Salmonella* infections, treatment with antibiotics does not prolong carriage of *Campylobacter* spp.; in contrast, it helps eliminate carriage in most patients. For example, treatment with erythromycin can eliminate carriage of *C. jejuni* within 72 hours (Blaser, 2000). Anti-intestinal motility agents should not be used for *Campylobacter* infections because they do not only prolong duration of symptoms, but they also have been associated with fatalities (Blaser, 2000; Smith and Blaser, 1985).

**Control and Prevention**

Control and prevention of *Campylobacter* infections in humans depend mainly on interruption of transmission of *Campylobacter* from risk factor sources such as farm and domestic animals, foods of animal origin, or contaminated environment to humans (Allos and Taylor, 1998; Blaser, 1986). As mentioned earlier, the major risk factor of *Campylobacter* infections in humans is consumption of undercooked poultry and/or other
foods that are cross-contaminated with raw poultry meat during food preparation. Therefore, careful food preparation habits in the kitchen seem to be one of the most important actions that will help reduce the risk of *Campylobacter* infections in humans (Allos, 1998; Allos, 2001). In addition, basic hygiene concepts also should be applied in the kitchen and food preparation area (Skirrow, 1982). Raw animal meats and animal products should be kept separately from other foods to avoid cross-contamination of cooked or ready-to-eat foods. All foods derived from animal sources particularly poultry should be thoroughly cooked (Allos, 1998; Allos, 2001; Skirrow, 1982). Since *Campylobacter* spp. are sensitive to heat (the decimal reduction time for *Campylobacter* spp. at 55 °C is about one minute), the organisms should not survive in food products brought to adequate cooking temperature. Thorough cooking of raw chicken and other meats; therefore, provides protection against food-borne campylobacteriosis (Doyle, 1990; Nachamkin, 1997). It is recommended that poultry should be heated to an internal temperature of 82 °C (180 °F) to kill *Campylobacter* organisms (Altekruse and Tollefson, 2003). So, it seems to be a good idea to use a meat thermometer when cooking meats to ensure that temperature is adequate to kill these organisms (Allos, 1998; Allos, 2001; Altekruse and Tollefson, 2003). Prevention of cross-contamination with raw poultry and other meats will help reduce the risk of *Campylobacter* infections as well. Cutting boards and other cooking utensils that are used in handling uncooked poultry or other meats should be washed with hot, soapy water before being used for preparation of salads or other foods that will be served without subsequent cooking (Allos, 1998; Allos, 2001).
Likewise, hands should be washed and food contact surfaces in the kitchen should be cleaned and disinfected every time after contact with raw meats and poultry (Altekruse and Tollefson, 2003).

In addition to proper handling and cooking of foods, reduction of *Campylobacter* contamination on the carcasses is also another important step that can help reduce the risk of food-borne campylobacteriosis (Altekruse and Tollefson, 2003). It has been reported that the microbial quality of broiler carcasses has been associated with the abattoir where the carcasses were processed (Altekruse and Tollefson, 2003; Wedderkopp et al., 2000). In general, the numbers of *Campylobacter* organisms on poultry carcasses seem to increase during defeathering and evisceration processes, but then decrease during scalding, washing, and chilling processes (Acuff et al., 1986; Altekruse et al., 1998; Berrang et al., 2000a; Berrang et al., 2000b; Berrang et al., 2001a; Berrang et al., 2001b; Whyte et al., 2001). Treatment of wash water with active chlorine, organic acids, sodium chloride, and tri-sodium phosphate seems to be an important control step that can help reduce carcass contamination by *Campylobacter* species (Altekruse et al., 1999; Altekruse and Tollefson, 2003). In one study, the use of electrolyzed water for washing poultry carcasses resulted in a 3 log₁₀ reduction of *C. jejuni* on chicken carcasses (Altekruse and Tollefson, 2003; Park et al., 2002). Another study also revealed that 10% oleic acid significantly reduced the numbers of *Campylobacter* on poultry skin (Altekruse and Tollefson, 2003; Hinton and Ingram, 2000). Since *Campylobacter* spp. are sensitive to active chlorine, the chlorination of carcass wash water definitely helps reduce the risk
of carcass contamination by *Campylobacter* species (Altekruse and Tollefson, 2003; Yang et al., 2001). In one processing plant, use of chlorinated sprays on equipment and working surfaces in combination with maintenance of clean working surfaces and reduction of carcass contact surfaces resulted in significant reduction of *Campylobacter* counts on packaged poultry (Altekruse et al., 1998; Altekruse et al., 1999; Mead et al., 1995). In addition, treatment of poultry chiller water containing sodium chloride or trisodium phosphate with an electrical current can reduce *C. jejuni* contamination on poultry carcasses as well (Altekruse et al., 1998; Altekruse et al., 1999; Li et al., 1995). Because *Campylobacter* species are sensitive to freezing at temperatures between – 20 °C to – 5 °C, it is not surprising that freezing poultry carcasses to – 20 °C would reduce *Campylobacter* counts by 2 log₁₀ (Altekruse and Tollefson, 2003; Doyle, 1990; Stern et al., 1985). Recent studies suggest that *Campylobacter* species are more radiation sensitive than other food-borne pathogens such as *Salmonella* or *Listeria monocytogenes* and the irradiation treatment that is effective against the latter organisms should be sufficient to kill *Campylobacter* spp. as well (Nachamkin, 1997). Currently, gamma irradiation and electron beam irradiation have been successfully used for elimination of *Campylobacter* spp. from poultry products; however, consumer acceptance rate for control of *Campylobacter* contamination on poultry carcasses and products by the irradiation method seems to be limited (Altekruse et al., 1998; Altekruse and Tollefson, 2003; Lewis et al., 2002; Nachamkin, 1997; Patterson, 1995; Skirrow, 1997). Since *Campylobacter* spp., particularly *C. jejuni* and *C. coli* are widespread in the intestinal tract of poultry and other food-producing animals, the reduction or elimination of colonization of poultry or
other food animals with *Campylobacter* spp. on the farm will definitely help reduce the risk of carcass contamination by *Campylobacter* spp. at the processing plant and in the kitchen, which unquestionably benefits consumers. Information on control and prevention of *Campylobacter* spp. at the farm level is discussed under the “*Campylobacter* spp. in poultry” section.

Because several outbreaks and sporadic cases of *Campylobacter* infections are caused by consumption of contaminated raw or unpasteurized milk and dairy products as well as untreated surface water, prevention of these outbreaks and sporadic cases can be easily accomplished by consuming only pasteurized milk and dairy products and treated water (Allos, 1998; Allos, 2001; Allos and Taylor, 1998; Blaser, 1986; Hoeprich et al., 1994). In addition, avoiding consumption of unpasteurized milk and dairy products and untreated water also should be emphasized to pregnant women, the elderly, immunocompromised persons, or other persons in whom *Campylobacter* infections may have serious consequences (Allos, 1998; Allos, 2001). As mentioned earlier, *Campylobacter* spp. are sensitive to heat and chlorine. Hence, they should be effectively destroyed by pasteurization and chlorination of milk and water, respectively (Doyle, 1990; Jay, 2000; Nachamkin, 1997; Skirrow, 1982). Because overflow of waste from farms and processing plants, entering rivers, has the potential to contaminate public water supplies, special care should also be taken to prevent contamination of community drinking water supplies from those sources as well as from fecal contamination by wild birds, domestic animals, or sewage effluent (Allos and Taylor, 1998; Jacobs-Reitsma, 2000; Skirrow, 1982). The safe disposal of sewage and the protection and purification of
water supplies are fundamental to control of most diseases due to enteric pathogens including *Campylobacter* spp. (Allos and Blaser, 1999). Although seafood such as shellfish may not be considered as an important source of *Campylobacter* infections in humans, avoiding consumption of raw or uncooked shellfish should help reduce the risk of food-borne campylobacteriosis at least in immunocompromised persons (Jacobs-Reitsma, 2000).

Since direct contact with animals especially household pets such as puppies and kittens as well as diarrheic animals has been identified as the risk factor for human campylobacteriosis, people who handle pets or other animals should wash their hands before eating or involving with any food activities (Allos, 1998; Allos, 2001; Altekruse and Tollefson, 2003). Young children in particular should be encouraged to wash their hands every time after playing with pets particularly before eating. Hand-washing after contact with animals is a prudent step in prevention of zoonotic transmission of *Campylobacter* spp. in household and occupational settings (Altekruse and Tollefson, 2003).

Although person-to-person transmission of *Campylobacter* infections is uncommon and has not been demonstrated, persons with any acute diarrheal illness should avoid preparation and handling of foods until their illness resolves (Allos, 1998; Allos, 2001; Hoeprich et al., 1994). As part of good general hygiene, all persons should wash their hands after using the bathroom, especially if they have diarrhea (Allos, 1998; Allos, 2001). Currently, there is no proven benefit of antibiotic prophylaxis to prevent *Campylobacter* infections in travelers, particularly the ones who travel to tropical
countries and in fact the routine use of antibiotic prophylaxis to prevent *Campylobacter* infections is not recommended. One thing that travelers can do to reduce the risk of *Campylobacter* infections is that they should be cautioned against drinking untreated water (Allos, 1998; Allos, 2001; Allos and Blaser, 1999). In addition, since there is no vaccine for prevention of *Campylobacter* infections at this stage, non-specific methods for prevention as mentioned above are very important (Allos and Blaser, 1999).

### 1.4 *Campylobacter* spp. in Poultry

#### 1.4.1 Prevalence in Poultry and Other Animals

Commercial poultry such as broilers, layers, turkeys, and ducks as well as free-living birds are considered to be natural reservoirs of thermophilic *Campylobacter* (Shane, 1992; Shane, 2000). However, the prevalence of *Campylobacter* spp. in different species of the birds seems to be different and this prevalence also varies among countries (Newell and Fearnley, 2003). In the United States, the prevalence of *Campylobacter* spp. in commercial broilers ranged from 20% to nearly 90% (Aho and Hirn, 1988; Harris et al., 1986a; Hiett et al., 2002b; Jones et al., 1991; Luangtongkum et al., 2005; Munroe et al., 1983; Saleha et al., 1998; Shane, 2000; Stern et al., 2001b), while the prevalence of this organism in other countries in North America such as Canada and in South America was around 45% to 48% and 20% to 96%, respectively (Aho and Hirn, 1988; Newell and Fearnley, 2003; Shane, 2000). In Europe, the proportion of commercial broiler flocks colonized with *Campylobacter* spp. varied from 2.9% to more than 92% (Aho and Hirn, 1988; Atanassova and Ring, 1999; Berndtson et al., 1996a; Bouwknegt et al., 2004;
Engvall et al., 1986; Evans and Sayers, 2000; Hald et al., 2000; Heuer et al., 2001; Humphrey et al., 1993; Jacobs-Reitsma et al., 1994a; Jacobs-Reitsma et al., 1995; Kapperud et al., 1993; Newell and Fearnley, 2003; Pearson et al., 1996; Perko-Makela et al., 2002; Refregier-Petton et al., 2001; Saleha et al., 1998; Shane, 2000; Stern et al., 2005; Van de Giessen et al., 1996; Wedderkopp et al., 2000; Wedderkopp et al., 2001) with the lowest flock prevalence (2.9%) observed in Finland (Perko-Makela et al., 2002). Likewise, the variation in the prevalence of thermophilic *Campylobacter* among commercial broiler flocks was also observed in other regions of the world ranging from 13.6% to 87% in Africa (Adekeye et al., 1989; Aho and Hirn, 1988; Cardinale et al., 2004; Kazwala et al., 1993; Sackey et al., 2001), 24% to 54% in Asia (Newell and Fearnley, 2003), and about 42% in Australia (Saleha et al., 1998; Shanker et al., 1982).

In addition to the prevalence of thermophilic *Campylobacter* in commercial broiler flocks, the prevalence of *Campylobacter* spp. on broiler chicken carcasses also varies among studies conducted in different countries. Several studies in the United States revealed that 22% to 98% of retail broiler chickens sold in the U.S. were contaminated with *Campylobacter* spp. (Baker et al., 1987; Berrang et al., 2001b; Harris et al., 1986a; Saleha et al., 1998; Shane, 2000; Smith et al., 1999; Willis and Murray, 1997; Zhao et al., 2001), while 38% of broiler carcasses sold in Canada and 79% to 84% of retail broiler chickens sold in South America were contaminated with this organism (Aho and Hirn, 1988; Shane, 2000). Likewise, the prevalence of *Campylobacter* spp. on broiler chicken carcasses sold in different European countries also varied from 14% to 88% with the lowest rates (14%) in Norway and the highest rates (88%) in the United
Kingdom (Aho and Hirn, 1988; Atanassova and Ring, 1999; Jorgensen et al., 2002; Shane, 2000; Wilson, 2002; Zanetti et al., 1996). In Asia, the prevalence of *Campylobacter* spp. on retail chicken meats sold in Japan, Malaysia, India, and Taiwan ranged from 46% to 100% (Ono and Yamamoto, 1999; Padungton and Kaneene, 2003; Saleha et al., 1998; Shane, 2000; Shih, 2000), whereas 45% and 77% of broiler chicken carcasses sold in Australia and Africa were contaminated with *Campylobacter* spp., respectively (Padungton and Kaneene, 2003; Shanker et al., 1982).

A high prevalence of *Campylobacter* spp. is observed not only in broilers, but it is also observed in other poultry species. Generally, the prevalence of thermophilic *Campylobacter* in commercial turkeys is quite high ranging from 50% to 100% (Acuff et al., 1986; Luangtongkum et al., 2005; Luechtfeld and Wang, 1981; Shane, 2000; Smith et al., 2004; Wallace et al., 1998; Wempe et al., 1983). In addition, the prevalence of this organism on turkey carcasses was also reported in a wide range from 14% to 94% (Acuff et al., 1986; Logue et al., 2003; Luechtfeld and Wang, 1981; Wallace et al., 1998; Zanetti et al., 1996; Zhao et al., 2001). Likewise, commercial layers are also frequently infected with thermophilic *Campylobacter* (Shane, 1992). The prevalence rates of commercial layer flocks colonized by *Campylobacter* spp. ranged from 13% to 62% (Doyle, 1984; Shane, 1992; Shane et al., 1986). A high prevalence of thermophilic *Campylobacter* is also observed in commercial ducks and geese (Aydin et al., 2001; Kasrazadeh and Genigeorgis, 1987; Tsai and Hsiang, 2004). *Campylobacter* spp. were isolated from 43.5% and 92% of ducks and duck farms, respectively (Tsai and Hsiang, 2004), whereas 100% of geese carried thermophilic *Campylobacter* in their intestinal
tracts (Aydin et al., 2001). For commercial pheasant, guinea fowl, and squab (young pigeon) farms, the isolation rates of \textit{Campylobacter} organisms from these pheasants, guinea fowl, and squabs were 25.9\%, 35.7\%, and 3.9\%, respectively (Adekeye et al., 1989; Atanassova and Ring, 1999; Jeffrey et al., 2001). Moreover, Adekeye et al. (1989) also reported that 66.7\% of guinea fowl flocks were positive for \textit{C. jejuni} (Adekeye et al., 1989). In addition, thermophilic \textit{Campylobacter} can be isolated from quails (Minakshi and Ayyagari, 1988; Shane, 1992) and ostriches (Ley et al., 2001; Stephens et al., 1998) as well.

As mentioned earlier, a wide range of free-living and migrating birds and waterfowl can serve as natural reservoirs of thermophilic \textit{Campylobacter} (Shane, 1992; Shane, 2000). Although \textit{Campylobacter} spp. can be isolated from free-living birds including sparrows, pigeons, crows, blue magpies, gray starlings, blackbirds, bulbuls, eastern turtledoves, owl goldeneye, and reed bunting, the highest prevalence of \textit{Campylobacter} species was found in crows, which 34\% to 89.8\% of the birds were \textit{Campylobacter} positive, followed by blue magpies (20\%), gray starlings (14\%), bulbuls (11\%), and eastern turtledoves (2\%) (Chuma et al., 2000; Fukuyama et al., 1986; Ito et al., 1988; Kapperud and Rosef, 1983; Kinjo et al., 1983; Luechtelfeld et al., 1981; Shane, 1992; Yogasundram et al., 1989). Likewise, the prevalence of these \textit{Campylobacter} organisms in pigeons has also been reported ranging from 4.2\% to 26\% (Ito et al., 1988; Kapperud and Rosef, 1983; Kinjo et al., 1983; Luechtelfeld et al., 1981; Shane, 1992). In addition to the avian species mentioned above, other wild birds as well as free-living and migratory waterfowl including passerines, Canada geese, mallards, shoveler, pintails,
American widgeons, green-winged teals, gadwalls, common gulls or seagulls, black-headed gulls, herring gulls, puffin, dunlins, sandpipers, and common terns also harbor thermophilic *Campylobacter* (Broman et al., 2002; Broman et al., 2004; Craven et al., 2000; Fallacara et al., 2001; Kapperud and Rosef, 1983; Kapperud et al., 1983; Kaneuchi et al., 1987; Luechtefeld et al., 1980; Shane, 1992; Waldenström et al., 2002; Yogasundram et al., 1989). The prevalence of *Campylobacter* spp. in Canada geese was about 52%, while the prevalence of this organism in mallards ranged between 34% and 40% (Fallacara et al., 2001; Luechtefeld et al., 1980). For other migratory waterfowl, the *Campylobacter* isolation rates were as the followings: 66% in shovelers, 50% in pintails, 42% in American widgeons, 16% in green-winged teals, and 15% in gadwalls (Luechtefeld et al., 1980). Similarly, a high frequency of *Campylobacter* spp. was also observed in shorebirds particularly in puffins. *Campylobacter* spp. were isolated from 51% to 78% of puffins, whereas only 5.6% of common terns were positive for *Campylobacter* species (Kapperud and Rosef, 1983; Kapperud et al., 1983). Among various species of gulls, the prevalence of thermophilic *Campylobacter* varied from 4.2% in herring gulls to 18.9% in common gulls and to 13.2% - 41.4% in black-headed gulls (Broman et al., 2002; Kapperud and Rosef, 1983; Kaneuchi et al., 1987).

In terms of the prevalence of thermophilic *Campylobacter* in free-range and organic poultry production systems, several studies revealed a high frequency of *Campylobacter* isolation from these free-range and organic broilers and turkeys (Avrain et al., 2003; El-Shibiny et al., 2005; Heuer et al., 2001; Kazwala et al., 1993; Luangtongkum et al., 2005; Saleha et al., 1998). In general, the prevalence of
Campylobacter spp. in free-range and organic broilers ranged from 68.5% to 100% (Avrain et al., 2003; El-Shibiny et al., 2005; Heuer et al., 2001; Kazwala et al., 1993; Luangtongkum et al., 2005; Saleha et al., 1998), while about 87% of organic turkeys were reported to be colonized by thermophilic Campylobacter (Luangtongkum et al., 2005).

Among Campylobacter-positive flocks, C. jejuni was the predominant species in poultry especially in commercial broilers. Many studies on the prevalence of Campylobacter spp. in commercial broilers have shown that 85% to 98% of commercial broiler flocks were colonized by C. jejuni, while about 2% - 11% and 1% - 5% were colonized by C. coli and C. lari, respectively (Avrain et al., 2003; Berndtson et al., 1996a; Berndtson et al., 1996b; Evans and Sayers, 2000; Hald et al., 2000; Heuer et al., 2001; Jorgensen et al., 2002; Luangtongkum et al., 2005; Nielsen et al., 1997; Refregier-Petton et al., 2001; Saleha et al., 1998; Wedderkopp et al., 2000). Interestingly, although several studies (Heuer et al., 2001; Luangtongkum et al., 2005; Saleha et al., 1998) indicated that C. jejuni was the major Campylobacter species isolated from free-range or organic broilers with the prevalence ranging from 72% to 91%, other studies (Avrain et al., 2003; El-Shibiny et al., 2005), on the other hand, reported that the majority (43% to 92%) of thermophilic Campylobacter colonized free-range or organic broilers were C. coli.

Unlike commercial broilers, the prevalence of C. jejuni and C. coli in commercial turkeys varies drastically among studies. Some studies (Lee et al., 2005; Smith et al., 2004) showed that 80% - 90% of Campylobacter strains isolated from turkeys were C.
coli, while other studies (Wallace et al., 1998; Van Looveren et al., 2001) found that almost 100% of Campylobacter isolated from commercial turkeys were C. jejuni. In one survey study, the difference in the prevalence of C. coli in turkey samples collected from 2 processing plants located in the Midwestern region of the United States was reported. The prevalence of C. coli in commercial turkeys collected from one processing plant was 40.5%, while the prevalence of this organism in commercial turkeys collected from another processing plant located in the same region was 14.6% (Logue et al., 2003). In addition, the recent study on the prevalence of thermophilic Campylobacter in commercial and organic turkeys revealed that about 46% and 54% of Campylobacter isolates from commercial turkeys were C. jejuni and C. coli, respectively; whereas about 66% of Campylobacter isolates from organic turkeys were identified as C. jejuni and about 34% of these isolates were identified as C. coli (Luangtongkum et al., 2005).

Among Campylobacter spp. isolates from other poultry species, C. jejuni was the predominant Campylobacter species isolated from ducks and geese (Aydin et al., 2001; Tsai and Hsiang, 2004). About 95% of Campylobacter isolates from ducks were identified as C. jejuni and 5.2% of these isolates were classified as C. coli (Tsai and Hsiang, 2004). Likewise, all Campylobacter isolates from geese were identified as C. jejuni (Aydin et al., 2001). C. jejuni and C. coli were found at a rate of 28% and 21% in pheasants, respectively (Atanassova and Ring, 1999). In addition, only C. jejuni was isolated from squabs and quails (Jeffrey et al., 2001; Minakshi and Ayyagari, 1988). For free-living and migrating birds and waterfowl, C. jejuni was the main Campylobacter species isolated from these birds (Broman et al., 2002; Broman et al., 2004; Chuma et al.,
2000; Craven et al., 2000; Fallacara et al., 2001; Ito et al., 1988; Kaneuchi et al., 1987; Kapperud and Rosef, 1983; Kapperud et al., 1983; Kinjo et al., 1983; Luechtfeld et al., 1980; Shane, 1992; Waldenström et al., 2002; Yogasundram et al., 1989), while a few Campylobacter isolates from pigeons and black-headed gulls were identified as C. coli (Broman et al., 2002; Kaneuchi et al., 1987; Kinjo et al., 1983). In addition, besides C. jejuni and C. coli, C. lari was also isolated from gulls; however, the isolation rate of this Campylobacter species was much lower than that of C. jejuni (less than 5% versus almost 95%) (Broman et al., 2002; Kaneuchi et al., 1987).

The prevalence of Campylobacter spp. in poultry particularly in broilers has been shown to be associated with the age of the birds at slaughter. In general, Campylobacter infection rates in commercial broiler flocks increase when age of the birds increases (Berndtson et al., 1996a; Berndtson et al., 1996b; Evans and Sayers, 2000; Genigeorgis et al., 1986; Lindblom et al., 1986; Luangtongkum et al., 2005; Newell and Fearnley, 2003; Newell and Wagenaar, 2000; Northcutt et al., 2003; Sahin et al., 2002; Willis and Murray, 1997). Colonization of broiler intestinal tract usually begins after the first or the second week, then this colonization rate increases with age and remains high or even peaks at the market age, which is normally about 6 to 7 weeks (Genigeorgis et al., 1986; Jacobs-Reitsma, 1997; Jacobs-Reitsma et al., 1995; Lindblom et al., 1986; Padunton and Kaneene, 2003). A study under commercial conditions revealed that the Campylobacter colonization rate of the birds increased from 2.3% in 10 days old birds to 81.8% in slaughter-age birds (Genigeorgis et al., 1986). Likewise, another study in the United Kingdom also found that 40% of commercial broiler flocks were colonized with
Campylobacter spp. by four weeks and up to 90% by seven weeks (Evans and Sayers, 2000). Similarly, turkeys also rapidly acquire Campylobacter infections (Kaneene and Potter, 2003). In one study, colonization of turkey chicks began at seven days after the birds arrived at the farm and this colonization rate reached 100% within three weeks (Wallace et al., 1998). In addition to the age of the birds at slaughter, the prevalence of Campylobacter spp. in broilers is also affected by season (Jacobs-Reitsma, 1997; Jacobs-Reitsma et al., 1994a; Kaneene and Potter, 2003; Kapperud et al., 1993; Newell and Wagenaar, 2000; Patrick et al., 2004; Refregier-Petton et al., 2001; Sahin et al., 2002; Shane, 2000; Wallace et al., 1997; Wedderkopp et al., 2001; Wedderkopp et al., 2000; Willis and Murray, 1997). Seasonal variation in presence of Campylobacter spp. in broiler flocks or broiler carcasses has been reported by several studies. Generally, the highest Campylobacter isolation rate was observed during summer/autumn months, while the lowest rate was observed during winter months (Jacobs-Reitsma, 1997; Jacobs-Reitsma et al., 1994a; Kaneene and Potter, 2003; Kapperud et al., 1993; Meldrum et al., 2005; Newell and Wagenaar, 2000; Patrick et al., 2004; Refregier-Petton et al., 2001; Sahin et al., 2002; Shane, 2000; Wallace et al., 1997; Wedderkopp et al., 2001; Wedderkopp et al., 2000; Willis and Murray, 1997). The seasonal variation in Campylobacter prevalence in broilers may correlate directly with temperature, relative humidity, or sunlight hours (Jacobs-Reitsma et al., 1994a; Kaneene and Potter, 2003; Patrick et al., 2004; Wallace et al., 1997; Willis and Murray, 1997). In addition, other sources of contamination within the farm environment that are temperature dependent
such as migratory birds, rodents, and insects, e.g. darkling beetles, may play some roles on the increased \textit{Campylobacter} carriage in broilers during the summer/autumn months as well (Jacobs-Reitsma, 1997; Jacobs-Reitsma et al., 1994a; Patrick et al., 2004).

In terms of the prevalence of \textit{Campylobacter} spp. in other animals, thermophilic \textit{Campylobacter} have been detected in many domestic and wild animals including cattle, sheep, swine, wildlife, and pet. \textit{Campylobacter} species often inhabit the bovine intestinal tract (Altekruse and Tollefson, 2003). Although prevalence rates of \textit{Campylobacter} spp. in cattle vary from less than 5% to more than 89% (Altekruse and Tollefson, 2003; Atabay and Corry, 1998; Beach et al., 2002; Busato et al., 1999; Cabrita et al., 1992; Giacoboni et al., 1993; Harvey et al., 2004; Nielsen, 2002; Rosef et al., 1983; Stanley et al., 1998c; Turkson et al., 1988; Wesley et al., 2000), a wide range of \textit{Campylobacter} prevalence is mainly observed in beef cattle, while the prevalence of this organism in dairy cows is usually less than 40% (Atabay and Corry, 1998; Harvey et al., 2004; Wesley et al., 2000). In addition, although cattle can be colonized by several \textit{Campylobacter} species, \textit{C. jejuni} is the most common thermophilic \textit{Campylobacter} species isolated from cattle (Busato et al., 1999; Harvey et al., 2004; Stanley et al., 1998c; Wesley et al., 2000). In general, \textit{C. jejuni} comprised 70% to 100% of thermophilic \textit{Campylobacter} in cattle (Harvey et al., 2004; Hoofar et al., 1999; Manser and Dalziel, 1985; Munroe et al., 1983; Nielsen et al., 1997; Stanley et al., 1998c; Wesley et al., 2000) except for one study, which reported that \textit{C. jejuni} comprised only 7% of the \textit{Campylobacter} isolates (Atabay and Corry, 1998). Besides \textit{C. jejuni}, \textit{C. hyointestinalis} is another \textit{Campylobacter} species that is commonly found in cattle (Nielsen et al., 1997).
The prevalence of this organism in intestinal tract of cattle could range from 24% to 88% (Grau, 1988; Nielsen et al., 1997). Risk factors that influence the prevalence of thermophilic *Campylobacter* in cattle include age of the animals and season of the year (Busato et al., 1999; Harvey et al., 2004; Stanley et al., 1998c; Wesley et al., 2000). Newborn calves were reported to be *Campylobacter* free at birth but became colonized rapidly within a few days via horizontal transmission from farm environment (Stanley et al., 1998c). It has been reported that high numbers of *Campylobacter* could be found in fecal samples of calves by 1 – 2 months of age and the overall prevalence of thermophilic *Campylobacter* in calves during the first 3 months of age was about 39% (Altekruse and Tollefson, 2003; Busato et al., 1999; Stanley et al., 1998c). In addition, calves are more frequently infected with *Campylobacter* spp. than adult cattle especially in dairy herds where young animals had a higher prevalence of *Campylobacter* than older animals (Altekruse and Tollefson, 2003; Nielsen 2002). Moreover, feedlot cattle are also more likely to be colonized by *Campylobacter* species than the cattle raised on pasture (Garcia et al., 1985; Grau, 1988; Stanley et al., 1998c). The significance of thermophilic *Campylobacter* colonization in cattle relates not only to the potential for contamination of milk at the farm or the carcass at slaughter, but also environmental contamination especially water contamination during disposal of abattoir effluents (Stanley et al., 1998a; Stanley et al., 1998c).

The prevalence of thermophilic *Campylobacter* in sheep and lambs varies significantly between studies. Although several studies reported that the prevalence of *Campylobacter* species in sheep and lambs at slaughter was about 92% (Altekruse and
Tollefson, 2003; Jones et al., 1999; Stanley et al., 1998b), other studies showed that the level of carriage of *Campylobacter* in sheep as well as the level of carcass contamination at slaughter was very low with 2% *Campylobacter* isolation rate (Turkson et al., 1988). Almost 90% of thermophilic *Campylobacter* isolated from sheep and lambs were identified as *C. jejuni*, while *C. coli* accounted for 8% to 10% of the isolates (Jones et al., 1999; Stanley et al., 1998b). In addition, a low frequency of other *Campylobacter* species such as *C. hyointestinalis* and *C. lari* could be isolated from sheep and lambs as well (Stanley et al., 1998b). Like poultry and cattle, seasonal variation in the numbers of thermophilic *Campylobacter* in the intestinal tract was also observed in sheep and lambs (Stanley et al., 1998b). When compared to other animals, the prevalence of *Campylobacter* spp. in goats is relatively low with the prevalence around 6% (Turkson et al., 1988).

Intestinal carriers of thermophilic *Campylobacter* are very common in swine. The isolation rate of *Campylobacter* spp. from swine generally ranged from 44% to 100% (Manser and Dalziel, 1985; Nielsen et al., 1997; Oosterom et al., 1985; Rosef et al., 1983; Turkson et al., 1988; Weijtens et al., 1993). Unlike other animals mentioned earlier that were mainly colonized by *C. jejuni*, *C. coli* is the predominant *Campylobacter* species that colonizes swine intestinal tract (Altekruse and Tollefson, 2003; Nielsen et al., 1997; Rosef et al., 1983; Turkson et al., 1988; Van Looveren et al., 2001). Since most studies have reported high carriage rates (almost 100%) of *C. coli* among healthy swine, this information provides evidence that *C. coli* may be a normal component of the intestinal
flora of this animal (Rosef et al., 1983; Sticht-Groh, 1982; Van Looveren et al., 2001). However, other *Campylobacter* species such as *C. jejuni* can be isolated from swine as well (Turkson et al., 1988).

As mentioned earlier, *Campylobacter* species are naturally found in domestic animals and pets (Svedhem and Kaijser, 1981). Since *Campylobacter* carriage in healthy pets is very common, it is not surprising that household pets especially puppies will be responsible for the transmission of these organisms to humans (Blaser et al., 1978). Several studies revealed that the prevalence of thermophilic *Campylobacter* in dogs ranged from 4.6% to 76.2% (Baker et al., 1999; Bruce et al., 1980; Burnens et al., 1992; Gondrosen et al., 1985; Hald and Madsen, 1997; Hald et al., 2004a; Olson and Sandstedt, 1987; Sandberg et al., 2002; Steinhauserova et al., 2000; Torre and Tello, 1993; Wright, 1982), while the prevalence of this organism in cats varied from 5% to 45% (Baker et al., 1999; Bruce et al., 1980; Burnens et al., 1992; Gondrosen et al., 1985; Hald and Madsen, 1997; Sandberg et al., 2002; Steinhauserova et al., 2000). Interestingly, the species distribution of *Campylobacter* isolates from household pets especially dogs differs considerably between studies; however, *C. jejuni* and *C. upsaliensis* are the two major species that account for more than 95% of thermophilic *Campylobacter* isolated from these animals (Burnens et al., 1992; Hald and Madsen, 1997; Hald et al., 2004a; Olson and Sandstedt, 1987; Sandberg et al., 2002). Several studies reported that the ratios of *C. upsaliensis* to *C. jejuni* can be either high; for example, 80% to 19% or low; e.g., 15% to 82% (Baker et al., 1999; Burnens et al., 1992; Hald and Madsen, 1997; Hald et al., 2004a; Sandberg et al., 2002). Unlike *C. jejuni*, *C. upsaliensis* has been reported to occur
only in dogs and cats (Hald and Madsen, 1997; Hald et al., 2004a; On, 1996). Other *Campylobacter* species that can be isolated from household pets include *C. coli* and *C. lari* (Hald and Madsen, 1997; Hald et al., 2004a). In addition, Hald et al. (2004a) revealed that the prevalence of thermophilic *Campylobacter* in dogs increased from 60% at 3 months of age to nearly 100% at 1 year of age, then the carrier rate decreased to 67% at 2 years of age. Moreover, Hald et al. (2004a) also reported that *C. jejuni* was isolated more frequently in dogs under 1 year of age than in the older dogs. This finding may help explain the higher prevalence of *C. jejuni* than that of *C. upsaliensis* (76% versus 19%) observed in puppies (Hald and Madsen, 1997).

Wildlife and zoo animals including bobcat, cheetah, capybaras, coati-mundis, hares, polar bear, red panda, tapir, llama, reindeer, roan antelope, wallaroos, wolves, and several species of primates have been documented to carry thermophilic *Campylobacter* (Luechtefeld et al., 1981; Rosef et al., 1983). However, the isolation rate of *Campylobacter* spp. from these animals is quite low with the prevalence less than 10% and *C. jejuni* is considered the main organism isolated from these animals (Luechtefeld et al., 1981; Rosef et al., 1983). In addition to wildlife and zoo animals, *Campylobacter* spp. can also be isolated from laboratory and wild rodents such as rats and bank voles, but not from rabbits, laboratory mice, hamsters, guinea-pigs, field mice or field voles (Fernie and Park, 1977).
1.4.2 Sources of Poultry Flock Colonization

In general, *Campylobacter* species are rarely detected in commercial broiler flocks under the age of 2 - 3 weeks old (Berndtson et al., 1996b; Engvall et al., 1986; Evans and Sayers, 2000; Jacobs-Reitsma, 1997; Jacobs-Reitsma et al., 1995; Newell and Fearnley, 2003; Pokamunski et al., 1986; Sahin et al., 2002; Stern, 1992; Stern et al., 1998). The explanation for this phenomenon is unclear; however, it is possible that the presence of *Campylobacter*-specific maternal antibodies in young chicks (Jacobs-Reitsma, 1997; Newell and Fearnley, 2003; Rice et al., 1997; Sahin et al., 2001; Sahin et al., 2002; Sahin et al., 2003b; Stern et al., 1998) or the presence of unique microbial flora in the intestinal tract especially in the cecum of these young chicks (competitive cecal microflora) (Humphrey et al., 1989; Sahin et al., 2002) may have an inhibitory effect or play a role on *Campylobacter* colonization during the first 2 weeks of life of these commercial broilers. Interestingly, once *Campylobacter* spp. are introduced into the flock, they tend to spread very rapidly and once these organisms are isolated from the flock, which is probably around 3 weeks of age, most or even all of the birds in that particular flock will become colonized and all environmental sources will be contaminated with *Campylobacter* spp. (Jacobs-Reitsma, 1997; Sahin et al., 2002; Saleha et al., 1998). In addition, *Campylobacter* isolation rates are also likely to increase and remain high until the birds are sent to the processing plant (Jacobs-Reitsma, 1997). Many studies suggest that horizontal transmission from environmental sources is the major mode of *Campylobacter* colonization in broiler flocks (Jacobs-Reitsma et al., 1995; Newell and Fearnley, 2003; Pearson et al., 1993; Stern, 1992); however, several findings
suggested that vertical transmission from breeders may also play a role in *Campylobacter* infection in broilers (Chuma et al., 1994; Chuma et al., 1997b; Doyle, 1984; Pearson et al., 1996; Shane et al., 1986; Shanker et al., 1986).

**Vertical transmission.** Several studies have shown that *Campylobacter* spp. can be isolated from the intestinal tracts and reproductive tracts of healthy laying hens and broiler breeder hens (Buhr et al., 2002; Camarda et al., 2000; Cox et al., 2002a; Jacobs-Reitsma, 1995; Pearson et al., 1996; Sahin et al., 2003a; Shanker et al., 1986) as well as from semen of broiler breeder roosters (Cox et al., 2002b; Hiett et al., 2003). In addition, some molecular studies also demonstrated that the genotypes of *Campylobacter* strains isolated from breeder flocks were similar to those of the isolates from their progeny flocks (Cox et al., 2002a; Pearson et al., 1996). This information in combination with the positive detection of *Campylobacter* DNA from cecal contents of newly hatched chicks (Chuma et al., 1994; Chuma et al., 1997b) suggest that vertical transmission of *Campylobacter* spp. from breeder flocks to broiler flocks through the egg may occur and this vertical transmission might be the source of *Campylobacter* colonization in broiler chickens (Jacobs-Reitsma, 1997; Newell and Fearnley, 2003; Sahin et al., 2002; Saleha et al., 1998). Although the findings mentioned above seem to support a vertical transmission, other studies suggest that vertical transmission of *Campylobacter* spp. via the egg is considered unlikely, mainly because of the difficulty or inability to culture *Campylobacter* spp. from naturally or experimentally infected eggs as well as from newly hatched chicks originating from infected breeder flocks (Acuff et al., 1982; Baker et al., 1987; Doyle, 1984; Hiett et al., 2002a; Jacobs-Reitsma, 1995; Jacobs-Reitsma et al., 1997).
1995; Jacobs-Reitsma, 1997; Jones et al., 1991; Newell and Fearnley, 2003; Sahin et al., 2002; Sahin et al., 2003a; Saleha et al., 1998; Shane et al., 1986; Shanker et al., 1986). Even though the detection of Campylobacter DNA in eggs, embryos, and cecal contents of newly hatched chicks has been shown in several investigations, none of these studies has been able to detect any live Campylobacter organisms from those samples (Chuma et al., 1994; Chuma et al., 1997b; Hiett et al., 2002a). In addition, several researchers also showed that isolation of Campylobacter species from broiler flocks before 2 or 3 weeks of age was hardly accomplished, even though the chicks were hatched from eggs obtained from infected parent flocks (Berndtson et al., 1996a; Jacobs-Reitsma, 1997; Newell and Fearnley, 2003; Sahin et al., 2002; Shanker et al., 1986; Van de Giessen et al., 1992). Moreover, broilers from the same parent flocks were also found to be colonized in one production cycle but Campylobacter-free in another cycle (Jacobs-Reitsma, 1995; Jacobs-Reitsma et al., 1995; Jacobs-Reitsma, 1997; Newell and Fearnley, 2003; Sahin et al., 2002). When Campylobacter strains from breeder flocks and broiler flocks were investigated, several studies revealed that Campylobacter strains infected broiler flocks were frequently found to be different from those infected breeder flocks (Chuma et al., 1997a; Petersen et al., 2001b; Sahin et al., 2002). In addition, Campylobacter strains isolated from different broiler flocks originating from the same breeder flocks did not always have the same serotypes, while Campylobacter strains isolated from broilers originating from different hatcheries but were raised in the same farm may be colonized with the same Campylobacter strains, indicating that the serotype pattern was more associated with the farm than with the origins of the birds (Berndtson et
Based on all information available currently, it is possible that vertical transmission of *Campylobacter* spp. through the egg may occur; however, it is probably a rare event and does not seem to play an important role in the introduction of *Campylobacter* species to broiler flocks (Sahin et al., 2002; Sahin et al., 2003a).

**Horizontal transmission.** Many studies have suggested that horizontal transmission of *Campylobacter* spp. from the rearing environment is likely to be the major source of *Campylobacter* infection in broiler flocks (Newell and Fearnley, 2003; Sahin et al., 2002; Saleha et al., 1998). Although many environmental sources including feed, litter, untreated water, insects, flies, rodents, other farm animals, domestic pets, wildlife species particularly wild birds, equipment and transport vehicles, and farm workers have been suspected to be the source of *Campylobacter* infection in broiler farms, none of these suspected sources has been identified conclusively as the actual source of infection and mode of transmission of this organism to broilers in the farms has been unclear (Sahin et al., 2002; Saleha et al., 1998). In general, *Campylobacter* spp. were usually detected from environmental sources after the broilers had become infected, suggesting that the birds might be the source of environmental contamination instead of being infected from environmental sources (Berndtson et al., 1996a; Jacobs-Reitsma et al., 1995; Kazwala et al., 1990; Sahin et al., 2002; Stern et al., 2001b).

As mentioned earlier, *Campylobacter* spp. usually have not been isolated from fresh litter and feed samples before the flock had been colonized with *Campylobacter* organisms (Gregory et al., 1997; Humphrey et al., 1993; Jacobs-Reitsma et al., 1995;
Pearson et al., 1993; Sahin et al., 2002). Because of the low water content of feed and litter, *Campylobacter* spp. are unlikely to survive for a long period of time in these environmental sources (Berndtson et al., 1996b; Evans, 1992; Jacobs-Reitsma, 1997; Jacobs-Reitsma et al., 1995; Kaneene and Potter, 2003; Newell and Fearnley, 2003; Pokamunski et al., 1986; Sahin et al., 2002; Shane, 2000). Therefore, feed and litter are not regarded as the major sources of *Campylobacter* infection in broiler flocks (Newell and Fearnley, 2003; Sahin et al., 2002; Shane, 2000). Although used litter that became contaminated with *Campylobacter* spp. may play a role in maintaining *Campylobacter* spp. in the farm environment and transmitting this organism to other broiler flocks (Montrose et al., 1985; Shane, 1992), a study by Payne et al. (1999) showed that the role of used litter in the transmission of *Campylobacter* species to successive flocks in the same poultry house was insignificant.

Several studies indicated that the use of untreated water such as groundwater or well water seemed to be an important risk factor for *Campylobacter* colonization in broiler flocks (Jacobs-Reitsma, 1997; Kapperud et al., 1993; Pearson et al., 1993). This is mainly because untreated water could be contaminated with *Campylobacter* species from other environmental sources, particularly from livestock or wild birds (Jones, 2001; Kaneene and Potter, 2003; Sahin et al., 2002; Stanley et al., 1998a). Contaminated or non-chlorinated water supplied to broilers has been demonstrated or implicated as a source of *Campylobacter* infection as well as a vehicle for transmission of *Campylobacter* spp. to broiler flocks in several countries (Engvall et al., 1986; Kaneene and Potter, 2003; Kapperud et al., 1993; Pearson et al., 1993; Sahin et al., 2002; Shane,
In general, fresh tap water from broiler farms was not found to be contaminated with *Campylobacter* spp. (Jacobs-Reitsma, 1997). However, water samples from the farms, particularly the ones collected from bell-shaped drinkers inside the broiler houses, could become *Campylobacter* positive especially after or at the same time that broilers in the houses were colonized by *Campylobacter* species (Berndtson et al., 1996a; Jacobs-Reitsma, 1997; Jacobs-Reitsma et al., 1995; Newell and Fearnley, 2003). This means water contamination usually follows rather than precedes *Campylobacter* colonization of a flock (Engvall et al., 1986; Kazwala et al., 1990; Linblom et al., 1986; Newell and Fearnley, 2003). In addition, the occurrence of viable but non-culturable form of *Campylobacter* species in water may be significant in introducing infection into broiler flocks as well (Newell and Fearnley, 2003; Shane, 2000). Since contaminated water can introduce *Campylobacter* infections into broiler houses and lead to subsequent dissemination of this organism within the flocks, water provided for the birds should be chlorinated and water supply system should be cleaned thoroughly and disinfected after the previous flock has been removed and before the new flock is introduced to the house (Engvall et al., 1986; Kapperud et al., 1993; Pearson et al., 1993; Saleha et al., 1998; Shane, 1992; Shanker et al., 1990; Smitherman et al., 1984).

Insects including flies (e.g. house flies, filth flies), darkling beetles, cockroaches, and mealworms in and around broiler houses can serve as mechanical vectors and may transmit or carry *Campylobacter* spp. from one location to another within or between the houses or farms as well as between successive broiler flocks (Bates et al., 2004; Berndtson et al., 1996a; Gregory et al., 1997; Hald et al., 2004b; Jacobs-Reitsma, 1997;
Jacobs-Reitsma et al., 1995; Kaneene and Potter, 2003; Newell and Fearnley, 2003; Rosef and Kapperud, 1983; Sahin et al., 2002; Shane, 2000; Shane et al., 1985; Skove et al., 2004; Szalanski et al., 2004). Interestingly, serotypes and genotypes of *Campylobacter* strains isolated from insects and broilers within the same broiler house were found to be identical; therefore, it was possible that an infection route from insects to broilers might occur (Berndtson et al., 1996a; Jacobs-Reitsma, 1997; Jacobs-Reitsma et al., 1995; Rosef et al., 1985; Sahin et al., 2002; Stern et al., 1997). However, since insects in broiler houses were usually not positive for *Campylobacter* spp. until the organism was isolated from the broilers (Berndtson et al., 1996a; Jacobs-Reitsma, 1997; Jacobs-Reitsma et al., 1995; Nesbit et al., 2001; Sahin et al., 2002), this finding suggested that an infection route was likely to occur from broilers to insects rather than from insects to broilers and the possibility that insects were an original source of *Campylobacter* infection in broiler flocks seemed to be very small (Sahin et al., 2002).

Although the presence of rodents such as rats and mice on a farm can lead to an increased risk of flock colonization with *Campylobacter* spp., this risk factor is considered unlikely to be significant especially for the farm that has an effective vermin control program (Berndtson et al., 1996b; Kapperud et al., 1993; Newell and Fearnley, 2003; Sahin et al., 2002). In addition, several studies also reported that they could not isolate *Campylobacter* species from trapped rats and mice on broiler farms with infected flocks (Gregory et al., 1997; Jones et al., 1991; Shane, 2000). Therefore, the role of rodents as a common source of *Campylobacter* infection in broiler flocks seems to be questionable (Evans and Sayers, 2000; Gregory et al., 1997; Sahin et al., 2002).
In addition to rodents, *Campylobacter* spp. can also be recovered from other wild animals including hares, raccoons, badgers, foxes, and deer (Newell and Fearnley, 2003; Sahin et al., 2002). And as mentioned earlier, a wide range of free-living and migrating birds and waterfowl can also serve as natural reservoirs of thermophilic *Campylobacter* (Broman et al., 2002; Broman et al., 2004; Craven et al., 2000; Fallacara et al., 2001; Kapperud and Rosef, 1983; Kapperud et al., 1983; Kaneuchi et al., 1987; Luechtefeld et al., 1980; Shane, 1992; Shane, 2000; Waldenström et al., 2002; Yogasundram et al., 1989). Therefore, it is not surprising that *Campylobacter* spp. are frequently isolated from feces of wild birds around broiler houses (Newell and Fearnley, 2003). Although *Campylobacter* strains isolated from wild birds around broiler houses were usually found to be different from those of broiler chickens, some epidemiological studies reported that these *Campylobacter* strains could occasionally be recovered from the intestinal tracts of of broilers in those houses (Gregory et al., 1997; Hiett et al., 2002b; Nesbit et al., 2001; Newell and Fearnley, 2003; Petersen et al., 2001a; Rosef et al., 1985; Sahin et al., 2002; Stern et al., 1997). Since wild animals, particularly wild birds seem to have a high carriage rate of *Campylobacter* species in their intestines, they should therefore be considered as a potential source of *Campylobacter* infection for broiler flocks as well (Craven et al., 2000; Sahin et al., 2002).

The presence of domestic livestock including cattle, sheep, and pigs as well as domestic pets such as dogs and cats on broiler farms has been found to be associated with an increased risk of *Campylobacter* infection in broiler flocks especially in the farms that do not have good or appropriate biosecurity procedures (Berndtson et al., 1996b; Gregory
et al., 1997; Kapperud et al., 1993; Newell and Fearnley, 2003; Rosef et al., 1985; Sahin et al., 2002; Shane, 2000; Stern et al., 1997; Van de Giessen et al., 1992; Van de Giessen et al., 1998). Farm animals can excrete substantial numbers of *Campylobacter* spp. in their feces and this can result in contamination of boots, other external clothing, and equipment used in the farm, which can lead to contamination of broiler flocks (Newell and Fearnley, 2003). Hence, it is not surprising that a mode of transmission of *Campylobacter* organisms from farm animals to broilers will be frequently associated with indirect mechanical transmission through farm personnel or farm equipment (Shane, 2000). In addition, farm animals especially cattle and sheep also have potential to contaminate pastures and surface waters, which in turn may act as a source of broiler flock colonization (Jones et al., 1999; Sahin et al., 2002; Stanley et al., 1998b; Stanley et al., 1998c). Like other environmental sources, some studies found that *Campylobacter* strains isolated from farm animals were similar to the isolates from broilers, while other studies reported that *Campylobacter* strains isolated from farm animals were different from those isolates recovered from broilers raised on the same farm (Gregory et al., 1997; Jacobs-Reitsma, 1997; Jacobs-Reitsma et al., 1995; Nesbit et al., 2001; Petersen et al., 2001a; Rosef et al., 1985; Stern et al., 1997; Van de Giessen et al., 1998). Likewise, several studies indicated the association between *Campylobacter* strains colonized broiler flocks and the presence of pigs on the same farm, whereas other studies did not observe this association (Jacobs-Reitsma, 1997; Jacobs-Reitsma et al., 1994; Jacobs-Reitsma et al., 1995; Kapperud et al., 1993; Rosef et al., 1985; Stern et al., 1997; Van de Giessen et al., 1992; Van de Giessen et al., 1998). However, since *C. coli* is mainly associated with
pigs, an increased proportion of *C. coli* in broiler flocks might indicate the transmission of these *Campylobacter* organisms from pigs to broilers (Jacobs-Reitsma, 1997). Although other domestic animals such as horses can also be infected with *Campylobacter* spp. (Stern, 1992), the potential role of these animals as a source of broiler flock infection has not yet been established (Sahin et al., 2002).

Since healthy carriers of *Campylobacter* spp. in humans are unlikely, farm workers are usually not regarded as direct sources of transmission (Jacobs-Reitsma, 1997). However, farm workers may carry and spread *Campylobacter* spp. from one flock to another by indirect mechanical transmission via boots/footwear, clothing, or equipment, which seems to occur easily (Berndtson et al., 1996b; Jacobs-Reitsma, 1997; Kazwala et al., 1990; Lindblom et al., 1986; Sahin et al., 2002; Saleha et al., 1998). Furthermore, farm workers taking care of broilers or other animals or loading birds for transport to slaughter can also increase the risk of *Campylobacter* infection in broiler flock especially if they enter the broiler house without changing clothes or boots (Berndtson et al., 1996b; Kapperud et al., 1993; Sahin et al., 2002; Saleha et al., 1998). Since *Campylobacter* spp. have been isolated from footbath water, boots, and transport crates (Hiett et al., 2002b; Jacobs-Reitsma, 1997; Kazwala et al., 1990; Newell et al., 2001; Slader et al., 2002; Stern et al., 2001b; Van de Giessen et al., 1998), movement of personal and equipment between flocks and farms, which seems to be associated with modern integrated production, is likely to contribute to introduction of *Campylobacter* infection in broiler flocks especially if clothing, boots, and equipment are contaminated with fresh fecal material from a flock excreting *Campylobacter* organisms (Newell and
Fearnley, 2003; Shane, 2000). However, a recent study showed that two adjacent broiler houses could be colonized with different *Campylobacter* genotypes even though these houses shared the same equipment and the farmer worked in both houses without changing any boots or clothes (Nesbit et al., 2001).

Other possible environmental sources of *Campylobacter* colonization in broiler flocks include aerosols, standing water, and soils around broiler houses (Jacobs-Reitsma, 1997; Newell and Fearnley, 2003). Although conditions for survival of *Campylobacter* species in air do not seem to be favorable, several studies could isolate *Campylobacter* spp. from air samples on broiler farms (Berndtson et al., 1996a; Engvall et al., 1986; Kazwala et al., 1990). Likewise, recent studies have shown that *Campylobacter* spp. could also be isolated from standing water around broiler houses before flock colonization and the same *Campylobacter* genotypes were subsequently recovered from broiler flock (Hiett et al., 2002b; Newell and Fearnley, 2003). However, the role of these environmental sources in introduction of *Campylobacter* spp. to broiler flocks is still unclear (Jacobs-Reitsma, 1997; Newell and Fearnley, 2003).

Based on information currently available, the source of broiler flock colonization is most likely mediated by multiple sources rather than specific single source. More details in sources of *Campylobacter* colonization in broiler flocks as well as information on horizontal and vertical transmission can be obtained from the review articles previously published by Newell and Fearnley (2003) and Sahin et al. (2002).
1.4.3 Control and Prevention at the Farm Level

The control and prevention of *Campylobacter* infection at the farm level are considered important factors in the reduction of *Campylobacter* spp. on poultry carcasses and even in the elimination of these organisms from the human food chain (Kapperud et al., 1992; Newell and Davison, 2003; White et al., 1997). However, since the sources of broiler flock colonization are unclear at this stage, the control and prevention of *Campylobacter* organisms at the farm level seem to be very difficult. The transmission of *Campylobacter* spp. in broiler flocks can occur very rapidly and once the flocks are infected, the incidence and levels of *Campylobacter* colonization in those flocks seem to remain similar for farms with both good and poor hygiene (Altekrause et al., 1998; Humphrey et al., 1993). Therefore, prevention of the first bird becoming infected with this organism is very important for successful control (Newell and Davison, 2003).

Currently, the measures for control and prevention of *Campylobacter* infection at the farm level can be classified into two major strategies: (i) biosecurity to exclude the organisms from the flock, and (ii) reduction or prevention of *Campylobacter* colonization of the birds by methods such as competitive exclusion and vaccination (Newell and Davison, 2003; Newell and Wagenaar, 2000; Rowe and Madden, 1999).

Although the sources of *Campylobacter* infection for broiler flocks are still unclear, it appears that the horizontal transmission of *Campylobacter* species from contaminated environment within or around the broiler house is likely to be the primary route for introduction of *Campylobacter* spp. to broiler flocks (Altekrause and Tollefson, 2003; Newell and Wagenaar, 2000). Some of the common risk factors include
contaminated water supplies, poor broiler house maintenance leading to insect or rodent infestation, insufficient cleaning and disinfection of broiler house between flocks, and poor hygienic practices such as lack of using boot dips and change of outer clothes before entering broiler house (Newell and Wagenaar, 2000). Since poor biosecurity is likely to be the main source of poultry flock colonization, the intervention strategy is primarily focused on maintaining and improving biosecurity measures in an attempt to prevent introduction of *Campylobacter* organisms into the broiler house (Altekruse and Tollefson, 2003; Gibbens et al., 2001; Shane, 1992; Van de Gissen et al., 1996). Good farm management practices including use of an all-in all-out system, cleansing and disinfection of building between flocks, control and keep buildings free of vermin such as insects, rodents, and wild birds, restricting other domestic animals on the farms as well as personnel contact with those animals, along with change of outer protective clothing and footwear at the entrance to broiler house, use of disinfectant footbaths and hand washing facilities, restriction on the staff and equipment entering broiler house, complete replacement of litter, and proper disposal of dead birds may help diminish introduction of *Campylobacter* spp. to broiler flocks (Altekruse et al., 1998; Altekruse and Tollefson, 2003; Gibbens et al., 2001; Humphrey et al., 1993; Kapperud et al., 1993; Kazwala et al., 1990; Newell and Davison, 2003; Newell and Wagenaar, 2000; Shane, 1992; Shane, 2000; Van de Gissen et al., 1996; White et al., 1997). As water has been demonstrated to be an important source of *Campylobacter* infection in broiler flocks, chlorination of drinking water, removal of biofilm in water supply lines, tanks, and drinkers using frequent cycles of flushing, and regular cleaning and disinfection of water supply system
can also reduce the proportion of birds colonized with *Campylobacter* spp. significantly although it may not totally eliminate carriage (Altekruse et al., 1998; Altekruse and Tollefson, 2003; Gibbens et al., 2001; Humphrey et al., 1993; Kapperud et al., 1993; Newell and Davison, 2003; Newell and Wagenaar, 2000; Pearson et al., 1993; Rowe and Madden, 1999; Shane, 2000; Stern et al., 2002; White et al., 1997). In addition, nipple drinkers rather than bell-shaped drinkers should be used in broiler production system in order to avoid fecal contamination of water (White et al., 1997). The association between chlorination of water supply and reduction in the colonization rates of broilers with *Campylobacter* spp. was revealed by Pearson et al. (1993). Because the hatchery is considered as a potential link in transmission of *Campylobacter* spp. from breeder flock to broilers, intensification of biosecurity procedures and decontamination of incubators at the hatchery should be emphasized as well (Shane, 2000).

As mentioned earlier, intensifying biosecurity can reduce but not eliminate the possibility of introduction of *Campylobacter* infection in broiler flocks (Shane, 2000). In addition, total prevention of flock exposure to *Campylobacter* spp. is unlikely to be accomplished by strict biosecurity measures alone (Newell and Davison, 2003). Therefore, other supportive measures such as competitive exclusion or vaccination seem to be required for control and prevention of *Campylobacter* colonization in broiler flocks (Newell and Davison, 2003; Shane, 2000). Although competitive exclusion has been a relatively successful approach for the control of *Salmonella*, its efficacy against *Campylobacter* colonization seems to be variable and unpredictable (Aho et al., 1992; Newell and Wagenaar, 2000). However, several studies reported that competitive
exclusion could help reduce the level and the prevalence of *Campylobacter* colonization in broilers and it could partly protect the birds from *Campylobacter* colonization in some experiments as well (Humphrey et al., 1989; Mead et al., 1996; Morishita et al., 1997; Newell and Wagenaar, 2000; Saleha et al., 1998; Schoeni and Doyle, 1992; Schoeni and Wong, 1994; Soerjadi et al., 1982; Soerjadi-Liem et al., 1984; Stern, 1994). In general, competitive exclusion materials are obtained from adult donor birds and given to the chicks by oral administration. These competitive exclusion materials can be fecal suspension, cecum-colonizing bacteria that produce anti-*Campylobacter* metabolites, or anaerobically harvested mucus from the cecal mucosa, either cultured in a laboratory medium or used as a diluted suspension (Humphrey et al., 1989; Mead et al., 1996; Newell and Wagenaar, 2000; Saleha et al., 1998; Schoeni and Doyle, 1992; Schoeni and Wong, 1994; Soerjadi et al., 1982; Soerjadi-Liem et al., 1984; Stern, 1994; Stern et al., 2001a). However, because these materials are derived from live birds, it is not surprising that they will be inconsistent and are mainly influenced by the status of the donor birds and the site from which the starter materials are collected (Laisney et al., 2004; Mead et al., 1996; Newell and Wagenaar, 2000; Schoeni and Doyle, 1992; Schoeni and Wong, 1994). Recently, several commercial probiotic preparations have been available such as Avian Pac Soluble, which is a mixture of *Lactobacillus acidophilus* and *Streptococcus faecium* (Chang and Chen, 2000; Morishita et al., 1997; Saleha et al., 1998). This avian-specific probiotic containing *L. acidophilus* and *S. faecium* has been found to be able to reduce the colonization and frequency of fecal shedding of *Campylobacter* spp. in broilers as well (Morishita et al., 1997). Besides *L. acidophilus* and *S. faecium*, other
microorganisms including a mixture of *Klebsiella pneumoniae*, *Citrobacter diversus*, and *Escherichia coli* (O13:H¹) and yeast *Saccharomyces boulardii* could also protect or at least reduce the level of *Campylobacter* colonization in cecum of broilers (Line et al., 1997; Line et al., 1998; Newell and Wagenaar, 2000; Rowe and Madden, 1999; Schoeni and Doyle, 1992). In addition, *Campylobacter* strains, which have a high colonizing potential but are nonpathogenic may be another interesting alternative to use as competitive excluders of pathogenic *Campylobacter* strains (Barrow and Page, 2000; Chen and Stern, 2001; Newell and Wagenaar, 2000). Although most of competitive exclusion materials mentioned above seem to have good results under experimental conditions, their abilities in control and prevention of *Campylobacter* colonization in broiler flocks under commercial field conditions still need to be investigated. In addition to competitive exclusion, vaccination seems to be another possible alternative approach for control and prevention of *Campylobacter* colonization; however, effective vaccine strategies directed against infection with *Campylobacter* spp. in broilers have yet to be developed (Newell and Wagenaar, 2000). Passive immunization by oral administration of anti-*Campylobacter* antibodies has also been reported to have both therapeutic and prophylactic properties in broilers (Newell and Wagenaar, 2000; Stern et al., 1990b; Tsubokura et al., 1997). Therefore, it may be possible to immunize parent flocks to produce passively protected chickens (Newell and Wagenaar, 2000). Based on the information currently available, vaccination of chickens is likely to help reduce rather than prevent colonization (Newell and Wagenaar, 2000). Other intervention strategies including use of inbred chicken lines, which are resistant to *Campylobacter* infection, use
of enzyme-supplemented or other supplemented diet, or administration of 
Campylobacter-specific bacteriophages may be useful for control and prevention of 
Campylobacter colonization in broiler flocks as well (Altekruse and Tollefson, 2003; 
Bailey, 1993; Connerton et al., 2004; El-Shibiny et al., 2005; Fernandez et al., 2000b; 
Heres et al., 2004; Hinton et al., 2002; Kassaify and Mine, 2004; Newell and Davison, 
2003; Newell and Wagenaar, 2000; Rowe and Madden, 1999; Shane, 2000; Stern et al., 
1990a).

1.5 Antimicrobial Resistance of Campylobacter Species

1.5.1 Antimicrobial Susceptibility Testing Methods

In general, antimicrobial susceptibility testing of Campylobacter species can be 
classified into two major tests, which are dilution and diffusion methods (Aarestrup and 
Engberg, 2001; Nachamkin et al., 2000b). Although a number of different diffusion and 
dilution methods have been used to measure susceptibility of bacterial species to various 
antimicrobial agents, the most commonly used diffusion and dilution methods for 
antimicrobial susceptibility testing of Campylobacter spp. include disk diffusion test, 
epsilometer test (E-test), agar dilution test, and broth microdilution test (Aarestrup and 
Engberg, 2001; Alfredson et al., 2003; Engberg et al., 1999; Fernandez et al., 2000a; 
Frediani-Wolf and Stephan, 2003; Gaudreau and Gilbert, 1997; Ge et al., 2002; Huang et 
al., 1992; Huysmans and Turnidge, 1997; Luber et al., 2003a; McDermott et al., 2004; 
Nachamkin et al., 2000b; Oncul et al., 2003).
The agar diffusion test (disk diffusion and E-test) is relatively easy to perform and is very useful especially when several antimicrobial agents need to be tested against a few isolates; however, in order to be reproducible, it requires a high level of standardization and quality control. In addition, the results (the zone of inhibition) of this test are directly influenced by the agar depth and the inoculum size (Acar and Goldstein, 1996; Caprioli et al., 2000; McDermott et al., 2004; Potz et al., 2004). Because of its convenience and low cost, the disk diffusion test has been widely used and probably be the most commonly used antimicrobial susceptibility testing method. However, only qualitative data can be obtained from this test (Acar and Goldstein, 1996; Caprioli et al., 2000; McDermott et al., 2004; Potz et al., 2004). The E-test, on the other hand, can provide quantitative minimal inhibitory concentration (MIC) values although its cost is much higher than that of the disk diffusion method (Acar and Goldstein, 1996). In terms of the dilution test (agar dilution and broth microdilution), although this method is very reliable, highly reproducible, and provides quantitative MIC values, it is labor-intensive, time-consuming, and quite expensive especially when compared to the disk diffusion method (Caprioli et al., 2000). Nevertheless, the agar dilution method has been recognized as a standard antimicrobial susceptibility testing method for *Campylobacter* species (McDermott et al., 2004; NCCLS, 2002a). The agreement between different antimicrobial susceptibility testing methods particularly between the agar dilution method and the E-test in determining antimicrobial resistance of *Campylobacter* spp. has been
reported by several studies (Alfredson et al., 2003; Engberg et al., 1999; Fernandez et al., 2000a; Frediani-Wolf and Stephan, 2003; Gaudreau and Gilbert, 1997; Ge et al., 2002; Huang et al., 1992; Huysmans and Turnidge, 1997; Luber et al., 2003a; Oncul et al., 2003).

Although antimicrobial susceptibility test of thermophilic *Campylobacter* by the agar dilution method has not been standardized yet, it is recommended that Mueller-Hinton agar supplemented with 5% defibrinated sheep blood should be used and these agar plates should be incubated at 42 °C for 24 hours or at 36 °C for 48 hours under a microaerophilic atmosphere (approximately 5% O₂, 10% CO₂, and 85% N₂). In addition, *Campylobacter jejuni* ATCC 33560 should also be used as a quality control organism (McDermott et al., 2004; NCCLS, 2002a). Since there are no internationally accepted standard resistance breakpoints specific for *Campylobacter* species available currently, the resistance breakpoints of enteric bacteria in the family *Enterobacteriaceae* have been used to determine antimicrobial resistance of thermophilic *Campylobacter* (Ge et al., 2002; Luber et al., 2003a; Nachamkin et al., 2000b). Due to the lack of a standardized method and the validated resistance breakpoints specific for thermophilic *Campylobacter*, different antimicrobial susceptibility tests and different resistance breakpoints have been used between studies; therefore, antimicrobial resistance of *Campylobacter* species reported from different countries in different studies should be analysed with caution.
1.5.2 Occurrence and Trends of Antimicrobial Resistance

A rapid increase in the proportion of *Campylobacter* strains resistant to antimicrobial agents particularly to fluoroquinolones has been observed in every region of the world. In the United States, the emergence of fluoroquinolone resistance among *Campylobacter* isolates was first noticed about 10 years ago. Prior to 1992 there were no reports of fluoroquinolone-resistant *Campylobacter* strains isolated from humans in the U.S. (Nachamkin et al., 2002; Wang et al. 1984). However, the prevalence of fluoroquinolone resistance among these *Campylobacter* isolates increased significantly from 1.3% in 1992 to 8% - 13% during 1996 - 1998 and this resistance trend has increased steady since 1998 (Gupta et al., 2004; Nachamkin et al., 2002; Smith et al., 1999). In 2001, the National Antimicrobial Resistance Monitoring System (NARMS) and Nachamkin et al. (2002) found that about 19% - 40% of *Campylobacter* strains isolated from humans were resistant to ciprofloxacin (Gupta et al., 2004; Nachamkin et al., 2002).

In Canada, no fluoroquinolone resistance was observed in *Campylobacter* isolates during 1980 to 1986 (Gaudreau and Gilbert, 1998; Lariavere et al., 1986). However, in 1992 - 1997, about 3.5% - 13.6% of these *Campylobacter* isolates became resistant to fluoroquinolones (Gaudreau and Gilbert, 1998; Harnett et al., 1995). In 1998 - 2000, the rate of ciprofloxacin resistance among *C. jejuni* in Canada rose to 10% - 27% and dramatically increased to 47% in 2001 (Gaudreau and Gilbert, 2003). In South America, 18.2% - 25% of *Campylobacter* strains isolated from humans and animals in Brazil were also resistant to fluoroquinolones (Aquino et al., 2002).
In Europe, particularly in the Netherlands where fluoroquinolone-resistant *Campylobacter* isolates were first reported, the prevalence of *Campylobacter* strains resistant to this antimicrobial agent increased from 0% in 1982 to 11% in 1989 for human isolates and to 14% for the isolates from poultry products (Endtz, 1991). In 1992, five years after enrofloxacin was licensed for use in poultry in the Netherlands, Jacobs-Reitsma et al. (1994c) found that about 31% of *Campylobacter* isolates from broilers were resistant to fluoroquinolones. Similarly, 29% of human *Campylobacter* isolates in 1997 were also resistant to these antibiotics (Talsma et al., 1999). Besides the Netherlands, the emergence of fluoroquinolone resistance was found in other European countries as well. In Belgium, 44%, 28%, and 35% of *C. jejuni* isolated from broilers, layers, and turkeys and 62%, 41%, and 28% of *C. coli* isolated from broilers, layers, and swine in 1998 were resistant to fluoroquinolones, respectively (Van Looveren et al., 2001). Likewise, 17% of *C. jejuni* and 40% *C. coli* isolated from broilers in France in 1999 were also found to be resistant to enrofloxacin (Avrain et al., 2001). In addition, a recent study in Italy showed that 42%, 53%, and 38% of *C. jejuni* isolated from broilers, chicken meat, and humans and 75%, 79%, and 55% of *C. coli* isolated from the same sources during 2000 - 2001 were resistant to fluoroquinolones, respectively (Pezzotti et al., 2003) In Switzerland, the prevalence of fluoroquinolone resistance in *Campylobacter* isolates from poultry carcasses increased from 0% in 1997 - 1998 (Frei et al., 2001) to 29% in 2002 (Ledergerber et al., 2003). Similarly, recent studies in Germany also revealed significant increases in the rates of fluoroquinolone resistance in both human and poultry isolates (Krausse and Ullmann, 2003; Luber et al., 2003b). Krausse and
Ullmann (2003) and Luber et al. (2003b) reported that fluoroquinolone-resistant *Campylobacter* strains isolated from humans in Germany rose from 0% in 1980 - 1982 to 4.9% in 1991 and to 31% - 45.1% during 2000 - 2002, while the prevalence of fluoroquinolone resistance in *Campylobacter* strains isolated from chickens and turkeys in Germany increased from 27.3% and 25.8% in 1991 to 45.6% and 33.3% in 2001 - 2002, respectively (Luber et al., 2003b). A significant increase in fluoroquinolone resistance among human *Campylobacter* isolates was observed in Greece as well. During 1987 - 1989, no fluoroquinolone-resistant *C. jejuni* isolates was reported in Greece; however, 30.6% of *Campylobacter* isolates in 1998 - 2000 were resistant to these antimicrobial agents (Chatzipanagiotou et al., 2002). In Spain, the resistance rates to fluoroquinolone antibiotics among *Campylobacter* isolates increased rapidly from 0% - 3% before 1988 to 7% - 14% in 1989 to 30% in 1990 - 1991 and to 31% - 57% in 1992 - 1993 (Navarro et al., 1993; Prats et al., 2000; Reina, 1992; Reina et al., 1992; Reina et al., 1994, Sanchez et al., 1994; Velazquez et al., 1995). During 1995 and 1998, an extremely high prevalence of fluoroquinolone resistance among *Campylobacter* isolates in Spain was reported. Approximately 99% of *Campylobacter* strains isolated from broilers were resistant to fluoroquinolones, while about 71% - 90% of human isolates were resistant to these antibiotics (Mirelis et al., 1999; Prats et al., 2000; Saenz et al., 2000). The emergence of fluoroquinolone resistance among *Campylobacter* isolates also occurred in the United Kingdom. Until the end of 1990, all *Campylobacter* isolates in the U.K. were susceptible to ciprofloxacin; however, since 1991 a steady increase in fluoroquinolone resistance in *Campylobacter* isolates in this country has been reported (Bowler, 1992;
Bowler et al., 1996; Gaunt and Piddock, 1996; McIntyre, 1993; Sam et al., 1999; Thwaites and Frost, 1999). Similarly, *Campylobacter* strains isolated from humans and poultry in Ireland and Northern Ireland were also resistant to fluoroquinolones (Fallon et al., 2003; Lucey et al., 2000; Lucey et al., 2002; Moore et al., 2001; Oza et al., 2003; Wilson 2003). In Ireland, fluoroquinolone resistance among *Campylobacter* isolates from humans increased from 0% in 1996 - 1998 to 34% in 2000, while fluoroquinolone resistance among *Campylobacter* isolates from poultry increased from 3.1% in 1996 - 1998 to 18% - 29% in 1999 - 2000 (Fallon et al., 2003; Lucey et al., 2000; Lucey et al., 2002). Prior to 1992, no *Campylobacter* isolates from humans in Northern Ireland was resistant to fluoroquinolones; however, fluoroquinolone resistance among these *Campylobacter* isolates increased to 10% in 1993 and to 17.4% - 23% in 1999 - 2000 (Moore et al., 2001). Interestingly, during the same period of time about 3% of *Campylobacter* isolates from broilers and 10% of the isolates from chicken meat were resistant to these antibiotics (Oza et al., 2003; Wilson 2003). Similar observation was also reported in Denmark, where 21% of human isolates, 6% of broiler isolates, and 13% of chicken meat isolates were resistant to ciprofloxacin (Anonymous, 2001). A high frequency of fluoroquinolone resistance among *Campylobacter* isolates was found not only in Denmark, but also in other Scandinavian countries (Aarestrup et al., 1997; Andreasen, 1987; Anonymous, 2001; Hakanen et al., 2003; Pedersen and Wedderkopp, 2003; Rautelin et al., 1991; Rautelin et al., 1993; Sjogren et al., 1992; Sjogren et al., 1997; Svedhem et al., 1981). In Sweden, the trend of fluoroquinolone resistance among *Campylobacter* isolates rose from 0% in 1981 to 6.1% in 1992 - 1995 (Sjogren et al.,
1992; Sjogren et al., 1997; Svedhem et al., 1981), whereas the prevalence of ciprofloxacin resistance among *Campylobacter* strains isolated from humans in Finland increased dramatically from 0% in 1978 - 1980 to 17% in 1993 and to 46% in 1995 - 2000 (Hakanen et al., 2003; Rautelin et al., 1991; Rautelin et al., 1993). Similar trends of antimicrobial resistance in *Campylobacter* strains isolated from humans and poultry were also observed in other European countries (Adler-Mosca et al., 1991; Engberg et al., 2001; Frediani-Wolf and Stephan, 2003; Hirschl et al., 1990; Ledergerber et al., 2003; Lekowska-Kochaniak et al., 1996; Nachamkin et al., 2000b; Smith et al., 2000).

The emergence of fluoroquinolone resistance in *Campylobacter* isolates was not restricted only in Europe and North and South America, but this problem also occurred in other continents of the world. A study in Egypt showed that fluoroquinolone-resistant *Campylobacter* strains isolated from humans increased rapidly from 13% in 1995 to 28% in 1996 to 35% in 1997 and to 48% - 50% in 1998 - 2000 (Putnam et al., 2003). In Asia, particularly in East and Southeast Asia, the high prevalence of fluoroquinolone resistance in *Campylobacter* isolates was also reported. Approximately 57% and 91% of *Campylobacter* strains isolated from humans and poultry in Taiwan during 1994 to 1996 were resistant to ciprofloxacin, respectively (Li et al., 1998). In Japan, no fluoroquinolone resistance among *Campylobacter* strains isolated from humans was detected in 1981 - 1982; however, since 1993 the frequency of fluoroquinolone-resistant *Campylobacter* strains increased remarkably. In addition, 32.4% of *Campylobacter* strains isolated from broilers in Japan during 1995 - 1999 were also resistant to fluoroquinolones (Chuma et al., 2001; Niwa et al., 2004; Sagara et al., 1987). Likewise,
the fluoroquinolone resistance among *Campylobacter* strains isolated from humans in Thailand was dramatically increased as well (Hoge et al., 1998; Isenbarger et al., 2002; Kuschner et al., 1995). Before 1991, all *Campylobacter* isolates in Thailand were uniformly susceptible to quinolones. In 1993, 40% - 50% of *Campylobacter* isolates were found to be resistant to fluoroquinolones (Hoge et al., 1998; Kuschner et al., 1995). By 1994 this resistance rate rose to 76% and during 1995 to 1999 about 77% - 84% of these *Campylobacter* isolates in Thailand were resistant to these antimicrobial agents (Hoge et al., 1998; Isenbarger et al., 2002). Similarly, the frequency of fluoroquinolone-resistant *Campylobacter* strains isolated from humans in Indonesia also increased from 0% in 1995 - 1997 to 22% - 43% in 1998 - 2001 (Tjaniadi et al., 2003). Although the emergence of fluoroquinolone resistance in *Campylobacter* isolates was also observed in Australia and New Zealand, the percentage of fluoroquinolone-resistant *Campylobacter* isolates was very low when compared to the isolates obtained from other countries (Dowling et al., 1998; Sharma et al., 2003).

The development of antimicrobial resistance of *Campylobacter* strains isolated from humans and animals to erythromycin, tetracycline, as well as other antimicrobial agents was also reported in many countries worldwide (Aarestrup et al., 1997; Alfredson et al., 2003; Avrain et al., 2001; Avrain et al., 2003; Bradbury and Munroe, 1985; Cabrita et al., 1992; Elharrif et al., 1985; Engberg et al., 2001; Fallon et al., 2003; Frediani-Wolf and Stephan, 2003; Gaudreau and Gilbert, 1998; Hoge et al., 1998; Isenbarger et al., 2002; Jacobs-Reitsma et al., 1994; Karmali et al., 1981; Krausse and Ullmann, 2003; Lariviere et al., 1986; Ledergerber et al., 2003; Lekowska-Kochaniak et al., 1996; Li et
al., 1998; Luber et al., 2003b; Lucey et al., 2000; Michel et al., 1983; Nachamkin et al., 2000b; Narvarro et al., 1993; Prats et al., 2000; Rautelin et al., 1991; Reina et al., 1992; Saenz et al., 2000; Sagara et al., 1987; Sanchez et al., 1994; Sjogren et al., 1992; Smith et al., 2000; Talsma, 1999; Tajada et al., 1996; Taylor et al., 1987; Vanhoof et al., 1982; Van Looveren et al., 2001; Wang et al., 1984).

1.5.3 Mechanisms of Antimicrobial Resistance

As mentioned in the previous section, the prevalence of antimicrobial resistance in *Campylobacter* species has increased drastically over the last decade and this problem has been reported from many countries in every continent of the world. In addition, a large number of *Campylobacter* isolates from both humans and animals were found to be resistant not only to fluoroquinolones, but also to other classes of antibiotics. The mechanisms of antimicrobial resistance in thermophilic *Campylobacter* to those antibiotics including to fluoroquinolones are summarized in this section. More details on the mechanisms of antibiotic resistance in *Campylobacter* species can be obtained from the previous review articles by Taylor and Courvalin (1988) and Trieber and Taylor (2000).

Fluoroquinolone resistance in *Campylobacter* species is mainly due to point mutations in the *gyrA* gene, which encodes the A subunit of the DNA gyrase enzyme (Aarestrup and Engberg, 2001; Engberg et al., 2001; Gibreel et al., 1998; Luo et al., 2003; Piddock et al., 2003; Wang et al., 1993; Zhang et al., 2003; Zirnstein et al., 1999). The mutations occurred in the *gyrA* gene at specific positions such as Thr-86, Asp-90,
and Ala-70 were not only responsible for fluoroquinolone resistance in *Campylobacter* species, but the positions of these mutations also have direct effect on the level of fluoroquinolone resistance in *Campylobacter* isolates; for example, the Thr-86-Ile mutation was associated with high-level fluoroquinolone resistance, while the mutations occurred at other positions such as Asp-90-Asn, Ala-70-Thr, and Thr-86-Lys mutations were associated with lower level of fluoroquinolone resistance (Aarestrup and Engberg, 2001; Engberg et al., 2001; Gootz and Martin, 1991; Luo et al., 2003; Wang et al., 1993; Zhang et al., 2003). Besides the point mutations in the *gyrA* gene, an active multi-drug efflux pump (CmeABC) is also linked to fluoroquinolone resistance in *Campylobacter* isolates (Charvalos et al., 1995; Lin et al., 2002; Luo et al., 2003; Pumbwe and Piddock, 2002; Pumbwe et al., 2004; Zhang et al., 2003).

Although the mechanism of macrolide resistance can occur through different mechanisms such as target modification by mutation or methylation; enzymatic inactivation by different types of macrolide-inactivating enzymes such as esterases, hydrolases, and transferases; or active efflux system, the mechanism of macrolide resistance in *Campylobacter* species seems to be associated with an alteration of the target site on the 23S rRNA of *Campylobacter* ribosomes or mutations at positions 2074 and 2075 of the ribosomal protein genes (23S rRNA) (Aarestrup and Engberg, 2001; Engberg et al., 2001; Niwa et al., 2001; Payot et al., 2004; Schwarz and Chaslus-Dancla, 2001; Trieber and Taylor, 2000; Yan and Taylor, 1991).

In general, the mechanism of aminoglycoside resistance is mainly due to enzymatic inactivation by aminoglycoside-modifying enzymes. As a result, these
aminoglycoside antibiotics are not able to interact with the ribosome, which is the target site of this antibiotic group (Aarestrup and Engberg, 2001; Schwarz and Chaslus-Dancla, 2001; Shaw et al., 1993; Trieber and Taylor, 2000; Trieu-Cuot and Courvalin, 1986). The aminoglycoside-modifying enzymes can be divided into three different groups based on the reaction that they mediate (phosphorylation or adenylation/nucleotidylation of a hydroxyl group or acetylation of an amino group). Three different types of these aminoglycoside-modifying enzymes include aminoglycoside phosphotransferases (APH), aminoglycoside adenyltransferases or aminoglycoside nucleotidyltransferases (AAD or ANT) and aminoglycoside acetyltransferases (AAC) (Aarestrup and Engberg, 2001; Schwarz and Chaslus-Dancla, 2001; Shaw et al., 1993; Trieber and Taylor, 2000; Trieu-Cuot and Courvalin, 1986). Each enzyme is also divided into various subgroups depending on the site at which the antibiotic is modified; for example, APHs, which phosphorelate the hydroxyl groups (O-phosphotransferases) at positions 4, 6, 3’, 2”, 3”, and 5” will be recognized as APH(4), APH(6), APH(3’), APH(2”), APH(3”), and APH(5”), respectively, while AADs, which adenylate the hydroxyl groups (O-adenyltransferases) at positions 6, 9, 4’, 2”, and 3” will be recognized as AAD(6), AAD(9), AAD(4’), AAD(2”), and AAD(3”)(9) and AACs, which acetylate the amino groups (N-acetyltransferases) at positions 1, 3, 2’, and 6’ will be recognized as AAC(1), AAC(3), AAC(2’), and AAC(6’), respectively (Schwarz and Chaslus-Dancla, 2001; Shaw et al., 1993; Trieber and Taylor, 2000; Trieu-Cuot and Courvalin, 1986). Although several aminoglycoside-modifying enzymes are associated with aminoglycoside resistance in Campylobacter species, the most common enzyme that involves kanamycin
and structurally related antibiotics such as neomycin resistance in *Campylobacter* species is 3’-aminoglycoside phosphotransferase type III [APH(3’)-III], which is encoded by the *aphA*-3 gene (Aarestrup and Engberg, 2001; Gibreel et al., 2004a; Ouellette et al., 1987; Papadopoulou and Courvalin, 1988; Saenz et al., 2000; Sagara et al., 1987; Taylor and Courvalin, 1988; Tenover and Elvrum, 1988; Tenover et al., 1992; Trieber and Taylor, 2000; Trieu-Cuot and Courvalin, 1986). However, the *aphA*-3 gene is not the only kanamycin resistance gene found in *Campylobacter* species. Other genes such as *aphA*-7 and *aphA*-1 are also associated with a high-level of kanamycin resistance in *Campylobacter* and *Campylobacter*-like organisms, respectively (Gibreel et al., 2004a; Ouellette et al., 1987; Taylor and Courvalin, 1988; Tenover and Elvrum, 1988; Tenover et al., 1992). The *aphA*-1 gene, which encodes 3’-aminoglycoside phosphotransferase type I [APH(3’)-I] is chromosomally located, whereas the *aphA*-3 and *aphA*-7 genes are generally found on the plasmid. Although both *aphA*-3 and *aphA*-7 genes are located on the plasmid, the *aphA*-3 gene is frequently detected on large plasmids (41 to 132 kb) that also encode other resistance determinants particularly tetracycline resistance determinant (*tetO* gene), while the *aphA*-7 gene seems to be located on smaller plasmids (9.5 to 11.5 kb) that apparently do not encode other resistance traits (Gibreel et al., 2004a; Ouellette et al., 1987; Papadopoulou and Courvalin, 1988; Saenz et al., 2000; Sagara et al., 1987; Taylor and Courvalin, 1988; Tenover and Elvrum, 1988; Tenover et al., 1992). In addition, kanamycin resistance in *Campylobacter* species especially in *C. coli* can be mediated by 3’-aminoglycoside phosphotransferase type IV [APH(3’)-IV] enzyme as well (Aarestrup and Engberg, 2001; Rivera et al., 1986; Trieber and Taylor, 2000).
Currently, four different mechanisms associated with tetracycline resistance in bacteria have been reported including (i) an energy-dependent efflux system, which helps reduce the intracellular concentration of tetracycline by an integral membrane protein; (ii) ribosomal protection protein, which protects the ribosomal binding site of tetracycline; (iii) inactivation of tetracycline by modifying enzymes, which occurs rarely; and (iv) mutation of the 16S rRNA, which found in propionibacteria (Aarestrup and Engberg, 2001; Chopra et al., 1992; Chopra and Roberts, 2001; Roberts, 1996; Ross et al., 1998; Schnappinger and Hillen, 1996; Speer et al., 1992; Taylor and Chau, 1996; Trieber and Taylor, 2000). However, only a ribosomal protection protein is associated with tetracycline resistance in *Campylobacter* species (Aarestrup and Engberg, 2001; Trieber and Taylor, 2000). As mentioned earlier, the mechanism of tetracycline resistance in *Campylobacter* is primarily associated with tet(O) gene, which encodes a ribosomal protection protein designated as Tet(O) (Taylor and Courvalin, 1988; Trieber and Taylor, 2000). Tet(O) protein interacts directly with the ribosome, the target of tetracycline, and displaces tetracycline from its primary binding site on the ribosome; thus, protecting the ribosome from the inhibitory effect of tetracycline (Connell et al., 2002; Connell et al., 2003; Gibreel et al., 2004b; Manavathu et al., 1990; Pratt and Korolik, 2005; Trieber and Taylor, 2000). Although tet(O) gene is usually carried on transmissible plasmids (Aarestrup and Engberg, 2001; Gibreel et al., 2004b; Lee et al., 1994; Pratt and Korolik, 2005; Sagara et al., 1987; Taylor and Courvalin, 1988; Trieber and Taylor, 2000), it has been found to be chromosomally-located as well (Gibreel et al., 2004b; Lee et al., 1994; Pratt and Korolik, 2005).
The main resistance mechanism of *C. jejuni* and *C. coli* isolates to β-lactam antimicrobial agents such as ampicillin seems to be associated with the production of enzyme β-lactamases, which break the β-lactam ring of β-lactam antibiotics (Aarestrup and Engberg, 2001; Lachance et al., 1991; Lachance et al., 1993; Tajada et al., 1996; Trieber and Taylor, 2000). Although approximately 90% of *C. jejuni* and 70% of *C. coli* produce this type of enzyme, a large number of *Campylobacter* isolates were found to be susceptible to ampicillin (Lachance et al., 1991; Lachance et al., 1993; Lariviere et al., 1986; Tajada et al., 1996; Trieber and Taylor, 2000). In addition, several ampicillin-resistant *Campylobacter* strains, on the other hand, were found to be negative for β-lactamase production (Saenz et al., 2000). This information indicated that other mechanisms of resistance such as alteration of penicillin-binding proteins or decreased permeability of the drug through modification of porins could be involved with ampicillin resistance in *Campylobacter* species as well (Aarestrup and Engberg, 2001; Saenz et al., 2000; Tajada et al., 1996; Trieber and Taylor, 2000).

Although the mechanism of multidrug resistance in *Campylobacter* species has not yet been clearly identified, it appears that the multidrug resistance mechanism in *Campylobacter* species seems to be associated with the presence of an active efflux system (Charvalos et al., 1995; Lin et al., 2002; Pumbwe and Piddock, 2002; Pumbwe et al., 2004; Trieber and Taylor, 2000).
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CHAPTER 2

THE EFFECT OF DIFFERENT POULTRY PRODUCTION PRACTICES ON ANTIBIOTIC RESISTANCE RATES OF \textit{CAMPYLOBACTER} IN POULTRY

2.1 ABSTRACT

Intestinal tracts of broilers and turkeys from 10 conventional poultry farms, where antibiotics were routinely used, and from 5 organic poultry farms, where antibiotics had never been used, were collected and cultured for \textit{Campylobacter} species. A total of 694 \textit{Campylobacter jejuni} and \textit{Campylobacter coli} isolates from the conventional and organic poultry operations were tested for antimicrobial resistance to nine antimicrobial agents by the agar dilution method. Although \textit{Campylobacter} spp. were highly prevalent in both conventional and organic poultry operations, the antibiotic resistance rates of the isolates were significantly different between the organic operations and the integrated conventional operations. Less than 2\% of \textit{Campylobacter} strains isolated from organically-raised poultry were resistant to fluoroquinolones, while 46\% and 67\% of \textit{Campylobacter} isolates from conventionally-raised broilers and turkeys were resistant to these antimicrobials. In addition, a high frequency of resistance to erythromycin (79.60\%), clindamycin (64.18\%), kanamycin (76.12\%), and ampicillin (31.34\%) was observed among \textit{Campylobacter} isolates from conventionally-raised turkeys. None of the
Campylobacter isolates obtained in this study was resistant to gentamicin, while a large number of the isolates from both conventional and organic poultry operations were resistant to tetracycline. Multidrug resistance was mainly observed among Campylobacter strains isolated from conventional turkey operation (81.09%). Findings from this study clearly indicate that different production practices significantly influence the antibiotic resistance rates of Campylobacter on poultry farms, which is likely due to the result of antimicrobial usage in conventional poultry production.

2.2 INTRODUCTION

Food-borne campylobacteriosis, a major public health concern in the United States and many countries worldwide, is caused mainly by Campylobacter jejuni (Mead et al., 1999). It is estimated that over 2 million cases of food-borne bacterial diarrhea that occur each year in the United States are caused by Campylobacter (Altekruse et al., 1999). In other industrialized countries, the numbers of Campylobacter infections exceeded those of Salmonella, Shigella, and E. coli 0157:H7 infections combined (Allos, 2001). Campylobacter jejuni is not only an important cause of bacterial gastroenteritis in humans, but it has also been associated with Guillane-Barré Syndrome (GBS), an acute immune-mediated demyelinating disorder of the peripheral nervous system (Blaser, 1997; Nachamkin et al., 1998).

Most Campylobacter infections in humans usually occur as sporadic cases and are associated with the ingestion of contaminated or improperly handled/cooked foods as well as milk or dairy products (Blaser, 1997; Altekruse and Tollefson, 2003). However,
consumption of undercooked poultry and/or other foods that are cross-contaminated with raw poultry meat during food preparation is considered a major risk factor for food-borne campylobacteriosis (Altekruse and Tollefson, 2003). As a commensal organism, thermophilic Campylobacter spp. including C. jejuni and C. coli are highly prevalent in chickens and turkeys (Newell and Fearnley, 2003; Newell and Wagenaar, 2000; Sahin et al., 2002). Due to the large number of Campylobacter present in feces, contamination of chicken carcasses by Campylobacter in slaughter houses is extensive and unavoidable, resulting in the potential transmission of Campylobacter from contaminated chicken meats to consumers.

Although most Campylobacter infections are mild, self-limiting, and usually resolve within a few days without any antimicrobial therapy, severe or prolonged infections can also occur under certain circumstances, in which an appropriate antimicrobial treatment is usually warranted (Blaser, 1997; Engberg et al., 2001). For instance, antibiotic therapy may be life saving and is required in invasive and/or systemic infections particularly in infants, elderly, or immunocompromised persons. Among a variety of antibiotics that are generally prescribed for treatment of bacterial gastrointestinal infections in human, macrolides (e.g. erythromycin) and fluoroquinolones (e.g. ciprofloxacin) are considered to be the drugs of choice for treatment of food-borne campylobacteriosis (Allos, 2001). However, these antibiotics are also used for treatment of diseases in livestock and poultry (Khachatourians, 1998; McEwen and Fedorka-Cray, 2002; Van den Bogaard and Stobberingh, 1999). Because of the widespread use of antimicrobial agents in humans and animals, the emergence of antimicrobial resistance
has increased dramatically over the last decade (Khachatourians, 1998; Threlfall et al., 2000; White et al., 2002). Antimicrobial-resistant *Campylobacter* strains isolated from humans and animals were reported in many countries worldwide (Engberg et al., 2001; Nachamkin et al., 2000b; Smith et al., 2000).

In the United States, the emergence of fluoroquinolone resistance among *Campylobacter* isolates was first noticed about 10 years ago. Prior to 1992 there were no reports of fluoroquinolone-resistant *Campylobacter* strains isolated from humans in the U.S. (Nachamkin et al., 2002). However, the prevalence of fluoroquinolone resistance among these *Campylobacter* isolates increased significantly from 1.3% in 1992 to 8% - 13% during 1996 - 1998 and this resistance trend has increased steadily since 1998 (Gupta et al., 2004; Nachamkin et al., 2002; Smith et al., 1999). In 2001, the National Antimicrobial Resistance Monitoring System (NARMS) and Nachamkin et al. (2002) found that about 19% - 40% of *Campylobacter* strains isolated from humans were resistant to ciprofloxacin (Gupta et al., 2004; Nachamkin et al., 2002). In other areas of North America, such as Canada, no fluoroquinolone resistance was observed in *Campylobacter* isolates during 1985 to 1986 (Gaudreau and Gilbert, 1998). However, in 1992 - 1997, 3.5% - 13.6% of *Campylobacter* isolates tested were resistant to fluoroquinolones (Gaudreau and Gilbert, 1998). In 1998-2000, the rate of ciprofloxacin resistance among *C. jejuni* in Canada rose to 10% - 27% and drastically increased to 47% in 2001 (Gaudreau and Gilbert, 2003).

In other regions of the world, the prevalence of fluoroquinolone resistance in *Campylobacter* strains isolated from humans and animals particularly poultry also
increased remarkably over the past decade (Engber et al., 2001; Nachamkin et al., 2000b). In Europe, the fluoroquinolone resistance rates among *Campylobacter* isolates increased from less than 3% before 1988 to more than 95% in some European countries during the late 1990s and the early twenty-first century (Avrain et al., 2001; Engber et al., 2001; Luber et al., 2003b; Nachamkin et al., 2000b; Saenz et al., 2000; Van Looveren et al., 2001). In Africa, fluoroquinolone-resistant *Campylobacter* strains isolated from humans increased from 13% in 1995 to 28% in 1996 to 35% in 1997 and to 48% - 50% in 1998 – 2000 (Putnam et al., 2003). In Asia, particularly in East and Southeast Asia, the fluoroquinolone resistance among *Campylobacter* strains isolated from humans and animals also remarkably increased. Since 1993, approximately 50% to 90% of *Campylobacter* isolates from this region were resistant to ciprofloxacin (Engberg et al., 2001; Li et al., 1998; Nachamkin et al., 2000b). Although the emergence of fluoroquinolone resistance in *Campylobacter* isolates was also observed in Australia, the percentage of fluoroquinolone-resistant *Campylobacter* isolates was very low when compared to the isolates obtained from other countries (Sharma et al., 2003).

Despite these advances in understanding the epidemiology of antibiotic-resistant *Campylobacter*, relatively little is known about the impact of different food animal production practices on the prevalence of antibiotic-resistant *Campylobacter*. Since contaminated poultry are considered an important source of *Campylobacter* infection in humans and the demand for organic animal produce is increasing in the U.S., we conducted a farm-based study to determine the antimicrobial resistance rates in *Campylobacter* isolates from both conventionally-raised and organically-raised broilers.
and turkeys. It is found that *Campylobacter* is highly prevalent in both organic and conventional production systems. However, the antibiotic resistance rates are significantly higher in conventional operations than in organic operations. This study also revealed that antibiotic-resistant *Campylobacter* strains are stable and can persist in the absence of antibiotic usage.

2.3 MATERIALS AND METHODS

**Sample collection.** This study focused on the prevalence of antibiotic-resistant *Campylobacter* in slaughter-age birds. The surveyed farms included 10 integrated conventional broiler operations, 10 integrated conventional turkey operations, 5 organic broiler farms, and 5 organic turkey farms. Intestinal samples were collected from slaughter houses during August 2000 to November 2002. At least 30 intestines from each conventional poultry farm and approximately 60 intestines from each organic poultry farm were collected at each collection period. A total of 345, 360, 355, and 230 intestinal tracts were obtained from conventional broilers, conventional turkeys, organic broilers, and organic turkeys, respectively.

According to the farmers, no antimicrobial agents were used in organic broiler or turkey operations from which the samples were collected. In contrast, antimicrobial agents were used in almost every conventional poultry farm. For conventionally-raised broilers, gentamicin was the most commonly used antibiotic. This antimicrobial agent was given to the birds at the hatchery to prevent early mortality due to *E. coli* infections. Lincomycin and amprolium were also used to control necrotic enteritis and coccidiosis in some
conventional broiler farms, respectively. In addition, bacitracin and virginiamycin, which were supplemented in broiler feed at sub-therapeutic levels in order to promote growth and improve feed efficiency, were also used in the conventional broiler farms in this study. For the conventional broiler flocks surveyed in this study, the birds were not exposed to treatments with fluoroquinolone antibiotics during the production period according to information obtained from the producers. For conventionally-raised turkeys, enrofloxacin was the drug routinely used for flocks with respiratory disease due to *E. coli* infections, while chlortetracycline was used only in the farms that had fowl cholera problem. Like conventional broiler production system, bacitracin was also used as feed additive for conventionally-raised turkeys.

**Bacterial isolation and identification.** The intestinal tracts were placed on ice and brought back to the laboratory within 3 hours of collection and cultured for *C. jejuni* and *C. coli*. Each intestine was aseptically opened and the cecal contents were directly streaked onto Campy CVA agar (BBL Becton Dickinson Microbiology Systems, Cockeysville, MD). The inoculated plates were then incubated at 42 °C for 48 hours under a microaerophilic environment (approximately 5% O2, 10% CO2, and 85% N2) in a CampyPak II anaerobic system jar with CampyPak gas generating system envelopes (BBL Becton Dickinson Microbiology Systems, Sparks, MD). Suspect *Campylobacter* colonies were identified by colony morphology characteristics, Gram-stain, oxidase test, catalase test, and *Campylobacter* culture-plate latex agglutination confirmation test (INDX-Campy [jel]; PanBio INDX, Inc., Baltimore, MD). In addition, the hippurate hydrolysis test was also
performed to differentiate between *C. jejuni* and *C. coli*. From each *Campylobacter*-positive sample, a single colony was used for antibiotic susceptibility tests. All *Campylobacter* isolates were stored in sterile cryovial tubes containing skim milk and 30% glycerol at –85 °C prior to antimicrobial susceptibility test.

**Antimicrobial susceptibility testing.** A total of 694 *C. jejuni* and *C. coli* isolates from conventional and organic poultry farms were tested for antimicrobial resistance to nine antimicrobial agents including ampicillin, tetracycline, gentamicin, kanamycin, clindamycin, erythromycin, ciprofloxacin, norfloxacin, and nalidixic acid by the agar dilution method (NCCLS, 2002a). All antimicrobial agents were obtained from Sigma Chemical Co., St. Louis, MO except ciprofloxacin (Serologicals Proteins, Inc., Kankakee, IL). The concentrations of most antimicrobial agents tested in this study ranged from 0.06 to 128 µg/ml except for ciprofloxacin (0.008 to 128 µg/ml) and for kanamycin and nalidixic acid (0.25 to 128 µg/ml) (Table 2.1). Briefly, *Campylobacter* isolates, grown on blood agar plates for at least 24 hours, were inoculated onto Mueller-Hinton (MH) broth (BBL Becton Dickinson Microbiology Systems, Cockeysville, MD) and then adjusted to a turbidity equivalent to 0.5 McFarland standard by a colorimeter (BioMérieux, Inc., Hazelwood, MO). A multipoint inoculator (a cathra replicator system) with 1 mm pins (Oxoid, Inc., Ogdensburg, NY) was used to inoculate approximately 10^4 CFU of samples onto Mueller-Hinton agar (BBL Becton Dickinson Microbiology Systems, Cockeysville, MD) containing a two-fold concentration series of antibiotics and supplemented with 5% defibrinated sheep blood (Cleveland Scientific, Cleveland, OH). *Campylobacter jejuni* ATCC 33560 was used
as the quality control organism. While QC ranges are not currently available for ampicillin, kanamycin, clindamycin, and norfloxacin, the MIC results for these drugs with \textit{C. jejuni} ATCC 33560 were consistent, falling within a three-dilution range throughout the study. The inoculated plates were incubated in a Forma Series II water-jacketed CO$_2$ incubator (Thermo Electron Corporation, Marietta, OH) at 42 °C for 24 hours under a microaerobic atmosphere of 5% O$_2$, 10% CO$_2$, and 85% N$_2$. The MIC was defined as the lowest concentration of antimicrobial agent that completely inhibited the visible growth on the plates. The resistance breakpoints for each antimicrobial agent were as follows: $\geq$ 4 µg/ml for ciprofloxacin and clindamycin; $\geq$ 8 µg/ml for erythromycin; $\geq$ 16 µg/ml for tetracycline, gentamicin, and norfloxacin; $\geq$ 32 µg/ml for ampicillin and nalidixic acid; and $\geq$ 64 µg/ml for kanamycin (Table 2.1) (CDC, 2003; NCCLS, 2002a; NCCLS, 2002b). If an isolate was resistant to three or more classes of antibiotics, it was defined as multidrug resistance.

\textbf{Pulsed-field gel electrophoresis (PFGE).} To determine the genetic diversity of \textit{Campylobacter} isolates from different poultry production systems, sample preparation and PFGE were performed using the protocol described previously (Huang et al., 2005). Restriction enzyme \textit{KpnI} was used for digestion of the gel plugs.

\textbf{Statistical analysis.} A Chi-square ($\chi^2$) test at a significance level of $P < 0.05$ (two-tailed) with Yates correction for continuity was used for statistical analysis of the data.
2.4 RESULTS

Prevalence of *Campylobacter*. From 345 intestinal tracts of conventionally-raised broilers, *Campylobacter* were obtained from 227 (65.80%) intestines. Among these 227 *Campylobacter* isolates, 220 (96.92%) and 7 (3.08%) were identified as *C. jejuni* and *C. coli*, respectively, on the basis of the hippurate hydrolysis test. For conventionally-raised turkeys, 299 (83.06%) *Campylobacter* isolates were obtained from 360 intestines. One hundred and thirty-seven (45.82%) isolates were classified as *C. jejuni*, while 162 (54.18%) isolates were classified as *C. coli*. In terms of the organic poultry production systems, a total of 317 (89.30%) and 201 (87.39%) *Campylobacter* isolates were obtained from 355 and 230 intestinal tracts of organically-raised broilers and turkeys, respectively. Two hundred and twenty-nine (72.24%) and 88 (27.76%) *Campylobacter* isolates from organically-raised broilers were identified as *C. jejuni* and *C. coli*, respectively, whereas 133 (66.17%) *Campylobacter* isolates from organically-raised turkeys were identified as *C. jejuni* and 68 (33.83%) were identified as *C. coli*. Although *Campylobacter* spp. could be isolated from every conventional and organic poultry farm, the prevalence of this organism varied among farms. For conventional production systems, the prevalence of *Campylobacter* spp. in conventional broiler farms ranged from 44% to 80%, while the prevalence of this organism in conventional turkey farms ranged from 63.33% to 97.78%. Likewise, the prevalence of *Campylobacter* spp. ranged from 69.57% to 100% in organic broiler farms and 5.56% to 100% in organic turkey farms.
**Antimicrobial susceptibility patterns.** The MIC distributions of ampicillin, tetracycline, gentamicin, kanamycin, clindamycin, erythromycin, ciprofloxacin, norfloxacin, and nalidixic acid against *C. jejuni* and *C. coli* isolates were summarized in Table 2.2 and Table 2.3, respectively. In general, a wider range of MICs were observed with isolates from conventional farms than from organic farms, except gentamicin, for which the MIC distributions of *Campylobacter* strains isolated from both types of productions were comparable. The MICs at which 50% and 90% of *Campylobacter* isolates were inhibited were shown in Table 2.4. The MIC$_{90}$ of fluoroquinolones (ciprofloxacin and norfloxacin) for the *Campylobacter* strains isolated from organic poultry farms were 4 to 16 times lower than the resistance breakpoints (Table 2.4). On the contrary, the MIC$_{90}$ of fluoroquinolones for *Campylobacter* strains isolated from conventional poultry farms were 8 times higher than the resistance breakpoints (Table 2.4). Among *Campylobacter* strains isolated from organic poultry farms, the MIC$_{50}$ and MIC$_{90}$ of erythromycin against *C. coli* isolates were slightly higher than those against *C. jejuni* isolates, while the MIC$_{50}$ and MIC$_{90}$ of clindamycin against *C. coli* isolates were similar to those against *C. jejuni* isolates (Table 2.4). In contrast, the MIC$_{50}$ of erythromycin and clindamycin against *C. coli* isolated from conventional poultry farms were more than 32-fold higher than those against *C. jejuni* isolates from the same operation types (> 128 µg/ml versus 2 µg/ml for erythromycin and 32 µg/ml versus 1 µg/ml for clindamycin). For the aminoglycoside, *Campylobacter* strains isolated from both conventional and organic poultry operations were uniformly susceptible to gentamicin with MIC$_{50}$ and MIC$_{90}$ ≤ 1 µg/ml. Interestingly, the MIC$_{50}$ and MIC$_{90}$ of
kanamycin (8 µg/ml and > 128 µg/ml, respectively) against C. jejuni isolates from both conventional and organic poultry farms were the same, while the MIC<sub>50</sub> against C. coli isolates from conventional poultry farms was more than 16 times higher than that against the C. coli isolates from organic poultry farms (> 128 µg/ml versus 8 µg/ml). In this study, tetracycline showed poor activity against Campylobacter strains isolated from both conventional and organic poultry farms, although the MIC<sub>50</sub> of tetracycline against the Campylobacter isolates (both C. jejuni and C. coli) from conventional farms was 4 times higher than that against the isolates from organic farms (Table 2.4). In terms of β-lactam antibiotics, ampicillin showed moderate activity against Campylobacter isolates from conventionally-raised and organically-raised poultry. For C. jejuni, the MIC<sub>50</sub> and MIC<sub>90</sub> of ampicillin against the isolates from conventional farms were two times higher than those against the isolates from organic farms, while the MIC<sub>50</sub> and MIC<sub>90</sub> against C. coli isolates from conventional farms were 2- and 8-fold higher, respectively, in comparison with the organic farms.

**Antimicrobial resistance rates.** The resistance rates of the Campylobacter strains were tabulated according to Campylobacter species (C. jejuni and C. coli), production types (conventional and organic), and poultry species (broiler and turkey) and were summarized in Tables 2.5 – 2.8. One of the most striking findings in this study was the difference in fluoroquinolone resistance between Campylobacter strains isolated from conventional poultry farms and organic poultry farms. Approximately 46% of Campylobacter strains isolated from conventionally-raised broilers and 67% of
Campylobacter strains isolated from conventionally-raised turkeys were resistant to ciprofloxacin, norfloxacin, and nalidixic acid. In contrast, none of Campylobacter strains isolated from organically-raised broilers and less than 2% of Campylobacter strains isolated from organically-raised turkeys were resistant to these antibiotics (Table 2.8). Besides the high resistance rates to fluoroquinolones, Campylobacter strains isolated from conventional turkey farms were also highly resistant to erythromycin (79.60%) and clindamycin (64.18%) (Table 2.8). Among C. coli isolated from conventionally-raised turkeys, 114 (90.48%) and 93 (73.81%) were resistant to erythromycin and clindamycin, respectively (Table 2.6). When compared to Campylobacter strains isolated from other poultry production systems, the isolates from conventional turkey operations were significantly more resistant to erythromycin and clindamycin (P < 0.001). Regardless of the sources of isolation, none of the Campylobacter strains tested in this study were resistant to gentamicin. However, 11.38% of Campylobacter strains isolated from conventionally-raised broilers and 76.12% of Campylobacter strains isolated from conventionally-raised turkeys were resistant to kanamycin. Interestingly, 16.97% and 31.06% of Campylobacter strains isolated from organically-raised broilers and turkeys were also resistant to this antimicrobial agent (Table 2.8). A high level of tetracycline resistance was observed among the C. jejuni and C. coli isolates, with resistance rates ranging from 50% - 92% in various production types (Table 2.8). For ampicillin, the resistance rates of C. jejuni and C. coli varied among the operation types, with the resistance rate in Campylobacter strains isolated from conventional turkey farms significantly higher (P < 0.001) than the resistance rates found in Campylobacter strains
isolated from other production systems (Table 2.8). Overall, the antimicrobial resistance rates in *Campylobacter* isolates from organic poultry farms were significantly lower than those from conventional poultry farms and the highest antibiotic resistance rates were observed in conventional turkey operation (Table 2.8).

**Multidrug resistance.** The occurrence of multidrug-resistant *Campylobacter* strains was mainly observed in strains from conventionally-raised turkeys, with 81.09% of these isolates showing resistance to three or more classes of antibiotics (Table 2.9). In comparison with the conventional turkey production, the multidrug resistance rates were significantly lower (P < 0.001) in *Campylobacter* strains isolated from other poultry production systems. Among multidrug-resistant *Campylobacter* strains isolated from conventionally-raised turkeys, 77 isolates (47.24%) were resistant to tetracycline, kanamycin, clindamycin, erythromycin, ciprofloxacin, norfloxacin, and nalidixic acid (Table 2.10). In addition, 117 (58.21%) of *Campylobacter* strains isolated from this operation type were also resistant to both erythromycin and ciprofloxacin, the preferred antimicrobials for the treatment of human campylobacteriosis, whereas none of *Campylobacter* strains isolated from conventionally-raised broilers and organically-raised broilers and turkeys were concomitantly resistant to both of these antibiotics.

**PFGE profiles.** Representative *C. jejuni* and *C. coli* isolates from different poultry operation types were analyzed by PFGE to determine the genetic diversity of the isolates. The PFGE patterns generated by *KpnI* digestion showed great differences among the
strains isolated from different farms, while the isolates from a single poultry farm tended to have similar or identical PFGE patterns (Fig. 2.1). Interestingly, a similar PFGE type was observed in isolates from two different conventional broiler farms, H (Lane 5; Fig. 2.1) and J (Lane 6 and 7; Fig. 2.1), indicating that both farms harbored a genetically-related *Campylobacter* strain. This PFGE result revealed diverse genotypes among *Campylobacter* species isolated from the conventional and organic poultry operations.

### 2.5 DISCUSSION

In this study, it is clearly shown that thermophilic *Campylobacter* is highly prevalent in both organic and conventional poultry production systems. However, the antimicrobial resistance rates vary significantly in different production types. In general, the conventional broiler and turkey farms harbor more antibiotic-resistant *Campylobacter* strains than organic poultry farms and the differences are especially obvious with fluoroquinolones. Little resistance was detected in *Campylobacter* strains isolated from organic poultry farms, whereas large numbers (45% - 67%) of the isolates from conventional broiler and turkey farms were resistant to these antimicrobials. Multidrug resistance to three or more classes of antibiotics in *Campylobacter* is high among the isolates from conventional turkey operations, which may be explained by relatively more antibiotic usage in turkey operations than in broiler operations due to the long production cycle of meat turkey and the need for more frequent antibiotic treatments of diseases.

The prevalence of *Campylobacter* species was significantly higher in organically-raised broilers than in conventionally-raised broilers (P < 0.001), while the prevalence of
this organism in organically-raised turkeys was not significantly different from that in conventionally-raised turkeys ($P = 0.19$). The high frequency of *Campylobacter* strains isolated from organic broiler production system seems to be associated with the increased age of the birds at slaughter since the average age of these organically-raised broilers at slaughter were about 8 to 12 weeks old compared to 6 weeks old for conventionally-raised broilers. On the other hand, both conventionally-raised and organically-raised turkeys were sent to the processing plant around the same age (18 to 20 weeks). This may explain why the prevalence of *Campylobacter* strains isolated from conventional and organic turkey farms was not significantly different. The association between *Campylobacter* colonization and age of the birds at slaughter was also noted by other studies (Berndtson et al., 1996b; Evans and Sayers, 2000; Newell and Wagenaar, 2000; Northcutt et al., 2003), which indicated that prevalence of *Campylobacter* in poultry elevated when the age of birds at the processing plant increased. In terms of the high prevalence of *Campylobacter* in organic poultry production systems, similar findings were also noted by other studies conducted in free-range and/or organic poultry operations (Avrain et al., 2003; Heuer et al., 2001; Kazwala et al., 1993).

Among *Campylobacter*-positive flocks, *C. jejuni* was the predominant species in both conventional and organic broiler production systems especially in conventional broiler farms, where almost 97% of *Campylobacter* isolates from our study were *C. jejuni*. This finding is in accordance with previous studies by other research groups, who reported that 85% to 98% of conventional broiler flocks were colonized by *C. jejuni* (Avrain et al., 2003; Berndtson et al., 1996b; Evans and Sayers, 2000; Hald et al., 2000;
Heuer et al., 2001; Jorgensen et al., 2002; Nielsen et al., 1997; Wedderkopp et al., 2000).

In this study, the distribution of *C. jejuni* and *C. coli* in conventional turkey production system is remarkably different from that in conventional broiler production system. About 46% of *Campylobacter* isolates from conventionally-raised turkeys were *C. jejuni*, whereas 54% of these isolates were *C. coli*. This finding is different from the previous study by Wallace et al. (1998), who reported that almost 100% of *Campylobacter* isolates from conventional turkey flocks were *C. jejuni*. In contrast, Smith et al. (2004) revealed that 80% - 90% of *Campylobacter* strains that colonized turkey flocks were *C. coli*. For organic poultry production systems, the proportion of *C. jejuni* and *C. coli* among *Campylobacter* isolates from organic broiler and organic turkey farms was not significantly different from each other (P = 0.171), with *C. jejuni* as the predominant *Campylobacter* species.

Although no fluoroquinolones were used in the conventional broiler flocks from which the samples were collected, we found that about 46% of *Campylobacter* isolates from conventionally-raised broilers were resistant to these antimicrobial agents (Table 2.8). The high resistance rate to fluoroquinolones may be associated with the previous use of these antibiotics on the broiler farms. Several studies have shown that *Campylobacter* displays a hypermutable phenotype to fluoroquinolone treatment in chickens and the treatment results in rapid occurrence of fluoroquinolone-resistant *Campylobacter* in the treated chickens (Griggs et al., 2005; Jacobs-Reitsma et al., 1994b; Luo et al., 2003; McDermott et al., 2002). Epidemiological surveys also suggested that certain quinolone-resistant clones were stable and able to persist on the farms during
several rotations even there had been no selective pressure on that farm for a long period of time (Pedersen and Wedderkopp, 2003; Price et al., 2005). Using clonally-related isolates and isogenic mutants, Luo et al. (2005) recently demonstrated that fluoroquinolone-resistant *Campylobacter* persisted and outcompeted fluoroquinolone-susceptible *Campylobacter* in the absence of antibiotic usage. Together, these findings underscore the difficulty in eliminating fluoroquinolone-resistant *Campylobacter* from poultry production. Since no fluoroquinolones were used in organic poultry operations, it is not surprising that there were little or no resistance to this class of antibiotics in *Campylobacter* strains isolated from organic poultry farms. The few fluoroquinolone-resistant *Campylobacter* isolates in organic turkey operation were likely the result of the transmission of fluoroquinolone-resistant *Campylobacter* from other sources.

In this study, a high level of erythromycin resistance was mainly observed in *Campylobacter* strains isolated from conventional turkey farms (Table 2.8). About 61% of *C. jejuni* and 90% of *C. coli* isolates from conventionally-raised turkeys were resistant to erythromycin (Table 2.6). This finding is consistent with the previous study by Ge et al. (2003), who reported that erythromycin resistance rates among *Campylobacter* strains isolated from processed turkeys were significantly higher than those from chickens. Consistent with findings in previously published work (Avrain et al., 2003; Ge et al., 2003; Li et al., 1998; Saenz et al., 2000; Van Looveren et al., 2001), erythromycin resistance was much more common in *C. coli* than in *C. jejuni* (Table 2.7). In addition, almost 80% of erythromycin-resistant *Campylobacter* isolates were also resistant to clindamycin, while all but two clindamycin-resistant *Campylobacter* strains were
resistant to erythromycin. A co-resistance between these two antibiotics was also observed in previous reports by others (Li et al., 1998; Saenz et al., 2000; Taylor and Courvalin, 1988). Similar to erythromycin, the prevalence of clindamycin resistance in our study was mainly observed among *C. coli* especially the isolates from conventionally-raised turkeys.

Interestingly, none of *C. jejuni* and *C. coli* isolates from both conventionally-raised and organically-raised broilers and turkeys in our study was resistant to gentamicin. This finding is in agreement with the previous studies by other research groups, who reported that no gentamicin resistance was observed among poultry isolates (Aarestrup et al., 1997; Li et al., 1998; Luber et al., 2003b). However, in some countries such as Spain, about 25% of *Campylobacter* strains isolated from broilers were resistant to this antimicrobial (Saenz et al., 2000). Although gentamicin was the most commonly used antibiotic in the conventional broiler production system in this study, it was given to the birds at the hatchery by subcutaneous injection in the neck region. Since *Campylobacter* is usually not present in the intestinal tracts of the birds during the first week of life, the injection of gentamicin may have very limited impact on selection of gentamicin resistance in *Campylobacter*. In terms of other aminoglycoside antibiotics, we found that *Campylobacter* isolates particularly the ones from turkeys appeared to be highly resistant to kanamycin (Table 2.8). In addition, we also noticed that kanamycin resistance seemed to be more prevalent among *C. coli* than *C. jejuni* (Table 2.7). Although 16.97% and 31.06% of *Campylobacter* strains isolated from organic broiler and turkey operations were resistant to kanamycin (Table 2.8), the resistant isolates were
limited only two organic poultry operations. Similarly, all kanamycin-resistant
Campylobacter isolates from conventionally-raised broilers were from a single
conventional broiler farm. In contrast, kanamycin-resistant Campylobacter was common
on different conventional turkey farms. The reason for this skewed distribution of
kanamycin-resistant Campylobacter on conventional broiler and organic poultry farms is
unclear at this stage.

High levels of tetracycline resistance among Campylobacter isolates were
observed from both conventionally-raised and organically-raised broilers and turkeys
(Table 2.8). Although tetracycline had never been used in those organic farms,
tetracycline-resistant Campylobacter strains were present in almost every organic poultry
farm surveyed in this study. This widespread distribution of tetracycline resistance is
different from that of kanamycin resistance for which only some farms showed resistant
strains. Since tetracyclines have been used as feed additives for livestock and poultry for
both therapeutic and subtherapeutic purposes for long period of time (Chopra and
Roberts, 2001; Fallon et al., 2003; Trieber and Taylor, 2000), it is possible that
Campylobacter may have evolutionally become resistant to this class of antibiotic,
leading to the widespread distribution of tetracycline-resistant Campylobacter in animal
reservoirs regardless of the production types. The high prevalence of tetracycline-
resistant Campylobacter in poultry was also reported in other studies (Aarestrup et al.,
1997; Avrain et al., 2001; Fallon et al., 2003; Li et al., 1998; Luber et al., 2003b; Saenz et
al., 2000; Van Looveren et al., 2001). For human Campylobacter isolates, the frequency
of tetracycline resistance showed a wide variation in different geographical regions ranging from 11% in Denmark to 95% in Taiwan (Aarestrup et al., 1997; Gaudreau and Gilbert, 2003; Li et al., 1998; Luber et al., 2003b).

The ampicillin resistance rates reported in this study are in agreement with the resistance rates reported by other studies (Aarestrup et al., 1997; Avrain et al., 2001; Fallon et al., 2003; Luber et al., 2003b). It is noticed that ampicillin resistance is more prevalent in conventional turkey operations than in other production types (Table 2.8). In addition, it appears that ampicillin resistance is more common in *C. jejuni* than in *C. coli* and more prevalent among turkey isolates than broiler isolates (Table 2.5 – 2.6).

This study also revealed a high frequency of multidrug resistance (81.09%) among *Campylobacter* strains isolated from conventionally-raised turkeys. On the other hand, less than 10% of *Campylobacter* strains isolated from other poultry production systems were resistant to three or more classes of antibiotics (Table 2.9). Multidrug-resistant strains among *Campylobacter* isolates from conventionally-raised turkeys were also reported by Lee et al. (2005). Interestingly, none of *Campylobacter* isolates from conventionally-raised broilers and organically-raised broilers and turkeys were resistant to both macrolides (e.g. erythromycin) and fluoroquinolones (e.g. ciprofloxacin), whereas more than 58% of *C. jejuni* and *C. coli* isolates from conventionally-raised turkeys were concomitantly resistant to these antibiotics. If these resistant turkey isolates transmit to humans, the concomitant resistance of the isolates to both macrolide and fluoroquinolone can potentially compromise the efficacy of antibiotic treatment because erythromycin and fluoroquinolones are the drugs of choice for the treatment of food-borne
campylobacteriosis in humans (Allos, 2001). Although the exact reason for multidrug resistance in *Campylobacter* isolated from conventionally-raised turkeys is unknown, it is possible that this phenotype is associated with the frequent antibiotic usage in turkey production.

In summary, this study revealed significant differences in antimicrobial-resistant *Campylobacter* in different poultry production systems. The results suggest that the practice of antimicrobial usage in poultry production systems influences the prevalence of antibiotic-resistant *Campylobacter* in poultry. However, antimicrobial usage alone may not solely be responsible for the increased antibiotic resistance in *Campylobacter* because even in the absence of antibiotic exposure, a high level of tetracycline resistance was observed in organic poultry. Similarly, the resistance rates to fluoroquinolones were also high in the surveyed conventional broiler flocks which did not get exposed to the class of antibiotics during the entire production period. These observations suggest that antibiotic-resistant *Campylobacter* are stable and able to transmit and persist in poultry even in the absence of selection pressure. Together, these findings reveal the complex nature in the spread of antibiotic resistance and further highlight the need for prudent measures to prevent the occurrence and transmission of antibiotic-resistant *Campylobacter* in the poultry reservoir.
2.6 ACKNOWLEDGEMENT

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2.7 REFERENCES


<table>
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<tr>
<th>Antimicrobial agents</th>
<th>Agar dilution test range (µg/ml)</th>
<th>MIC quality control ranges of <em>C. jejuni</em> ATCC 33560&lt;sup&gt;e&lt;/sup&gt; (µg/ml)</th>
<th>MIC breakpoints&lt;sup&gt;e&lt;/sup&gt; (µg/ml)</th>
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<sup>a</sup> MIC breakpoints for enteric bacteria used by the National Antimicrobial Resistance Monitoring System (NARMS)

<sup>b</sup> MIC breakpoints for Enterobacteriaceae recommended by the National Committee for Clinical Laboratory Standard (NCCLS)

<sup>c</sup> Tentative agar dilution quality control ranges of *C. jejuni* ATCC 33560 approved by the NCCLS

<sup>d</sup> No data available

<sup>e</sup> S, susceptible; I, intermediate; R, resistant

Table 2.1 Antimicrobial test ranges, MIC quality control ranges, and MIC breakpoints used for antimicrobial susceptibility testing
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* Thin vertical lines indicate the breakpoint between susceptible and intermediate strains. Thick vertical lines indicate the breakpoint between intermediate and resistant strains (except for Nalidixic acid that indicates between susceptible and resistant strains).

b C. jejuni isolates from conventional poultry farms (n = 240)

c C. jejuni isolates from organic poultry farms (n = 211)

Table 2.2 MIC distributions of C. jejuni isolated from conventional and organic poultry farms
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</table>

a Thin vertical lines indicate the breakpoint between susceptible and intermediate strains. Thick vertical lines indicate the breakpoint between intermediate and resistant strains (except for Nalidixic acid that indicates between susceptible and resistant strains).
b C. coli isolates from conventional poultry farms (n = 128)
c C. coli isolates from organic poultry farms (n = 115)

Table 2.3 MIC distributions of C. coli isolated from conventional and organic poultry farms
<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Agar dilution test range (µg/ml)</th>
<th>MIC breakpoints (µg/ml)</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt;/MIC&lt;sub&gt;90&lt;/sub&gt; (µg/ml) of Campylobacter strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C. jejuni</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Conventional (n=240)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0.06 - 128</td>
<td>≥ 32</td>
<td>8/32</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.06 - 128</td>
<td>≥ 16</td>
<td>64/128</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.06 - 128</td>
<td>≥ 16</td>
<td>1/1</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>0.25 - 128</td>
<td>≥ 64</td>
<td>8/&gt;128</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.06 - 128</td>
<td>≥ 4</td>
<td>1/32</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.06 - 128</td>
<td>≥ 8</td>
<td>2/&gt;128</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.008 - 128</td>
<td>≥ 4</td>
<td>8/32</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>0.06 - 128</td>
<td>≥ 16</td>
<td>64/128</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>0.25 - 128</td>
<td>≥ 32</td>
<td>128/&gt;128</td>
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</table>

Table 2.4 MICs of nine antimicrobial agents against *C. jejuni* and *C. coli* isolated from conventional and organic poultry farms.
<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Number (%) of resistant <em>Campylobacter</em> strains</th>
<th>C. jejuni</th>
<th>C. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conventional (n=165)</td>
<td>Organic (n=108)</td>
<td>Conventional (n=2)†</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4 (3.7)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>139 (84.24)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>69 (63.89)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>17 (10.30)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12 (11.11)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>1 (0.61)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3 (2.78)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3 (2.78)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>76 (46.06)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>77 (46.67)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>77 (46.67)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
</tr>
</tbody>
</table>

† *C. coli* isolates from conventional broiler farms are excluded from chi-square analysis because of low sample size.
*Numbers in the same row with different superscripts are significantly different (P<0.05); numbers with the same superscript do not differ significantly (a chi-square test with Yates correction for continuity).

Table 2.5 Resistance rates of *C. jejuni* and *C. coli* isolated from conventional and organic broiler farms
<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Number (%) of resistant <em>Campylobacter</em> strains</th>
<th>C. <em>jejuni</em></th>
<th>C. <em>coli</em></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Conventional (n=75)</td>
<td>Organic (n=103)</td>
<td>Conventional (n=126)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>30 (40)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7 (6.80)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33 (26.19)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>74 (98.67)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41 (39.81)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>112 (88.89)&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Gentamicin</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>46 (61.33)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17 (16.50)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>107 (84.92)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>36 (48)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2 (1.94)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>93 (73.81)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>46 (61.33)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2 (1.94)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>114 (90.48)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>52 (69.33)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2 (1.94)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>84 (66.67)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>52 (69.33)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2 (1.94)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>82 (65.08)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>53 (70.67)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2 (1.94)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>82 (65.08)&lt;sup&gt;a&lt;/sup&gt;</td>
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</tbody>
</table>

* Numbers in the same row with different superscripts are significantly different (P<0.05); numbers with the same superscript do not differ significantly (a chi-square test with Yates correction for continuity).

Table 2.6 Resistance rates of *C. jejuni* and *C. coli* isolated from conventional and organic turkey farms
<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Number (%) of resistant <em>Campylobacter</em> strains</th>
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<td>C. <em>jejuni</em></td>
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<tr>
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<td>Conventional (n=240)</td>
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<tr>
<td>Ampicillin</td>
<td>30 (12.5)(^a)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>213 (88.75)(^a)</td>
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<tr>
<td>Gentamicin</td>
<td>0</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>63 (26.25)(^a)</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>37 (15.42)(^a)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>46 (19.17)(^a)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>128 (53.33)(^a)</td>
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<tr>
<td>Norfloxacin</td>
<td>129 (53.75)(^a)</td>
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<tr>
<td>Nalidixic acid</td>
<td>130 (54.17)(^a)</td>
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* Numbers in the same row with different superscripts are significantly different (P<0.05); numbers with the same superscript do not differ significantly (a chi-square test with Yates correction for continuity).

Table 2.7 Resistance rates of *C. jejuni* and *C. coli* isolated from conventional and organic poultry farms
<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Conventional broiler farms (n=167)</th>
<th>Organic broiler farms (n=165)</th>
<th>Conventional turkey farms (n=201)</th>
<th>Organic turkey farms (n=161)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>0(^a)</td>
<td>5 (3.03)(^a)</td>
<td>63 (31.34)(^b)</td>
<td>10 (6.21)(^a)</td>
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<tr>
<td>Tetracycline</td>
<td>141 (84.43)(^a)</td>
<td>99 (60)(^b)</td>
<td>186 (92.54)(^c)</td>
<td>81 (50.31)(^b)</td>
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<td>Gentamicin</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>19 (11.38)(^a)</td>
<td>28 (16.97)(^a)</td>
<td>153 (76.12)(^b)</td>
<td>50 (31.06)(^c)</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>2 (1.20)(^a)</td>
<td>9 (5.45)(^a)</td>
<td>129 (64.18)(^b)</td>
<td>5 (3.11)(^a)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0(^a)</td>
<td>15 (9.09)(^b)</td>
<td>160 (79.60)(^c)</td>
<td>5 (3.11)(^d)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>76 (45.51)(^a)</td>
<td>0(^b)</td>
<td>136 (67.66)(^c)</td>
<td>3 (1.86)(^b)</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>77 (46.11)(^a)</td>
<td>0(^b)</td>
<td>134 (66.67)(^c)</td>
<td>3 (1.86)(^b)</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>77 (46.11)(^a)</td>
<td>0(^b)</td>
<td>135 (67.16)(^c)</td>
<td>3 (1.86)(^b)</td>
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</table>

\(^a\) Numbers in the same row with different superscripts are significantly different (P<0.05); numbers with the same superscript do not differ significantly (a chi-square test with Yates correction for continuity).

Table 2.8 Resistance rates of *Campylobacter* strains isolated from different poultry production systems
<table>
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<tr>
<th>Operation types</th>
<th>Number (%) of Campylobacter strains resistant to different groups of antibiotics</th>
<th>Number (%) of multidrug-resistant Campylobacter strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional broiler farms (n=167)</td>
<td>9 (5.39) 94 (56.29) 49 (29.34) 15 (8.98) - - -</td>
<td>15 (8.98)</td>
</tr>
<tr>
<td>Organic broiler farms (n=165)</td>
<td>56 (33.94) 83 (50.30) 15 (9.09) 2 (1.21) 9 (5.45) - -</td>
<td>11 (6.67)</td>
</tr>
<tr>
<td>Conventional turkey farms (n=201)</td>
<td>- 13 (6.47) 25 (12.44) 23 (11.44) 30 (14.93) 87 (43.28) 23 (11.44)</td>
<td>163 (81.09)</td>
</tr>
<tr>
<td>Organic turkey farms (n=161)</td>
<td>55 (34.16) 74 (45.96) 24 (14.91) 2 (1.24) 4 (2.48) 2 (1.24) -</td>
<td>8 (4.97)</td>
</tr>
</tbody>
</table>

Table 2.9 Multidrug resistance of *Campylobacter* strains isolated from conventional and organic poultry farms
<table>
<thead>
<tr>
<th>Operation types</th>
<th>Major resistance patterns&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number (%) of resistant Campylobacter strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C. jejuni</td>
</tr>
<tr>
<td>Conventional broiler farms (n=167)</td>
<td>TET-KAN-CIP/NOR/NAL</td>
<td>15 (8.98)</td>
</tr>
<tr>
<td></td>
<td>TET-KAN-CLI-ERY</td>
<td>3 (1.82)</td>
</tr>
<tr>
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<td>AMP-TET-KAN</td>
<td>1 (0.61)</td>
</tr>
<tr>
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<td>TET-KAN-CLI-ERY-CIP/NOR/NAL</td>
<td>19 (9.45)</td>
</tr>
<tr>
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<td>AMP-TET-KAN-CLI-ERY-CIP/NOR/NAL</td>
<td>7 (3.48)</td>
</tr>
<tr>
<td></td>
<td>AMP-TET-CLI-ERY</td>
<td>5 (2.49)</td>
</tr>
<tr>
<td></td>
<td>AMP-TET-KAN-ERY-CIP/NOR/NAL</td>
<td>4 (1.99)</td>
</tr>
<tr>
<td>Organic broiler farms (n=165)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AMP-TET-CIP/NOR/NAL</td>
<td>5 (2.49)</td>
</tr>
<tr>
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<td>TET-KAN-CLI-ERY</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>TET-KAN-ERY-CIP/NOR/NAL</td>
<td>4 (1.99)</td>
</tr>
<tr>
<td>Conventional turkey farms (n=201)</td>
<td>TET-KAN-CLI-ERY</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>TET-KAN-ERY-CIP/NOR/NAL</td>
<td>4 (1.99)</td>
</tr>
<tr>
<td></td>
<td>AMP-TET-CIP/NOR/NAL</td>
<td>5 (2.49)</td>
</tr>
<tr>
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<td>TET-KAN-CIP/NOR/NAL</td>
<td>5 (2.49)</td>
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<td>AMP-TET-KAN-CLI-ERY</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>TET-KAN-ERY</td>
<td>2 (0.99)</td>
</tr>
<tr>
<td>Organic turkey farms (n=161)</td>
<td>TET-KAN-CLI-ERY</td>
<td>1 (0.62)</td>
</tr>
</tbody>
</table>

<sup>a</sup>AMP, ampicillin; CIP, ciprofloxacin; CLI, clindamycin; ERY, erythromycin; KAN, kanamycin; NAL, nalidixic acid; NOR, norfloxacin; TET, tetracycline

Table 2.10 Major multidrug resistance patterns of *C. jejuni* and *C. coli* isolated from conventional and organic poultry farms
Figure 2.1. PFGE patterns of *Campylobacter* strains isolated from conventional broiler and turkey farms and from organic broiler and turkey farms. Lane 1 and 15, molecular weight marker (Promega-makers® Lambda ladders); lane 2, *C. coli* isolate from conventional turkey farm A; lane 3, *C. coli* isolate from conventional turkey farm B; lane 4, *C. jejuni* isolate from organic turkey farm D; lane 5, *C. jejuni* isolate from conventional broiler farm H; lane 6-8, *C. jejuni* isolates from conventional broiler farm J; lane 9-10, *C. coli* isolates from organic broiler farm A; lane 11, *C. jejuni* isolate from organic broiler farm A; lane 12-13, *C. coli* isolates from organic broiler farm C; lane 14, *C. jejuni* isolate from organic broiler farm C.
CHAPTER 3

ANTIMICROBIAL SUSCEPTIBILITY TESTING OF CAMPYLOBACTER BY
THE AGAR DILUTION AND THE AGAR DISK DIFFUSION METHODS

3.1 ABSTRACT

The agreement between the agar disk diffusion method and the standardized agar
dilution method for antimicrobial susceptibility testing of Campylobacter was
investigated. Six hundred and sixty-eight Campylobacter strains (431 C. jejuni and 237
C. coli) isolated from intestinal tracts of healthy slaughter-age broilers and turkeys with
different histories of antibiotic exposure were included in this study. In general, if only
susceptible and resistant Campylobacter strains were included in the data analysis, the
percent agreement between the agar dilution method and the disk diffusion method in
identification of susceptible and resistant Campylobacter isolates would be very high
ranging from 87.79% for ampicillin to 99.85% for gentamicin with the average percent
agreement around 95.72% for other antibiotics. In addition, the kappa statistic also
showed almost perfect agreement (kappa > 0.8) between these two tests for almost every
class of antibiotics except for ampicillin and tetracycline. Because of a high level of
agreement between the agar dilution and the agar disk diffusion methods in determining
fluoroquinolones (e.g. ciprofloxacin) and aminoglycosides (e.g. gentamicin) susceptible
or resistant *Campylobacter* isolates, the agar disk diffusion method is likely to be an interesting alternative for antimicrobial susceptibility testing of thermophilic *Campylobacter* to these classes of antimicrobial agent. However, until the standard resistance breakpoints specific for *Campylobacter* is established and validated, the agar dilution method should be used to determine antimicrobial resistance of *Campylobacter* spp. to ampicillin, tetracycline, clindamycin, and erythromycin.

### 3.2 INTRODUCTION

*Campylobacter* species particularly *C. jejuni* has been recognized as an important cause of food-borne bacterial diarrhea in human worldwide (Allos, 2001). This organism is generally carried in the intestinal tracts of food animals especially poultry and it is often present on food of animal origin through fecal contamination during processing (Jacobs-Reitsma, 2000). Although most patients with *Campylobacter* infections do not require treatment, antimicrobial therapy seems to be essential in patients with systemic, severe or prolonged infections (Allos, 2001; Blaser, 1997). In this circumstance, macrolides (e.g. erythromycin) and fluoroquinolones (e.g. ciprofloxacin) are considered to be the drugs of choice (Allos, 2001; Blaser, 2000). However, other antibiotics such as gentamicin, tetracycline, clindamycin, and ampicillin may be listed as alternative drugs for treatment of systemic *Campylobacter* infections as well (Blaser, 2000). In general, antimicrobial susceptibility testing prior to treatment of *Campylobacter* infections may be unnecessary; however, it may be useful especially in the area where the prevalence of antimicrobial resistance in *Campylobacter* isolates is high. Several antimicrobial
susceptibility testing methods including agar dilution, broth microdilution, epsilometer test (E-test), and disk diffusion test have been used to test for antimicrobial resistance of *Campylobacter* species (Alfredson et al., 2003; Engberg et al., 1999; Fernandez et al., 2000a; Frediani-Wolf and Stephan, 2003; Gaudreau and Gilbert, 1997; Ge et al., 2002; Huang et al., 1992; Huysmans and Turnidge, 1997; Luber et al., 2003a; McDermott et al., 2004; Oncul et al., 2003). For the disk diffusion method, an interpretation criterion indicating whether the isolate is susceptible or resistant to antibiotics is based on the diameter of the zone of inhibition around the antibiotic disk (Caprioli et al., 2000). In contrast, a criterion to interpret whether the isolate is susceptible or resistant to antibiotics by the agar dilution method relies on the growth of the organism on a medium containing a two-fold concentration series of antibiotics (Caprioli et al., 2000). The lowest concentration of antimicrobial agent that completely inhibits the visible growth is defined as the minimal inhibitory concentration (MIC) (Caprioli et al., 2000; NCCLS, 2002a). Unfortunately, there are no antimicrobial resistance breakpoints specific for *Campylobacter* available currently. Hence, the resistance breakpoints of enteric bacteria in the family Enterobacteriaceae have been used to determine antimicrobial resistance of *Campylobacter* spp. (Ge et al., 2002; Luber et al., 2003a; Nachamkin et al., 2000b). However, the resistance breakpoints for Enterobacteriaceae approved by different organizations, e.g., the National Committee for Clinical Laboratory Standard (NCCLS), the British Society for Antimicrobial Therapy (BSAC), the World Health Organization (WHO), the Société Francaise de Microbiologie (SFM), the Deutsches Institute fur Normung (DIN), the Swedish Reference Group for Antibiotics (SIR), and the Werkgroep
Richtlijnen Gevoeligheidsbepalingen (WRG) seem to be different (Acar and Goldstein, 1996; Andrews, 2004; NCCLS, 2002a; SFM Antibiogram Committee, 2003). Since there are no internationally accepted standard resistance breakpoints specific for Campylobacter species, different resistance breakpoints have been used making a comparison of antimicrobial resistance results from various studies more difficult; for example, the breakpoints for susceptible Campylobacter strains to erythromycin varied from $\leq 0.5$ to $\leq 8 \mu g/ml$, while the breakpoints for erythromycin-resistant Campylobacter strains ranged from $\geq 2$ to $\geq 64 \mu g/ml$ (Acar and Goldstein, 1996; Engberg et al., 1999; Huysmans and Turnidge, 1997; King, 2001; NCCLS, 2002a; SFM Antibiogram Committee, 2003; Shanker and Sorrell, 1983). In order to compare the antimicrobial susceptibility patterns of thermophilic Campylobacter isolated from food animals, food of animal origin, and humans from different laboratories, the same standardized methods and interpretation criteria should be applied (Engberg et al., 1999; Nachamkin et al., 2000b). Recently, the agar dilution method is considered as a standard antimicrobial susceptibility testing method for Campylobacter species and Campylobacter jejuni ATCC 33560 is recommended to use as a quality control organism (McDermott et al., 2004; NCCLS, 2002a). Although the agar dilution method is very reliable, highly reproducible, and provides quantitative MIC values, it is labor-intensive, time-consuming, and quite expensive (Caprioli et al., 2000). On the other hand, the agar diffusion test such as disk diffusion method seems to be relatively easy to perform, not expensive, and can be reproducible if it is conducted carefully with a high level of standardization and quality control (Caprioli et al., 2000; Potz et al., 2004). Over the last
ten years, several comparison studies of the agreement between different antimicrobial susceptibility testing methods for *Campylobacter* species have been conducted (Alfredson et al., 2003; Engberg et al., 1999; Fernandez et al., 2000a; Frediani-Wolf and Stephan, 2003; Gaudreau and Gilbert, 1997; Ge et al., 2002; Huang et al., 1992; Huysmans and Turnidge, 1997; Luber et al., 2003a; Oncul et al., 2003). However, only a few studies have compared the results obtained from the agar dilution and the disk diffusion methods. Especially, after the standardized agar dilution method for *Campylobacter* has been proposed, no information on the agreement between the standardized agar dilution method and the agar disk diffusion method has been reported yet. Hence, the aim of this study was to determine whether the agar disk diffusion test could be used as a reliable method for antimicrobial susceptibility testing of *Campylobacter* by analyzing the agreement between antimicrobial resistance results obtained from the disk diffusion test and the results obtained from the standardized agar dilution test.

### 3.3 MATERIALS AND METHODS

**Bacterial isolates.** Six hundred and sixty-eight *Campylobacter* strains isolated from the intestinal tracts of healthy slaughter-age broilers and turkeys with different histories of antibiotic exposure were selected for antimicrobial susceptibility testing. These *Campylobacter* strains were isolated during August 2000 to November 2002 by the standard microbiological method and were stored in skim milk and glycerol at -85 °C until the antimicrobial susceptibility test was performed. One isolate per intestinal tract
was included in this study. These *Campylobacter* strains consisted of 431 *C. jejuni* and 237 *C. coli* on the basis of the hippurate hydrolysis test. In addition, reference standard bacterial strains including *C. jejuni* ATCC 33560 and *E. coli* ATCC 25922 were also tested concurrently as quality control organisms in this study.

**Antimicrobial agents.** Nine antimicrobial agents tested in this study included ampicillin, tetracycline, gentamicin, kanamycin, clindamycin, erythromycin, ciprofloxacin, norfloxacin, and nalidixic acid. For the agar dilution method, all antimicrobial agents were obtained from Sigma Chemical Co., St. Louis, MO except ciprofloxacin (Serologicals Proteins, Inc., Kankakee, IL). The concentrations of most antimicrobial agents tested in this study ranged from 0.06 to 128 µg/ml except for ciprofloxacin (0.008 to 128 µg/ml) and for kanamycin and nalidixic acid (0.25 to 128 µg/ml). For the disk diffusion method, all antibiotics, e.g., ampicillin (10 µg), tetracycline (30 µg), gentamicin (10 µg), kanamycin (30 µg), clindamycin (2 µg), erythromycin (15 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), and nalidixic acid (30 µg) were obtained from Becton Dickinson and Company, Sparks, MD.

**Antimicrobial susceptibility testing.** All *Campylobacter* isolates were thawed and resubcultured onto blood agar plates and then incubated at 42 °C for at least 24 hours under a microaerophilic environment (approximately 5% O₂, 10% CO₂, and 85% N₂) prior to antimicrobial susceptibility testing. A few *Campylobacter* colonies were randomly selected and inoculated into Mueller-Hinton (MH) broth (BBL Becton
Dickinson Microbiology Systems, Cockeysville, MD) and then adjusted to a turbidity equivalent to 0.5 McFarland standard by a colorimeter (BioMérieux, Inc., Hazelwood, MO).

For the agar dilution method, approximately $10^4$ CFU of adjusted samples were applied onto Mueller-Hinton agar (BBL Becton Dickinson Microbiology Systems, Cockeysville, MD) containing a two-fold concentration series of antibiotics and supplemented with 5% defibrinated sheep blood (Cleveland Scientific, Cleveland, OH) by a multipoint inoculator (a cathra replicator system) with 1 mm pins (Oxoid, Inc., Ogdensburg, NY). The inoculated plates were incubated in a Forma Series II water-jacketed CO$_2$ incubator (Thermo Electron Corporation, Marietta, OH) at 42 °C for 24 hours under a microaerophilic condition. The MIC breakpoints of ampicillin, tetracycline, gentamicin, kanamycin, clindamycin, erythromycin, ciprofloxacin, and nalidixic acid were determined according to the breakpoints used by the National Antimicrobial Resistance Monitoring System (NARMS) (CDC, 2003). For norfloxacin, the breakpoints defined by the National Committee for Clinical Laboratory Standard (NCCLS) for Enterobacteriaceae were used (NCCLS, 2002a; NCCLS, 2002b). The resistance breakpoints for each antimicrobial agent were as the followings: $\geq 4$ µg/ml for ciprofloxacin and clindamycin; $\geq 8$ µg/ml for erythromycin; $\geq 16$ µg/ml for tetracycline, gentamicin, and norfloxacin; $\geq 32$ µg/ml for ampicillin and nalidixic acid; and $\geq 64$ µg/ml for kanamycin (Table 3.1).

For the disk diffusion method, the adjusted samples were evenly streaked in three directions onto Mueller-Hinton II agar (BBL Becton Dickinson Microbiology Systems,
Cockeysville, MD) to produce confluent lawn of bacterial growth by sterile cotton swabs. Once the inoculum on the plates dried, antibiotic disks were distributed over the inoculated plates using a BBL Sensi-disc dispenser (BBL Becton Dickinson Microbiology Systems, Cockeysville, MD). These plates were then incubated at 42 °C under the same condition as the agar dilution method. Zone of inhibition around each antibiotic disk was measured in millimeters and recorded. Zone diameter breakpoints indicating susceptible, intermediate, or resistant were determined according to the NCCLS established guidelines for Enterobacteriaceae and bacteria isolated from animals (NCCLS, 2002a; NCCLS, 2002b). The resistance breakpoints for each antimicrobial agent were as the followings: ≤ 12 mm for gentamicin and norfloxacin; ≤ 13 mm for ampicillin, kanamycin, erythromycin, and nalidixic acid; ≤ 14 mm for tetracycline and clindamycin; and ≤ 15 mm for ciprofloxacin (Table 3.1).

Data analysis. To measure the level of agreement between the results obtained from the agar dilution method and the disk diffusion method, the percent agreement and the kappa statistic were calculated as previously described by Luber et al. (2003a) and Dohoo et al. (2003).

3.4 RESULTS

The results of antimicrobial susceptibility testing of *Campylobacter* spp. using the agar dilution and the disk diffusion methods for each antibiotic were summarized in Table 3.2. According to the resistance breakpoints currently used by the NARMS and the
NCCLS, a majority of *Campylobacter* isolates were classified as either susceptible or resistant to ciprofloxacin, norfloxacin, gentamicin, and kanamycin by the agar dilution and by the disk diffusion methods (Table 3.2). For erythromycin, clindamycin, and ampicillin, a large number of *Campylobacter* isolates were classified as intermediate to these antimicrobial agents by both methods; for example, 413, 366, and 154 *Campylobacter* isolates were identified as intermediate by the agar dilution method to erythromycin, clindamycin, and ampicillin, respectively (Table 3.2). Although 49 and 31 *Campylobacter* isolates were recognized as intermediate to tetracycline and nalidixic acid by the disk diffusion method, respectively, only four *Campylobacter* isolates were classified as intermediate to tetracycline and none of the isolates was classified as intermediate to nalidixic acid by the agar dilution method (Table 3.2).

The agreement between the agar dilution and the disk diffusion methods for antimicrobial susceptibility testing of thermophilic *Campylobacter* was summarized in Table 3.3 – 3.5. In general, the percent identification of resistant isolates by both methods was quite similar except for clindamycin and erythromycin that the percent of resistant strains identified by the agar dilution method was much higher than that identified by the disk diffusion method (76.20% versus 51.20% for clindamycin and 88.62% versus 37.13% for erythromycin) when the intermediate strains reclassified as resistant strains were included in the calculation (Table 3.4). The percent agreement between the agar dilution method and the disk diffusion method in identification of susceptible and resistant isolates ranged from 87.79% for ampicillin to 99.85% for gentamicin with the average percent agreement around 95.72% for other antibiotics when no intermediate
strains were included in the data analysis (Table 3.3). However, when the intermediate strains reclassified as resistant strains were included in the calculation, the percent agreement between these two methods changed significantly from 47.90% for erythromycin to 99.85% for gentamicin (Table 3.4). On the other hand, when the intermediate strains reclassified as susceptible strains were included in the analysis, the percent agreement between the agar dilution and the disk diffusion methods varied from 83.21% for tetracycline to 99.85% for gentamicin (Table 3.5). In terms of the kappa value, if only susceptible and resistant *Campylobacter* strains or the intermediate strains that were reclassified as susceptible strains were included in the kappa calculation, the kappa revealed almost perfect agreement (kappa > 0.8) between the agar dilution and the disk diffusion methods for susceptibility testing of *C. jejuni* and *C. coli* to almost every class of antibiotics tested in this study except for ampicillin and tetracycline (Table 3.3 and 3.5). However, when the intermediate strains reclassified as resistant strains were included in the kappa analysis, the weak agreement between these two methods for susceptibility testing of thermophilic *Campylobacter* to erythromycin, clindamycin, and ampicillin was observed with kappa 0.13, 0.30, and 0.40, respectively (Table 3.4). For gentamicin, the kappa value could not be calculated because none of the isolates in this study was resistant to this antibiotic causing the lack of the essential value for kappa statistic calculation.
3.5 DISCUSSION

When the results of antimicrobial susceptibility testing of *Campylobacter* isolates obtained from the disk diffusion method were compared to the results obtained from the standardized agar dilution method, this study shows that the disk diffusion test may be able to use as a reliable screening method for determining antimicrobial resistance of *Campylobacter* isolates from poultry to fluoroquinolones (e.g. ciprofloxacin) and aminoglycosides (e.g. gentamicin and kanamycin). However, the disk diffusion method may not be a good alternative to use for susceptibility testing of thermophilic *Campylobacter* to ampicillin and tetracycline because the percent agreement between the agar dilution and the disk diffusion methods in identification of susceptible and resistant *Campylobacter* isolates to these antimicrobials as well as the kappa value of ampicillin and tetracycline between these two tests were not very high when compared to those of other antibiotics. Since a large number of *Campylobacter* isolates were classified as intermediate to erythromycin and clindamycin by the standardized agar dilution method but they were classified as either intermediate or susceptible to these antimicrobial agents by the disk diffusion method, until the standard resistance breakpoints specific for *Campylobacter* is established, it is going to be difficult to determine whether the disk diffusion method is a reliable alternative for susceptibility testing of *Campylobacter* to erythromycin and clindamycin or not.

In this study, *Campylobacter* strains that were resistant to fluoroquinolones and aminoglycosides by the standardized agar dilution method were also resistant to these antimicrobial agents by the disk diffusion method. Interestingly, no zone of inhibition
around these antibiotic disks was observed among the resistant *Campylobacter* strains, while a large clear zone of inhibition averaged more than 37 mm in diameter was observed around the disks of the susceptible *Campylobacter* strains. The drastic difference in the zone of inhibition diameter between the susceptible and resistant *Campylobacter* strains to fluoroquinolone and aminoglycoside antibiotics in combination with the high percent agreement in identification of susceptible and resistant isolates and a high kappa value between the agar dilution and the disk diffusion methods indicate that the disk diffusion test may be able to use as a reliable screening method for antimicrobial susceptibility testing of thermophilic *Campylobacter* to these classes of antimicrobial agents. This finding is correlated well with the previous study by Gaudreau and Gilbert (1997), who reported a complete agreement between the agar dilution method and the disk diffusion method for susceptibility testing of *C. jejuni* and *C. coli* to ciprofloxacin. In addition, the previous study by Vanhoof et al. (1984) also showed a good correlation between the disk diffusion and the agar dilution methods for antimicrobial susceptibility testing of *C. jejuni* to many antibiotics. Likewise, Frediani-Wolf and Stephan (2003) suggested that the disk diffusion method can be used as a reliable and easy tool for monitoring the prevalence of ciprofloxacin-resistant *C. jejuni* strains although they found a weak correlation between the MIC and zone diameter results for ciprofloxacin-susceptible strains.

The MICs of erythromycin and clindamycin for most *Campylobacter* strains tested in this study ranged between 1 and 4 µg/ml for erythromycin and between 1 and 2 µg/ml for clindamycin, which were in the intermediate category according to the current
resistance breakpoints. However, if the interpretation criteria for resistance change, the numbers of *Campylobacter* isolates that are susceptible, intermediate, or resistant to these antibiotics will change. For example, if the tentative resistance breakpoints of erythromycin for thermophilic *Campylobacter* proposed by Huysmans and Turnidge (1997) are used, the numbers of intermediate strains in this study will reduce from 413 to 19 isolates for the agar dilution method and from 62 to 15 isolates for the disk diffusion method, whereas the numbers of susceptible strains will increase to 489 isolates by both methods and the numbers of resistant isolates will change to 160 for the agar dilution method and to 164 for the disk diffusion method. In addition, the percent agreement in identification of susceptible and resistant isolates to erythromycin as well as the kappa value will also change to 97.96% and 0.95, respectively. Likewise, as shown in Table 3.4 and 3.5, if the intermediate strains were reclassified as resistant or susceptible strains, the percent agreement between the agar dilution method and the disk diffusion method in identification of erythromycin-susceptible and erythromycin-resistant isolates as well as the kappa value would change significantly. Particularly, when the intermediate strains were reclassified as resistant strains, the percent agreement between these two methods changed dramatically from 96.14% to 47.90% and the kappa also reduced significantly from 0.91 to only 0.13 (Table 3.4). However, this drastic change was not really surprising because most of the isolates classified as intermediate to erythromycin by the agar dilution method were classified as susceptible or intermediate by the disk diffusion method with the zone of inhibition diameter around 32 mm. Similar change was also observed on clindamycin. If the MIC and zone diameter breakpoints of clindamycin
recommended by the Société Francaise de Microbiologie (SFM) are used as the interpretation criteria (Acar and Goldstein, 1996), the numbers of intermediate strains will not be present, while the numbers of susceptible and resistant strains to clindamycin will change to 525 and 143 isolates by the agar dilution method and to 506 and 162 isolates by the disk diffusion method, respectively. In addition, the percent agreement between the agar dilution and the disk diffusion methods and the kappa value will change to 92.96% and 0.80, respectively as well. Likewise, when the intermediate strains were reclassified as resistant strains, the percent agreement in identification of clindamycin-susceptible and clindamycin-resistant strains between these two tests reduced significantly from 97.34% to 65.42%, while the kappa value also extremely reduced from 0.95 to 0.30 when compared to the percent agreement and the kappa value calculated without including the intermediate strains (Table 3.3 and 3.4).

Since the MICs and the zone of inhibition diameter between susceptible and resistant Campylobacter isolates to erythromycin and especially clindamycin were not drastically different like the case of fluoroquinolones, the resistance breakpoints used to determine whether the isolates were susceptible, intermediate, or resistant to erythromycin and clindamycin were very crucial. Unfortunately, the standardized interpretation criteria specific for Campylobacter have not been established yet. In addition, as mentioned earlier, the numbers of susceptible and resistant Campylobacter strains to erythromycin and clindamycin changed significantly when different resistance breakpoints were used. Therefore, it is quite difficult at this stage to determine whether the disk diffusion method is a reliable screening test for monitoring antimicrobial
resistance of thermophilic *Campylobacter* to erythromycin and clindamycin or not. However, if the intermediate strains are not included in the data analysis, the disk diffusion method seems to correlate well with the agar dilution method (Table 3.3). Similarly, if the interpretation criteria proposed by Huysmans and Turnidge (1997) are used to determine erythromycin resistance of *Campylobacter* isolates in this study, the percent agreement between the agar dilution method and the disk diffusion method as well as the kappa value of these two tests are also very high. A good correlation between the agar dilution method and the disk diffusion method for screening erythromycin-resistant *Campylobacter* strains was also previously reported by Alfredson et al. (2003) and Frediani-Wolf and Stephan (2003). Since 95% of the quality control organism *C. jejuni* ATCC 33560 and the majority of *Campylobacter* isolates tested in this study as well as in other studies (Baker, 1992; Gaudreau and Gilbert, 1997) had the MICs of erythromycin in the intermediate category (between 1 and 4 µg/ml) when the current NCCLS interpretation criteria were applied, it might be a better idea if the MIC breakpoints for susceptible *Campylobacter* isolates are raised to less than or equivalent to 4 µg/ml instead of less than or equivalent to 0.5 µg/ml as currently use.

For ampicillin and tetracycline, the disk diffusion method may not be a reliable method to use for susceptibility testing of *Campylobacter* to these antibiotics because the percent agreement in identification of susceptible and resistant isolates between the agar dilution and the disk diffusion methods and the kappa value between these two tests were not very high even though different resistance breakpoints or different interpretation criteria have been used. For example, if the interpretation criteria for ampicillin
recommended by the British Society for Antimicrobial Therapy (BSAC) (Andrews, 2004) are used to determine antimicrobial resistance of *Campylobacter* isolates in this study, the percent agreement in identification of ampicillin-susceptible and ampicillin-resistant *Campylobacter* isolates between the agar dilution and the disk diffusion methods will be 85.82%, whereas the kappa value between these tests will be only 0.41%. As shown in Table 3.3 – 3.5, it does not matter whether the intermediate strains were reclassified as either susceptible or resistant strains or whether no intermediate strains were included in the statistical calculation, the kappa value of ampicillin ranged only between 0.39 and 0.56 (Table 3.3 – 3.5). Although the kappa value of tetracycline was not as low as that of ampicillin, it was still lower than those of fluoroquinolones and aminoglycosides tested in this study with the kappa ranging from 0.61 to 0.75 (Table 3.3 – 3.5). Since a moderate number of tetracycline-resistant *Campylobacter* isolates determined by the agar dilution method were classified as intermediate strains by the disk diffusion method, it was not usual that the percent agreement and the kappa value between these two tests were not very high. The previous study by Alfredson et al. (2003) also showed a low level of agreement (77%) between the agar dilution method and the disk diffusion method when they were used to determine ampicillin-resistant *Campylobacter* isolates. However, this research group found that these two methods correlated well when they were used for screening of tetracycline-resistant *Campylobacter* strains.

Although a correlation coefficient and a linear regression analysis have been the most commonly used statistics for determining the agreement between different
antimicrobial susceptibility testing methods, these statistics may not be the best statistics to use because they measure the degree of association to which the results obtained from one test varies linearly with the results obtained from the other test not the agreement between the tests and they also highly influenced by the variation between individuals (Altman and Bland, 1983; Dohoo et al., 2003). In addition, McDermott et al. (2004) also suggested that it was impossible to correlate the disk diffusion results with the agar dilution results by the linear regression analysis.

In conclusion, this study reveals that the disk diffusion method can be used as a reliable alternative method for susceptibility testing of thermophilic *Campylobacter* to fluoroquinolone and aminoglycoside antibiotics especially for monitoring the prevalence of resistant *Campylobacter* strains to these classes of antibiotics. Since the percent agreement and the kappa value of ampicillin and tetracycline between the standardized agar dilution method and the disk diffusion method obtained from this present study were not very high, the disk diffusion method should not be used for susceptibility testing of *Campylobacter* to these classes of antimicrobials. When the current NCCLS resistance breakpoints for erythromycin and clindamycin were used, a large number of *Campylobacter* isolates in this study were classified as intermediate by the agar dilution method. Since the numbers of *Campylobacter* strains susceptible, intermediate, or resistant to erythromycin and clindamycin changed drastically when the different resistance breakpoints were used, it is difficult at this stage to determine whether the disk diffusion method is a reliable alternative for susceptibility testing of *Campylobacter* to erythromycin and clindamycin or not. Until the standard resistance breakpoints specific
for *Campylobacter* is established and validated, the agar dilution method seems to be a better method for antimicrobial susceptibility testing of *Campylobacter* to erythromycin and clindamycin. Nevertheless, we feel that the disk diffusion method may be able to use as a reliable screening method for susceptibility testing of thermophilic *Campylobacter* to erythromycin. Although the disk diffusion method is not complicated to perform as the agar dilution method and provides reliable results for several classes of antimicrobials, only qualitative data can be obtained from this method. In addition, another drawback of the disk diffusion method that we noticed in this study was the poor growth of *Campylobacter* isolates on the plates causing difficulty in interpreting the results that sometimes led to the retest. However, this method seems to be very useful especially when several antimicrobial agents need to be tested against a few isolates. If the quantitative data is required, other methods such as the agar dilution method or the E-test should be performed.

### 3.6 ACKNOWLEDGEMENT

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3.7 REFERENCES


<table>
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<tr>
<th>Antimicrobial agents</th>
<th>Agar dilution method</th>
<th>Disk diffusion method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC breakpoints (µg/ml)</td>
<td>Disk concentration (µg)</td>
</tr>
<tr>
<td></td>
<td>S  I  R</td>
<td>S  I  R</td>
</tr>
<tr>
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<td>0.06-128 ≤ 8 16 ≥ 32</td>
<td>10 ≥ 17 14-16 ≤ 13</td>
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<td>0.06-128 ≤ 4 8 ≥ 16</td>
<td>30 ≥ 19 15-18 ≤ 14</td>
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<td>Nalidixic acid</td>
<td>0.25-128 ≤ 16 ≥ 32</td>
<td>30 ≥ 19 14-18 ≤ 13</td>
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</table>

*a* MIC breakpoints for enteric bacteria used by the NARMS and the NCCLS; S, susceptible; I, intermediate; R, resistance

*b* Zone diameter breakpoints for Enterobacteriaceae approved by the NCCLS; S, susceptible; I, intermediate; R, resistance

Table 3.1 Breakpoints of the agar dilution method and the disk diffusion method used to determine antimicrobial susceptibility of *Campylobacter* isolates
Table 3.2 Antimicrobial susceptibility patterns of *Campylobacter* spp. identified by the agar dilution method and the disk diffusion method

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Number of susceptible-, intermediate-, resistant- <em>Campylobacter</em> isolates identified by</th>
</tr>
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<tr>
<td></td>
<td>Agar dilution method&lt;sup&gt;a&lt;/sup&gt;</td>
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<sup>a</sup> S, susceptible; I, intermediate; R, resistant
<table>
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<th>% identification of resistant isolates by AD method</th>
<th>% identification of resistant isolates by DD method</th>
<th>% agreement&lt;sup&gt;b&lt;/sup&gt; between AD and DD methods</th>
<th>Kappa&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>Gentamicin</td>
<td>667</td>
<td>37.75</td>
<td>36.99</td>
<td>98.94</td>
<td>n/a&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>408</td>
<td>51.71</td>
<td>49.05</td>
<td>97.34</td>
<td>0.95</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>127</td>
<td>68.24</td>
<td>64.38</td>
<td>96.14</td>
<td>0.91</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>74</td>
<td>32.62</td>
<td>34.91</td>
<td>96.19</td>
<td>0.92</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>422</td>
<td>32.08</td>
<td>34.49</td>
<td>96.39</td>
<td>0.92</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>431</td>
<td>30.14</td>
<td>31.87</td>
<td>95.48</td>
<td>0.90</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>425</td>
<td>30.14</td>
<td>31.87</td>
<td>95.48</td>
<td>0.90</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of susceptible or resistant *Campylobacter* isolates identified by the agar dilution (AD) and the disk diffusion (DD) methods.

<sup>b</sup> % agreement in identification of susceptible and resistant *Campylobacter* isolates by the agar dilution (AD) and the disk diffusion (DD) methods.

<sup>c</sup> The magnitude of kappa indicates the level of agreement between the two tests as the followings: < 0.2, slight agreement; 0.2 to 0.4, fair agreement; 0.4 to 0.6, moderate agreement; 0.6 to 0.8, substantial agreement; > 0.8, almost perfect agreement.

<sup>d</sup> Kappa value of gentamicin could not be calculated because none of the isolates was resistant to this antibiotic causing the lack of representative value essential for statistical calculation.

Table 3.3 The agreement between the agar dilution method and the disk diffusion method for antimicrobial susceptibility testing of *Campylobacter* (no intermediate strains were included)
<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Number of Campylobacter isolates&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% identification of resistant isolates by AD method</th>
<th>% identification of resistant isolates by DD method</th>
<th>% agreement&lt;sup&gt;b&lt;/sup&gt; between AD and DD methods</th>
<th>Kappa&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>337</td>
<td>100</td>
<td>143</td>
<td>34.09</td>
<td>36.65</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>143</td>
<td>15</td>
<td>457</td>
<td>76.31</td>
<td>70.77</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>667</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>408</td>
<td>10</td>
<td>243</td>
<td>37.43</td>
<td>37.87</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>127</td>
<td>32</td>
<td>310</td>
<td>76.20</td>
<td>51.20</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>74</td>
<td>2</td>
<td>246</td>
<td>88.62</td>
<td>37.13</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>422</td>
<td>22</td>
<td>209</td>
<td>33.53</td>
<td>34.58</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>431</td>
<td>20</td>
<td>209</td>
<td>32.49</td>
<td>34.28</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>425</td>
<td>29</td>
<td>205</td>
<td>32.04</td>
<td>35.03</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of susceptible or resistant *Campylobacter* isolates identified by the agar dilution (AD) and the disk diffusion (DD) methods.

<sup>b</sup> % agreement in identification of susceptible and resistant *Campylobacter* isolates by the agar dilution (AD) and the disk diffusion (DD) methods.

<sup>c</sup> The magnitude of kappa indicates the level of agreement between the two tests as the followings: < 0.2, slight agreement; 0.2 to 0.4, fair agreement; 0.4 to 0.6, moderate agreement; 0.6 to 0.8, substantial agreement; > 0.8, almost perfect agreement.

<sup>d</sup> Kappa value of gentamicin could not be calculated because none of the isolates was resistant to this antibiotic causing the lack of representative value essential for statistical calculation.

Table 3.4 The agreement between the agar dilution method and the disk diffusion method for antimicrobial susceptibility testing of *Campylobacter* (intermediate strains were classified as resistant strains)
<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Number of <em>Campylobacter</em> isolates&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% identification of resistant isolates by AD method</th>
<th>% identification of resistant isolates by DD method</th>
<th>% agreement&lt;sup&gt;b&lt;/sup&gt; between AD and DD methods</th>
<th>Kappa&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>524</td>
<td>11.31</td>
<td>16.14</td>
<td>85.52</td>
<td>0.39</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>147</td>
<td>75.71</td>
<td>63.42</td>
<td>83.21</td>
<td>0.61</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>667</td>
<td>0</td>
<td>0</td>
<td>99.85</td>
<td>n/a&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>419</td>
<td>37.13</td>
<td>36.38</td>
<td>98.95</td>
<td>0.98</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>492</td>
<td>21.41</td>
<td>24.25</td>
<td>92.96</td>
<td>0.80</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>482</td>
<td>26.80</td>
<td>23.50</td>
<td>94.61</td>
<td>0.86</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>434</td>
<td>32.04</td>
<td>34.28</td>
<td>96.26</td>
<td>0.92</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>435</td>
<td>31.89</td>
<td>34.28</td>
<td>96.41</td>
<td>0.92</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>434</td>
<td>32.04</td>
<td>30.39</td>
<td>92.37</td>
<td>0.82</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of susceptible or resistant *Campylobacter* isolates identified by the agar dilution (AD) and the disk diffusion (DD) methods.

<sup>b</sup> % agreement in identification of susceptible and resistant *Campylobacter* isolates by the agar dilution (AD) and the disk diffusion (DD) methods.

<sup>c</sup> The magnitude of kappa indicates the level of agreement between the two tests as the followings: < 0.2, slight agreement; 0.2 to 0.4, fair agreement; 0.4 to 0.6, moderate agreement; 0.6 to 0.8, substantial agreement; > 0.8, almost perfect agreement.

<sup>d</sup> Kappa value of gentamicin could not be calculated because none of the isolates was resistant to this antibiotic causing the lack of representative value essential for statistical calculation.

Table 3.5 The agreement between the agar dilution method and the disk diffusion method for antimicrobial susceptibility testing of *Campylobacter* (intermediate strains were classified as susceptible strains)
CHAPTER 4

TETRACYCLINE RESISTANCE OF CAMPYLOBACTER ISOLATES ON ORGANIC POULTRY FARMS

4.1 ABSTRACT

A total of 245 pooled samples from one organic broiler farm were collected weekly from the first week until the end of the production cycle. Similarly, 585 pooled samples originated from 5 organic broiler farms and 3 organic turkey farms were also collected and cultured for Campylobacter. Tetracycline resistance of these Campylobacter isolates were identified by the agar dilution method, whereas the presence of tet(O) gene were determined by the PCR method. The changes of tetracycline resistance rates on the organic broiler farm were observed during the production cycle. Campylobacter strains isolated from the first few weeks of the production cycle were susceptible to tetracycline, while tetracycline resistance was first noticed among Campylobacter isolates from week 5 of the production cycle. The resistance rates reached to 100% at week 6 and 7 then dropped to 33.33% at week 10 of the production cycle. Interestingly, only 13.79% of Campylobacter isolates from intestinal tracts of this flock were resistant to tetracycline. In addition, tetracycline resistance rates were different among organic broiler and turkey farms. The presence of tet(O) gene was detected in
97.79% of tetracycline-resistant *Campylobacter* isolates, while a few DNA samples directly extracted from unexposed environmental samples were positive for *tet*(O) gene. This study reveals the complex nature of tetracycline resistance among *Campylobacter* isolates on organic poultry farms.

4.2 INTRODUCTION

*Campylobacter jejuni*, a leading cause of bacterial gastroenterocolitis in humans, is responsible for over 2 million cases of food-borne bacterial diarrhea each year in the United States (Altekruse et al., 1999). Most illnesses associated with *Campylobacter* infections are usually due to consumption of contaminated foods or foods that are cross-contaminated with raw or undercooked poultry (Altekruse and Tollefson, 2003). In general, *Campylobacter* is carried in intestinal tract of domestic poultry and other livestock as commensal organisms and it is often present on foods of animal origin through fecal contamination during processing (Altekruse et al., 1999; Altekruse and Tollefson, 2003). Since poultry is considered to be a major source of human *Campylobacter* infections (Newell and Wagenaar, 2000), a development of antimicrobial resistance in *Campylobacter* isolated from poultry is a matter of concern.

Tetracycline is a broad-spectrum antibiotic that has been widely used in human and veterinary medicine since the late 1940s and the early 1950s (Chopra et al., 1992; Chopra and Roberts, 2001; Roberts, 1996). This antibiotic inhibits protein synthesis in gram-positive and gram-negative bacteria by preventing the binding of aminoacyl-tRNA to the ribosomal acceptor (A) site on the 30S ribosomal subunit (Chopra et al., 1992;
Chopra and Roberts, 2001; Roberts, 1996; Speer et al., 1992). Currently, four different mechanisms associated with tetracycline resistance in bacteria have been reported including (i) an energy-dependent efflux system, which helps reduce the intracellular concentration of tetracycline by an integral membrane protein; (ii) ribosomal protection protein, which protects the ribosomal binding site of tetracycline; (iii) inactivation of tetracycline by modifying enzymes, which occurs rarely; and (iv) mutation of the 16S rRNA, which found in propionibacteria (Aarestrup and Engberg, 2001; Chopra et al., 1992; Chopra and Roberts, 2001; Roberts, 1996; Ross et al., 1998; Schnappinger and Hillen, 1996; Speer et al., 1992; Taylor and Chau, 1996; Trieber and Taylor, 2000). However, only a ribosomal protection protein is associated with tetracycline resistance in *Campylobacter* species (Aarestrup and Engberg, 2001; Trieber and Taylor, 2000). In general, the mechanism of tetracycline resistance in *Campylobacter* is primarily associated with *tet*(O) gene, which encodes a ribosomal protection protein designated as Tet(O) (Taylor and Courvalin, 1988; Trieber and Taylor, 2000). Tet(O) protein interacts directly with the ribosome, the target of tetracycline, and displaces tetracycline from its primary binding site on the ribosome; thus, protecting the ribosome from the inhibitory effect of tetracycline (Connell et al., 2002; Connell et al., 2003; Gibreel et al., 2004b; Manavathu et al., 1990; Pratt and Korolik, 2005; Trieber and Taylor, 2000). Although *tet*(O) gene is usually carried on transmissible plasmids (Aarestrup and Engberg, 2001; Gibreel et al., 2004b; Lee et al., 1994; Pratt and Korolik, 2005; Sagara et al., 1987;
Taylor and Courvalin, 1988; Trieber and Taylor, 2000), it has been found to be chromosomally-located as well (Gibreel et al., 2004b; Lee et al., 1994; Pratt and Korolik, 2005).

Over the last decade, the emergence of tetracycline resistance in *C. jejuni* and *C. coli* has increased drastically in many countries worldwide. For example, in Canada, the prevalence of tetracycline resistance in *Campylobacter* isolates increased significantly from 19.1% during 1985 - 1986 to 43%-68% during 1998-2001 (Gaudreau and Gilbert, 1998; Gaudreau and Gilbert, 2003). Similarly, a rapid increase in tetracycline resistance was also observed among clinical *Campylobacter* isolates in Spain, where the prevalence of tetracycline resistance increased dramatically from 23% during 1985 - 1987 to 72% during 1995 - 1998 for *C. jejuni* isolates and from 15.2% to 97% for *C. coli* isolates during the same period of time (Mirelis et al., 1999; Prats et al., 2000; Reina et al., 1994). The prevalence of tetracycline resistance was also reported from other countries located in different geographical regions of the world. In the United States, the prevalence of tetracycline resistance in human isolates was around 43% (Gupta et al., 2004), while the prevalence of tetracycline-resistant *C. jejuni* isolates in Europe ranged from 11% in Denmark (Aarestrup et al., 1997) to 46% in Finland (Hakanen et al., 2003). In other European countries, about 30% - 42% of *C. jejuni* and *C. coli* isolates from humans were resistant to tetracycline (Luber et al., 2003b; Pezzotti et al., 2003). A high frequency of tetracycline resistance was mainly observed in Asia, where 55% of *C. jejuni* isolates in Japan and 95% of *C. jejuni* and 85% of *C. coli* isolates in Taiwan were resistant to this antibiotic (Li et al., 1998; Sagara et al., 1987). In terms of poultry isolates, about 69% -
81% of *C. jejuni* and 50% - 77% of *C. coli* isolates from poultry meat in the United States were resistant to tetracycline (Ge et al., 2003; Gupta et al., 2004). Interestingly, a wide variation of tetracycline-resistant *C. jejuni* and *C. coli* isolates from broilers and turkeys as well as from poultry meat was observed in Europe ranging from 2% in Denmark to 76.3% in Italy (Aarestrup et al., 1997; Avrain et al., 2003; Luber et al., 2003b; Pezzotti et al., 2003; Van Looveren et al., 2001). As mentioned earlier, a large number of *Campylobacter* isolates from Asia were resistant to tetracycline. So, it was not unusual that 83% of *C. jejuni* and 96% of *C. coli* isolates from poultry meat in Taiwan were resistant to tetracycline (Li et al., 1998).

The emergence of antimicrobial resistance in zoonotic food-borne bacterial pathogens including *Campylobacter* seems to be an undesired consequence of antimicrobial usage in animal agriculture (Khachatourians, 1998; McEwen and Fedorka-Cray, 2002; Witte, 1998). Many antibiotics including tetracycline have been widely used in animal production systems to control and prevent diseases as well as to promote growth and improve feed efficiency (Chopra and Roberts, 2001; Khachatourians, 1998; McEwen and Fedorka-Cray, 2002; Roberts, 1996). Unfortunately, the extensive use of antibiotics for non-therapeutic purposes such as feeding of sub-therapeutic level of chlortetracycline to food-producing animals may have led to the development of antimicrobial resistance not only in pathogenic bacteria but also in non-pathogenic bacteria in the intestinal tracts of these animals (Chopra and Roberts, 2001; Khachatourians, 1998; McEwen and Fedorka-Cray, 2002; Van den Bogaard and Stobberingh, 1999; Witte, 1998). In addition, this practice may also lead to the
establishment of antimicrobial resistance gene in the environment (McEwen and Fedorka-Cray, 2002; Van den Bogaard and Stobberingh, 1999). Since no antibiotics have been used in the organic production system, bacterial isolates from organically-raised animals should be susceptible to almost every class of antimicrobial agents, to which they are not intrinsically resistant. Unfortunately, from our previous study, we found that about 50% to 60% of \textit{C. jejuni} and \textit{C. coli} isolates from organically-raised broilers and turkeys were resistant to tetracycline (Luangtongkum et al., 2005). In addition, the study by Sato et al. (2004) also indicated that a large number of \textit{Campylobacter} isolates from organic dairy farms was resistant to tetracycline. Since tetracyclines have been used as feed additives for livestock and poultry for both therapeutic and sub-therapeutic purposes for long period of time (Chopra and Roberts, 2001; McEwen and Fedorka-Cray, 2002), it is possible that bacteria including \textit{Campylobacter} may evolutionally become resistant to this class of antibiotic regardless of the sources of the isolates. However, there is no scientific information to support this assumption. The purpose of this study was to investigate tetracycline resistance of thermophilic \textit{Campylobacter} on organic poultry farms in order to get some scientific information that may help elucidate a high prevalence of tetracycline resistance found among \textit{Campylobacter} isolates from organic production system. In addition, this study may help identify the source of tetracycline resistance on organic poultry operations as well.
4.3 MATERIALS AND METHODS

Sample collection. This study focused on tetracycline resistance of thermophilic *Campylobacter* on organic poultry farms. Five organic broiler farms and three organic turkey farms were utilized in this study. For one organic broiler farm, the samples were collected every week from the first week until the end of the production cycle. Environmental samples such as feed, water, litter, and grass as well as fresh fecal samples were randomly collected throughout the farm to ensure that samples represented the whole production system and the whole-flock exposure and placed into sterile Nasco Whirl-Pak™ (Nasco Agriculture Sciences, Fort Atkinson, WI) and sterile specimen containers (Fisher Scientific, Pittsburgh, PA) and brought back to the laboratory within three hours of collection to culture for *Campylobacter*. Generally, each type of environmental samples was collected before and after coming in contact with the birds. Approximately 1,075 environmental and fecal samples obtained from the whole production cycle of one organic broiler farm and about 1,630 environmental and fecal samples obtained from 5 organic broiler farms and 3 organic turkey farms were collected from August 2004 to November 2004. In addition, at the end of the production cycle, at least 30 intestinal tracts from each organic poultry farm were also collected and brought back to the laboratory within three hours of collection to culture for *Campylobacter*.

Bacterial isolation and identification. A total of 215 pooled environmental and fecal samples obtained from the whole production cycle of one organic broiler farm and 326
pooled environmental and fecal samples obtained from 5 organic broiler farms and 3 organic turkey farms as well as 283 intestinal tracts of organically-raised broilers and turkeys were cultured for *Campylobacter* by the standard microbiological method.

Since *Campylobacter* is susceptible to a variety of environmental conditions and is unlikely to survive for long period of time outside the host’s intestinal tract, the presence of *Campylobacter* in environmental samples was determined by a selective enrichment method, whereas the presence of this organism in poultry intestines was determined by a direct plating method. In addition, for water samples, besides a selective enrichment method, a direct plating method and a filtration method were also performed to increase a chance of *Campylobacter* detection from water samples. Likewise, both selective enrichment and direct plating methods were also conducted for fecal samples.

For direct plating method, each sample was directly streaked onto Campy CVA agar (BBL Becton Dickinson Microbiology Systems, Cockeysville, MD). The inoculated plates were then incubated at 42 °C for 48 hours under a microaerophilic environment (approximately 5% O₂, 10% CO₂, and 85% N₂) in a CampyPak II anaerobic system jar with CampyPak gas generating system envelopes (BBL Becton Dickinson Microbiology Systems, Sparks, MD).

For selective enrichment method, the procedure was performed according to the protocol previously published by Denis et al. (2001) with some modifications. Briefly, 10 grams of samples were added to 90 ml of Preston broth containing Nutrient broth No. 2 (CM0067) (Oxoid LTD., Basingstoke, Hampshire, England), *Campylobacter* growth supplement (SR084E/SR0232E) (Oxoid LTD., Basingstoke, Hampshire, England),
Preston *Campylobacter* selective supplement (SR0117E) (Oxoid LTD., Basingstoke, Hampshire, England), and 5% laked horse blood (SR0048C) (Oxoid LTD., Basingstoke, Hampshire, England) and homogenized for 30 seconds using a Seward stomacher® lab blender (model 400) (Seward Medical Limited, London, England). These samples were then incubated at 42 °C overnight under a microaerophilic environment in a CampyPak II anaerobic system jar with CampyPak gas generating system envelopes (BBL Becton Dickinson Microbiology Systems, Sparks, MD). After incubation, the enriched samples were subcultured onto Campy CVA agar (BBL Becton Dickinson Microbiology Systems, Cockeysville, MD) and incubated at 42 °C for another 48 hours under a microaerophilic environment in a CampyPak II anaerobic system jar with CampyPak gas generating system envelopes (BBL Becton Dickinson Microbiology Systems, Sparks, MD).

For filtration method, the procedure was performed according to the previously published protocols by Blaser and Cody (1986) and Pearson et al. (1993) with modifications. Briefly, 100 ml of water sample was filtered through a Nalgene® analytical test filter funnel with 0.45-µm pore size, 47-mm diameter cellulose nitrate membrane filter (Nalge Nunc International, Rochester, NY). Each filter was placed facedown onto Campy CVA agar (BBL Becton Dickinson Microbiology Systems, Cockeysville, MD) and incubated overnight at 42 °C under a microaerophilic condition in a Forma Series II water-jacketed CO₂ incubator (Thermo Electron Corporation, Marietta, OH). After incubation, the filter was removed and the Campy CVA agar plate was resubcultured and then incubated at 42 °C for another 48 hours under a microaerophilic environment in a CampyPak II anaerobic system jar with CampyPak gas generating system envelopes
(BBL Becton Dickinson Microbiology Systems, Sparks, MD). If there were no suspect *Campylobacter* colonies on the plates, these Campy CVA agar plates would be re-incubated for up to four days before being reported as negative.

Suspect *Campylobacter* colonies were identified by colony morphology characteristics as well as biochemical characteristics to Gram-stain, oxidase test, catalase test, and *Campylobacter* culture-plate latex agglutination confirmation test (INDX-Campy [jcl]; PanBio INDX, Inc., Baltimore, MD). In addition, the hippurate hydrolysis test was also performed to differentiate between *C. jejuni* and *C. coli*.

**Tetracycline resistance determination.** The agar dilution method was used to determine the minimal inhibitory concentration (MIC) of tetracycline for *Campylobacter* isolates in this study as recommended by the National Committee for Clinical Laboratory Standard (NCCLS) (NCCLS, 2002). Briefly, *Campylobacter* isolates grown on blood agar plates for at least 24 hours were inoculated into Mueller-Hinton (MH) broth (BBL Becton Dickinson Microbiology Systems, Cockeysville, MD) and then adjusted to a turbidity equivalent to 0.5 McFarland standard by a colorimeter (BioMérieux, Inc., Hazelwood, MO). Approximately $10^4$ CFU of adjusted *Campylobacter* suspensions were applied onto Mueller-Hinton agar (BBL Becton Dickinson Microbiology Systems, Cockeysville, MD) containing a two-fold concentration series (0.06-128 µg/ml) of tetracycline (Sigma Chemical Co., St. Louis, MO) and supplemented with 5% defibrinated sheep blood (Cleveland Scientific, Cleveland, OH) by a multipoint inoculator (a Cathra replicator system®) with 1 mm pins (Oxoid, Inc., Ogdensburg, NY). *Campylobacter jejuni* ATCC
33560 was also inoculated onto each plate to serve as a quality control organism. The inoculated plates were incubated in a Forma Series II water-jacketed CO₂ incubator (Thermo Electron Corporation, Marietta, OH) at 42 °C under a microaerophilic condition for 24 hours. The MIC was defined as the lowest concentration of antimicrobial agent that completely inhibited the visible growth on the plates. The MIC breakpoint for tetracycline resistance, which is ≥ 16 µg/ml, was determined according to the NCCLS established guideline for bacteria isolated from animals (NCCLS, 2002a).

**DNA extraction.** In order to detect the presence of *tet*(O) gene in environmental and fecal samples as well as in bacterial isolates, DNA was extracted from these samples by the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI) according to the manufacturer’s protocol and the protocol previously published by Denis et al. (2001). Briefly, after the enrichment step, 1.5 ml of Preston broth was centrifuged at 5500 rpm for 1 minute to precipitate a residue using a 5590 Microcentrifuge-HP (Thermo Electron Corporation, Marietta, OH). Then, the supernatant or the suspension of bacterial cells was centrifuged at 13,500 rpm for 10 minutes to pellet the cells. After the supernatant was discarded, bacterial cells were suspended in 600 µl nuclei lysis solution and then incubated at 80 °C in a water bath for 5 minutes. After that 3 µl of RNase solution were added to the tube and then incubated at 37 °C in a water bath for another 40 minutes. Then, 200 µl of protein precipitation solution were added to the tube. The tube was then vortexed and placed on ice for 5 minutes. After centrifugation at 13,500 rpm for 3 minutes, the supernatant was transferred to a new microcentrifuge tube containing 600
µl of isopropanol and then centrifuged at 13,500 rpm for another 2 minutes. The pellet was then washed with 70% ethanol and centrifuged again at 13,500 rpm for 2 minutes. The ethanol was aspirated and the pellet was air-dried for 15-20 minutes or until it was completely dried. Finally, the DNA pellet was rehydrated in 100 µl of DNA rehydration solution overnight at 4 °C. The DNA samples were stored at -20 °C until the detection of tet(O) gene was performed.

**Polymerase chain reaction (PCR).** The presence of tet(O) and major outer membrane protein (MOMP) of *Campylobacter* genes in environmental and fecal samples as well as in bacterial isolates was determined by the PCR method. The PCR conditions for tet(O) gene detection were performed according to the protocol previously published by Aminov et al. (2001), while the PCR conditions for MOMP gene detection were followed the protocol previously published by Zhang et al. (2000). Briefly, PCR was performed in a volume of 50 µl with a final concentration of each component as the followings: 1x PCR buffer, 2.5 mM MgSO₄, 0.2 mM each deoxynucleoside triphosphate (dNTPs), 0.2 µM primers, and 1.25 U of Taq DNA polymerase. All PCR components except primers (IDT® Integrated DNA Technologies, Inc., Coralville, IA) (Table 4.1) were obtained from Promega Corporation, Madison, WI. PCR amplification was performed with a GeneAmp® 2400 PCR System (Perkin-Elmer Corporation, Norwalk, CT) as the followings: initial denaturation at 94 °C for 5 minutes, followed by 25 cycles of 94 °C for 30 seconds, 30 seconds of annealing temperature at 60 °C, and 30 seconds of extension at 72 °C for tet(O) gene detection, or 30 cycles of 94 °C for 1 minute, 1 minute of annealing
temperature at 58 °C, and 1 minute of extension at 72 °C for MOMP gene detection, and then a final extension step at 72 °C for 10 minutes. This PCR amplification generated 170 bp and 1,400 bp DNA fragment of tet(O) and MOMP genes, respectively. PCR products were determined by electrophoresis on a 2% agarose gel containing 0.5 µg/ml of ethidium bromide solution (Promega Corporation, Madison, WI) and visualized with a UV transilluminator and photographed by a digital imaging system (Alpha Innotech Corporation, San Leandro, CA).

**Statistical analysis.** A Chi-square ($\chi^2$) test at a significance level of $P < 0.05$ (two-tailed) with Yates correction for continuity was used for statistical analysis of tetracycline resistance rates of *Campylobacter* spp. between different sources, different farms and different studies.

**4.4 RESULTS**

For the organic broiler farm that was followed for the whole production cycle, *Campylobacter* was not recovered from any environmental or fecal samples collected during the first and second week of the rearing cycle (Table 4.2). At the third week, *Campylobacter* was first isolated from environmental and fecal samples with 73.33% of these samples positive for *Campylobacter* (Table 4.2). During the fourth to the tenth week of the production cycle, the prevalence of *Campylobacter* ranged from 61.11% to 88% (Table 4.2). At the end of the production cycle, *Campylobacter* was isolated from all
30 intestinal tracts (100%) of these organic broilers (Table 4.2). Interestingly, all *Campylobacter* isolates from this organic broiler farm were *C. jejuni* on the basis of hippurate hydrolysis test.

One of the most interesting findings of this study was the changes in tetracycline resistance rates among *Campylobacter* isolates from each week of the production cycle. Neither *Campylobacter* isolates from the third nor the fourth week of the production cycle were resistant to tetracycline, while more than 65% of the isolates from the fifth week were resistant to this antibiotic (Table 4.2). Tetracycline resistance rates reached to 100% during the sixth and seventh week then dropped to 72.22% and 81.82% in the eighth and ninth week of the production cycle, respectively (Table 4.2). At week 10, a few days before the birds were sent to the processing plant only 33.33% of these *Campylobacter* isolates were resistant to tetracycline, whereas only 13.79% of *Campylobacter* isolates from the intestinal tracts of these organically-raised broilers were resistant to this antibiotic (Table 4.2). In addition, all tetracycline-resistant *Campylobacter* isolates except one isolate from the fifth week of the production cycle were positive for *tet*(O) gene, while none of the randomly selected tetracycline-susceptible *Campylobacter* isolates was positive for *tet*(O) gene when determined by PCR method (Figure 4.1).

In terms of the prevalence and tetracycline resistance rates of *Campylobacter* isolates from other organic broiler and turkey farms, we found that the prevalence of *Campylobacter* isolated from environmental and fecal samples of organic broiler farms ranged from 20.31% to 47.37%, whereas the prevalence of *Campylobacter* isolated from
environmental and fecal samples of organic turkey farms ranged from 46.15% to 59.38% (Table 4.3 and 4.4). In addition, more than 90% of intestinal tracts of both organically-raised broilers and turkeys were positive for *Campylobacter* (Table 4.3 and 4.4). As mentioned earlier, although a majority of *Campylobacter* isolates from organic broiler farms were *C. jejuni*, a proportion of *C. jejuni* and *C. coli* in organic turkey farms seemed to be different from farm to farm (Table 4.3 and 4.4). For example, none of *Campylobacter* isolates from organic turkey farm A was *C. coli*, while 33.33% and 63.33% of *Campylobacter* isolated from environmental and fecal samples and from intestinal tracts of organic turkey farm C were *C. coli*, respectively (Table 4.4). Interestingly, although none of *Campylobacter* isolated from environmental and fecal samples of organic turkey farm B was classified as *C. coli*, 35.71% of *Campylobacter* isolated from intestinal samples of this organic turkey farm B were classified as *C. coli* on the basis of hippurate hydrolysis test (Table 4.4).

When tetracycline resistance rates between organic broiler farms were compared, the resistance rates varied drastically from 0% in organic broiler farm D to 100% in organic broiler farm A (Table 4.3). Interestingly, although 100% of *Campylobacter* isolated from environmental and fecal samples of organic broiler farm A were resistant to tetracycline, only 7.14% of *Campylobacter* isolated from intestinal samples of this organic broiler farm A were resistant to this antibiotic (Table 4.3). In contrast, only 15.39% of *Campylobacter* isolated from environmental and fecal samples of organic broiler farm C were resistant to tetracycline, while 90% of *Campylobacter* isolated from intestinal tracts of the birds in this farm were resistant to tetracycline (Table 4.3). For
organic broiler farm B and farm E, although tetracycline resistance rates between 
*Campylobacter* isolates from environmental/fecal samples and intestinal samples were 
not extremely different as those of organic broiler farm A and farm C, the tetracycline 
resistance rates between *Campylobacter* isolates from these two sources were still 
significantly different from each other (*P* < 0.05) with a higher resistance rates observed 
among the isolates from environmental and fecal samples for organic broiler farm B and 
among the isolates from intestinal samples for organic broiler farm E (Table 4.3). Like 
tetracycline resistance rates noticed in organic broiler farms, the resistance rates in 
organic turkey farms were also varied from farm to farm with 100% and 11.77% of 
*Campylobacter* isolated from environmental and fecal samples of organic turkey farm B 
and farm C resistant to tetracycline, respectively (Table 4.4). As mentioned earlier, more 
than 97% of tetracycline-resistant *Campylobacter* isolates from both organic broiler and 
organic turkey farms also harbored *tet*(O) gene (Table 4.3 and 4.4).

In order to investigate the source of tetracycline resistance on organic poultry 
farms, environmental samples such as feed, grit, litter, grass, and water samples from 
separate storage places before coming in contact with the birds were collected and tested 
for the presence of *Campylobacter* as well as *tet*(O) gene. In addition, other potential 
sources of tetracycline resistance such as fecal samples of other animals in the farm were 
also collected and tested for the presence of *Campylobacter* and *tet*(O) gene as well. 
From 105 DNA samples directly extracted from enrichment broth of environmental 
samples prior to coming in contact with the birds, 5 DNA samples from organic broiler 
farm A and 2 DNA samples from organic broiler farm B were positive for *tet*(O) gene;
however, none of these tet(O) gene positive DNA samples were positive for MOMP gene (Table 4.5). In addition, neither tet(O) gene nor MOMP gene was detected in DNA samples extracted from other organic broiler and turkey farms (Table 4.5). Although Campylobacter was isolated from some of these clean environmental samples, all of them except one isolate from organic broiler farm C were susceptible to tetracycline (Table 4.5). Thus, it was unlikely that these environmental samples were the sources of tetracycline resistance on organic poultry farms; however, for organic broiler farm C, it is possible that tetracycline-resistant Campylobacter isolated from grass on the pasture may be the source of tetracycline resistance on this particular organic broiler farm.

4.5 DISCUSSION

For one organic broiler farm that was followed for the whole production cycle, the changes in tetracycline resistance rate of Campylobacter isolates were observed. None of Campylobacter isolates from the third and the fourth week of the production cycle was resistant to tetracycline (Table 4.2). Tetracycline resistance was first noticed in Campylobacter isolates during the fifth week of the production cycle (Table 4.2). During the sixth and seventh week, all Campylobacter isolates became resistant to tetracycline then this resistance rate dropped to 33.33% during the last week of the production cycle (Table 4.2). Two days after the last environmental and fecal samples were collected from the farm; the intestinal tracts of these organically-raised broilers were collected and cultured for thermophilic Campylobacter. Interestingly, only 13.79% of these Campylobacter isolates were resistant to tetracycline (Table 4.2). The similar change of
tetracycline resistance rate was also observed in another flock of this organic broiler farm A, where 100% of *Campylobacter* isolated from environmental and fecal samples of nine weeks old birds were resistant to tetracycline, while only 7.14% of *Campylobacter* isolated from the intestinal tracts of the ten and a half weeks old birds were resistant to this antibiotic (Table 4.3 and 4.6). The association between age of the birds and tetracycline resistance rates were also observed among *Campylobacter* isolates from organic broiler farm E. Most of *Campylobacter* isolates from environmental and fecal samples of three weeks old birds in organic broiler farm E were susceptible to tetracycline, while more than 90% of *Campylobacter* isolates from six and seven weeks old birds in organic broiler farm E were resistant to this antibiotic (Table 4.3 and 4.6). Likewise, less than 41% of *Campylobacter* isolates from intestinal tracts of 10 weeks or older birds (farm A and farm B) were resistant to tetracycline, while as high as 92% of *Campylobacter* isolates from intestinal tracts of 7 to 9 weeks old birds (farm C and farm E) were resistant to this antibiotic (Table 4.3 and 4.6). This observation suggests that age of the organic broilers is likely to affect the prevalence of tetracycline resistance of *Campylobacter* spp. on organic broiler farms. Since the birds from both organic broiler farm A and farm E were obtained from the same hatchery, it is interesting to see that *Campylobacter* isolates from both farms have similar changes of tetracycline resistance patterns. For other organic broiler farms where the birds were obtained from different hatcheries, different tetracycline resistance patterns were observed.

When tetracycline resistance rates observed in this recent study were compared to the resistance rates observed in the previous study (Luangtongkum et al., 2005), we
notice that tetracycline resistance rates in *Campylobacter* isolates on organic broiler farms seemed to decrease, while the resistance rates in organic turkey farms seemed to increase (Table 4.7). One of the most drastic changes in tetracycline resistance rates between our previous and recent studies was observed in *Campylobacter* isolates from organic broiler farm D where the resistance rates to tetracycline extremely decreased from 94.12% to 0% (Table 4.7).

In terms of the level of tetracycline resistance, most tetracycline-resistant *Campylobacter* isolates in this study had MICs ranging between 32 and 128 µg/ml except for the isolates from organic broiler farm E that the majority of tetracycline-resistant isolates had MICs higher than 128 µg/ml. Similar observation was also observed by Taylor et al. (1986), who reported that the MICs of tetracycline for tetracycline-resistant *Campylobacter* isolates in Canada varied between 32 and 128 µg/ml. Recently, Gibreel et al. (2004b) found that 37% of clinical *Campylobacter* isolates in Canada had MICs ranging between 256 and 512 µg/ml, while Pratt and Korolik (2005) revealed that tetracycline-resistant *C. jejuni* and *C. coli* isolates in Australia had MICs ranging from 32 to >256 µg/ml. In addition, these previous studies (Taylor et al., 1986; Gibreel et al., 2004b) also demonstrated that plasmid that harbored tet(O) gene could confer high-level of resistance to tetracycline with MICs of 128 to 512 µg/ml.

In general, tetracycline resistance in *Campylobacter* spp. has been reported to be plasmid-mediated. Around 70-100% of tetracycline-resistant *Campylobacter* isolates carried plasmid ranging in size from 42 to 133 kb (Gibreel et al., 2004b; Lee et al., 1994; Pratt and Korolik, 2005; Sagara et al., 1987; Taylor and Courvalin, 1988; Tenover et al.,
1985). However, the rates of tet(O)-harboring plasmid seemed to vary significantly between studies ranging from 32.3% to 96% (Lee et al., 1994; Pratt and Korolik, 2005). In addition, plasmid-free tetracycline-resistant *Campylobacter* strains were also reported by previous studies (Lee et al., 1994; Pratt and Korolik, 2005; Sagara et al., 1987). Although the plasmid-encoded tet(O) gene is the primary tetracycline resistance determinant in *Campylobacter* species, the chromosomally-encoded tet(O) gene may play an important role in tetracycline resistance in *C. jejuni* and *C. coli* isolates as well especially among the isolates that lacked plasmids (Gibreel et al., 2004b; Lee et al., 1994; Pratt and Korolik, 2005). Since tet(O) gene detected by PCR in this study originated from total genomic DNA, the location of tet(O) gene whether it was plasmid-encoded tet(O) gene or chromosomally-encoded tet(O) gene could not be identified in the present study.

As previously reported (Gibreel et al., 2004b; Pratt and Korolik, 2005), almost 100% (265 of 271) of tetracycline-resistant *Campylobacter* isolates in this study were positive for tet(O) gene. Since the mechanism of tetracycline resistance in *Campylobacter* spp. is mainly dependent on tet(O) gene, it is not surprising that most tetracycline-resistant *Campylobacter* isolates in this study harbor tet(O) gene. However, when the presence of tet(O) gene in DNA directly extracted from enrichment broth was investigated, some of these samples especially the ones directly extracted from clean environmental samples prior to coming in contact with the birds were positive for tet(O) gene, but they were negative for MOMP gene (Table 4.5). This finding suggests that these environmental samples may be contaminated with other bacteria that can also harbor tet(O) gene. At present, neither species nor the ability of these bacteria in
transferring \textit{tet}(O) gene to \textit{Campylobacter} is known. Although previous studies (Chopra and Roberts, 2001; Roberts et al., 1993; Taylor and Courvalin, 1988; Widdowson et al., 1996; Zilhao et al., 1988) reported that other bacteria particularly gram-positive bacteria including \textit{Enterococcus} spp., \textit{Streptococcus} spp., \textit{Staphylococcus}, \textit{Lactobacillus}, \textit{Molbiluncus}, \textit{Aerococcus}, and \textit{Peptostreptococcus} can also harbor \textit{tet}(O) gene, no information whether these bacteria can transfer \textit{tet}(O) gene to \textit{Campylobacter} was available. However, it is believed that \textit{Campylobacter} species acquired \textit{tet}(O) gene most likely from gram-positive coccus (Taylor and Courvalin, 1988).

In terms of the prevalence of \textit{Campylobacter} in organic poultry farms, the present study along with other previous studies (Heuer et al., 2001; Luangtongkum et al., 2005) found that the prevalence of \textit{Campylobacter} in organic poultry farms was very high. On the other hand, El-Shibiny et al. (2005) reported that only 68.5% of \textit{Campylobacter} were isolated from organic chickens in the United Kingdom. In addition, \textit{Campylobacter} spp. in the present study were first isolated from organic broilers at 3 weeks of age, while El-Shibiny et al. (2005) found that not until 31 days of age that \textit{Campylobacter} from organic chicken flock were first isolated. When compared to our previous study (Luangtongkum et al., 2005), the prevalence of \textit{C. jejuni} isolated from intestinal tracts of organically-raised broilers and turkeys in this study is higher than that in the previous study except for the organic turkey farm C where the prevalence of \textit{C. jejuni} in the recent study is lower than that in the previous study (Table 4.7). For \textit{C. coli}, the prevalence of this organism in the previous and recent studies is opposite to that of \textit{C. jejuni} (Table 4.7). In general, \textit{C. jejuni} was the main organism isolated from both organic broiler and turkey.
farms in this study except for organic turkey farm C where *C. jejuni* was predominant in environmental and fecal samples, while *C. coli* was predominant in intestinal samples (Table 4.4). The prevalence of *C. jejuni* and *C. coli* in turkeys seems to vary significantly among the studies. Some studies (Lee et al., 2005; Smith et al., 2004) reported that *C. coli* was the main organism isolated from turkeys. In contrast, other studies (Wallace et al., 1998; Van Looveren et al., 2001) found that almost 100% of *Campylobacter* isolated from turkeys were *C. jejuni*. In addition, one survey study in the Midwest showed that 40.5% of *Campylobacter* isolated from turkeys from one processing plant were *C. coli*, while only 14.6% of the isolates from another processing plant were *C. coli* (Logue et al., 2003).

Another interesting observation from this study was the association between the prevalence of *C. jejuni* and *C. coli* and the species of turkey (white or bronze turkey). Fourteen intestinal tracts of white turkeys and 30 intestinal tracts of bronze turkeys were collected from organic turkey farm B and cultured for *Campylobacter*. Among 14 intestinal tracts of white turkeys, 12 intestines were positive for *Campylobacter*, while *Campylobacter* was recovered from all 30 intestinal tracts of bronze turkeys. When the species of *Campylobacter* isolated from intestinal tracts of white turkeys were determined by the hippurate hydrolysis test, 11 *C. jejuni* and 1 *C. coli* were identified. In contrast, the majority of *Campylobacter* isolates from bronze turkeys were identified as *C. coli* on the basis of hippurate hydrolysis test. Although the prevalence of *Campylobacter* spp. between white and bronze turkeys is not different, *C. jejuni* seems to be more prevalent in white turkeys, while *C. coli* seems to be more prevalent in bronze turkeys.
Since *Campylobacter* is unlikely to survive for long period of time outside intestinal tract, the selective enrichment method was used to culture *Campylobacter* from environmental and fecal samples in this study in order to increase *Campylobacter* recovery rate. The results of this study showed that there was no significant difference (P > 0.05) between the direct plating method and the selective enrichment method for *Campylobacter* isolation from fecal samples; however, the selective enrichment method seemed to be very useful especially for isolation of *Campylobacter* from samples that seemed to have low numbers of *Campylobacter* contamination such as feed or water samples. In addition, when the selective enrichment method was compared to the filtration method for recovering *Campylobacter* from water samples, we noticed that both methods were not significantly different (P > 0.05) from each other although the selective enrichment method might be a little bit better than the filtration method.

In summary, this study reveals the complex nature of tetracycline resistance of *Campylobacter* spp. on organic poultry farms. The changes of tetracycline resistance rates in *Campylobacter* species were observed during the production cycle of one organic broiler flock. This information indicates that tetracycline resistance phenotype among *Campylobacter* isolates from organic broilers is not stable. Since other bacteria that contaminate outside environment may harbor tet(O) gene, it might be useful to identify the genus and species of these bacteria as well as to investigate the ability of these bacteria in transferring tet(O) gene to *Campylobacter* species.
4.6 ACKNOWLEDGEMENT

The authors gratefully acknowledge the organic poultry farms and processing plant for participating in the study. The authors would like to thank Mr. Jerrel C. Meitzler and Mr. Jeremiah J. Meeks for their technical assistance on PCR. The authors would also like to thank Dr. Amna B. El-Tayeb, Ms. Elisabeth J. Angrick, Ms. Marisa Ames, and fellow colleagues at the Avian Disease Investigation Laboratory at The Ohio State University for their help, advice, and technical support.

4.7 REFERENCES


<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>PCR annealing temperature (°C)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TetO-FW</td>
<td>5’ACGGARAGTTTATTGTATACC3’</td>
<td>60</td>
<td>171</td>
</tr>
<tr>
<td>TetO-RV</td>
<td>5’TGGCGTATCTATAATGTTGAC3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOMP-FW</td>
<td>5’ATGAAACTAGTTAAACTTTAAGCGCCAAG3’</td>
<td>58</td>
<td>1400</td>
</tr>
<tr>
<td>MOMP-RV</td>
<td>5’GAATTTGTAAGAGCCTGAAG3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1 PCR primers and annealing temperature (°C)
A number of Campylobacter isolates tested for tetracycline resistance were less than the number of Campylobacter isolated from samples because some isolates could not be recovered after they were stored at -85°C.

Table 4.2 Campylobacter and tetracycline resistance rates on an organic broiler farm from the first week until the end of the production cycle

<table>
<thead>
<tr>
<th>Production period</th>
<th>No. of samples positive for Campylobacter/ No. of samples cultured for Campylobacter (%)</th>
<th>No. (%) of samples positive for C. jejuni</th>
<th>No. (%) of samples positive for C. coli</th>
<th>No. of Campylobacter resistant to tetracycline/ No. of Campylobacter isolates tested (%)</th>
<th>No. (%) of tetracycline-resistant Campylobacter positive for tet(O) gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wk 1</td>
<td>0/9 (0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Wk 2</td>
<td>0/14 (0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Wk 3</td>
<td>11/15 (73.33)</td>
<td>11 (100)</td>
<td>0</td>
<td>0/11 (0)</td>
<td>0</td>
</tr>
<tr>
<td>Wk 4</td>
<td>24/30 (80)</td>
<td>24 (100)</td>
<td>0</td>
<td>0/24 (0)</td>
<td>0</td>
</tr>
<tr>
<td>Wk 5</td>
<td>21/30 (70)</td>
<td>21 (100)</td>
<td>0</td>
<td>14/21 (66.67)</td>
<td>13 (92.86)</td>
</tr>
<tr>
<td>Wk 6</td>
<td>21/24 (87.50)</td>
<td>21 (100)</td>
<td>0</td>
<td>18/18 (100)</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Wk 7</td>
<td>24/28 (85.71)</td>
<td>24 (100)</td>
<td>0</td>
<td>24/24 (100)</td>
<td>24 (100)</td>
</tr>
<tr>
<td>Wk 8</td>
<td>18/22 (81.82)</td>
<td>18 (100)</td>
<td>0</td>
<td>13/18 (72.22)</td>
<td>13 (100)</td>
</tr>
<tr>
<td>Wk 9</td>
<td>11/18 (61.11)</td>
<td>11 (100)</td>
<td>0</td>
<td>9/11 (81.82)</td>
<td>9 (100)</td>
</tr>
<tr>
<td>Wk 10</td>
<td>22/25 (88)</td>
<td>22 (100)</td>
<td>0</td>
<td>7/21 (33.33)</td>
<td>7 (100)</td>
</tr>
<tr>
<td>Intestine</td>
<td>30/30 (100)</td>
<td>30 (100)</td>
<td>0</td>
<td>4/29 (13.79)</td>
<td>4 (100)</td>
</tr>
</tbody>
</table>

* Number of Campylobacter isolates tested for tetracycline resistance were less than the number of Campylobacter isolated from samples because some isolates could not be recovered after they were stored at -85°C.
A number of *Campylobacter* isolates tested for tetracycline resistance were less than the number of *Campylobacter* isolated from samples because some isolates could not be recovered after they were stored at -85 °C.

Table 4.3 *Campylobacter* and tetracycline resistance rates on five organic broiler farms

<table>
<thead>
<tr>
<th>Farm Sample</th>
<th>No. (%) of samples positive for <em>Campylobacter</em></th>
<th>No. (%) of samples positive for C. <em>jejuni</em></th>
<th>No. (%) of samples positive for C. <em>coli</em></th>
<th>No. (%) of tetracycline-resistant <em>Campylobacter</em> isolates tested</th>
<th>No. (%) of tetracycline-resistant <em>Campylobacter</em> positive for tet(O) gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Environment</td>
<td>5/24 (20.83)</td>
<td>5 (100)</td>
<td>0</td>
<td>5/5 (100)</td>
<td>5 (100)</td>
</tr>
<tr>
<td>Intestine</td>
<td>28/30 (93.33)</td>
<td>28 (100)</td>
<td>0</td>
<td>2/28 (7.14)</td>
<td>2 (100)</td>
</tr>
<tr>
<td>B Environment</td>
<td>25/64 (39.06)</td>
<td>21 (84)</td>
<td>4 (16)</td>
<td>19/23 (82.61)*</td>
<td>19 (100)</td>
</tr>
<tr>
<td>Intestine</td>
<td>27/30 (90)</td>
<td>24 (88.89)</td>
<td>3 (11.11)</td>
<td>11/27 (40.74)</td>
<td>11 (100)</td>
</tr>
<tr>
<td>C Environment</td>
<td>13/41 (31.71)</td>
<td>9 (69.23)</td>
<td>4 (30.77)</td>
<td>2/13 (15.39)</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Intestine</td>
<td>30/30 (100)</td>
<td>30 (100)</td>
<td>0</td>
<td>27/30 (90)</td>
<td>27 (100)</td>
</tr>
<tr>
<td>D Environment</td>
<td>15/37 (40.54)</td>
<td>15 (100)</td>
<td>0</td>
<td>0/15 (0)</td>
<td>0</td>
</tr>
<tr>
<td>Intestine</td>
<td>30/30 (100)</td>
<td>15 (100)</td>
<td>0</td>
<td>0/30 (0)</td>
<td>0</td>
</tr>
<tr>
<td>E Environment</td>
<td>18/38 (47.37)</td>
<td>17 (94.44)</td>
<td>1 (5.56)</td>
<td>13/18 (72.22)</td>
<td>11 (84.62)</td>
</tr>
<tr>
<td>Intestine</td>
<td>27/30 (90)</td>
<td>27 (100)</td>
<td>0</td>
<td>25/27 (92.59)</td>
<td>25 (100)</td>
</tr>
</tbody>
</table>

* Number of *Campylobacter* isolates tested for tetracycline resistance were less than the number of *Campylobacter* isolated from samples because some isolates could not be recovered after they were stored at -85 °C.
<table>
<thead>
<tr>
<th>Farm</th>
<th>Sample</th>
<th>No. of samples positive for <em>Campylobacter</em> (%)</th>
<th>No. (%) of samples positive for <em>C. jejuni</em></th>
<th>C. coli</th>
<th>No. of <em>Campylobacter</em> resistant to tetracycline/No. of <em>Campylobacter</em> isolates tested (%)</th>
<th>No. (%) of tetracycline-resistant <em>Campylobacter</em> positive for tet(O) gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Environment</td>
<td>19/32 (59.38)</td>
<td>19 (100)</td>
<td>0</td>
<td>5/19 (26.32)</td>
<td>5 (100)</td>
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<tr>
<td></td>
<td>Intestine</td>
<td>29/30 (96.67)</td>
<td>29 (100)</td>
<td>0</td>
<td>13/29 (44.83)</td>
<td>12 (92.31)</td>
</tr>
<tr>
<td>B</td>
<td>Environment</td>
<td>33/56 (58.93)</td>
<td>33 (100)</td>
<td>0</td>
<td>14/14 (100)</td>
<td>14 (100)</td>
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<tr>
<td></td>
<td>Intestine</td>
<td>42/44 (95.46)</td>
<td>27 (64.29)</td>
<td>15 (35.71)</td>
<td>31/42 (73.81)</td>
<td>29 (93.55)</td>
</tr>
<tr>
<td>C</td>
<td>Environment</td>
<td>18/39 (46.15)</td>
<td>12 (66.67)</td>
<td>6 (33.33)</td>
<td>2/17 (11.77)</td>
<td>2 (100)</td>
</tr>
<tr>
<td></td>
<td>Intestine</td>
<td>30/30 (100)</td>
<td>11 (36.67)</td>
<td>19 (63.33)</td>
<td>13/21 (61.91)</td>
<td>13 (100)</td>
</tr>
</tbody>
</table>

* Number of *Campylobacter* isolates tested for tetracycline resistance were less than the number of *Campylobacter* isolated from samples because some isolates could not be recovered after they were stored at -85 °C.

Table 4.4 *Campylobacter* and tetracycline resistance rates on three organic turkey farms.
<table>
<thead>
<tr>
<th>Operation type</th>
<th>Farm</th>
<th>No. of pooled samples collected(^a)</th>
<th>No. of samples positive for <em>Campylobacter</em> by standard culture method</th>
<th>No. of tetracycline-resistant <em>Campylobacter</em> isolates</th>
<th>No. of samples positive for (^b) tet(O) gene</th>
<th>tet(O) and MOMP gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic broiler</td>
<td>A*</td>
<td>18</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Organic turkey</td>
<td>A</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>9</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) Number of environmental samples before coming in contact with the organically-raised broilers and turkeys were collected.

\(^b\) The presence of tet(O) and MOMP genes in DNA directly extracted from enrichment broth detected by PCR method.

* Organic broiler farm A followed for the whole production cycle.

Table 4.5 The presence of *Campylobacter* and tet(O) gene in environmental samples before coming in contact with the organically-raised broilers and turkeys.
<table>
<thead>
<tr>
<th>Operation type</th>
<th>Farm</th>
<th>Age (week) of the birds when samples were collected from</th>
<th>Number of samples positive for tetracycline-resistant <em>Campylobacter</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Environmental and fecal samples</td>
<td>Intestinal samples</td>
</tr>
<tr>
<td>Organic broiler</td>
<td>A</td>
<td>9</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>B&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2, 3, 4-12</td>
<td>10.5-12.5</td>
</tr>
<tr>
<td></td>
<td>C&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5, 6, 7, 8</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>D&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3, 7, 8</td>
<td>8-9</td>
</tr>
<tr>
<td></td>
<td>E&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3, 6</td>
<td>7.5</td>
</tr>
<tr>
<td>Organic turkey</td>
<td>A</td>
<td>19</td>
<td>19.5</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>20-24</td>
<td>21.5-25.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> The birds in organic broiler farm B were raised in three separate areas: nursery barn for 2 weeks old birds, nursery barn for 3 weeks old birds, and pasture for 4 to 12 weeks old birds, while the birds in organic broiler farm E were raised in two separate areas: nursery barn for 3 weeks old birds and pasture for 6 weeks old birds.

<sup>b</sup> The birds in organic broiler farm C and D were raised in the same area but separate pens. For organic broiler farm C, samples were collected from pen#1 and #2 (8 weeks old), pen#3 and #4 (7 weeks old), pen#5 (6 weeks old), and pen#6 (5 weeks old). For organic broiler farm D, samples were collected from pen#1 (8 weeks old), pen#2 (7 weeks old), and pen#3 (3 weeks old).

<sup>c</sup> Tetracycline-resistant *Campylobacter* strains were isolated from all three areas that samples were collected.

<sup>d</sup> Only samples from 8 weeks old birds were positive for tetracycline-resistant *Campylobacter* strains.

<sup>e</sup> Tetracycline-resistant *Campylobacter* strains were isolated from 6 weeks old birds.

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Table 4.6 Age of the birds when environmental and fecal samples and intestinal samples were collected from each organic poultry farm
<table>
<thead>
<tr>
<th>Operation type</th>
<th>Farm</th>
<th>Study</th>
<th>Percent of tetracycline resistance</th>
<th>Percent of intestinal samples positive for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Campylobacter spp.</td>
</tr>
<tr>
<td>Organic broiler</td>
<td>B</td>
<td>Recent</td>
<td>40.74</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Previous</td>
<td>50</td>
<td>81.48</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>Recent</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Previous</td>
<td>100</td>
<td>88.17</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>Recent</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Previous</td>
<td>94.12</td>
<td>91.40</td>
</tr>
<tr>
<td>Organic turkey</td>
<td>B</td>
<td>Recent</td>
<td>73.81</td>
<td>95.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Previous</td>
<td>37.50</td>
<td>93.62</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>Recent</td>
<td>61.91</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Previous</td>
<td>57.14</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 4.7 Prevalence of Campylobacter and tetracycline resistance in organically-raised broilers and turkeys between our previous and recent studies.
Figure 4.1. Tetracycline resistance gene [tet(O) gene] of Campylobacter strains isolated from organically-raised broilers and turkeys. Lane 1, 2, 4, 5, 6, 7, 8, 11, and 12, tetracycline-resistant Campylobacter strains; lane 3, 9, and 10, tetracycline-susceptible Campylobacter strains; lane 13, positive control (C. jejuni ATCC 43503); lane 14, 100 bp DNA ladder (Promega Corporation, Madison, WI).


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