KIDON SUNG
A Reverse Transcriptase-Polymerase Chain Reaction Assay for Detection of Viable *Campylobacter* spp.
(Under the direction of NORMAN JEFFREY STERN)

The study focused on the development and application of a reverse transcriptase-PCR (RT-PCR), which specifically detects mRNA, for the detection of viable *Campylobacter*. A diversity of *Campylobacter* spp. was tested, however, our RT-PCR technique was specific for *C. jejuni*, *C. coli*, and *C. lari*. Messenger RNA from the four genes was detected for varying intervals after the cells had been killed by heat, but gradually the message disappeared when heat-treated cells were held at 37^{o}C. We observed that the durability of mRNA species detected by our RT-PCR technique depends significantly on the individual *Campylobacter* strain tested, the condition of heat treatment and post-treatment holding time, and the transcript targeted. The intensity of the RT-PCR products decreased as the temperature of heat treatment increased and as the subsequent holding time extended. The 256 bp amplicon was determined to be the most stable mRNA species tested. mRNA of the 256 bp amplicon was detectable even after *Campylobacter* spp. had been killed at temperatures of 95 to 99^{o}C. Using DNA-based PCR, the four genes could be amplified after 48 h holding time after each heat inactivation, indicating that the chromosomal DNA was minimally influenced by the heat treatment. PCR products from the 256 bp amplicon were detected at 10^2 to 10^3 *C. jejuni* CFU per ml, exhibiting the highest level of sensitivity among the genes tested

The main goal of the second study was to determine whether the ability of coccoid and VBNC cells of *Campylobacter* spp. were infectious after passage through day-old chicks. Further, this study was conducted to confirm the non-recovery of heat-killed *Campylobacter* spp. correlated with mRNA message detection through chicken challenge.

The levels of four *Campylobacter* spp., previously isolated from poultry feces, declined progressively over time and loss of culturability occurred after 6 to 7 weeks incubation in phosphate buffed saline (PBS) at 4^{o}C. Cold-stored, nonculturable and heat-inactivated (60^{o}C for

10 min) *Campylobacter* spp. produced inconsistent amplified products from RT-PCR assay depending on the target genes and strains used, though all fresh cultures showed mRNA signals. Mostly, signals of mRNA species from viable but nonculturable (VBNC) and heat-killed *Campylobacter* spp. AH-1, AH-2 and CH-3 persisted. RT-PCR amplification of *tkt*, *porA*, and a 256 bp amplicon from a previously described putative haem-copper oxidase provided consistent signal while *flaA* did not. Presumed VBNC and heat-inactivated *Campylobacter* spp., which produced positive mRNA signal but was not culturable by conventional culture-based methods, did not establish colonization in the intestine of chicks seven days after challenge.

This study suggests that while RT-PCR is generally a good technique to distinguish between viable and nonviable cells, the assay does not appear to be useful for *Campylobacter* spp. Furthermore, the results question the correlationship between mRNA durability as assayed by RT-PCR and cell viability, as well as the significance of the VBNC cells in environmental transmission of *Campylobacter* spp.

INDEX WORDS: Reverse Transcriptase-PCR, *Campylobacter*, Messenger RNA, Viable But Nonculturable, Cell Viability, Colonization

A REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION ASSAY FOR DETECTION OF VIABLE CAMPYLOBACTER SPP.

by

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DEDICATION

This dissertation is dedicated to

Wonderful God

My parents:

Mr. Ui-Kyoung Sung and Mrs. Jung-Lim Lee

and Family

For constant support, encouragement, and

unconditional love.

To

My beloved wife

Soon-Young Park

For her patience and encouragement.

AND

To

My beloved son and daughter

Gee-Yong Sung and Ye-Eun Sung, who are a constant source of joy and inspiration

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CHAPTER I

INTRODUCTION

Campylobacter spp. are considered to be a leading bacterial cause of foodborne diarrheal illness with a frequency that exceeds salmonellosis and shigellosis combined in the United States (Lior, 1994; Skirrow, 1990). Epidemiological investigations have implicated poultry, raw milk and untreated water in disease transmission. Sensitive enrichment methods traditionally used to detect low levels of *Campylobacter* spp. in food require at least two days of incubation followed by selective plating and two days of incubation on complex culture media containing a combination of antibiotics (Goossens and Butzler, 1992). *Campylobacter* may also enter the viable but nonculturable (VBNC) status due to starvation and physical stress, which may account for the failure of culture techniques to isolate the organism (Jones et al., 1991; Rollins and Colwell, 1986). Rapid, sensitive, and specific methods for detection of viable campylobacters are essential to further understand the epidemiology of infection and to enable tracking of the organisms.

Recently, new specific detection techniques such as enzyme-linked immunosorbent assay (ELISA), DNA/RNA probes, and polymerase chain reaction (PCR) assays have been developed for the detection of *Campylobacter*. Although immunological approaches are rapid, nonspecific detection due to cross-reactivity may occur. While PCR-based assays are rapid, sensitive, and specific, the possibility exists that positive PCR amplifications may arise from either dead or noninfectious cells because PCR methods detect chromosomal gene sequences, which may be present in

nonviable cells. Therefore, a method, which combines sensitivity and specificity together with the ability to differentiate between viable and nonviable cells, is desirable.

mRNA is documented to be more rapidly destroyed in cells than genomic DNA. We reasoned that a technique targeting bacterial mRNA would provide a better estimation for viability than previously reported DNA amplification tests. Several studies have recently investigated the detection of viable pathogens including *Salmonella enterica*, *S. enteritidis*, *Campylobacter* spp., *Vibrio cholerae*, *Listeria monocytogenes* and *Escherichia coli* using RT-PCR. However, due to the difficulties in determining suitable target genes and low efficiency of RNA purification, these approaches remain in the developmental stage.

Campylobacter spp. normally grows well in the digestive tract of poultry. When they are excreted into the environment, they are exposed to various environmental stresses such as nutrient starvation, osmotic shock, oxygen stress, temperature variation, oxidative stress (Saha et al., 1991). Under unfavorable conditions for growth, cells of *Campylobacter* spp. change from their physiological spiral form to coccoidal form. Such phenomenon has been characterized by a so-called viable but nonculturable (VBNC) state. The VBNC state has been documented in numerous human pathogens, including *Escherichia coli*, *Salmonella enteritis*, *Vibrio cholerae*, *Legionella pneumophila*, and *Campylobacter jejuni*. Jones *et al*. (Jones et al., 1991) reported on the VBNC form of various suspended *C. jejuni* strains following 18 to 28 days of holding in sterile water and incubating at 4ºC. Its significance for infection of animals and as the cause of disease in humans remains controversial. Beumer *et al*. (Beumer et al., 1992) could not recover *Campylobacter* from rabbits, mice or humans after infection with VBNC cells. Also,

VBNC *C. jejuni* was not isolated from embryonated eggs or the ceca of chicks after incubation for 7 days (Medema et al., 1992). However, Rollins *et al*. (Rollins et al., 1986) reported that nonculturable cells of *C. jejuni* in a stream water microcosm could be recovered after animal passage. This was confirmed by Stern *et al*. (Stern et al., 1994), who showed that some isolates of VBNC *C. jejuni* were able to colonize 5 of 79 challenged chicks.

One difficulty in elucidating the potential hazard of VBNC cells is the inability to detect such cells by conventional bacteriological methods. In view of the potential epidemiological importance of such VBNC cells, it would be important to establish, unequivocally, the viability of VBNC cells. Such an assay would contribute to an understanding of the processes leading to the loss of culturability and develop methods for VBNC detection. Since animal experiments are complex and difficult to control, it would be desirable to develop *in vitro* systems to detect the presence of VBNC cells.

This dissertation is divided into five chapters. Chapter II is a literature review, which describes history, background, and general characteristics of *Campylobacter* spp., VBNC cells, epidemiology, poultry colonization by *C. jejuni*, prevention and control, pathogenesis, clinical aspects in humans, mode of detection, epidemiological typing system, and previous studies related to this research.

In Chapter III, the main purpose of the study was to validate the use of mRNA as a marker for *Campylobacter* spp. viability and to demonstrate the ability of RT-PCR to discriminate between viable and nonviable cells. The expression of four genes, including *flaA*, *tkt*, *porA*, and a 256 bp amplicon of previously described as encoding for a putative haem-copper oxidase, in *Campylobacter* spp. were targeted. Cells were treated by heat

and examined to determine whether these were appropriate targets for RT-PCR amplification. The correlationship between persistence of RT-PCR products and cell viability after heat treatment was verified by conducting a time-course heat treatment experiment. The time-temperature relationship to extinguish mRNA signal was determined and RNA half-life was assessed according to holding time at 37°C. Additionally, sensitivity of amplification of *Campylobacter* spp. using the primers of these four genes was determined by conventional PCR assays.

In Chapter IV, we investigated whether the ability of coccoid and VBNC cells of *Campylobacter* spp. were infectious after passage through day-old chicks. Further, this study was conducted to confirm the non-recovery of heat-killed *Campylobacter* spp. correlated with mRNA message detection through chicken challenge.

A summary and conclusion of the two studies reported in Chapter III through Chapter IV are presented in chapter V.

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CHAPTER II

LITERATURE REVIEW

History and Background

Campylobacter species were not appreciated as a cause of diarrhea in humans until 1957 (King 1957), and their impact in terms of sheer numbers of human infections emerged only in the past 20 years. In 1886, Escherich observed organisms resembling campylobacters in stool samples of children with diarrhea. In 1913, McFadyean and Stockman (McFadyean 1913) isolated *Vibrio*-like bacteria from aborted ovine fetuses. Five years later, Smith (Smith 1918) discovered spiral bacteria in aborted bovine fetuses and concluded that these strains and the vibrios of McFadyean and Stockman belonged to the same species. A definitive link to human infection was established in 1957 when King observed bacteria in blood cultures taken from humans with enterocolitis (King 1957). These bacteria were designated as "related Vibrios". In 1963, Sebald and Véron (Sebald 1963) transferred *V. fetus* and *V. bubulus* into a new genus, *Campylobacter* because of their low DNA base composition, their microaerophilic growth requirements, and their nonfermentative metabolism. In the early 1970s, Butzler and coworkers applied a filtration method, thereby using the small cell size and the vigorous motility of *Campylobacter* cells, to selectively isolate them from stools of humans with diarrhea (Butzler 1973). The main breakthrough, however, was provided a few years later by Skirrow, who described a selective supplement comprising a mixture of vancomycin, polymyxin B, and trimethoprim, that was added to a basal medium (Skirrow 1977). Since that time, our understanding of these organisms has expanded substantially and *C. jejuni* have been recognized as major human enteropathogen with an incidence that exceeds that of *Salmonella* or *Shigella* (Griffiths 1990; Taylor 1992).

After the genus *Campylobacter* was first proposed in 1963, its taxonomic structure has changed extensively because many diverse phenotypic and genotypic characteristics were recognized. The status of *C. jejuni* as a valid species has been clearly established based on biochemical criteria (Smibert 1984), 16 S rRNA sequencing (Thompson 1988) and DNA hybridization (Vandamme 1991). Use of DNA-rRNA hybridizations provided the basis of the taxonomic structure used at present, and delineated *Campylobacter* spp. as a diverse yet phylogenetically distinct group, rRNA superfamily VI. This comprised rRNA homology groups I (*Campylobacter* and *Bacteroides ureolyticus*), II (*Arcobacter*) and III (*Helicobacter* and *Wolinella succinogenes*). At present, the genus *Campylobacter* contains 16 species and six subspecies. *Campylobacter jejuni* subsp. *jejuni*, *C. jejuni* subsp. *doylei*, *C. coli, C. lari, C. upsaliensis* and *C. helveticus* form a genetically close group of species which (*C. jejuni* subsp. *doylei* aside) are the most commonly isolated from human and animal diarrhea. Three thermophilic *Campylobacter* species, *C. jejuni*, *C. coli* (Munroe 1983), and *C. lari* (Kaneuchi 1987) may all be associated with the avian intestinal tract and foodborne infection, but *C. jejuni* is the predominant pathogen of human health significance (Griffiths 1990).

General Characteristics

Campylobacter is a small, spiral or curved, non-sporeforming Gram-negative rod, 0.2 to 0.8 μm in width and 0.5 to 5μm in length. Cells in old cultures may form coccoid bodies which are considered degenerative forms rather than a dormant stage of the organism (Hazeleger 1994). The bacteria are motile with a characteristic corkscrew-like motion by means of a single polar unsheathed flagellum at one or both ends. *Campylobacter* spp. are thermophilic, growing best in 42^{o}C, and microaerophilic, requiring an O_2 concentration of 3-5 % with a CO_2 concentration of 3-15 % (Ketley 1995). *C. jejuni* grows well in the range of pH 5.5-8.0, with an optimum pH of 6.5-7.5. Its metabolism is characterized by inability to utilize carbohydrates. It utilizes amino acids or intermediates of the Krebs cycle as its energy source and is extremely sensitive to hydrogen

peroxide and superoxide anions that are produced in the culture medium when it is exposed to air and light (Humphrey 1988). They are oxidase, catalase positive, indole negative and reduce selenite. *C. jejuni* can be differentiated from *C. coli* by its ability to hydrolyze hippurate. *C. lari* is characterized by its resistance to nalidixic acid (Skirrow 1980). The growth of *Campylobacter* in the environment is limited by its thermophilic and microaerophilic nature and the organism is susceptible to environmental stress. Survival of *Campylobacter* is better at lower (e.g. 4^{o}C) than higher (e.g. 20^{o}C) temperatures. Other factors that influence their survival are: pH (better at pH 6.4 than 5.8) (Gill 1982; Gill 1984); sodium chloride levels (worse with >0.5 % NaCl) (Doyle 1982; Hänninen 1981); oxygen levels (better in atmospheres without oxygen at 4°C, but no difference at 21°C) (Phebus 1991). They are also susceptible to common disinfectants and gamma irradiation (Radomyski 1994). Campylobacters can persist, but not grow, in biofilms, where they are protected from biocidal activity (Buswell 1998).

Campylobacter species have small genomes of approximately 1.6x10^{6} bp, which is less than half the size of that of *Escherichia coli* (Chang 1990). The G+C content of *Campylobacter* DNA is on average 30-35 %, which can cause problems when cloning these A+T rich sequences in *E. coli* (Ketley 1997). Natural populations of *C. jejuni* are genetically diverse and the genome of strains can change during the course of colonization of poultry (Thomas 1997). This is likely due to the fact that *Campylobacter* is naturally transformable and appears to undergo recombination with a high frequency. The difficulty of studying the genetics of *Campylobacter* has been attributed to instability due to the high AT ratio leading to collapsing inserts, the lack of expression and necessary accessory factors in *E. coli*, different codon usage patterns in *E. coli*, different DNA methylation patterns from *E. coli*, or genomic rearrangements (Ketley 1995; Wassenaar 1998).

Viable But Nonculturable (VBNC) *Campylobacter*

Campylobacter spp. grow well in the intestinal tract of poultry and when they are released in the environment, they are exposed to various environmental stresses, such as nutrient starvation, osmotic shock, temperature variation, or oxidative stress (Saha 1991). Thus, the persistence of bacteria in the environment is to a great part determined by their ability to endure these stresses. The formation of viable but nonculturable (VBNC) cells may be a survival strategy and an active process involving the induction of global control networks leading to sequentially regulated differentiation responses (Oliver 1993). These cells should therefore be able to reverse this program and resuscitate when growth conditions become more favorable. Conversely, others argue that the VNBC state is a moribund condition in which cells become progressively debilitated until cell death finally occurs. These cells may maintain signs of metabolic activity or respiration for some time but are not able to resuscitate.

VBNC cells have been described for many food and waterborne pathogens, including *Listeria monocytogenes*, *Vibrio cholerae*, *Helicobacter pylori*, *Salmonella typhi*, *Escherichia coli*, and *Campylobacter jejuni*. Changes of cells entering the VBNC state involve stabilization of the cell wall and membrane, thereby increasing the stability of the cell. VBNC cells appear to maintain gross membrane integrity although changes in membrane composition have been reported (Linder 1989; Morgan 1991). These changes in the cell wall and membrane allow for long-term stability and persistence. Electron micrographs of VBNC *C. jejuni* revealed intact but asymmetric membrane structures (Rollins 1986). Boucher *et al*. (Boucher 1994) showed that the presence of the antibiotic did not affect the rate of shape transformation from spiral to coccoid cells while nutrient limitation, aeration of the medium and presence of free-radical scavengers had major effects. Hazeleger *et al*. (Hazeleger 1995) demonstrated that there was a marked

difference in membrane fatty acid composition of coccoid cells that were formed at 4°C in comparison with those formed at 25°C and concluded that the formation of cocci was passive rather than active process.

A number of studies have focused on the nucleic acids of VBNC cells. In some cases, VBNC cells are reported to maintain normal amount of DNA while in other cases DNA amounts decrease. Most studies, however, report decreases of RNA content in VBNC cells. *Micrococcus luteus* maintained a constant DNA content but showed a 50 % decrease in RNA amount when becoming VBNC (Mukamolova 1995). VBNC cells of *Vibro vulnificus* possess reduced ribosomal and nucleic acid materials (Oliver 1993). Prolonged cold exposure of VBNC *V. vulnificus* cells leads to a gradual degradation of DNA and RNA (Weichart 1997). Yamamoto *et al*. (Yamamoto 1996) found that a majority of VBNC cells of *Legionella pneumophila* contained degraded nucleic acids. Lazaro *et al*. (Lazaro 1999) demonstrated that nonculturable cells after 116 days in PBS buffer at 4°C maintained intact chromosomal DNA. Based on these observations the formation of VBNC cells is considered to be composed of two phases, transition to the VBNC state with concomitant loss of culturability while cellular integrity and intact nucleic acids are maintained, and gradual loss of cellular integrity and degradation of RNA and DNA ultimately leading to loss of viability. A number of studies have focused on nucleic acids associated with VBNC cells as an indirect measure of cell viability. Variations in rapid amplified polymorphic DNA (RAPD) profiles occurred in *Vibro* during the first few days of entry into a VBNC state, followed by loss of RAPD profile. Upon resuscitation, starved and VBNC cells regained RAPD profiles (Wang 1996). This suggests that, under stress conditions, DNA still exists in the cell but either DNA binding proteins or chromosomal supercoiling, or a combination of both, prevents DNA amplification. The *stx* gene in VBNC *Shigella dysenteria* (Rahman 1996) and the cholera toxin gene (*ctx*) in VBNC *V. cholerae* (Hasan 1994) were successfully amplified by PCR without any difficulties. Rahman *et al*. (Rahman 1996) reported that VBNC *S. dysenteria* maintained not only the *stx* gene, but also biologically active toxin.

Many methods have been used to assess the viability of VBNC cells, all of which have advantages and disadvantages. Some methods access viability by demonstration of metabolic activity or maintenance of cellular structures. Techniques which have been used as indicator of cellular metabolic activity include the use of microautoradiograph (Rahman 1994) as well as inducible enzyme activity (Nwoguh 1995) as an indicator of *de novo* protein synthesis, the direct viable count (DVC) method (Kogure 1979) which is based on the enlargement of cells upon addition of nutrient, and the reduction of tetrazolium salts as an indication of an active election transport chain (Rodriguez 1992). Cell viability assays have also been developed based on the staining of cells with fluorochromes. These methods assess viability by the maintenance of stable cellular structures. Acridine orange direct counts (Korgaonkar 1966) and 4′, 6-diamidino-2-phenylindole (DAPI) staining (Porter 1980) have been used as an indication of the maintenance of intact nucleic acids. Rhodamine 123 has been used widely as an indicator of membrane potential and with the development of flow cytometry, there has been a surge of methods for characterization of the physiological status of the cells (Davey 1996). Several investigators have studied VBNC cells in *C. jejuni* by employing a vital staining method incorporating fluorescence microscopy and image analysis (Rowe 1998) and 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC)–4′, 6-diamidino-2-phenylindele (DAPI) dihydrochloride double staining (Cappelier 1997).

There are numerous reports of *in vitro* resuscitation induced by mechanisms such as nutrient addition (Rozak 1984), transfer to fresh medium (Evdokinova 1994), temperature upshift (Nilsson 1991), and heat shock (Ravel 1995). In most of these reports, resuscitation could only be accomplished with those cultures which had been VBNC for a short period of time. Rollins *et al*. (Rollins 1987) demonstrated that nonculturable forms for *C. jejuni* could return to cultuable forms under appropriate conditions. *V. vulnificus* was shown to resuscitate when placed *in situ* in a warm marine water (Oliver 1995). Roth *et al*. (Roth 1988) showed that cells were recovered to the control level within 2 h after loss of culturability. *V. cholerae* had been nonculturable for up to 86 days and became culturable on solid media after heat shock at 45^{o}C for 1 min (Wai 1996).

In contrast, other researchers suggested the coccoid form is nonviable and a degenerate state of the spiral (Buck 1983; Moran 1986). The fact that presence of viable cells in a VBNC population consisting of viable, nonculturable and dead cells before resuscitation is required for the recovery of dormant cells may explain the inconsistency of resuscitation experiments (Votyakova 1994). This requirement may be due to either the presence of the cells themselves or the secretion of some signal from viable cells to stimulate the resuscitation of dormant cells.

Concerning the potential infectivity of VBNC-stage bacterial strains produced in laboratories, experimental studies have been contradictory. VBNC of *V. cholerae* and enteropathogenic *E. coli* were shown to regain culturability after animal passage (Colwell 1985). Grimes *et al*. (Grimes 1986) could resuscitate *E. coli* VBNC cells by introducing into ligated rabbit ileal loops. Culturable cells were isolated from feces of human volunteers who ingested cells which had been VBNC for 23 days, but not from those ingesting cells of *V. cholerae* that had been VBNC for 4 weeks (Colwell 1996). This result indicate that cells in the first stage maintain the potential for resuscitation and pathogenicity, while cells in the later stage are in the process of gradual loss of cellular integrity and are thus not capable of colonization. In another study, suspensions of *C. jejuni* strains, which were shown to be nonculturable were able to colonize mice (Jones 1991). Similarly, VBNC *C. jejuni* were resuscitated upon passage through rat gut (Saha 1991) and were shown to colonize one-week-old chicks (Stern 1994b). However, other researchers have demonstrated a lack of colonization of the intestines of one-day-old chicks when VBNC cells of *C. jejuni* were administered orally (Medema 1992). Likewise, no resuscitation was observed in simulated stomach, ileal, or colon environments (Beumer 1992). The studies discussed above indicate that it is not clear if VBNC cells are sufficient to cause disease or if they are not. In view of the potential epidemiological importance of VBNC cells, it would be important to establish unequivocally that they exist and to understand the process leading to the loss and subsequent recovery of culturability. Such research should focus on how to

quantify the mechanisms in environment which stimulate the commencement of VBNC stages and how best to control and eliminate these to ensure safety issues.

Epidemiology

C. jejuni has become recognized as a major human enteric pathogen with an incidence that exceeds that of *Salmonella* or *Shigella* (Griffiths 1990; Newell 1984). In the United States, *Campylobacter* is the most commonly isolated gastrointestinal pathogen (Tauxe 1992); an estimated 2.1 to 2.4 million cases of human campylobacteriosis occur each year. Similar rates are also reported for Europe and the United Kingdom (Skirrow 1991). Some studies have shown an increased incidence in recent years. However, these findings may reflect increased awareness and detection rather than a true increase in the incidence of disease (Ketley 1997; Skirrow 1992).

The age and sex distributions of *Campylobacter* infections are unique among bacterial enteric pathogens. A bimodal age distribution is observed in industrialized countries with peaks of incidence occurring among infants and young adults. In developing countries, only the infant peak is observed. Furthermore, there is a preponderance of males among infected persons, which begins during early childhood and persists until old age (Friedman 2000). The reasons for these age and sex distribution remain unknown. Since the beginning of national reporting on *Campylobacter* in the early 1980s, the infections have demonstrated a marked seasonal distribution, with a surge that begins in May and peaks in August (Friedman 2000). Most cases of human campylobacteriosis are sporadic. Outbreaks have different epidemiological characteristics from sporadic infections (Tauxe 1992). Outbreaks are predominantly related to consumption of contaminated water or raw milk while sporadic disease is generally associated with the mishandling and the consumption of raw or undercooked poultry (Altekruse 1998; Altekruse 1999; Tauxe 1992). Other risk factors accounting for a smaller proportion of sporadic illnesses include drinking untreated water (Hopkins 1984); traveling abroad (Norkrans 1982); eating barbequed pork (Oosterom 1984) or sausage (Kapperud 1992a); drinking raw milk (Hopkins

1984; Schmid 1987) or milk from bird-pecked bottles (Lighton 1991); and contact with dogs (Kapperud 1992a) and cats (Deming 1987; Hopkins 1984), particularly juvenile pets or pets with diarrhea (Norkrans 1982; Saeed 1993).

Campylobacter spp. frequently are present in the intestinal flora of commercially raised birds and such wild birds as pigeons, sea gulls, crows, ravens, cranes, ducks, and geese. The organism is also found in rodents and insects (Cabrita 1992; Glunder 1992; Luetchefeld 1980). The intestines of poultry are easily colonized with *C. jejuni*. Day-old chicks can be colonized with as few as 35 organisms (Kaino 1988). Most chickens in commercial operations are colonized by 4 weeks (Humphrey 1993; Kapperud 1993). Vertical transmission from breeder flocks to progeny has been suggested in one study but is not widely accepted (Pearson 1996). Potential routes of entry of *Campylobacter* into a flock include infection of newborn chicks from older birds, unchlorinated water, wild or game birds, beetles and farm workers (Jacobs-Reitsma 1995; Humphrey 1993; Kapperud 1993; Kazwala 1990; Pearson 1996).

During the slaughtering process, *C. jejuni* from the intestinal contents of processed birds spread to the carcasses. *C. jejuni* has been recovered from the scald-tank water, feathers, offal flumes, chiller and equipment (Blaser 1986; Wempe 1983). Processing steps that spread contamination include: transport to the plant, electrical stunning, immersion scalding, defeathering, evisceration, continuous water bath chilling and use of recycled water. Wempe *et al*. (Wempe 1983) demonstrated that chilling and feather picker water were the major sites for cross-contamination. *C. jejuni* appears to be a normal commensal of all classes of bovine. In one outbreak, 525 specimens were obtained from 100 slaughtered beef cattle and examined for the presence of *C. jejuni* and *C. coli* of the 100 animals, 50 were positive for *C. jejuni* and 1 was positive for *C. coli* (Sjögren 1996). The organism can be isolated from gallbladders, large intestines, small intestines and livers. Carcasses may become contaminated with intestinal contents but this is infrequent. *Campylobacter* can be present in raw milk at levels of 4.5 – 9 % (Beumer 1988). Mechanism of raw milk contamination is presumed to occur through fecal origin

or mastitis infection (Lander 1980). Carcass contamination by *Campylobacter* species appears more common in swine carcasses than in cattle, sheep, or goats. Swine commonly carry *C. coli* and occasionally *C. jejuni* as intestinal commensal inhabitants (Luechtefeld 1982; Svedhem 1981). Contact with domestic pets is also an important source of human campylobacteriosis. The incidence of human *Campylobacter* infection correlates with the seasonal pattern of canine births (Evans 1993). Support for this correlation is that fecal shedding of *C. jejuni* from both dogs and cats appears to be age dependent, peaking in the first year and declining thereafter (Altekruse 1994).

Outbreaks of waterborne campylobacteriosis in the United States have been traced to the drinking, during outdoor recreational activities, of contaminated spring water or other water not meant for drinking. Domestic or wild animals and birds potentially are significant sources of *Campylobacter* found in polluted water (US Department 1983). In temperate regions, recovery rates of *Campylobacter* are highest during the cold season (Bolton 1987; Carter 1987). Survival in cold water is important in the life cycle of *Campylobacter*. Unchlorinated drinking water can introduce *Campylobacter* into the farm environment (Humphrey 1987; Pearson 1993).

The significance of food handlers in transmission of *Campylobacter* infection has not been fully defined, nor has the role of person-to-person contact. Two cooks who were asymptomatic excreters of *C. jejuni* did not transmit the pathogen to prepared foods (Norkrans 1982). However, *Campylobacter* were isolated from the hands of a food handler suffering from campylobacteriosis (Blaser 1982). Human-to-human transmission of *Campylobacter* can occur and some studies demonstrated that transmission from ill children to family members occurred frequently (Jones 1981; Pai 1979). By contrast, in two outbreaks among Japanese children secondary transmission was not observed (Yanagisawa 1980).

Prenatal transmission of *Campylobacter* infection is possible, although rare. Pregnant women infected with *Campylobacter*-associated bacteremia may pass on a severe systemic infection to the fetus (Blaser 1983). It is also possible for neonates to acquire infection during or

shortly after birth. Anders *et al*. (Anders 1981) reported seven cases of *Campylobacter* enteritis in neonates over a 2-year period in Denver in which symptoms began from 2 to 11 days after birth. Although the reported incidence of *Campylobacter* infection among homosexual men is almost 40 times greater than in the general population (Sovillo 1991), recent analysis shows the rate is not higher than among heterosexual men of a similar age (Friedmann 2000).

The epidemiology of *Campylobacter* infection is quite different in developing countries than in the industrialized world. *Campylobacter* infections are hyperendemic among children, especially those aged under two years in tropical developing countries. Asymptomatic infections occur commonly in both children and adults, but are unusual in developed countries (Blaser 1981). In addition, outbreaks of infection are uncommon and the illness lacks the marked seasonal nature observed in industrialized nations. Nevertheless, in both developed and developing countries, *Campylobacter* remains one of the most common bacterial causes of diarrhea.

Poultry Colonization by *Campylobacter jejuni*

Much of the world's poultry production is contaminated with *Campylobacter* and this has been implicated as a major source of human infections. Removal of these organisms from the poultry food chain or at least reduction in their frequency, has now become a significant objective of *Campylobacter* research. Evidence for poultry meat being the prime source of human *Campylobacter* infection is mostly indirect, because most cases are sporadic, and not traced to a specific source. A few outbreaks have been attributed to poultry meat, usually undercooked, or cross-contaminated from raw poultry (Pebody 1997; Rosenfield 1985). Certainly, a significant proportion of chicken meat is contaminated with *Campylobacter* at retail, and at least some of the serotypes of these poultry strains are identical to those found in humans (Nielsen 1997; Nielsen 1999). However, the pathogenicity of all poultry *Campylobacter* is debatable. The distribution of serotypes in *C. jejuni* strains from poultry and humans are not necessarily the same and recent

data obtained by using genotypic techniques support some host specificity among clones of *Campylobacter*. Genotypes by *flaA* typing frequently present in poultry strains are not always detectable in strains of human isolates (Clow 1998).

C. jejuni usually is not isolated from the hatchery, nor is it typically isolated from the production environment during the first two weeks after chicks are placed. However, by the third or fourth week of production, most flocks are contaminated to some extent and the bacterium eventually spreads to almost all members of the flocks. The intestinal tract of chickens may colonize up to 10^7 CFU/ml without any clinical disease in the birds (Stern 1988). The seasonality generally shows a higher rate of infection in summer than in winter (Jacobs-Reitsma 1994). The reason for this seasonal variation is unknown but probably reflects levels of environmental contamination. Poultry houses have more ventilation in the summer, increasing the contact of the birds with the outside environment. Rates of isolation appear similar among in intensive-reared and free-ranged bird flocks when *C. jejuni* was isolated from cloacal swabs (Adekeye 1989). Colonization is found in the ceca, large intestine, and mucus with the cloacal crypts. The preferential attraction to the cecal crypts may be due to chemotactic attraction to the mucin (Hugdahl 1988). Investigations suggest that *Campylobacter* do not directly adhere to the intestinal surface but rather rapidly track along the intestinal mucus (Lee 1986). *Campylobacter* have also been recovered from extraintestinal sites, including the liver, and spleen, even in asymptomatic birds (Beery 1988). It is generally accepted that chickens, once colonized with *Campylobacter*, remain chronically infected for many weeks. For broiler chickens, this condition continues until slaughter.

Prevention and Control

Because most *Campylobacter* infections are acquired by consuming or handling poultry, the best way to control human infections would be to limit contamination of poultry flocks. However, the near-universal contamination of poultry with *Campylobacter* and the heavy

bacterial burden in these flocks make elimination of *Campylobacter* in chickens impractical. Current mass production and distribution of chicken may amplify the bacterial load. Several intervention strategies at the poultry production level are currently being developed. The most effective way will probably be a stratified one with initial measures to minimize exposure of the flock to the organism and subsequent measures to prevent any such exposure from leading to maximal colonization.

Several epidemiological studies have identified risk factors associated with flock infection (Jacobs-Reitsma 1997; Humphrey 1993; Kapperud 1993; van de Giessen 1992). These risk factors included poor broiler house maintenance, inadequate staff training, insufficient cleansing and disinfections between flocks, close location to other poultry sites or farm animals, and contaminated water supplies. Rapid dissemination of organisms throughout the flock is then facilitated by communal drinkers and feed lines. Therefore, most studies have concluded that the most significant measures in preventing infection are associated with biosecurity. This approach has been relatively empirical, focusing on simple intervention measures such as use of closed houses, treatment of drinking water, control of rodents, flies and wild birds, restriction on the staff and equipment entering the house, and improved hygiene procedures by poultry house workers, concentrating on changing of outer clothes and efficient dipping of boots. Significant reduction in *Campylobacter* infection in broilers after the application of strict hygienic measures has been reported (van de Giessen 1998). Improved methods of detection would help to elucidate modes of infection. Polymerase chain reaction (PCR)-based techniques undoubtedly detect more *Campylobacter* (Studer 1999), and it is possible to identify and type strains, but such methods do not distinguish between live and dead or VBNC organisms.

Efficacy of competitive exclusion against *Campylobacter* colonization appears variable. Stern *et al*. (Stern 1994a) observed that mucosal competitive exclusion flora derivatization was inconsistently effective against chicken colonization by *C. jejuni.* Chen *et al*. (Chen 2001) demonstrated that it is possible to use combination of *C. jejuni* chicken isolates as a defined

bacterial preparation for the competitive exclusion of human-pathogenic *C. jejuni* in poultry. However, in other experiments, similar agents had no effect despite showing efficacy against *Salmonella* (Aho 1992). The oral administration of the yeast *Saccharomyces boulardii* to *Campylobacter*-infected broiler reduces the level, although not the prevalence of cecal colonization (Line 1997). Similar results have been obtained with a mixture of *Lactobacillus acidophilu*s and *Streptococcus faecium* (Morishita 1997). The success of competitive exclusion approach is dependent on the identification of highly colonizing strains, which will exclude or replace other strains, the identifications and elimination of virulence factors from these strains, and the stability of this nonpathogenic phenotype. Vaccination strategies employed to date include passive immunization, inactivated whole cell vaccines, gene vaccines, subunit vaccines, and antigens presented in microparticulate form (Baqar 1995; Pawelec 1997; Rice 1997; Scott 1997; Widders 1996). Inactivated bacterial whole-cell vaccines have been the most widely used treatment against *Campylobacter* in poultry (Rice 1997).

Slaughter and processing provide opportunities for reducing *C. jejuni* counts on food-animal carcasses. Bacterial counts in carcasses can increase during slaughter and processing steps. Stern *et al*. (Stern 1995) reported that *Campylobacter* on carcasses increased up to 1,000-fold during transportation to slaughter. Scalding reduced carcass counts of *Campylobacter* to near or below detectable levels in Texas turkey plants (Acuff 1986). Addition of sodium chloride or trisodium phosphate to the chiller water in presence of an electrical current reduced *C. jejuni* contamination of chiller water by 2 $\log_{10}$ units (Li 1995). Use of chlorine in water sprays and in chill water has been found useful in reducing numbers but is not generally thought useful for eliminating *Campylobacter* from the final product (Cason 1997; Mead 1995). A risk-analysis study for *Campylobacter* in fresh chicken concluded that chlorination of chill tank water could reduce the risk of *Campylobacter* infection by 25 % (Fazil 1999). Campylobacters are more sensitive to gamma irradiation than most Gram-negative bacteria, including *Salmonella* and *E. coli* O157:H7. A radiation dose of 2.5 kGy reduced *C. jejuni* levels on retail poultry by 10 $\log_{10}$

Units (Patterson 1995). Irradiation has an advantage that it would inactivate organisms in skin surface, skin folds, crevices, and feather follicles unlike most other decontamination treatments. Small quantities of irradiated poultry are sold through a few outlets in the United States (Mulder 1999).

Pathogenesis

The pathogenesis of *Campylobacter* gastrointestinal infection is not well understood, perhaps due in part to the lack of a good animal model. Though it was demonstrated that *Campylobacter* enteritis can be developed by ingestion of as few as 500 cells by a volunteer (Robinson 1981), the dose for causing diarrhea is varied with a range of $8x10^2$ to $2x10^9$ cells and development of disease was not always dose-dependent (Black 1988). After ingestion, *Campylobacter* colonizes the distal ileum and colon via penetration of the intestinal mucus or adhesion to intestinal cell surfaces. The bacteria disrupt intestinal absorption and can produce intestinal damage and associated inflammatory infiltrates (Ketly 1997; Wooldridge 1997). Evidence suggests that they also may invade intestinal epithelial cells and spread transiently to the bloodstream (Skirrow 1994; Wooldridge 1997).

Many pathogen-specific virulence determinants may contribute to the pathogenesis of *C. jejuni* infection, but none has a proven role. Colonization of the intestinal requires the ability to move into the mucus layer covering the intestinal cells. *Campylobacter* motility is conferred by the polar flagella, and combined with their cork-screw form allows them to penetrate this mucus barrier (Newell 1985; Szymanski 1995). The flagellum of *C. jejuni* consists of an unsheathed polymer of flagellum subunits, which are encoded by the adjacent *flaA* and *flaB* genes (Nuijten 1990). Mutants of *flaA* have been shown to be unable to invade human intestinal epithelial cells *in vitro* (Wassenaar 1991) or to colonize 3-day-old chicks (Nachamkin 1993). Chemotaxis is essential for *C. jejuni* colonization, as non-chemotactic mutants were unable to colonize the intestine in animal models (Takata 1992). *C. jejuni* is attracted to mucins, L-serine and L-fucose,

whereas bile acids are repellants (Hugdahl 1988). *C. jejuni* and *C. coli* adhere to cell lines *in vitro* and several molecules have been examined for adhesive properties, but there is no definitive evidence for a specific adhesin *in vivo* (Ketley 1997). *C. jejuni* crosses the mucus layer covering the epithelial cells and adhere to these cells, and a subpopulation subsequently invades the epithelial cells. The invasion of epithelial cells can cause the mucosal damage and inflammation, but it is unknown whether inflammation has a direct role in epithelial damage and/or diarrhea. Adhesion and invasion are dependent on both motility and flagella expression (Yao 1994) and adhesion is often mediated by fimbrial structures (Doig 1996). Damage to the intestinal epithelium may result from invasion, toxin production, induction of host inflammatory mediators, or some combination of these phenomena.

Campylobacter species have been reported to produce several different toxins, including toxins similar to cholera toxin, shiga toxin, and hemolysin (Ketley 1997; Wassenaar 1997). Recently a cytolethal distending toxin has been described in *C. jejuni* (Whitehouse 1998). It functions by inhibiting host cell division by blocking cell at the G_2 phase (Whitehouse 1998). The ability to acquire the essential nutrient iron from the host contributes to bacterial pathogenesis. *C. jejuni* expresses several ferric iron acquisition systems upon growth in iron-restricted conditions. A haemin/haemoglobin uptake system (Rock 1999) and an enterochelin transport system lacking an outer membrane receptor (Richardson 1995) have been identified and verified to be involved in iron acquisition. Lipooligosaccharide (LOS) and lipopolysaccharide (LPS) form a major component of the Gram-negative outer membrane, and are important virulence factors involved in serum resistance, endotoxicity and adhesion. The cloning of a *C. jejuni* operon containing genes thought to be involved in LOS/LPS biosynthesis (Wood 1999), as expression of these genes in *E. coli* led to the production of an O-antigen recognized by antisera to *C. jejuni* LPS (Korolik 1997).

C. jejuni must be able to respond to temperature change, as they can be found in the avian gut, where the temperature is 42^{o}C, as well as temperature in human host (37^{o}C) and during

transmission in water, milk or on meat at 40^{o}C or varying temperatures. The heat shock protein (hsp) has a significant role in themotolerance as well as in the response to other stresses by acting as chaperons to promote the folding of most cellular proteins and proteolysis of potentially deleterious, misfolded proteins. Several heat shock proteins have been identified in *C. jejuni*, including GroESL, DnaJ, DnaK, and ClpB proteins (Konkel 1998; Thies 1999a; Thies 1999b; Thies 1999c). However, a function in *C. jejuni* pathogenesis has only been demonstrated for the DnaJ protein, as a *C. jejuni dnaJ* mutant was unable to colonize chickens (Konkel 1998).

Clinical Aspects in Humans

Most typically, infection with *C. jejuni* results in an acute, self-limited gastrointestinal illness characterized by diarrhea, fever, and abdominal cramps. Most affected individuals experience abdominal pain and diarrhea with 1 to 5 days of exposure. Vomiting, stools containing blood, mucus, and inflammatory exudate cells, and transient bacteremia also are associated with *Campylobacter* infection (Ketley 1997; Skirrow 1993). The diarrhea is either loose and watery or grossly bloody. Symptoms typically last for 4 to 5 days and usually resolve within a week or two. Occasionally, however, patients can develop a longer, relapsing diarrhea illness that lasts several weeks (Kapperud 1992b). *C. jejuni* is seldom isolated from normal individuals in developed countries, but usually isolated from healthy individuals in developing countries (Nachamkin 1998). The reactive arthritis that sometimes follows *Campylobacter* enteritis is no different from that associated with *Salmonella*. Multiple joints can be affected, particularly knee joint. Pain and incapacitation can last for months or become chronic. The mean interval between the onset of bowel symptoms and the appearance of painful swelling of the joint was 14 days, with a range of 3 days to 6 weeks (Peterson 1994). The most important sequelae of *C. jejuni* infection is the Guillain-Barré syndrome (GBS), which is an acute demyelinating disease of the peripheral nervous system that affects 1 to 2 persons per 100,000 population in the United States each year. Up to 40 % of patients with the syndrome have evidence of recent *Campylobacter* infection

(Allos 1997). Approximately 20 % of patients with GBS are left with some disability, and approximately 5 % die despite advances in respiratory care. O serotyping has been especially important in linking particular types with GBS. Penner's serotype O:19 as well as O:41 is often involved in GBS and the antigen has core oligosaccharide LPS structures that mimic ganglioside structures such as GMI and GD1a, a component of motor neurons (Kuroki 1993; Misawa 1998). Among GBS patients, those with preceding *C. jejuni* infection are more likely to have anti-GMI antibodies (Rees 1995; Visser 1995). Other studies showed that patients with antibodies to *C. jejuni* and GMI are more likely have axonal destruction and more severe disease (Gregson 1993; Rees 1995). Host characteristics may play a role in the pathogenesis of *C. jejuni*-induced GBS. A familial tendency for development of GBS has been noted in several reports (Davidson 1992; Luijckx 1997; Ropper 1991). Additionally, two reports described *C. jejuni* O:19 infections followed by GBS in sibling pairs (Aspinall 1994; Yuki 1995), further supporting the hypothesis that there is individual inherited susceptibility to *C. jejuni*-induced GBS.

Mode of Detection

Most food products require the use of enrichment methods to detect the small numbers of *Campylobacter*. Raw poultry products harboring sufficiently large numbers of the organism can be detected by employing direct plating. Appropriate sample care, a large sample size and the use of a selective enrichment broth, proper incubation conditions including appropriate microaerobic conditions, elevated temperature (42^{o}C) and the plating of enrichments onto appropriate selective agar media are essential for the isolation of *Campylobacter* (Stern 1982a; Stern 1992a). Samples for analysis should be protected from exposure to air, transported under refrigeration, and analyzed as soon as possible after sampling. Most *Campylobacter* species require a microaerobic atmosphere containing approximately 5 % oxygen, 10 % carbon dioxide, and 85 % nitrogen for optimal recovery. Oxygen at atmospheric condition (21 %) is toxic to *Campylobacter* and oxygen tolerance of the bacteria depends greatly on the species and strains (Chynoweth 1998; Stern

1982b). Luechtefeld *et al*. (Luechtefeld 1982) observed that the atmosphere of 5 % oxygen and 8 % carbon dioxide recovered more *C. jejuni* counts than that of 17 % oxygen and 3 % carbon dioxide. Most of *Campylobacter* media contain a basal medium consisting of beef extract, peptone, yeast extract, and sodium chloride to which supplements such as lysed horse or sheep blood are added. The addition of reducing agents, such as 0.025 % each of ferrous sulfate, sodium metabisulfate, and sodium pyruvate (FBP) enhances the recovery of *Campylobacter* (George 1978). These reducing agents are considered to play a role in quenching toxic superoxide anions. Most of selective media contain one or more antimicrobial agents, mainly cefoperazone, as the primary inhibitor of enteric bacterial flora. These antibiotics, such as cefoperazon, amphotericin B, cycloheximide, polymyxin B, rifampicin, trimethoprim lactate, and vancomycin improve the selective growth of *Campylobacter*. Typical morphology of colonies on agar plate appears smooth, convex, and glistening with a distinct edge or flat, shiny, translucent, and spreading with an irregular edge. The colonies are colorless to grayish or light cream and may range from pinpoint to 4 to 5 mm in diameter (Stern 1992b). Oxidase-positive colonies showing Gram-negative, curved to S-shaped rods by Gram stain on selective media at 42^{o}C under microaerobic conditions can be presumptively reported as *Campylobacter* spp. until other biochemical tests are conducted. Although separation of *C. jejuni* and *C. coli* may not be necessary, two strains may be differentiated by hippurate hydrolysis test; *C. jejuni* is positive (Skirrow 1980). Phenotypic assays still remain the most useful methods for the routine differentiation of *Campylobacter*. The most useful tests for initial identification include growth temperature studies, catalase production, hippurate hydrolysis, indoxyl acetate hydrolysis, nitrate reduction, production of H_2S, and antibiotic susceptibility (Barrett 1988). Because many *Campylobacter* species are difficult to identify, molecular methods have been developed to differentiate a number of species. *C. jejuni* and *C. coli* have been reported to be differentiated based on hippurate hydrolysis test (Skirrow 1980), polymorphisms in the *ceuE* gene (Gonzalez 1997), a GTPase gene (VanDoorn 1997), the

16 S ribosomal DNA gene (Cardarelli 1996; Linton 1997), and the 23 S rDNA gene (Hurtado 1997) by PCR and/or PCR-restriction fragment length polymorphism analysis.

The immunomagnetic-PCR assay was developed for the detection of *C. jejuni* in milk and chicken products (Docherty 1996). Target bacteria were captured from the food sample by magnetic particles coated with a specific antibody and the bound bacteria subjected to PCR. The technique could detect 420 CFU/g of chicken after 18 h, 42 CFU/g after 24 h, and 4.2 CFU/g after 36 h enrichment. Lamoureux *et al*. (Lamoureux 1997) detected thermophilic *Campylobacter* after enrichment step by using immunomagnetic separation (IMS)-hybridization assay. In one of the methods, the captured cells were lysed and the 23 S rRNA was reacted with a microtitre plate-immobilized rDNA probe specific for the *Campylobacter*. In the other methods, the genomic DNA of captured cells was reacted with a microtitre plate-immobilized RNA probe after lysis. The detection limit of the IMS rRNA probe-rDNA hybridization with cells in pure culture was 10^4 CFU/ml; the detection limit of the more specific IMS DNA probe-RNA was 10^8 CFU/ml. Two alternative immunomagnetic beads (IMB) were tested for direct detection of *C. jejuni* in artificially contaminated ground poultry meats. Polyclonal anti-*Campylobacter* antibodies were used to coat tosylactivated Dynabeads, and conjugated with biotin that was used to label streptavidin-coated beads. Without preenrichment, this approach could detect 10^4 CFU/g of ground poultry meats. The major theoretical advantage of IMS includes the concentration of the target, improved assay speed, and an increase in recovery of sublethally damaged cells. Possible drawbacks to IMS are aggregation of bacterial cells and recovery of high level of competing microflora.

PCR-based detection methods are rapid, sensitive, and specific though they cannot differentiate between viable and dead cells. Nested primer PCR amplification was developed to detect *C. jejuni* in poultry rinses without the need for an enrichment step (Winters 1997; Winters 1998). A portion of the reaction mixture containing the primary PCR product was added to a second PCR mixture containing the same forward primer and a different reverse primer

complementary to a region contained within ('nested') the 3'end of the first 159 bp primary PCR product. The secondary PCR assay produced a 122 bp product with *C. jejuni*, but not *C. coli*. The nested PCR assay detected as low as 100 cells per ml within 24 h. Stonnet *et al*. (Stonnet 1995) successfully detected all tested the *C. jejuni* and *C. coli* strains without any false positive by using a PCR amplification. Flagellin genes have often been used as target sequence for the detection of *C. jejuni* and *C. coli* resulting in a higher level of sensitivity and specificity than DNA probe-based method (Comi 1995). A PCR method detected *Campylobacter* spp. from cloacal swabs of chicken by employing *flaA* gene (Oyofo 1997). The PCR product was detected on southern blots after hybridization of a digoxigenin-labelled probe. Between 35 and 120 cells of *C. jejuni* per ml of cloacal sample were detected. A multiplex PCR assay, which allows several DNA segments to be amplified by using multiple pairs of primers, has been developed for *C. jejuni* detection. The technique could distinguish *C. jejuni* and *C. coli* by which one set primer amplifies a conserved region of the flagellin genes in *C. jejuni* and *C. coli* and the other set amplifies DNA of an unknown gene present in *C. jejuni* (Harmon. 1997). The specificity and sensitivity of the PCR technique are ideal for the detection of a target organism but the presence of amplification products does not imply that the organisms were viable. An alternative system, nucleic acid sequence-based amplification (NASBA®) was reported targeting RNA sequences by isothermal transcription (Kievits 1981). The NASBA® process involves the concerted action of three enzymes (reverse transcriptase, RNase H, and T7 RNA polymerase) as well as two primers to amplify a RNA target sequence. Uyttendaele *et al*. (Uyttendaele 1995) detected pathogenic campylobacters by using a 16S rRNA target and demonstrated that it was specific and sensitive.

mRNA is turned over rapidly in living bacterial cells, with most mRNA species having a half-life of only a few minutes (Alifano 1994; Belasco 1993). Because mRNA is more rapidly degraded in cells than rRNA or chromosomal DNA, an assay targeting bacterial mRNA would provide a better guide to bacterial viability than amplification tests directed at DNA or rRNA targets. Bej *et al*. (Bej 1996) used reverse transcriptase-PCR (PT-PCR) to examine *Vibrio*

cholerae exposed to heat or starvation and detected mRNA only in viable cells. Patel *et al*. assessed the viability of heat-inactivated *Mycobacterium leprae*, detecting heat shock protein only living cells (Patel 1993). Klein and Juneja developed an RT-PCR method for viable *Listeria monocytogenes* and successfully applied it to the detection of the pathogen in food (Klein 1997). Limitation of RT-PCR as an indicator of cell viability was explored in *E. coli*, and the investigators found that the persistence of mRNA in dead cells depended on the mRNA species, the method of cell inactivation and the subsequent holding conditions of the cell (Sheridan 1998). Sails *et al*. (Sails 1998) developed RT-PCR method to detect *C. jejuni, C. coli* and *C. upsaliensis*. The detection of mRNA was possible by RT-PCR immediately after heat inactivation but became undetectable within 4 h when the cells were held at 37^{o}C. A recent study has demonstrated that the presence of viable but nonculturable cells may be detected by RT-PCR analysis if an appropriate assay target is utilized. The expression of several genes under thermal treatment and VBNC state was examined to determine a suitable mRNA target for RT-PCR amplification in *E. coli* O157:H7 (Yaron 2002). In this study, they observed that 16 S rRNA, *mobA*, *rfbE* and *stx1* are good targets for the detection of the presence of VBNC *E. coli* O157:H7. mRNA of *stx1* and *rfbE* were detected after autoclaving (121^{o}C for 15 min) but *fliC*, *rfbE* and *stx1* mRNAs were degraded after heat treatment at 60^{o}C for 20 min. Birch *et al*. (Birch 2001) assessed three amplification techniques, PCR, RT-PCR and NASBA®, for their ability to detect nucleic acid persistence in *E. coli* following heat killing and suggested NASBA® provided the greatest sensitivity of the three methods tested. The presence of mRNA was detected by NASBA® for up to 30 h post-death but by RT-PCR for up to 7 h post-death.

Microorganisms have been analyzed by mass spectrometry (MS). Matrix-assisted Laser Desorption Ionization Time-of-shift (MALDI-TOF), a soft-ionization MS technique, is very useful for analyzing proteins and other molecules sensitively and accurately (Arnold 1998; Wang 1998). Approximately 100 strains of *C. jejuni, C. coli*, and nonpathogenic *Campylobacter* spp. obtained from clinical and poultry isolates have been analyzed by MALDI-TOF to identify

species-specific ions (Haddon 1998). A comparison of the ion profiles of three strains revealed a number of species-specific ions, in addition to common ions present in both *C. jejuni* and *C. coli* strains.

Epidemiological Typing Systems

The subtyping is essential to epidemiological studies of the genes, providing information on relationships between isolates, determining the extent of disease outbreaks, and identifying sources of the organism and modes of transmission. Typing methods can be divided into two main categories, phenotype-based and genotype-based schemes.

Serotyping

Two methods of serotyping are commonly used for *C. jejuni*, the heat-stable or somatic O antigen (Penner) and the heat-labile antigen (Lior) schemes (Penner 1980; Lior 1982). In the Penner scheme, passive haemagglutination was introduced as the detection system in attempt to eliminate non-specific agglutination reactions. However, reproducibility problems can occur as a result of variation in the source, age, concentration and condition of the red blood cells (Fricker 1987). In order to address these problems, a modified serotyping scheme based on direct bacterial agglutination and using absorbed antisera was developed in the Laboratory of Enteric Pathogens (LEP) (Frost 1998). The LEP typing scheme improves reproducibility problems due to variations in blood cells and non-specific reactions were eliminated by increased reaction temperature to 50^{o}C with shaking.

Multilocus Enzyme Electrophoresis (MEE)

MEE has been used for many years in eukaryotic population genetics and recently it has been applied to bacterial diversity (Selander 1986). The mechanism of MEE is based on the electrophoretic migration distance of enzyme present in bacteria. Enzyme mobility differences relate directly to allelic variation in the structural gene locus for each enzyme. Aeschbacher and Piffaretti (Aeschbacher 1989) reported the finding of 50 MEE electrophoretic types (ETs) among

105 *C. jejuni* isolates and 14 ETs among 21 *C. coli* strains. Fraser *et al*. (Fraser 1992) demonstrated that 27 strains of *C. coli* were clustered into 9 different ETs and 17 of them grouped into ETI. MEE is relatively time-consuming, highly complex and depends on the number of enzyme analyzed and the use of appropriate electrophoretic parameters.

Multi Locus Sequence Typing (MLST)

MLST is an unambiguous procedure for characterizing bacterial isolates using the sequence of internal fragments of seven house keeping genes (Maiden 1998). It is based on the principles of MEE, but differs in that it assigns alleles at multiple house keeping loci directly by DNA sequencing, rather than indirectly via the electophoretic mobility of their gene products. The great merit of MLST is that sequence data is unambiguous and the allelic profiles of isolates can easily be compared to those in a large central database via the Internet. Dingle *et al*. (Dingle 2001) showed that *C. jejuni* was genetically diverse with a weakly clonal population structure and horizontal genetic exchange had a major effect on the structure and evolution of *Campylobacter* populations by performing MLST.

Random Amplified Polymorphic DNA (RAPD)

The RAPD method is based on the use of arbitrary primers, which are allowed to bind to target DNA and then amplify sections by normal PCR (Welsh 1990; Williams 1990). Species-specific probes have been developed to identify *C. jejuni, C. coli* and *C. lari* from arbitrarily primed PCR products (Day 1997; Giesendorf 1993). Hernandez *et al*. (Hernandez 1995) found that 178 among 208 strains were typeable whereas Madden *et al*. (Madden 1996) observed that all of 200 *C. coli* porcine isolates and 76 *C. jejuni* clinical isolates were typeable. RAPD is faster and cheaper than PFGE and does not require a complex apparatus. It is also less sensitive to genetic instability than *fla* typing. However, the analysis has poor reproducibility.

Pulsed Field Gel Electrophoresis (PFGE)

PFGE is a modification of the more traditional restriction fragment length polymorphism (RFLP) methods, used for the subtyping of many bacterial genera including *Campylobacter*

species (Yan 1991). In PFGE fingerprinting bacterial cells are embedded chromosomal-grade agarose and lysed *in situ* to prevent DNA shearing. Then "rare cutting" enzymes are used to cut the target DNA into relatively few fragments of comparatively large size. The DNA fragments are then separated by alternating electric field in more than one direction through gel. PFGE has been successfully used to group *C. jejuni* isolates from human isolates (Gibson 1995; Iriarte 1996), the same poultry flock (On 1998), and cattle and their drinking water supply (Hanninen 1998). There are some cases of *Campylobacter* that cannot be subtyped by PFGE. This may be due to DNase production and overcome by formaldehyde treatment (Gibson 1994; Owen 1995). Although the discriminatory potential is excellent, several disadvantages are noted: 1) Preparation of the DNA-containing agarose blocks is time-consuming and tedious. 2) The DNase of some *Campylobacter* strains must be inactivated to ensure that DNA samples do not degrade before electrophoresis. 3) The apparatus used for the electrophoresis is specialized and expensive. 4) Interpretation of the result can be difficult since genetic instability can lead to minor or major changes in profiles.

Amplified Fragment Length Polymorphism (AFLP)

AFLP is a new high-resolution genotyping method whose value in the typing of campylobacters has been reported (Duim 1999; Kokotovic 1999). AFLP analysis involves the digestion of chromosomal DNA with two restriction enzymes and the use of specific oligonucleotide adapters and primers to amplify a subset of DNA fragments from the whole genome. The oligonucleotides should allow ligation compatible with the restriction enzymes used for digestion, eliminate the restriction site after ligation, and create a template sequence for subsequent PCR amplification. Dendograms constructed by using AFLP banding profiles showed highly relatedness between some Dutch human poultry isolates (Duim 1999). Similar studies in Denmark identified isolates of *C. jejuni* from human, poultry and cattle (Kokotovic 1999). AFLP analysis was the highly discriminatory method, identifying 41 different groups, while PFGE showed 38 distinct types, *flaA* typing separated 31 different genotypes, and ribotyping

discriminated 26 distinct groups (de Boer 2000). The problems of AFLP analysis include high cost, standardized procedures must be established, and it is a complicated technique. Nevertheless, it seems likely that AFLP analysis will be more widely used in the near future (Wassenaar 2000).

Ribotyping

The presence of multiple copies of the rRNA genes coding for 16 S and 23 S rRNA at different positions on the genome and the strong conservation of these genes among bacteria make them suitable for typing purpose. The most widely used technique is Southern blot hybridization of chromosomal DNA, digested with a six-cutter restriction enzyme and hybridized with a common rRNA probe. A 16 S ribotyping scheme using a combination of *HaeIII* and *PstI* digestion identified over 80 types within *C. jejuni* (Fayos 1992). One unique property of *Campylobacter* strain is that it has fewer ribosomal genes than other bacteria, which reduces the discriminatory power of the method. The RiboPrinter system operates a completely automated and reproducible ribotyping procedure for identification and characterization of bacteria. Automated ribotyping with *PstI* as the restriction enzyme could identify 29 different types (ribogroups) in a set of 48 reference *Campylobacter* strains isolated from geographically unrelated poultry flocks (Thomas 1997). Automated ribotyping provides a unique opportunity for the interlaboratory exchange of data from a standard typing system. However, the high cost for equipment and kits, and the low throughput (24 samples per day) restrict the use of this technology.

fla Typing

The expression of flagella filament is composed of two genes, *flaA* and *flaB*, which are arranged in tandem in both *C. jejuni* and *C. coli* (Wassenaar 1991). Since both highly conserved and variable regions are present, this locus is suitable for restriction fragment length polymorphism (RFLP) analysis of PCR product (Meinersmann 1997).

Primers are synthesized based on the conserved sequence and a product incorporating both the conserved and variable regions can be prepared by PCR. This product can then be digested with appropriate restriction endonuclease to reveal RFLP after the products are separated by gel electrophoresis. The selection of enzymes has a significant effect on discrimination, and the combination of enzymes such as *DdeI* and *HinfI* has been shown to improve discrimination substantially in veterinary isolates (Ayling 1996). *fla* typing has been successfully applied to *Campylobacter* from broiler flock (Ayling 1996; Stern 1997; Thomas 1997) in an effort to understand and control their manner of infection. Similarly, *Campylobacter* from foodstuffs (Madden 1998) and from animals and water (Koenraad 1995; Lorenz 1998) have been investigated. A short variable region (SVR) of approximately 150 bp has been described (Meinersmann 1997) and sequencing of this region enables fingerprinting of strains. Application of *fla* typing is relatively simple and fast and involves widely available reagents and equipment. Potentially one of the most significant disadvantages of *fla* typing is related to genetic instability. There is strong evidence for recombination between *flaA* genes of different strains (Harrington 1997) and between the *flaA* and *flaB* gene of individual strains. The frequency of such events under natural conditions is currently unknown, but *fla* typing cannot be considered a particularly stable long-term typing method. *fla* typing therefore needs to be used in conjunction with other phenotypic or genotypic methods.

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CHAPTER III

HEAT-TREATED CAMPYLOBACTER SPP. AND MRNA STABILITY

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ABSTRACT

Polymerase chain reaction (PCR) is a rapid, specific, and sensitive technique for detecting pathogenic bacteria. However, the technique cannot distinguish between viable and nonviable cells. Bacterial mRNA may be as an indicator for cell viability as it expresses a component of ongoing metabolic activity. Based on this principle, this study focused on the development and application of a reverse transcriptase-PCR (RT-PCR), which specifically detects mRNA, for the detection of viable *Campylobacter*. The expression of four genes, *flaA*, *tkt*, *porA* and a 256 bp amplicon of a putative haem-copper oxidase domain downstream from a previously uncharacterized *C. jejuni* two-component regulator, in heat-inactivated *Campylobacter* spp. was explored to determine an optimum target for RT-PCR amplification. Furthermore, the relationship between time and temperature to ablate the mRNA signal was determined by RT-PCR. mRNA half-life was assessed according to post-heating holding time at 37^{o}C. An amplification sensitivity of *Campylobacter* spp. was estimated by PCR.

A diversity of *Campylobacter* spp. was tested indicating that the RT-PCR technique was specific for *C. jejuni*, *C. coli*, and *C. lari*. Messenger RNA from the four genes was detected for varying intervals after the cells had been killed by heat, but gradually the message disappeared when heat-treated cells were held at 37^{o}C. We observed that the durability of mRNA species detected by our RT-PCR technique depends significantly on the individual *Campylobacter* strain tested, the condition of heat treatment and post-treatment holding time, and the transcript targeted. The intensity of the RT-PCR products decreased as the temperature of heat treatment increased and as the subsequent holding time extended. The 256 bp amplicon was determined to be the most stable mRNA species tested. mRNA of the 256 bp amplicon was detectable even after *Campylobacter* spp. had been killed at 95 to 99^{o}C. Using DNA-based PCR, the four genes could be amplified after 48 h holding time after each heat inactivation, indicating that the chromosomal DNA was minimally influenced by the heat treatment. PCR products from the 256 bp amplicon were detected at 10^2 to 10^3 *C. jejuni* CFU per ml, exhibiting the highest level of

sensitivity among the genes tested. This study suggests that while RT-PCR is generally a good technique to distinguish between viable and nonviable cells, the assay does not appear to be useful for *Campylobacter* spp.

INTRODUCTION

Campylobacter spp. are a leading bacterial cause of foodborne diarrheal illness with a frequency that exceeds salmonellosis and shigellosis combined in the United States. [11, 26] *Campylobacter jejuni* and *C. coli* account for the majority of the reported human cases. Epidemiological investigations have implicated poultry,[5] raw milk[19] and untreated water[17] in disease transmission. As few as 500 cells[10] may cause disease. Under *in vitro* conditions, *Campylobacter* growth is slow. Sensitive enrichment methods traditionally used to detect low levels of *Campylobacter* spp. in food require at least two days of incubation followed by selective plating and two days of incubation on complex culture media containing a combination of antibiotics.[4] *Campylobacter* may also enter the viable but nonculturable (VBNC) status due to starvation and physical stress, which may account for the failure of culture techniques to isolate the organism.[8, 20] Rapid, sensitive, and specific methods for detection of viable campylobacters are essential to further understand the epidemiology of infection and to enable tracking of the organisms.

Recently, new specific detection techniques such as enzyme-linked immunosorbent assay (ELISA), DNA/RNA probes, and polymerase chain reaction (PCR) assays have been developed for the detection of *Campylobacter*. Although immunological approaches are rapid, nonspecific detection due to cross-reactivity may occur. Additionally, immunological methods may not be adequate for detection of injured or stressed bacteria since the organism may not express all antigens required for immunological detection. While PCR-based assays are rapid, sensitive, and specific, the possibility exists that positive PCR amplifications may arise from either dead or noninfectious cells because PCR methods detect chromosomal gene sequences, which may be present in nonviable cells. Therefore, a method, which combines sensitivity and specificity together with the ability to differentiate between viable and nonviable cells, is desirable.

mRNA is documented to be more rapidly destroyed in cells than genomic DNA. We reasoned that a technique targeting bacterial mRNA would provide a better estimation for

viability than previously reported DNA amplification tests. Several studies have recently investigated the detection of viable pathogens including *Salmonella enterica*,[25] *S. enteritidis*,[29] *Campylobacter* spp.,[22] *Vibrio cholerae*,[1] *Listeria monocytogenes*[9] and *Escherichia coli*[23, 24] using RT-PCR. However, due to the difficulties in determining suitable target genes and low efficiency of RNA purification, these approaches remain in the developmental stage.

The central purpose of the present study was to validate the use of mRNA as a marker for *Campylobacter* spp. viability and to demonstrate the ability of RT-PCR to discriminate between viable and nonviable cells. The expression of four genes, including *flaA*, [14] *tkt*, [2] *porA*, [32] and a 256 bp amplicon of previously described as encoding for a putative haem-copper oxidase, [7] in *Campylobacter* spp. were targeted. Cells were treated by heat and examined to determine whether these were appropriate targets for RT-PCR amplification. The correlationship between persistence of RT-PCR products and cell viability after heat treatment was verified by conducting a time-course heat treatment experiment. The time-temperature relationship to extinguish mRNA signal was determined and RNA half-life was assessed according to holding time at 37°C. Additionally, sensitivity of amplification of *Campylobacter* spp. using the primers of these four genes was determined by conventional PCR assays.

MATERIALS AND METHODS

Bacterial strains and growth conditions

A list of bacteria used in this investigation is presented in Table 1. *C. jejuni* A74/O and A74/C have been previously described.[27] All strains of *Campylobacter* except *C. fetus* and *C. upsaliensis* were grown on Brucella-FBP agar (Difco, USA) containing 0.5 g per l of ferrous sulfate, 0.2 g per l of sodium bisulfate, and 0.5 g per l of pyruvic acid at 42ºC for 24 h in a sealed plastic bag containing 5 % oxygen, 10 % carbon dioxide, and 85 % nitrogen. *C. fetus* and *C. upsaliensis* were grown overnight on Trypticase Soy Agar (Difco, USA) with 5 % defibrinated sheep blood at 37 ºC in the above microaerobic conditions. Estimates of viable cell numbers were obtained by serially diluting cell suspensions in phosphate buffered saline (PBS) and plating onto Campy-Cefex agar.[28] Poultry-associated strains (Table 1) were grown on their appropriate media under suitable condition for each organism.

Heat treatment

All experiments were conducted in duplicate. *Campylobacter* spp. were grown overnight on Brucella-FBP agar at 42°C to obtain a cell density of approximately 10^8 CFU per ml. *Campylobacter* cultures (1 ml) were harvested, suspended and pelleted by centrifugation at 5,000 x g for 10 min at 4°C, washed, resuspended in diethyl pyrocarbonate (DEPC)-treated PBS, and transferred to 1.5 ml microcentrifuge tubes. Cell suspensions were subjected to heat treatment by placing tightly capped tubes in a water bath held at the appropriate temperature and subsequently held at 37°C for selected time intervals to test mRNA half-life until mRNA RT-PCR signal was extinguished. To monitor for the presence of culturable cells in heat-treated bacterial suspensions used in the RT-PCR assay, 1 ml of each sample was removed at the same time as the samples were removed for RNA extraction. The removed cells were centrifuged at 5,000 x g for 10 min at 4°C, inoculated into Bolton enrichment broth (Dalynn Biologicals, Canada), and incubated at

37°C for 4 h and then at 42°C for 72 h under microaerobic conditions. The broths were subcultured onto Campy-Cefex agar and incubated at 42°C for 48 h.

Nucleic acid isolation

Total RNA was extracted from cells using the RNeasy Mini kit (Qaigen, USA) as specified by the manufacturer. An one-milliliter of cell culture was centrifuged at 5,000 x g for 10 min at 4°C. The pellet was disrupted by incubation in 100 µl of TE (10 mM Tris-HCl [PH 8.0], 1 mM EDTA [PH 8.0]) buffer containing 400 µg of lysozyme per ml for 5 min. To remove contaminating DNA, RNase-Free DNase (Qaigen, USA) was applied on-column according to manufacturer instructions. Extracted RNA was resuspended in 40 µl of nuclease-free water and stored at –70°C for further use. The quantity and purity of total RNA were determined using optical density measurement at 260 nm by GeneQuant RNA/DNA Calculator (Amersham Biosciences, USA). An aliquot of detectable RNA was loaded with RNA sample loading buffer (Sigma, USA) and analyzed in a 1.2 % agarose gel containing 0.66 M formaldehyde, as needed. Chromosomal DNA was extracted from 1 ml of cells by using the QIAamp DNA Mini Kit (Qiagen, USA), according to the manufacturer's instructions. Isolated DNA was used as the template for PCR assay.

RT-PCR and PCR

The primers (Table 2) were selected to amplify *flaA* (flagellin A),[14] *tkt* (transketolase),[2] *porA* (major outer membrane protein),[32] and a 256 bp amplicon of putative haem-copper oxidase domain downstream from a previously uncharacterized *C. jejuni* two-component regulator gene.[7] The primers were analyzed in a single-step procedure using Qiagen OneStep RT-PCR Kit (Qiagen, USA), which includes both reverse transcriptase and DNA polymerase in one tube. To test for contaminating DNA, conventional PCR was conducted using AmpliTaq polymerase (Roche Molecular Biochemicals, USA) on fractions containing isolated RNA. Additionally, RT-PCR reactions without cDNA synthesis were conducted as a DNA contamination control.

RT-PCR was conducted in 50 µl volumes containing the following: 2 µl of RNA; 10 µl of 5x OneStep RT-PCR buffer containing 12.5 mM $MgCl_2$; 2 µl of dNTP mix containing 10 mM of each dNTP, 0.6 µM each primers; 2 µl OneStep RT-PCR enzyme mix contained both reverse transcriptase and HotStarTaq DNA polymerase; and 10 U RNase inhibitor. To increase the specificity of RT-PCR, a touchdown PCR cycling program was applied. [3] RT-PCR amplification conditions consisted of 1 cycle at 50°C for 30 min for reverse transcription of the RNA followed by the initial PCR activation step at 95°C for 15 min. Amplification was conducted for 20 cycles consisting of 94°C for 20 s, 68°C for 20 s, and 72°C for 30 s by decreasing the annealing temperature by 0.5°C every cycle. Fifteen cycles of denaturation at 94°C for 20 s, annealing at 53°C for 20 s, and extension at 72°C for 30 s followed. After 15 cycles, a final extension at 72°C for 5 min was conducted and the tubes were cooled to 4°C.

For PCR amplification, the reaction mixture (total volume, 100µl) consisted of the following: 5 µl of genomic DNA; 10 µl of 10x PCR reaction buffer; 6 µl of 25 mM $MgCl_2$; 8 µl of 10 mM dNTP mix; 0.5 µg per µl of each primer; and 2.5 U of AmpliTaq DNA polymerase (Roche Molecular Biochemicals, USA). The temperature cycling for denaturation, annealing and extension was as described above. A 10 µl aliquot of each reaction was electrophoresed on a 1 % agarose gel and examined by ethidium bromide staining. Limits in sensitivity for PCR were determined by amplifying a 10-fold dilution series of genomic DNA extract with primers directed to the four target amplicons, *flaA*, *tkt*, *porA*, and the 256 bp amplicon.

RESULTS AND DISCUSSIONS

Many studies have shown that bacteria may remain viable and maintain active metabolism after ceasing to divide and loss of the ability to be cultured.[15, 30] An acceptable definition of viable cells has been suggested: cells considered capable of taking up amino acids or sugars,[13, 18] protein synthesis,[12] maintenance of intact DNA,[21] and respiration.[12, 18] The presence of nucleic acids in bacteria might be a good indicator of viability if they are present only in viable cells, the kinetics of their disappearance is associated with loss of viability, or they disappear from cells soon after death.[23] Since DNA from dead cells may be amplified by PCR, targeting of genomic sequences may be inadequate for determining the viability of bacteria. Since the detection of mRNA in microorganisms by RT-PCR has previously been associated with viability, this assay was applied for the detection of *Campylobacter* cells, which were inactivated by heat, to determine whether the assay could differentiate between viable and dead cells.

Total RNA, 90 to 150 μg per ml, was recovered from approximately 10^8 CFU per ml of untreated *Campylobacter* spp. using the Qiagen RNeasy Mini Kit (Qiagen, USA). The Qiagen OneStep RT-PCR enzyme mix contains both reverse transcriptase and DNA polymerase, thus DNA present in the RNA extract can be amplified and lead to false positive results. To confirm that resulting amplicons were derived from RNA only, rather than contaminating genomic DNA, control reactions without cDNA synthesis steps were conducted. All control reactions in this study were negative, indicating that the amplified product originated from mRNA. As an additional control, conventional PCR was also conducted on samples. All PCR controls were negative, showing that chromosomal DNA was successfully removed from the RNA extract. To further confirm that the RT-PCR assay was detecting mRNA, i.e. cDNA, RNase A, 10 μg per ml, added to each reaction. No RT-PCR product was produced in these controls, indicating that RNase A completely extinguished the message and mRNA was the target of the RT-PCR assay.

The four different mRNA species employed in this study were selected to optimize *Campylobacter* spp. detection. These included the *flaA*, which is involved in the expression of *Campylobacter* flagellin protein, *tkt*, which encodes for an abundant housekeeping protein, *porA*, which involves in expression of a *Campylobacter* major outer membrane protein, and a 256 bp product, which is originated from the sequence of an open-reading frame adjacent and downstream of a novel of two-component regulatory gene. Specificity of all poultry-related microorganisms was tested using PCR and that the primers were specific for *Campylobacter* only (data not shown).

The suitability of each of the four genes to provide a sensitive target for detection of *Campylobacter* spp. was examined by conducting PCR on a 10-fold dilution series of chromosomal DNA extract (Table 3). Detection of the *porA* gene by PCR demonstrated the lowest level of sensitivity (10^6 CFU per ml) among the four genes tested. The 402 bp *flaA*-specific product was detected at levels between 10^5 to 10^6 CFU per ml. The *tkt*-specific fragment was detected by PCR from levels of 10^3 to 10^4 CFU per ml. PCR products of the 256 bp amplicon were present at levels of 10^2 to 10^3 CFU per ml for *C. jejuni*; the amplicon was detected from *C. coli*, *C. lari* 35221, and *C. lari* 43675 at levels of 10^6, 10^8, and 10^9 CFU per ml, respectively. Because the primers tested did not amplify *C. fetus* subsp. *fetus* and *C. upsaliensis* and *C. lari*, which demonstrated a very low limit of sensitivity, these strains were not included in the subsequent heat-inactivation experiments.

The correlationship between persistence of RT-PCR products and cell viability after heat treatment was investigated by conducting a time-course heat treatment experiment. Time-temperature relationships to ablate all mRNA signal were determined and then the RNA half-life was tested by holding samples at 37°C for selected intervals. The results of this experiment are summarized in Table 4. Although an occasional faint amplification signal was obtained at other times, these were considered negative when the observance of bands was not consistent. Resuscitation of the heat-treated cells using extended enrichment culture (72 h) yielded no cell

growth on Campy-Cefex agar, indicating that the cells could not be recovered by current cultural methods.

Detection of *flaA* in heat-treated cells is shown in Figures 1 and 2. Heat treatment of *C. jejuni* 49943 held at 95°C for 10 min resulted in *flaA* transcript presence up to 4 h post-treatment. *flaA* transcript of heat-treated *C. jejuni* 11168 and A74/C at 60°C for 10 min persisted up to 30 min post heat treatment. In *C. jejuni* A74/O, detectable *flaA* transcript was present 5 h after heat treatment at 95°C for 20 min. As shown in Figures 3 and 4, for *C. jejuni* 49943, no amplified product of *tkt* mRNA could be detected 4 h after heat treatment of the cells at 85°C for 10 min. *tkt* transcript from *C. jejuni* 11168, A74/O, and A74/C could be detected up to 8 h after heat treatment at 75°C for 10 min, 95°C for 20 min, 95°C for 10 min, respectively. Results from amplification of *porA* transcript are presented in Figures 5, 6, and 7. Target *porA* mRNA was detectable after 10 h, 2 h, and 5 h holding time from *C. jejuni* 11168, A74/O, and A74/C inactivated at 65°C for 10 min, 85°C for 10 min, and 70°C for 10 min, respectively. The 256 bp putative haem-copper oxidase transcript of heat-treated *C. jejuni* 49943 and A74/O at 99°C for 1.5 h persisted up to 30 min and 1 h post heat treatment, respectively (Figures 8 and 9). The 256 bp amplicon from *C. jejuni* A74/C, which was treated at 99°C for 50 min, was observed after 1 h holding at 37°C. *C. coli* heat-treated at 95°C for 1 h resulted in no amplicon after 2 h at 37°C using the 256 bp putative haem-copper oxidase primers. Additionally, RT-PCR signals from the 256 bp primers were positive in all *C. jejuni* after heat treatment at 95^{o}C for 30 min and 24 h holding at 37^{o}C; no 256 bp transcripts were present when all *C. jejuni* strains were autoclaved at 121°C for 15 min (data not shown). The observation that the 256 bp transcript continued to amplify after all heat treatment tested, except for autoclaving, may be due to the stability of the mRNA sequences targeted by the primer or due to the relative abundance of the transcript. Converse to our findings, Sails *et al*.[22] demonstrated that the mRNA signal of the 256 bp amplicon of *Campylobacter* spp. treated at 72°C for 5 min was negative within 4 h when the cells

were held at 37°C. The four genes were detected by DNA-based PCR at 48 h holding after each heat treatment (data not shown), showing that bacterial genomic DNA was not affected by the heat treatment.

The most important element for the detection of viable cells by RT-PCR is the identification of genes that are specific to the target pathogen, which are constituitively transcribed by the cell and have a short half-life in nonviable cells.[31] Previous investigations have demonstrated that the ability to amplify mRNA by RT-PCR correlates well with the presence of viable organisms in some situations.[6, 16] However, the results in the present study indicate that mRNA from *Campylobacter* spp. may persist in a form that is detectable by RT-PCR amplification for an extended period after heat treatment, demonstrating a poor correlation between mRNA detection and cell viability. To our knowledge, this is the first report regarding RT-PCR amplification using different strains (*C. jejuni* 49943, 11168, A74/O, and A74/C) of the same genus, determining time-temperature relationship to ablate mRNA signal, and subsequently testing RNA persistence according to holding time. The persistence of mRNA, generally, decreased as the temperature of heat treatment increased and as the subsequent holding time extended. We observed that the longevity of mRNA species as detected by RT-PCR of *Campylobacter* was greatly dependent on the individual bacterial strains, the temperature of the heat treatment and the duration of the holding time, and the region of the transcript targeted. Even though mRNA is not an absolute marker of viability, the relatively rapid degradation of mRNA from heat-inactivated cells showed that mRNA is generally a much better indicator of cell viability than was DNA. However, results from this investigation demonstrate that mRNA must be carefully characterized before RT-PCR should be used as an indication of cell viability.

Table 3-1. Bacterial strains used in this study and their origins

Gram staining	Organisms	Origin
Gram negative	*Acinetobacter baumannii*	WT* (chicken carcass rinse)
	Arcobacter butzleri ATCC**49616	human diarrheal stool
	Bordetella avium ATCC 35086	respiratory tract of turkey poults
	Brevundimonas diminuta	WT (chicken carcass rinse)
	Campylobacter coli ATCC 49941	unknown
	Campylobacter fetus subsp. *fetus* ATCC 29428	diarrheic stool of child
	Campylobacter jejuni A74/O (CDC***)	human isolate
	Campylobacter jejuni A74/C	originated from *C. jejuni* A74/O by chicken challenge
	Campylobacter jejuni ATCC 11168	unknown
	Campylobacter jejuni ATCC 49943	unknown
	Campylobacter lari ATCC 35221	herring gull cloacal swab
	Campylobacter lari ATCC 43675	human feces
	Campylobacter upsaliensis ATCC 43953	dog feces
	Citrobacter freundii ATCC 43864	unknown
	Enterobacter cloacae ATCC 23355	unknown
	Escherichia coli ATCC 25922	clinical isolate
	Flavobacterium odoratum	WT (chicken carcass rinse)
	Haemophilus paragallinarum ATCC 29545	infraorbital sinus of a chick
	Klebsiella pneumoniae ATCC 13883	unknown
	Ochrobactrum spp.	WT (chicken carcass rinse)
	Pasteurella multocida ATCC 9659	calf
	Proteus vulgaris ATCC 13315	unknown
	Pseudomonas aeruginosa ATCC 27853	blood culture
	Salmonella enteritidis BL9-14 3-1-5C	WT (chicken carcass rinse)
	Salmonella typhimurium ATCC 14028	unknown
	Serratia marcescens ATCC 8100	unknown
	Shigella sonnei ATCC 9290	unknown
	Sphingomonas paucimobilis	WT (chicken carcass rinse)
	Vibrio parahaemolyticus ATCC 49398	clinical isolate
Gram positive	*Clostridium perfringens* ATCC 3624	unknown
	Enterococcus faecalis ATCC 7080	meat involved in food poisoning
	Listeria monocytogenes ATCC 19111	poultry
	Staphylococcus aureus ATCC 25923	clinical isolate
	Staphylococcus epidermidis ATCC 12228	unknown
	Staphylococcus gallinarum ATCC 35539	chicken nares
	Streptococcus pyogenes ATCC 19615	pharynx of child

*WT: Wild Type
**ATCC: American Type Culture Collection
***CDC: Center for Disease Control

Table 3-2. Primers used for the RT-PCR and PCR detection of mRNA and contaminating DNA

Primers	Sequences (5′ to 3′)	Target gene	Size of amplified product
flaAR651F	CTATGGATGAGCAATTWAAAAT	*flaA* (flagellin A)	402 bp
flaAR652R	CAAGWCCTGTTCCWACTGAAG		
tktR653F	GCAAACTCAGGACACCCAGG	*tkt* (transketolase)	1,133 bp
tktR654R	AAAGCATTGTTAATGGCTGC		
porAR655F	ATGAAACTAGTTAAACTTAGTTTA	*porA* (major outer membrane protein)	1,272 bp
porAR656R	GAATTTGTAAAGAGCTTGAAG		
BOR657F	AGAACACGCGGACCTATATA	256 bp amplicon (putative haem-copper oxidase)	256 bp
BOR658R	CGATGCATCCAGGTAATGTAT		

Table 3-3. Amplification sensitivity of *Campylobacter* spp. in PCR

Strains	*fla*A	*tkt*	*porA*	256 bp amplicon
C. jejuni 49943	10^5*	10^3	10^6	10^3
C. jejuni 11168	10^6	10^3	10^6	10^2
C. jejuni A74/O	10^5	10^4	10^6	10^3
C. jejuni A74/C	10^5	10^3	10^6	10^2
C. coli 49941	ND**	ND	10^6	10^6
C. lari 43675	ND	ND	ND	10^8
C. lari 35221	ND	ND	ND	10^9

* CFU/ml

** Nondetectable

Table 3-4. Half-life Measurement of *Campylobacter* spp. by RT-PCR using *fla*A, *tkt*, *porA* and a 256 bp amplicon

	*fla*A		*tkt*		*porA*		256 bp amplicon	
	Temp-time*	Holding time**	Temp-time	Holding time	Temp-time	Holding time	Temp-time	Holding time
C. jejuni 49943	95°C – 10 min	4 h	85°C – 10 min	4 h	60°C – 10 min	8 h	99°C – 90 min	30 min
C. jejuni 11168	60°C – 10 min	30 min	75°C – 10 min	30 min	65°C – 10 min	10 h	99°C – 60 min	30 min
C. jejuni A74/O	95°C – 20 min	5 h	95°C – 20 min	5 h	85°C – 10 min	2h	99°C – 90 min	1 h
C. jejuni A74/C	60°C – 10 min	30 min	95°C – 10 min	8 h	70°C – 10 min	5 h	99°C – 50 min	1 h
C. coli 49941	ND***	ND	ND	ND	60°C – 10 min	8 h	95°C – 60 min	2 h

* Temperature (°C) and time (min) conditions for heat inactivation
**Subsequent holding time to assess mRNA half-life
***Nondetectable

Figure 3-1. RT-PCR detection of *flaA* mRNA in heat-treated (95^{o}C, 10 min) *C. jejuni* 49943 (lane 1 to 6) and heat-treated (60^{o}C, 10 min) *C. jejuni* 11168 (lane 7 and 8). *C. jejuni* 49943 was held at 37^{o}C for 0 min (lane 1), 30 min (lane 2), 1 h (lane 3), 2 h (lane 4), 3 h (lane 5), and 4 h (lane 6). *C. jejuni* 11168 was held at 37^{o}C for 0 min (lane 7) and 30 min (lane 8). Lane M contains 1 Kb DNA ladder marker and Lane + contains positive control.

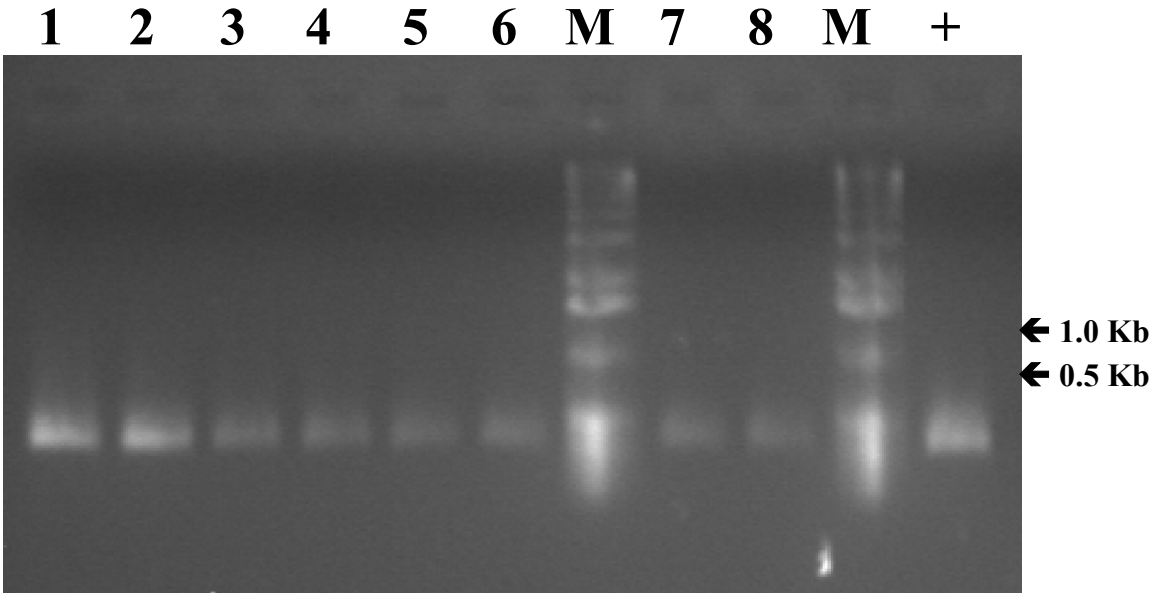


Figure 3-2. RT-PCR detection of *flaA* mRNA in heat-treated (95^{o}C, 20 min) *C. jejuni* A74/O (lane 1 to 7) and heat-treated (60^{o}C, 10 min) *C. jejuni* A74/C (lane 8 and 9). *C. jejuni* A74/O was held at 37^{o}C for 0 min (lane 1), 30 min (lane 2), 1 h (lane 3), 2 h (lane 4), 3 h (lane 5), 4 h (lane 6), and 5 h (lane 7). *C. jejuni* A74/C was held at 37^{o}C for 0 min (lane 8) and 30 min (lane 9). Lane M contains 1 Kb DNA ladder marker and Lane + contains positive control.

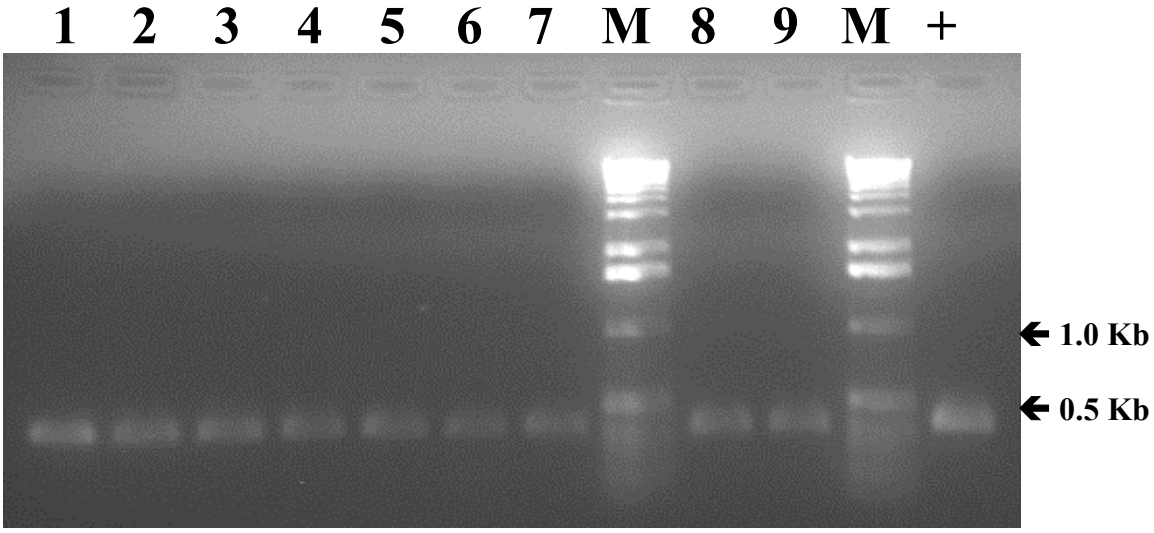


Figure 3-3. RT-PCR detection of *tkt* mRNA in heat-treated (85^{o}C, 10 min) *C. jejuni* 49943 (lane 1 to 6) and heat-treated (75^{o}C, 10 min) *C. jejuni* 11168 (lane 7 and 8). *C. jejuni* 49943 was held at 37^{o}C for 0 min (lane 1), 30 min (lane 2), 1 h (lane 3), 2 h (lane 4), 3 h (lane 5), and 4 h (lane 6). *C. jejuni* 11168 was held at 37^{o}C for 0 min (lane 7) and 30 min (lane 8). Lane M contains 1 Kb DNA ladder marker and Lane + contains positive control.

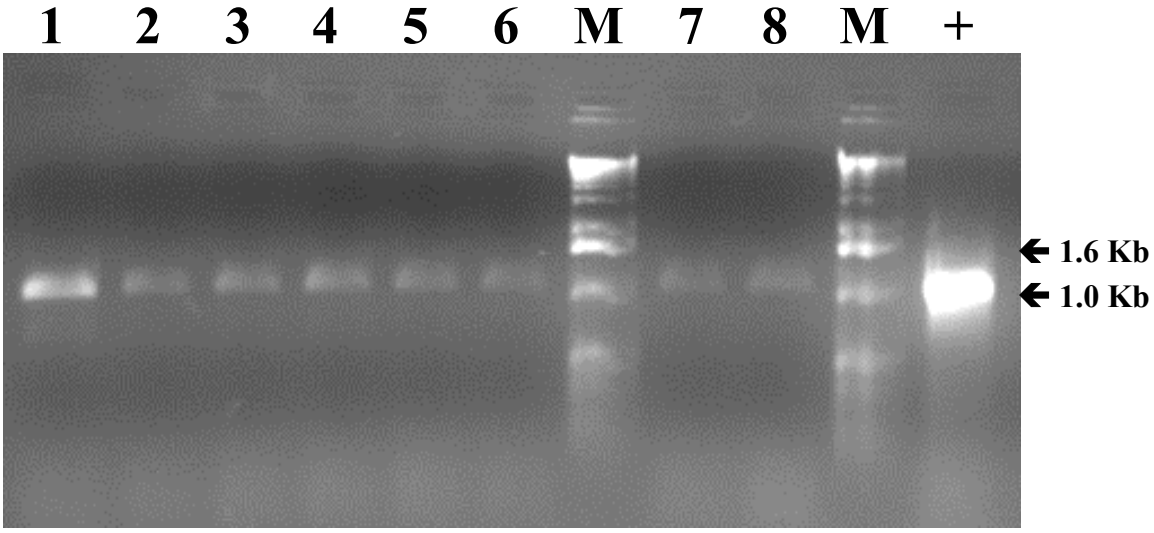


Figure 3-4. RT-PCR detection of *tkt* mRNA in heat-treated (95^{o}C, 20 min) *C. jejuni* A74/O (lane 1 to 7) and heat-treated (95^{o}C, 10 min) *C. jejuni* A74/C (lane 8 and 12). *C. jejuni* A74/O was held at 37^{o}C for 0 min (lane 1), 30 min (lane 2), 1 h (lane 3), 2 h (lane 4), 3 h (lane 5), 4 h (lane 6), and 5 h (lane 7). *C. jejuni* A74/C was held at 37^{o}C for 0 min (lane 8), 2 h (lane 9), 4 h (lane 10), 6 h (lane 11), and 8 h (lane 12). Lane M contains 1 Kb DNA ladder marker and Lane + contains positive control.

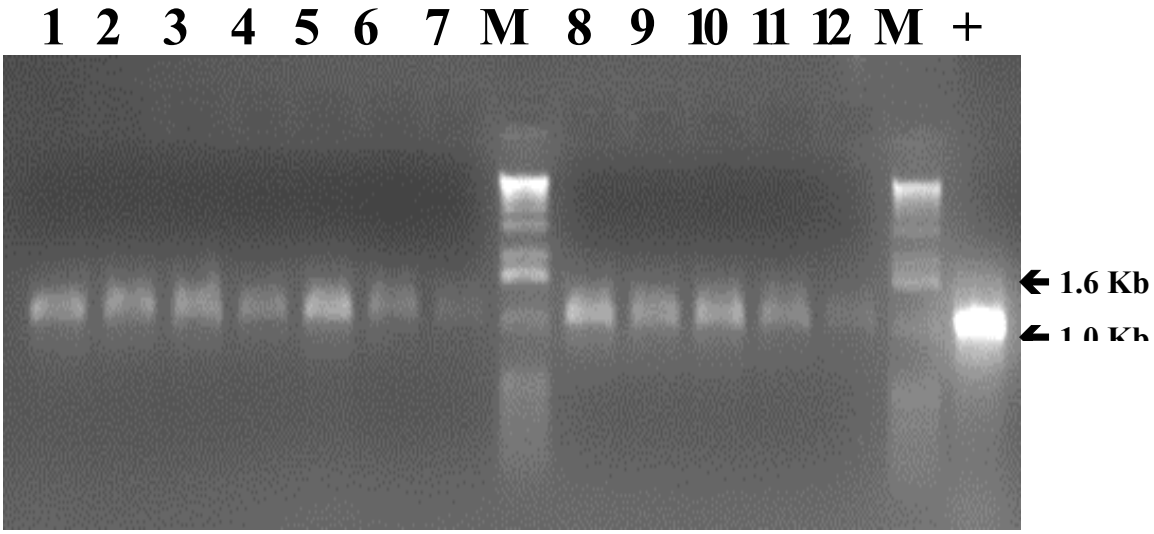


Figure 3-5. RT-PCR detection of *porA* mRNA in heat-treated (60°C, 10 min) *C. jejuni* 49943 (lane 1 to 5) and heat-treated (65°C, 10 min) *C. jejuni* 11168 (lane 6 and 11). *C. jejuni* 49943 was held at 37°C for 0 min (lane 1), 2 h (lane 2), 4 h (lane 3), 6 h (lane 4), and 8 h (lane 5). *C. jejuni* 11168 was held at 37°C for 0 min (lane 6), 2 h (lane 7), 4 h (lane 8), 6 h (lane 9), 8 h (lane 10), and 10 h (lane 11). Lane M contains 1 Kb DNA ladder marker and Lane + contains positive control.

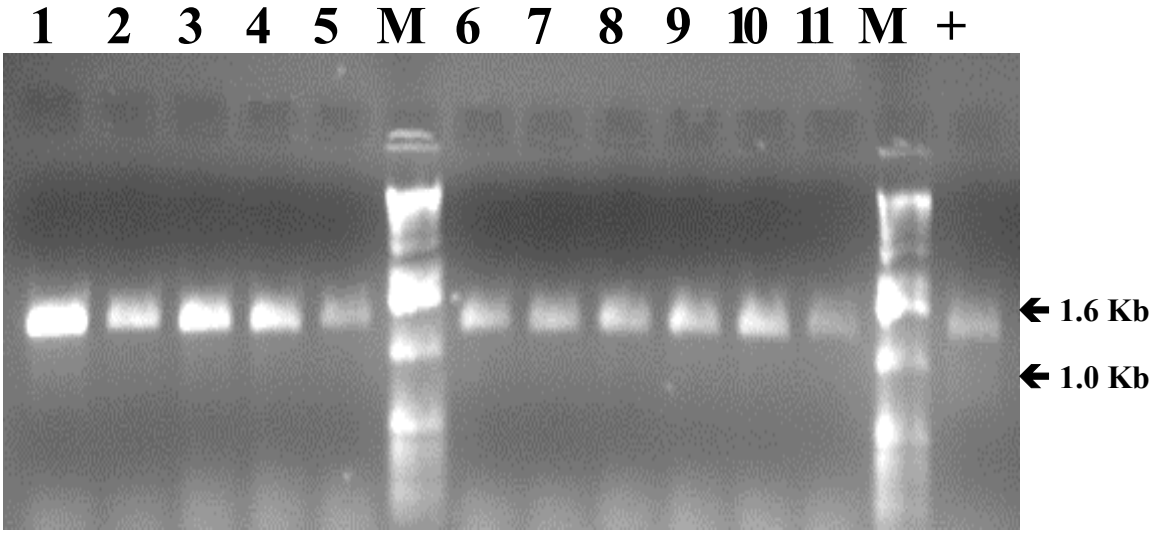


Figure 3-6. RT-PCR detection of *porA* mRNA in heat-treated (85°C, 10 min) *C. jejuni* A74/O (lane 1 to 4) and heat-treated (70°C, 10 min) *C. jejuni* A74/C (lane 5 and 11). *C. jejuni* A74/O was held at 37°C for 0 min (lane 1), 30 min (lane 2), 1 h (lane 3), and 2 h (lane 4). *C. jejuni* A74/C was held at 37°C for 0 min (lane 5), 30 min (lane 6), 1 h (lane 7), 2 h (lane 8), 3 h (lane 9), 4 h (lane 10), and 5 h (lane 11). Lane M contains 1 Kb DNA ladder marker and Lane + contains positive control.

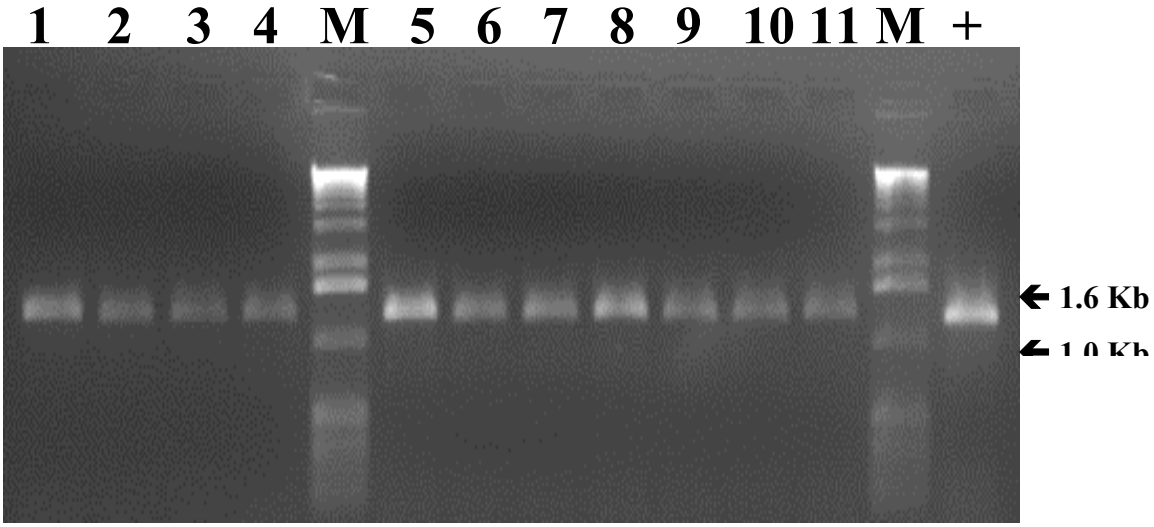


Figure 3-7. RT-PCR detection of *porA* mRNA in heat-treated (60^{o}C, 10 min) *C. coli* (lane 1 to 5). *C. coli* was held at 37^{o}C for 0 min (lane 1), 2 h (lane 2), 4 h (lane 3), 6 h (lane 4), and 8 h (lane 5). Lane M contains 1 Kb DNA ladder marker and Lane + contains positive control.

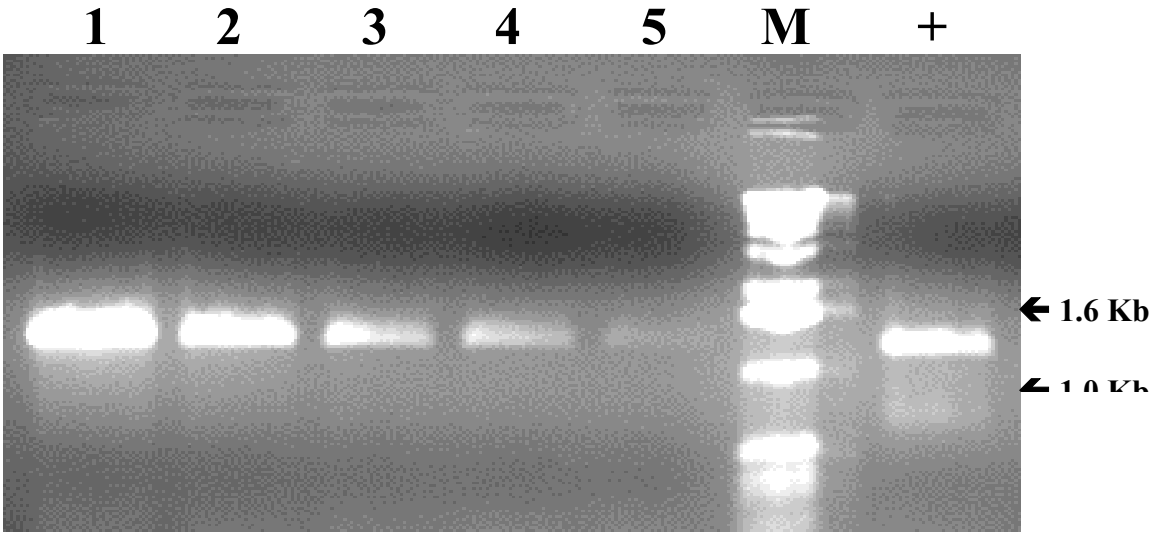


Figure 3-8. RT-PCR detection of a 256 bp amplicon mRNA in heat-treated (99^{o}C, 90 min) *C. jejuni* 49943 (lane 1 to 3) and heat-treated (99^{o}C, 60 min) *C. jejuni* 11168 (lane 4 and 6). *C. jejuni* 49943 was held at 37^{o}C for 0 min (lane 1), 10 min (lane 2), and 30 min (lane 3). *C. jejuni* 11168 was held at 37^{o}C for 0 min (lane 4), 10 min (lane 5), and 30 min (lane 6). Lane M contains 1 Kb DNA ladder marker and Lane + contains positive control.

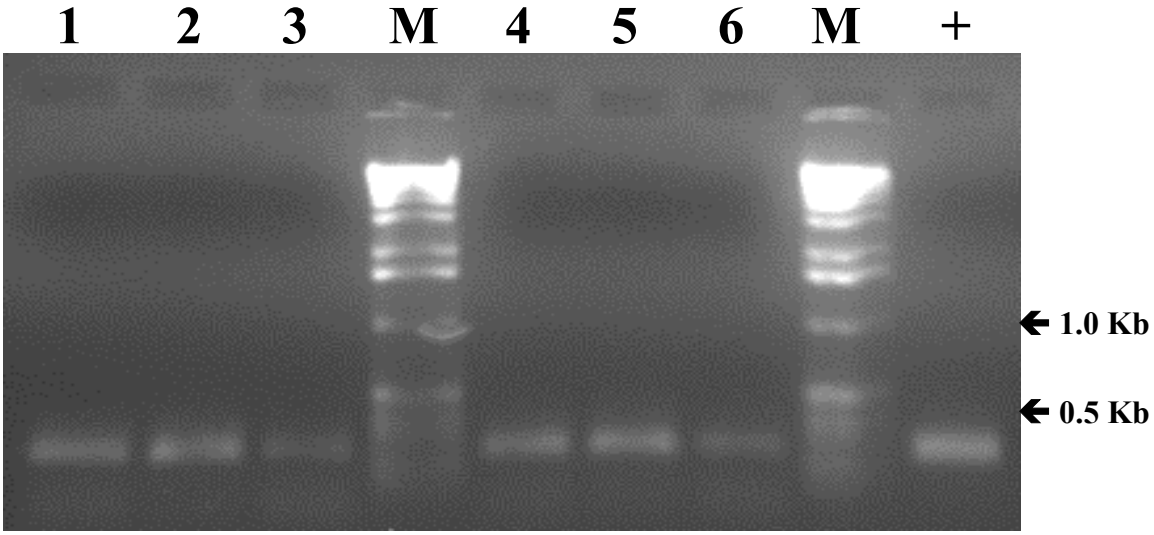
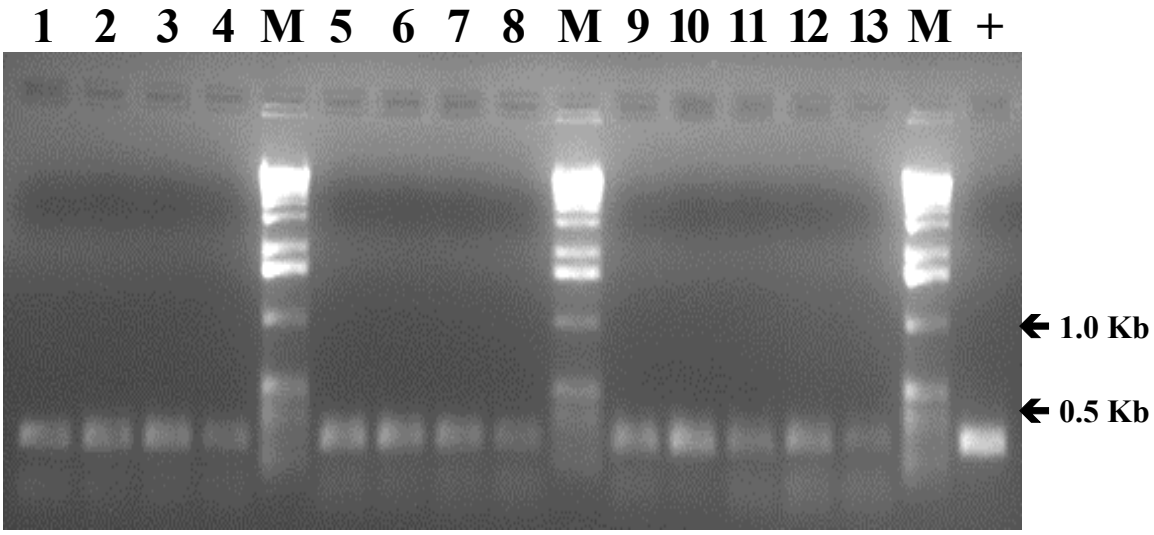


Figure 3-9. RT-PCR detection of a 256 bp amplicon mRNA in heat-treated (99°C, 90 min) *C. jejuni* A74/O (lane 1 to 4), heat-treated (99°C, 50 min) *C. jejuni* A74/C (lane 5 and 8), and heat-treated (95°C, 60 min) *C. coli* (lane 9 to 13). *C. jejuni* A74/O was held at 37°C for 0 min (lane 1), 10 min (lane 2), 30 min (lane 3), and 1 h (lane 4). *C. jejuni* A74/C was held at 37°C for 0 min (lane 5), 10 min (lane 6), 30 min (lane 7), and 1 h (lane 8). *C. coli* was at 37°C for 0 min (lane 9), 10 min (lane 10), 30 min (lane 11), 1 h (lane 12), and 2 h (lane 13). Lane M contains 1 Kb DNA ladder marker and Lane + contains positive control.



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CHAPTER IV

RELATION OF MRNA REVERSE TRANSCRIPTASE-PCR SIGNAL WITH CAMPYLOBACTER SPP. COLONIZATION OF CHICKS

[1]Sung, K. D., N. J. Stern, and K. L. Hiett. To be submitted to *Avian Disease*

ABSTRACT

Discrimination of viable from dead cells is of importance in the development of bacterial detection methods. A positive RT-PCR amplification signal has been considered to indicate the presence of viable cells because mRNA has an extremely short half-life. However, some researchers have suggested that the presence of mRNA is not well correlated with cell viability following heat inactivation as mRNA might also be amplified by Reverse Transcriptase-PCR (RT-PCR) after cells are dead. Chicken colonization by cells having positive mRNA signal, but which are nonculturable, would provide a measure to characterize the correlation between cell viability and persistence of mRNA. In addition, the role of a viable but nonculturable (VBNC) form of *Campylobacter* spp. for infection of poultry could be verified. The levels of four *Campylobacter* spp., previously isolated from poultry feces, declined progressively over time and loss of culturability occurred after 6 to 7 weeks incubation in phosphate buffed saline (PBS) at 4^{o}C. Cold-stored, nonculturable and heat-inactivated (60^{o}C for 10 min) *Campylobacter* spp. produced inconsistent amplified products from RT-PCR assay depending on the target genes and strains used, though all fresh cultures showed mRNA signals. Mostly, signals of mRNA species from VBNC and heat-killed *Campylobacter* spp. AH-1, AH-2 and CH-3 persisted. RT-PCR amplification of *tkt*, *porA*, and a 256 bp amplicon from a previously described putative haem-copper oxidase provided consistent signal while *flaA* did not. Presumed VBNC and heat-inactivated *Campylobacter* spp., which produced positive mRNA signal but was not culturable by conventional culture-based methods, did not establish colonization in the intestine of chicks seven days after challenge. These results question the correlationship between mRNA durability as assayed by RT-PCR and cell viability, as well as the significance of the VBNC cells in environmental transmission of *Campylobacter* spp.

INTRODUCTION

Worldwide *Campylobacter jejuni* infection is the most commonly identified bacterial cause of acute gastroenteritis. *Campylobacter* causes human diarrheal disease at rates more than 2 to 7 times as frequently as *Salmonella*, *Shigella* or *E. coli* 0157:H7 (5, 27). *Campylobacter* spp. grows well in the digestive tract of poultry. When they are excreted into the environment, they are exposed to various environmental stresses such as nutrient starvation, osmotic shock, oxygen stress, temperature variation, oxidative stress (24). Under unfavorable conditions for growth, cells of *Campylobacter* spp. change from their physiological spiral form to coccoidal form. This transformation occurs in conjunction with reduction in maximum growth rate and viable count (11). The survival of bacterial cells in cold environments could sustain viability longer than culturability. Such phenomenon has been characterized by a viable but nonculturable (VBNC) state. The VBNC state has been documented in numerous human pathogens, including *Escherichia coli* (31), *Salmonella enteritis* (22), *Vibrio cholerae* (7), *Legionella pneumophila* (13), and *Campylobacter jejuni* (12). Jones *et al*. (16) reported on the VBNC form of various suspended *C. jejuni* strains following 18 to 28 days of holding in sterile water and incubating at 4ºC. Hazeleger *et al*. (12) observed the VBNC state with various *C. jejuni* strains within 6 weeks of inoculation into brain heart infusion incubated at 4ºC. The existence of VBNC forms of *C. jejuni* might explain why infections have occurred in the absence of a detectable environmental source (7). However, its significance for infection of animals and as the cause of disease in humans remains controversial. Beumer *et al*. (3) could not recover *Campylobacter* from rabbits, mice or humans after infection with VBNC cells. Also, VBNC *C. jejuni* was not isolated from embryonated eggs or the ceca of chicks after incubation for 7 days (19). However, Jones *et al*. (16) reported that two of four strains of *C. jejuni,* which were unculturable following storage in water for 6 weeks, could be recovered after feeding to suckling mice. This was confirmed by Stern *et al*. (28), who showed that some isolates of VBNC *C. jejuni* were able to colonize 5 of 79

challenged chicks. Cells rendered unculturable by freeze injury were similarly recovered by animal passage (23). VBNC cells of *C. jejuni* appeared capable of colonization in the yolk sacs of embryonated eggs (6).

One difficulty in elucidating the potential hazard of VBNC cells is the inability to detect such cells by conventional bacteriological methods. In view of the potential epidemiological importance of such VBNC cells, it would be important to establish, unequivocally, the viability of VBNC cells. Such an assay would contribute to an understanding of the processes leading to the loss of culturability and develop methods for VBNC detection. Since animal experiments are complex and difficult to control, it would be desirable to develop *in vitro* systems to detect the presence of VBNC cells. Islam *et al*. (14) used polymerase chain reaction (PCR) to detect the VBNC form of *Shigella dysenteria* from water microcosms. Although this technique is rapid, sensitive and specific, the use of PCR under these conditions is unable to differentiate between the VBNC and nonviable cell.

Bacterial mRNAs have been proposed as markers for cell viability because they are very unstable molecules with short half-lives inside the cell (24). Thus, it would be expected that as long as VBNC bacteria are alive, they might produce selected mRNA molecules. In this study, we investigated the expression of four genes, *flaA* (20), *tkt* (9), *porA* (32), and a putative haem-copper oxidase domain downstream from a previously uncharacterized *C. jejuni* two-component regulator (13) genes, of *Campylobacter* spp. by reverse transcriptase-PCR (RT-PCR) assay. Cells were inactivated by heat treatment at 60ºC for 10 min based on their D value (27) and VBNC cells were obtained by inoculating *Campylobacter* spp. in PBS and incubating these at 4ºC. The main goal of the study was to determine whether the ability of coccoid and VBNC cells of *Campylobacter* spp. were infectious after passage through day-old chicks. Further, this study was conducted to confirm the non-recovery of heat-killed *Campylobacter* spp. correlated with mRNA message detection through chicken challenge.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Four isolates (TF-1, AH-1, AH-2, and CH-3) of *Campylobacter* spp. were used in this study. TF-1 was isolated from a fecal sample on a broiler farm in North Georgia and other isolates were obtained from broiler fecal samples in Iceland. The strains were grown on Brucella-FBP agar (Difco, Detroit, MI) supplemented with 0.5 g per l of ferrous sulfate, 0.2 g per l of sodium bisulfate, and 0.5 g per l of pyruvic acid. Cultures were incubated for 24 h at 42ºC in a microaerobic atmosphere (5 % oxygen, 10 % carbon dioxide, and 85 % nitrogen). Before the strains were stored at 4ºC, a subset of the strains was orally gavaged into ten day-of-hatch chicks at the level of 10^5 CFU per 100 µl and recovered seven days later from the ceca of the animals to confirm the ability of the isolates to colonize. Isolates recovered from Campy-Cefex agar (29) were purified, amplified, and suspended in individual flasks containing 500 ml of phosphate buffered saline (PBS: pH 7.2) at ca. 10^7 to 10^8 CFU per ml. Cultures were stored in parafilmed, screw-capped media bottles in the dark at 4ºC. Culturability was demonstrated by spread plating of serial dilutions onto Campy-Cefex agar and incubation at 42ºC for 48 h under microaerobic conditions, initially, and at weekly intervals thereafter. When the bacteria were no longer detectable by direct plating, 50 ml of the suspensions were centrifuged at 5,000 x g for 10 min, inoculated onto Bolton enrichment broth (Dalynn Biologicals, Calgary, Canada), and incubated at 37ºC for 4 h and subsequently at 42ºC for 72 h under microaerobic conditions. The broths were subcultured onto Campy-Cefex agar and plates incubated at 42ºC for 48 h.

Heat treatment

Campylobacter spp. were grown on Brucella-FBP agar at 42ºC for 24 h, suspended and 10^8 CFU per ml were centrifuged at 5,000 x g for 10 min at 4ºC, washed, resuspended in PBS, and a 10 ml PBS suspension of 10^{7-9} per ml of cells were transferred to a 500 ml, cotton-plugged Erlenmeyer flask. The flasks were held in a water bath at 60ºC for 10 min. The suspensions were

cultured by protocol described above to confirm colony-forming capacity by conventional microbiological method.

DNA and RNA isolation

In cold-stored, nonculturable *Campylobacter* spp., 200 ml suspensions were centrifuged at 5,000 x g for 10 min and the pellet resuspended in 3 ml diethyl pyrocarbonate (DEPC)-treated PBS. Chromosomal DNA was extracted from 1 ml of resuspended cells using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Isolated DNA was used as the template for PCR assay.

Total RNA was isolated from cold-stored, nonculturable *Campylobacter* isolates using the RNeasy Mini Kit (Qiagen, Valencia, CA) as directed by the manufacturer. RNase-Free DNase (Qiagen, Valencia, CA) was treated on-column as specified by the manufacturer to completely digest any contaminating DNA. Genomic DNA and RNA were extracted from heat-inactivated cells following the same procedure described above.

PCR and RT-PCR

Four different oligonucleotides were synthesized by the Molecular Genetics Instrumentation Facility (MGIF) located at the University of Georgia (Table 1). These primers were selected to amplify *flaA*, flagellin A (20), *tkt*, transketolase (9), *porA*, major outer membrane protein (32), and a 256 bp amplicon of putative haem-copper oxidase domain downstream from a previously uncharacterized *C. jejuni* two-component regulator gene (15). The PCR amplification reaction was conducted in a volume of 100 µl, containing: 5 µl of genomic DNA; 10 µl of 10x PCR reaction buffer; 6 µl of 25 mM $MgCl_2$; 8 µl of 10 mM dNTP mix; 0.5 µg per µl of each primer; and 2.5 U of AmpliTaq DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN). Based on touchdown PCR (10), the reaction was conducted for first 20 cycles as follows: 94ºC for 20 s, 68ºC for 20 s, and 72ºC for 30 s by reducing the annealing temperature by 0.5ºC every cycle. Next, 15 cycles of the amplification step included denaturation at 94ºC for 20 s,

annealing at 53ºC for 20 s, and extension at 72ºC for 30 s was continued. A final extension at 72ºC for 5 min was conducted and the tubes were held at 4ºC.

RT-PCR using Qiagen OneStep RT-PCR Kit (Qiagen, Valencia, CA) was conducted in 50 µl volumes containing the following: 2 µl of RNA; 10 µl of 5x OneStep RT-PCR buffer; 2 µl of dNTP mix; 0.6 µM each primer; 2 µl OneStep RT-PCR enzyme mix containing both reverse transcriptase and HotStarTaq DNA polymerase; and 10 U RNase inhibitor (Sigma, St. Louis, NJ). Positive and negative, DNA contamination, and RNase controls were included in every RT-PCR assay. RT-PCR amplification and testing for the presence of mRNA consisted of reverse transcription, initial PCR activation step, a three-step cycling of denaturation, annealing, and extension, and final extension. One cycle at 50ºC for 30 min was conducted for reverse transcription of RNA and the initial PCR activation step was followed at 95ºC for 15 min. Then, touchdown PCR and the amplification cycling were continued as described above.

Chick challenge

Day-of-hatch pathogen-free chicks were procured from Select Laboratory (Gainsville, GA). Chicks were placed in raised wire-floor isolation units ventilated with positive pressure, filtered air at the poultry experiment facility of the Poultry Microbiological Safety Research Unit (PMSRU), USDA, ARS, Athens, GA. The isolation units were equipped with heating elements providing optimum temperatures (37^{o}C) for the chicks, watering facilities, and *ad libitum* access to standard, unmedicated commercial feed. From heat-killed and cold-stored, nonculturable cell suspensions, each 10 ml and 200 ml suspensions were concentrated by centrifugation at 5,000 x g for 10 min and resuspended in 3 ml PBS. Ten day-old chicks per strain, held in individual isolation units, were gavaged with 0.1 ml of the (1) nontreated, cold-stored, nonculturable and (2) heat-inactivated *Campylobacter* spp. suspensions described above, and ten chicks served as negative controls without any *Campylobacter* challenge. At 7 days of age, birds were killed by cervical dislocation and the carcasses were washed in 70 % ethanol to prevent external bacterial contamination. Ceca from individual chicks were aseptically removed and placed into a sterile

plastic stomacher bag. The ceca were weighed and diluted 1:3 (wt: vol) with PBS. After pummeling with a stomacher for 30 s, direct and decimal serial dilutions were streaked onto Campy-Cefex agar and inoculated into Bolton enrichment broth. Plates were incubated at 42ºC for 24 h in microaerobic environment (5 % O_2, 10 % CO_2, and 85 % N_2) prior to confirming characteristic *Campylobacter* colonies by phase-contrast microscopy and latex agglutination. Enrichment culture in Bolton enrichment broth was incubated as described above.

RESULTS

Culturability of *Campylobacter* spp.

Campylobacter spp. suspended in PBS were incubated in the dark at 4ºC at a level ranging from 10^7 to 10^8 CFU per ml and the survival data is presented in Figure 1. *Campylobacter* spp. TF-1 and AH-1 lost their culturability as detected by conventional bacteriological methods after 6 weeks storage. *Campylobacter* spp. AH-2 and CH-3 became nonculturable after 7 weeks of storage at refrigerated temperature. The culturability of cells declined progressively over time and each of the *Campylobacter* strains showed similar survival curves. Microscopic observation of cold-stored cells by phase contrast microscopy showed a transition in cell morphology over time (Data not shown). Initially, the great majority of the cells consisted of motile, morphologically typical curved or spiral rods. Concomitant with the decline in plate counts an increasing proportion of the population changed to a coccoidal shape. Heat-treated *Campylobacter* spp. at 60ºC for 10 min were never recovered by enrichment-based technique.

RT-PCR

RT-PCR results of fresh viable, VBNC, and heat-killed *Campylobacter* spp. are summarized in Table 2. RT-PCR amplification of total RNA extracted from nontreated fresh viable *Campylobacter* spp. produced positive signals from all genes, *flaA*, *tkt*, *porA* and the specified 256 bp amplicon (Figures 2 and 3). Messages of *flaA* of refrigeration stored, VBNC *Campylobacter* spp. were not expressed among isolates TF-1, AH-1 and AH-2 or amplified very weakly for CH-3 in RT-PCR assays (Figure 4). mRNA species of VBNC *Campylobacter* AH-1, AH-2 and CH-3 persisted with RT-PCR amplification of *tkt*, *porA* and a 256 bp amplicon as shown in Figures 4 and 5. However, mRNA target of VBNC *Campylobacter* TF-1 was negative for *flaA*, *tkt*, and *porA* genes, and expressed a very faint signal for the 256 bp amplicon in RT-PCR amplifications. Based on the D-value reported previously (27), wild-type *Campylobacter* spp. were inactivated by the heat treatment at 60ºC for 10 min. Cell non-culturability was

confirmed after incubation in Bolton enrichment broth. mRNA-targeted amplification of VBNC and heat-inactivated *Campylobacter* spp. exhibited very similar results from RT-PCR assays except with the *porA*-targeted *Campylobacter* CH-3 (Figures 4 and 5). All four genes were detected by RT-PCR amplification after the *Campylobacter* spp. strains were killed by heat treatment at 60ºC for 10 min or had reached VBNC state, though the *flaA* gene was amplified very limitedly.

Chick colonization

The results of chick challenge are summarized in Table 3. One week following challenge, chicks were readily colonized by an oral dose of 10^5 CFU per 100 µl challenge of all fresh *Campylobacter* spp. cultures. *Campylobacter* spp. AH-1, AH-2, and CH-3 showed a high frequency of colonization with 80-90 % but the recovery frequency of TF-1 was 50 %. However, cold-stored, nonculturable and, heat-inactivated *Campylobacter* spp. did not establish colonization in the intestinal tracts of chicks seven days after challenge. Ten negative control chicks, likewise, were never colonized by *Campylobacter*.

DISCUSSION

Storage of wild-type *Campylobacter* spp. in PBS at 4ºC became nonculturable after 6 to 7 weeks while their corresponding plate counts decreased gradually over time. The results indicate that VBNC cells can be generated by exposure to nutrient depletion and incubation at 4ºC. Conversely, Medema *et al*. (19) showed the inability to culture *Campylobacter* cells was less than 3 days when the cells were stored at 25ºC. Jones *et al*. (16) reported VBNC cells from *C. jejuni* suspended in sterile surface water and held at 4ºC after 18 to 28 days, depending on the strain. All of *Campylobacter* spp. in the present study were morphologically similarly manifesting uniform spiral or gull-wing cells when in the logarithmic phase of growth. Examination by phase-contrast microscopy after cells reached nonculturable status revealed that the coccoidal form constituted the majority of morphology. The question of how curved, rod-shaped *Campylobacter* change into coccoidal morphology and what significance this change has for the *Campylobacter* infection is yet unresolved.

While any single marker of metabolic activity dose not conclusively determine the state of cell viability, metabolic indicators such as membrane potential, the ability to produce catabolic reducing power, or the ability to undertake DNA synthesis are likely determinants of viability (1, 17). Since mRNA is thought to be a short-lived molecule due to the presence of nucleases that normally degrade it very rapidly (24), the presence of mRNA might be regarded as a valid criterion for assessing cell viability (2, 18, 21, 24, 30). However, Birch *et al*. (4) confirmed the potential for poor correlation between mRNA detection and viability by observing that mRNA might exist in a detectable form manifested by nucleic acid sequence based amplification (NASBA) for up to 30 h after heat inactivation. Targets of RT-PCR amplification would be appropriate if they are transcribed abundantly, expressed throughout the cycle, and not under transcriptional regulation. In this present study, we chose four targets for mRNA amplification encoding for flagellin A protein (*flaA*), housekeeping protein (*tkt*), major outer membrane protein

(*porA*), and the 256 bp amplicon of putative haem-copper oxidase domain downstream from a previously uncharacterized *C. jejuni* two-component regulator gene. Even though all fresh cultures of *Campylobacter* exhibited mRNA signals, VBNC and heat-inactivated *Campylobacter* spp. produced inconsistent or reduced amplified products in RT-PCR assays depending on genes and strains. mRNA isolated from cold-stored, nonculturable and heat-inactivated *Campylobacter* TF-1 did not show any persistence for *flaA*, *tkt*, and *porA* genes or produced a very weak signal from the selected 256 bp amplicon when these were analyzed by RT-PCR assays. mRNA messages of *flaA* in VBNC and heat-inactivated *Campylobacter* spp. were not expressed for TF-1, AH-1 and AH-2 or produced a weaker response for CH-3 in RT-PCR assays. The results suggest that expression of flagellin A protein may be significantly influenced by physiological state or heat treatment. Persistence of mRNA species of VBNC and heat-killed *Campylobacter* spp. AH-1, AH-2, and CH-3 were observed from RT-PCR amplification of *tkt*, *porA*, and a 256 amplicon except for *porA*-targeted cells from heat-treated *Campylobacter* CH-3. The four genes were detected by PCR amplification of both VBNC and heat-killed *Campylobacter* spp.

We confirmed that mRNA was more susceptible than genomic DNA to degrade and persisted in cells killed by heat inactivation. Therefore, mRNA may not be an unambiguous indicator of cell viability provided that the heat-inactivated suspensions do not contain any viable cells. The durability of mRNA may change considerably depending on the environmental conditions (8, 25), the method of cell death (24), and the physiological state of the cell population prior to killing. Therefore, mRNA can remain undamaged for longer periods if cells are destroyed by treatments that inactivate RNase but not the message.

Our results prove that *tkt*, *porA*, and the 256 bp amplicon are good mRNA targets for the detection of the presence of VBNC *Campylobacter* spp. However, there is no unequivocal difference in the ability to amplify mRNA between heat-killed and viable but nonculturable cells. Nucleic acid amplification did not permit unequivocal discrimination of cell viability. Thus, detection of a positive RT-PCR signal did not necessarily imply the existence of viable cells, and

the cell viability still must be confirmed by an appropriate analytical method. In the present study, we investigated the potential of heat inactivated and VBNC *Campylobacter* spp. to infect chicks without success. Additionally, heat-inactivated and cold-stored, potential VBNC *Campylobacter* spp. that retained mRNA message were used to challenge chicks. Fresh viable *Campylobacter* spp. colonized the ceca of chicks after 7 days of incubation. However, none of the cold-stored, nonculturable or heat-killed *Campylobacter* spp. could establish colonization in the ceca of chicks.

These findings question the significance of the VBNC cells in environmental transmission of *Campylobacter* spp. and, the relationship of persistent mRNA signal as determined by an RT-PCR assay with cell viability. mRNA may be a significant and integral candidate as a marker of cell viability in many bacterial species. Further work is required to characterize the degradation rates of *Campylobacter* mRNA under various conditions. Also needed is the ability to identify conditions under which mRNA disappears very rapidly. Such ability might potentiate application of a useful *Campylobacter* RT-PCR.

Table 4-1. RT-PCR primers used for the detection of mRNA

Primers	Sequences (5′ to 3′)	Target gene	Size of amplified product
flaAR651F	CTATGGATGAGCAATTWAAAAT	*flaA* (flagellin A)	402 bp
flaAR652R	CAAGWCCTGTTCCWACTGAAG		
tktR653F	GCAAACTCAGGACACCCAGG	*tkt* (transketolase)	1,133 bp
tktR654R	AAAGCATTGTTAATGGCTGC		
porAR655F	ATGAAACTAGTTAAACTTAGTTTA	*porA* (major outer membrane protein)	1,272 bp
porAR656R	GAATTTGTAAAGAGCTTGAAG		
BOR657F	AGAACACGCGGACCTATATA	256 bp amplicon (putative haem-copper oxidase)	256 bp
BOR658R	CGATGCATCCAGGTAATGTAT		

Table 4-2. RT-PCR amplification of target genes from (1) fresh viable, (2) cold-stored nonculturable, and (3) heat-inactivated *Campylobacter* spp.

Treatments	Strains	*fla*A	*tkt*	*porA*	256 bp amplicon
Fresh viable *Campylobacter* spp.	TF-1	+	+	+	+
	AH-1	+	+	+	+
	AH-2	+	+	+	+
	CH-3	+	+	+	+
Cold-stored, nonculturable *Campylobacter* spp.	TF-1	-	-	-	+
	AH-1	-	+	+	+
	AH-2	-	+	+	+
	CH-3	+	+	+	+
Heat-inactivated *Campylobacter* spp.	TF-1	-	-	-	+
	AH-1	-	+	+	+
	AH-2	-	+	+	+
	CH-3	+	+	-	+

Table 4-3. Colonization of 7 day-old chicks following day of hatch challenge by *Campylobacter* spp.

	Campylobacter strain			
	TF-1	AH-1	AH-2	CH-3
Fresh culture	5/10*	9/10	9/10	8/10
Cold-stored, nonculturable	0/10	0/10	0/10	0/10
Heat-inactivated	0/10	0/10	0/10	0/10

* Number of chicks colonized per number of chicks challenged

Figure 4-1. Survival of *Campylobacter* spp. during incubation in PBS at 4°C. Culturable cells were enumerated using spread plates of Campy-Cefex agar.

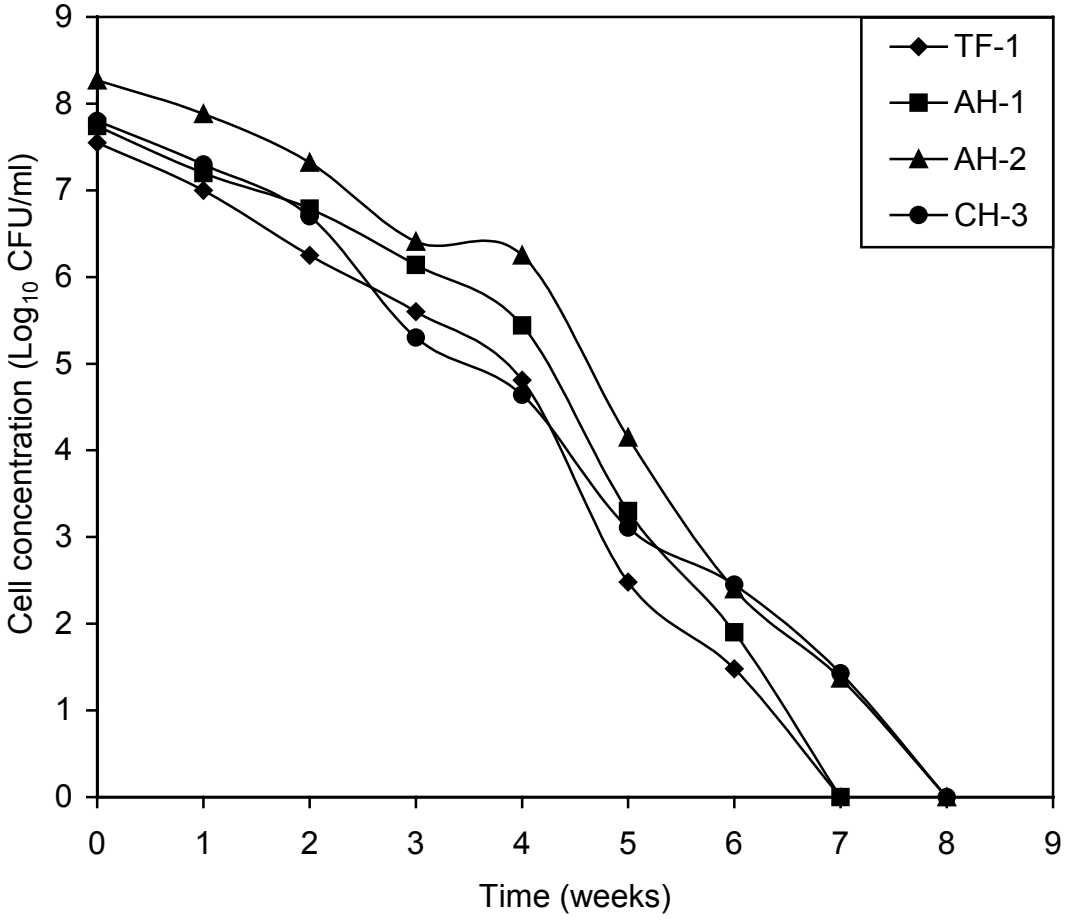


Figure 4-2. RT-PCR detection of *flaA* and *tkt* mRNA in fresh viable *C. jejuni* spp. Lane 1 to 4 shows products of *flaA* (Lane 1; TF-1, lane 2; AH-1, lane 3; AH-2, lane 4; CH-3), and lane 5 to lane 8 shows products of *tkt* (Lane 5; TF-1, lane 6; AH-1, lane 7; AH-2, lane 8; CH-3). Lane M contains 1 Kb DNA ladder marker and Lane + contains positive control.

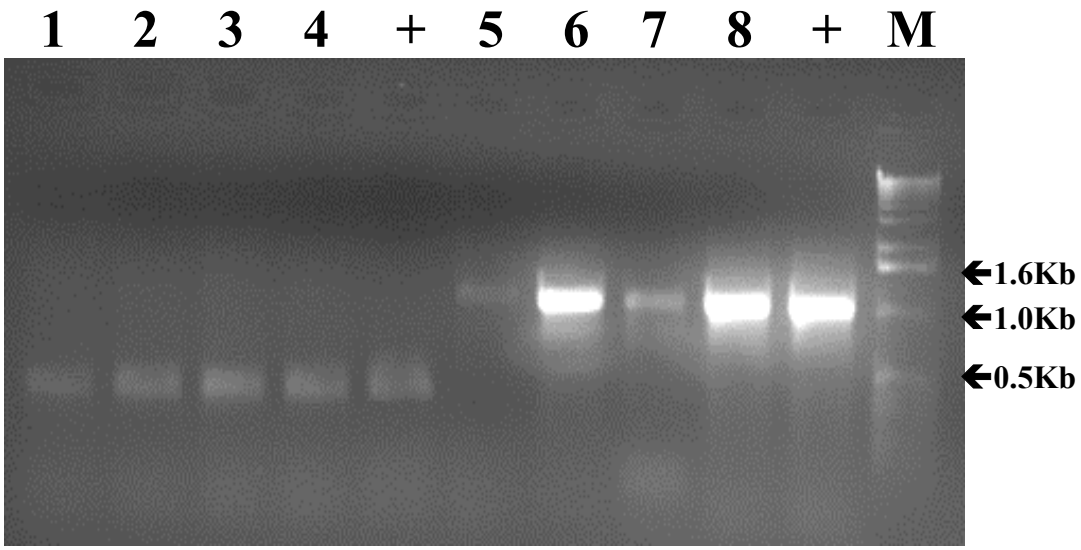


Figure 4-3. RT-PCR detection of *porA* and a 256 bp amplicon mRNA in fresh viable *C. jejuni* spp. Lane 1 to 4 shows products of *porA* (Lane 1; TF-1, lane 2; AH-1, lane 3; AH-2, lane 4; CH-3), and lane 5 to lane 8 shows products of a 256 bp amplicon (Lane 5; TF-1, lane 6; AH-1, lane 7; AH-2, lane 8; CH-3). Lane M contains 1 Kb DNA ladder marker and Lane + contains positive control.

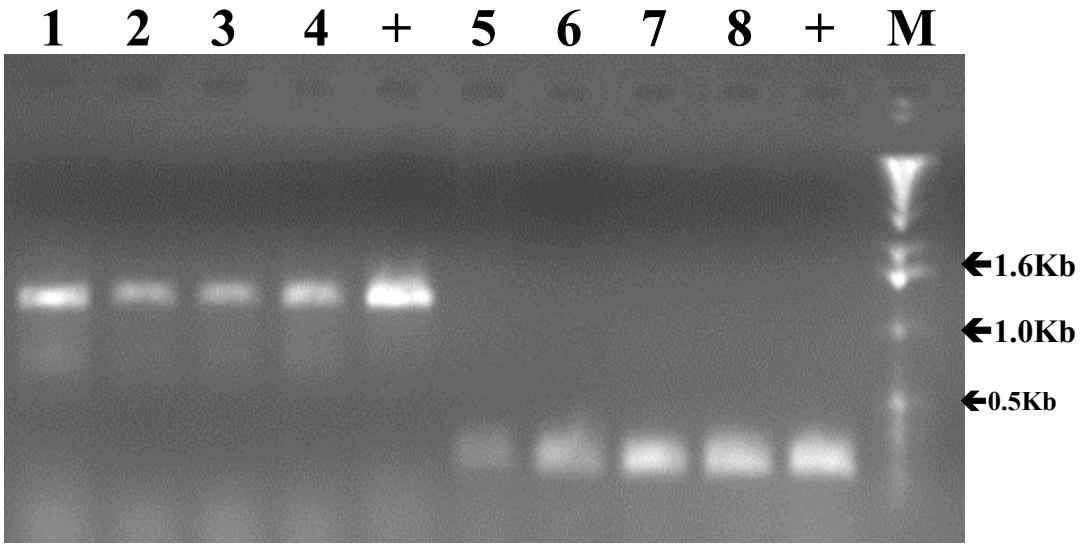


Figure 4-4. RT-PCR detection of *flaA* and *tkt* mRNA in VBNC and heat-inactivated (60^{o}C, 10 min) *C. jejuni* spp. Lane 1 to 4 shows products of *flaA* in VBNC cells (Lane 1; TF-1, lane 2; AH-1, lane 3; AH-2, lane 4; CH-3), and lane 5 to lane 8 shows products of *flaA* in heat-inactivated cells (Lane 5; TF-1, lane 6; AH-1, lane 7; AH-2, lane 8; CH-3). Lane 9 to 12 shows products of *tkt* in VBNC cells (Lane 9; TF-1, lane 10; AH-1, lane 11; AH-2, lane 12; CH-3), and lane 13 to lane 16 shows products of *tkt* in heat-inactivated cells (Lane 13; TF-1, lane 14; AH-1, lane 15; AH-2, lane 16; CH-3). Lane M contains 1 Kb DNA ladder marker and Lane + contains positive control.

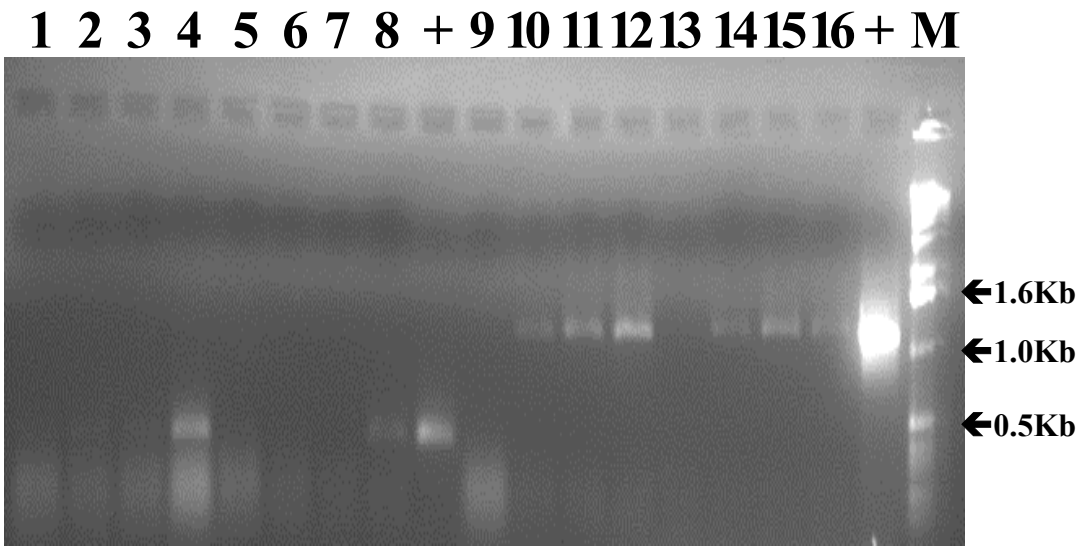
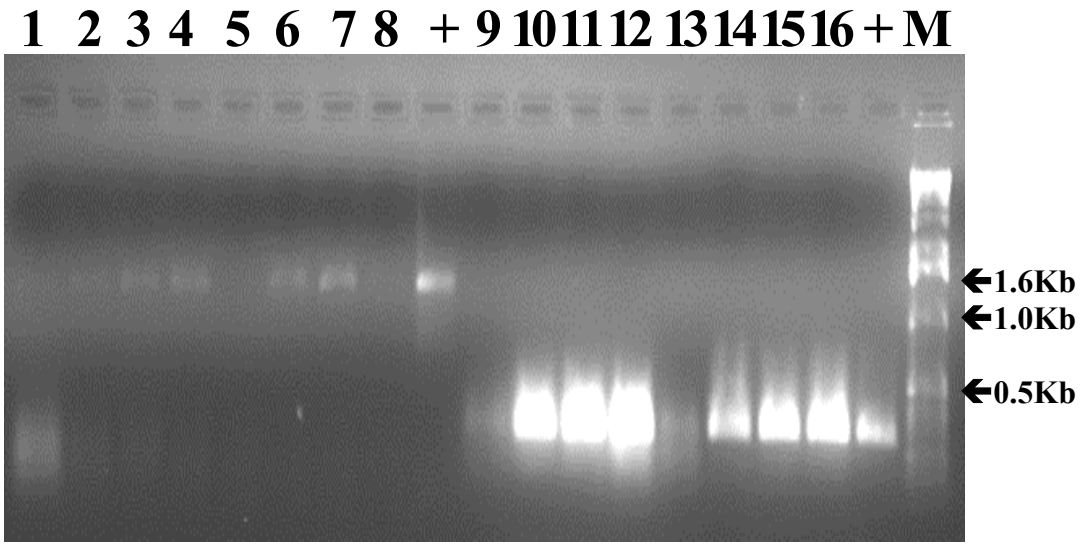


Figure 4-5. RT-PCR detection of *porA* and a 256 bp amplicon mRNA in VBNC and heat-inactivated (60^{o}C, 10 min) *C. jejuni* spp. Lane 1 to 4 shows products of *porA* in VBNC cells (Lane 1; TF-1, lane 2; AH-1, lane 3; AH-2, lane 4; CH-3), and lane 5 to lane 8 shows products of *porA* in heat-inactivated cells (Lane 5; TF-1, lane 6; AH-1, lane 7; AH-2, lane 8; CH-3). Lane 9 to 12 shows products of a 256 bp amplicon in VBNC cells (Lane 9; TF-1, lane 10; AH-1, lane 11; AH-2, lane 12; CH-3), and lane 13 to lane 16 shows products of a 256 bp amplicon in heat-inactivated cells (Lane 13; TF-1, lane 14; AH-1, lane 15; AH-2, lane 16; CH-3). Lane M contains 1 Kb DNA ladder marker and Lane + contains positive control.



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CHAPTER V

SUMMARY AND CONCLUSIONS

The main purpose of the study was to use mRNA as a marker for viability and to demonstrate the ability of RT-PCR to discriminate live and dead *Campylobacter* spp. The relationship between RT-PCR products and viability after heat-treatment was verified by a time-course experiment. Time-temperature relations needed to ablate mRNA signal was determined and mRNA half-life was assessed. Additionally, sensitivity of amplification of *Campylobacter* spp. using the primers of these four genes was determined by conventional PCR assays. The results of the first study can be summarized as follows:

1) Our RT-PCR technique was specific for *C. jejuni*, *C. coli*, and *C. lari*.
2) Longevity of the mRNA species (detected by RT-PCR) depended on the strain, the inactivation treatment, and the region of the transcript.
3) The 256 bp amplicon was determined to be the most stable mRNA species tested. mRNA of the 256 bp amplicon was detectable even after *Campylobacter* spp. had been killed at temperatures of 95 to 99^{o}C.
4) Using DNA-based PCR, the four genes could be amplified after 48 h holding time after each heat inactivation, indicating that the chromosomal DNA was minimally influenced by the heat treatment.
5) PCR products from the 256 bp amplicon were detected at 10^2 to 10^3 *C. jejuni* CFU per ml, exhibiting the highest level of sensitivity among the genes tested.

The main goal of the second study was to determine whether the ability of coccoid and VBNC cells of *Campylobacter* spp. were infectious after passage through day-old chicks. Further, this study was conducted to confirm the non-recovery of heat-killed *Campylobacter* spp.

correlated with mRNA message detection through chicken challenge. We investigated the expression of the four genes of *Campylobacter* spp., previously isolated from poultry feces, by reverse transcriptase-PCR (RT-PCR) assay. Cells were inactivated by heat treatment at 60ºC for 10 min based on their D value and VBNC cells were obtained by inoculating *Campylobacter* spp. in PBS and incubating these at 4ºC. The results of the second study can be summarized as follows:

1) Loss of culturability of four *Campylobacter* spp. occurred after 6 to 7 weeks incubation in phosphate buffed saline (PBS) at 4^{o}C.

2) Cold-stored, nonculturable and heat-inactivated *Campylobacter* spp. produced inconsistent amplified products from RT-PCR assay depending on the target genes and strains used.

3) Mostly, signals of mRNA species from VBNC and heat-killed *Campylobacter* spp. AH-1, AH-2 and CH-3 persisted.

4) VBNC and heat-inactivated *Campylobacter* spp., which produced positive mRNA signal but was not culturable by conventional culture-based methods, did not establish colonization in the intestine of chicks seven days after challenge.

In conclusion, the study suggests that the comparatively rapid degradation of mRNA provides a better measure of *Campylobacter* viability than does DNA-based PCR. In addition, the persistence of *Campylobacter* mRNA, as detected by RT-PCR following heat-treatment, was unexpected and is atypical among bacteria. Furthermore, the results question the correlationship between mRNA durability as assayed by RT-PCR and cell viability, as well as the significance of the VBNC cells in environmental transmission of *Campylobacter* spp.

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