THE DEVELOPMENT OF REAL-TIME POLYMERASE CHAIN REACTION FOR

THE DETECTION OF *CAMPYLOBACTER JEJUNI*

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THE DEVELOPMENT OF REAL-TIME POLYMERASE CHAIN REACTION FOR THE DETECTION OF *CAMPYLOBACTER JEJUNI*

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THE DEVELOPMENT OF REAL-TIME POLYMERASE CHAIN REACTION FOR

THE DETECTION OF *CAMPYLOBACTER JEJUNI*

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Lin Liu, daughter of Wanshen Liu and Yumei Wang, was born September 10, 1975, in Huaibei city, Anhui, China. She graduated from China Agricultural University (former Beijing Agricultural University) with a Bachelor's degree in 1997. She then worked in a Sino-Swiss joint company as a food quality manager for 2 years. In August 2006, she was admitted into Department of Poultry Science at Auburn University as a graduate research assistant.

THESIS ABSTRACT

THE DEVELOPMENT OF REAL-TIME POLYMERASE CHAIN REACTION FOR THE DETECTION OF *CAMPYLOBACTER JEJUNI*

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Campylobacter jejuni (and related species) has been recognized as the most common pathogen that causes human bacterial gastroenteritis worldwide. It is the major cause of food-borne illnesses in the developed countries. Undercook poultry has been reported associated with the outbreak of campylobacter infection in human. Additionally, it has a very low infective dose, which can be as low as few hundred viable cells. Because of its leading role in causing food-borne illness in human, it is of paramount importance to develop methods that can be used to detection and differentiate *C. jejuni*. Traditional detection methods for *Campylobacter jejuni* require the enrichment of the food samples to reach a high number of bacterial cells. Therefore a rapid, specific and sensitive detection methods needs to be developed.

In this study, two real-time PCR protocols have been developed to target the *C. jejuni*-specific *HipO* gene. The first one was a SYBR Green I based real time PCR. A primer set was designed from the conserved region of the benzoyglycine amidohydrolase (hippuricase) gene of *C. jejuni*. This assay had a detection limit of 10 CFU/ml, was also capable of differentiating *C. jejuni* from closely related species, such as *C. coli* by melting curve analysis in addition to the highly specific detection of *C. jejuni*.

The second was a dual labeled hydrolysis probe (TaqMan®). A different set of primers was designed from the conserved region of the *HipO* gene of *C. jejuni*. The specificity of the assay was then tested with several *Campylobacter* strain and other Gram-negative bacteria. The PCR protocol was highly specific and only DNA from *C. jejuni* strains were amplified. Quantitative detection were conducted after generating the standard curve with serially diluted DNA extracted from pure culture and was found to be linear over 9 log units, with a standard curve correlation coefficient of 0.998. The detection limit for the current assay is 4.6 colony forming unit (CFU) per milliliter in pure cultures. This real-time PCR was also been used to tested with inoculated retail samples, and the detection limit of the assay was 100 CFU per milliliter. This assay may reduce the time for detection of *C. jejuni* in retail broiler samples.

Key words: *C. jejuni*, real-time PCR, SYBR Green I, hydrolysis probe, hippuricase gene, retail broiler meat.

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CHAPTER I: GENERAL INTRODUCTION

Campylobacters, especially *C. jejuni* and *C. coli*, are the most common pathogens that can cause human bacterial gastroenteritis worldwide. They are the major cause of food-borne illnesses in the developed countries (Altekruse et al., 1999; Coker et al., 2002; Moore et al., 2005; Tauxe, 2002). In the US alone, there are 15 out every 100,000 people diagnosed with campylobacteriosis annually, with more unreported cases (CDC, 2005). In 2005, the reported *Campylobacter* infections in humans increased 7.8% compared to the previous year. Campylobacteriosis overtook salmonellosis as the most reported zoonotic disease in the EU (MeatNews.com, 2007).

Campylobacters are found in the intestinal tract of both domestic and wild animals as commensal bacteria. Campylobacters are also the leading cause of the bacterial diarrheal disease worldwide, especially in developed countries. Among all the *Campylobacter* species, *C. jejuni* and *C. coli* account for at least 95% of gastrointestinal infections in human. Other species, such as *C. upsaliensis* and *C. lari*, are also associated with other forms of campylobacteriosis. In addition, Campylobacters have very low infectious doses as low as few hundred cells (Black et al., 1988). The incubation period is up to 10 days with typical symptoms related to enteritis, with diarrhea, cramps, abdominal pain and fever. For young children, the elderly, or people with immunosuppressive diseases, such as AIDS and cancer, the infection could be fatal. *Campylobacter* infections are also

associated with a severe paralytic disease to the peripheral nervous system known as Guillian-Barre syndrome (Allos et al., 1998; Bar and Fricke, 1987).

Early and accurate detection of Campylobacters from food sources, especially broiler meat that is one of the most consumed meat sources in the US, is critical for the control and reduction of campylobacteriosis. Currently, the common detection and diagnostic methods for campylobacters are conventional culture-based procedures, which are slow, sometimes labor intensive and may not be accurate. The phenotype identification to species level is difficult due to the lack of informative biochemical test. These limitations have led to the development of alternative detection methods for *Campylobacter* detection, including enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) methods.

Real-time PCR is a relatively new experimental technique derived from traditional PCR. It incorporates fluorescence reporters, which enable monitoring the amplification of specific target DNA in real-time. The high sensitivity and specificity of real-time PCR assays have resulted in several methods for pathogen detection in clinical samples. In the following literature review, the discussion will focus on the characteristics of Campylobacters, especially *C. jejuni*, and the possible application of real-time PCR in the detection of *C. jejuni*. Subsequently, two newly developed real-time PCR protocols for the detection of *C. jejuni* will be reported. One is SYBR Green I based and the other method is based on the TaqMan probe system.

CHAPTER II: LITERATURE REVIEW

1. The history of Campylobacters

Since the recognition as one of human pathogens in the 1970s, campylobacters have gained growing attention in public health. Microbiologists then realized that in fact *Campylobacter*-associated diseases might have been reported about a hundred years before that time (Kist, 1985). In 1886, the German scientist Theodore Escherich reported isolation of a spiral-shape bacterium from the colons of infants died of "cholera infantum". However, these bacteria were deemed unculturable and might not be the cause of the infant deaths. Our early knowledge of Campylobacters was mostly from veterinary medicine, which has reported *Campylobacter* infections since early 20th century. At that time, these spiral-shaped bacteria were known to cause epizootic abortion in ewes and cattle, thus they were categorized as *Vibrio fetus* subsp. *jejuni*. *V. fetus* has also been associated with abortion in human in late 1947 (Skirrow and Butzler, 2000). The first well-documented *V. jejuni* related human gastroenteritis was in 1938, when an outbreak of diarrheal disease caused by contaminated milk affected 355 prisoners (Lavy, 1946).

Scientists gradually realized that these bacteria have different chemical and antigenic characteristics from the vibrios, therefore they were termed "related vibrios" until the 1963, when it was renamed to *Campylobacter* by Sebald and Veron (Butzler, 2004; Sebald and Veron, 1963). As the result, *Vibrio fetus* subsp. *jejuni* was reclassified as

Campylobacter jejuni (Veron and Chatelain, 1973). Owing to the development of *Campylobacter* isolation methods and molecular techniques, more species have been isolated and categorized into the genus *Campylobacter*, such as *C. coli*, *C. lari*, *C. upsaliensis*, and *C. hyointestinalis* (Dekeyser et al., 1972; Lawson et al., 1997). In the past 40 years, Campylobacters has gradually changed their roles in public health from a group of mysterious bacteria to major disease causing food-borne pathogens.

2. Characteristics of Campylobacters

The genus Campylobacters belongs to the family *Campylobacteriaceae*, which is comprised of 18 species and 6 sub-species (Humphrey et al., 2007). Campylobacters are small (0.2~0.9 μm in width and 0.2~5.0 μm in length), slender, spiral curved rod. They are Gram-negative bacteria with a polar flagellum at one or both ends of the cell, which gives them high motility. These microscopic characteristics can be used to distinguish them from other foodborne pathogens.

Campylobacters are urease negative and catalase and oxidase positive, which means that they can catalyze hydrogen peroxide to produce oxygen and water. They grow under microaerobic conditions, meaning that they require 5-10% of oxygen and 3-5% of carbon dioxide for growth. They are also thermophilic microorganisms and can grow at temperatures 42 ℃ with an optimal growth temperature between 37 and 42 ℃. The growth at high temperatures was considered as an adaptation to the environment of the intestinal tracts of wild birds (van Vliet and Ketley, 2001).

The normal spiral shaped in campylobacters can transform to a coccoid form in aged cultures, upon exposure to the atmospheric conditions or other stresses. It is not clear of

these coccoid forms are not culturable. The most recent research in the area suggests that the coccoid form is merely a degenerative state rather than a dormant stage of the bacteria, due to the difficulty in establishing the viable non-culturable status (Hazeleger et al., 1994; Hazeleger et al., 1998; Rollins and Colwell, 1986). The role of these coccoid forms in *Campylobacter* life cycle, transmission, and colonization are still not fully understood.

The first complete sequencing of *C. jejuni* strain NCTC 11168 was published in 2000, which revealed that, *C. jejuni* genome is 1,641,481 nucleotides in length with a G+C content of 30% (Parkhill et al., 2000). This is consistent with previous reports that Campylobacters genome's high A+T contents also makes cloning and sequencing of *Campylobacter* genes exceedingly difficult (Chang and Taylor, 1990; Taylor et al., 1992). In addition, *C. jejuni* and *C. coli* genomes are comparatively smaller than many other common enteropathogens, such as *Escherichia coli* (4.5 Mbp), which might explain the lacking of some functions of these bacteria. For example, campylobacters are not capable of fermenting carbohydrates and degrading some complex substances. As the result, they require complex growth medium, which makes them difficult to work in laboratory conditions (van Vliet and Ketley, 2001).

By screening the open reading frames from the sequence, a total of 1,705 genes were discovered, with 1,629 functional proteins (Parkhill et al., 2000). This detailed information has provided scientists with opportunities for further studies with campylobacters. As a result, there is an explosion of research on the genetics aspects of *C. jejuni* and their relationships with its physiology and pathogenesis.

3. The epidemiology of *Campylobacter jejuni*

Because *C. jejuni* and *C. coli* are the most common causes of human diseases, the rest of this review will be specific for these two species unless specifically mentioned.

Campylobacters are zoonotic pathogens, which are carried in the intestinal tract of many domestic and wild animals commensal bacterial flora. These animals serve as the reservoirs for the bacteria and spread the pathogens to the environment and other animals. Domestic animals, especially pork and poultry, can contract the pathogens by consuming contaminated water and feed. The meat products from these production animals could be contaminated at slaughtering plants. Other sources of contamination for humans are water, milk, or pets. In the US, bacteria are most likely transmitted to human via raw or undercooked food, especially poultry meat (Deming et al., 1987), and there few outbreaks have been reported to be associated with the consumption of undercooked chicken meat. If contaminated, very low dosage is needed, as one study reported 500-800 CFU to be sufficient to cause typical campylobacterosis symptoms (Black et al., 1988).

Interestingly, the clinical aspects of the diseases caused by campylobacters are quite different between industrialized and developing nations. In industrialized countries, the most common symptoms are inflammatory diarrhea, severe cramping in young adults and young children, and sometime the elderly. In developing countries, it manifests in more of an asymptomatic carriage, or milder clinical signs of watery, non-inflammatory diarrhea in young children Seasonal aspects of the disease are also more prominent in industrialized countries than in developing countries. These differences are believed to reflect the less than optimal public health conditions, which lead to early and constant

exposure to the pathogen in developing countries and the developing of immunity against the disease in the population (Calva et al., 1988; Ketley, 1997; Taylor et al., 1993).

4. The pathogenesis of *Campylobacter jejuni*

After successful colonization of the epithelial cell in the intestine, Campylobacters can express a number of virulence factors to invade and damage the epithelial cells

Campylobacters are highly motile and can penetrate the mucus membrane and invade the epithelium (Szymanski et al., 1995). They are able to recognize specific chemical compounds (chemotaxis). For instance, they can recognize L-serine and L-fucose from the mucin layer and avoid the harmful ones such as bile acids (Hugdahl et al., 1988). Adhesion and invasion play critical roles in *Campylobacter* pathogenesis. Subsequent to penetrating the mucus layer, some campylobacters can invade the epithelial cells after colonizing on the cell surface. The breaking and entering of epithelial cells may lead to the mucosal damage and inflammation commonly seen in *Campylobacter* infections (Ketley, 1997).

As one of the important mechanisms of their pathogenicity, Campylobacters can produce at least two exotoxins: a heat-labile enterotoxin and a cytotoxin, which its virulence characteristics not yet known (Johnson and Lior, 1984). The heat-labile enterotoxin is known to be a large protein, which has a molecular weight of 60~70kDa and can be inactivated at 96˚C for 10min. This cholera toxin like protein can cause watery diarrhea by activating the adenylate cyclase activity with the intestinal mucosal epithelial cells, which in turns disrupts the normal ion transportation chain (Ruiz-Palacios et al., 1983). Interestingly, the production of the enterotoxin is stimulated by the iron contents in the growth media, and Campylobacters can lost their enterotoxin producing

activity when growth media is lacking iron supplementation (Walker et al., 1986). However, recent detailed search in the NCTC 11168 genome only resulted in a homolog of *cdt* gene, which is known to encode for cytolethal distending toxin (CDT). It has been proposed that CDT may interfere with the development and maturation of crypt cells into functional villus cells, thus results in erosion of the epithelial cells and the disruption of absorption (Whitehouse et al., 1998).

Oxidative and temperature stress defense mechanisms help *Campylobacter* spp. survive the host defense system. Iron acquisition mechanism facilitates the organism's ability to acquire essential iron from the host and contribute to the pathogenesis of *Campylobacter*.

5. Isolation and identification of *Campylobacter jejuni*

C. jejuni is strictly a microaerobic organism; therefore, a successful isolation method has to provide an atmosphere with reduced oxygen content (~5%), a carbon dioxide concentration of 10%, and nitrogen concentration of 85%. The isolation and identification was not chemically possible before the first *Campylobacter* medium developed with blood agar containing vancomycin (10mg/l), polymyxin B (2.5IU/ml), and trimethoprim (5mg/l) (Skirrow, 1977). The blood in the growth media (usually 5~10% v/v) is to reduce toxic oxygen compounds, such as hydrogen peroxide synthesis in the media that can result from the exposure to light. Furthermore, the addition of blood appears to help in the neutralization of trimethoprim antagonists that can develop when using certain enrichment broths (Corry et al., 1995). The growth media may also

contain supplements such as ferrous sulfate, sodium metabisulfite, and sodium pyruvate, which can increase the oxygen tolerance of *C. jejuni*.

The inclusion of antibiotics in a typical growth media is critical for the isolation and recovery of campylobacters. This is because Campylobacters are resistant to vancomycin, trimethoprim, and cephalosporins, which inhibit Gram-negative cocci, such as *Escherichia coli* and *Salmonella, Enterobacteriaceae* and Gram-positive cocci, such as, *Clostridium* and *Straphylococcus*, respectively.

Commonly, samples collected from food and environment contains only low numbers of Campylobacters. Therefore, it is important to enrich the samples before transferring to agar plates. The most popular enrichment media for Campylobacters are Bolton and broths, which are commonly supplemented with 5% (v/v) lysed horse or sheep blood and a combination of antibiotics for the control of competing organisms. The enrichment cultures are subsequently plated onto selective media and then incubated at 42˚C for up to 48 hrs. The selective media and the microaerobic condition may result in a detection limit of less than 1CFU of *C. jejuni* per 25 g of food product.

6. Hippuricase activity of *Campylobacter jejuni*

Because of its leading role in causing food-borne illness in human, it is of paramount importance to differentiate *C. jejuni* from other *Campylobacter* species. However, the limited biochemical properties and similarities in microscopic appearances, *C. jejuni* cannot be easily distinguished from *C. coli*.

The first reliable biochemical assay to identify *C. jejuni* from other *Campylobacter* species was established by Harvey (1980). This assay utilized the unique *N*-

benzoylglycine amidohydrolase (hippuricase) activity of *C. jejuni* to hydrolyze *N*-benzoylglycine (hippuric acid) by cleaving it glycine and benzoic acid. The glycine formation can be detected by a ninhydrin-based reagent system to produce a purple color (Harvey, 1980). In addition, the presence of benzoic acid can also be determined by precipitation with ferric chloride reagent (Facklam et al., 1974). This simple and reliable biochemical test has become the most popular phenotypic characterization test used to distinguish between *C. jejuni* and other species (Bar and Fricke, 1987; Hani and Chan, 1995; Penner, 1988).

However, *C. jejuni* hippruicase negative can occur. Studies have indicated that *C. jejuni* may fail to hydrolyze hippuric acid. In addition, the assay is unable to detect low level of hippuricase product, and the expression of *HipO* is depend on the inoculums can cause the unsuccessful differentiation of *C. jejuni* from other closely related species (Eyers et al., 1993; Linton et al., 1997; On and Holmes, 1991; Totten et al., 1987).

This *C. jejuni*-specific enzyme activity is encoded by hippuricase (*HipO*) gene, which contains a single open reading frame of 1149bp, and sequence analyses indicated that it is absent from other *Campylobacter* species (Hani and Chan, 1995). Further PCR-RFLP study also indicated that *HipO* gene is highly conserved amongst *C. jejuni* strains. Therefore, detection of *HipO* gene using PCR-based methods can be a reliable alternative for the identification of *C. jejuni* (Slater and Owen, 1997).

7. Polymerase chain reaction

Invented by Dr. Mullis in 1983, the polymerase chain reaction (PCR) technique is an indispensable tool in molecular biology, medicine, genetics, criminal investigations, etc.

With PCR, we are now able to produce a selective amplification of a specific DNA sequence by a factor of 10^6, which gives generates a large number of target materials to work with (Saiki et al., 1988). Two specific oligonucleotide primers are involved in common PCR amplifications. Each of the primers is complementary to the 5' end of one of the strand and they flank the target DNA sequence to be amplified. The amplification procedures include repeated cycles (30~40), and each cycle consists of the denaturation of DNA at high temperature (~95˚C), the annealing of the primers to the complementary strands (~45-60˚C), and the extension of the annealed primers by the DNA polymerase (~72˚C). The amplified products (amplicons) are also complimentary to the primers, so the next cycle of reaction continues as the temperature changes, and results in doubled amount of the DNA amplified from the previous cycle. Theoretically, the amplicons accumulate at an exponential rate, which can reach 2^n with "*n*" being the number of the cycles (Saiki et al., 1988).

Several essential components and reagents are included for a basic PCR reaction to be carried out: DNA template (contains the target region), primers (complimentary to the 5' and 3' end of the target DNA), DNA polymerase, deoxynucleotide phosphates (dNTPs, used by DNA polymerase for synthesizing a new DNA strand), buffer solution (to provide a suitable chemical environment for optimum activity and stability of the DNA polymerase, and magnesium (co-factor of the DNA polymerase). DNA polymerases derived from *E. coli* DNA polymerase I with low thermo stability were used in PCR at the early stage, but the discovery of the thermostable *Taq* polymerase DNA polymerase, purified from thermophilic bacteria *Thermus aquaticus*, was the real milestone in the evolution of PCR. Taq polymerase can survive the extended incubation of 95˚C and

there is no need for replenishment at each cycle. Thus, the fully automated PCR was developed (Saiki et al., 1988).

The PCR assays have been used in many areas, including isolation of specific region of genomic DNA, in combination with restriction enzymes to create the restriction fragment length polymorphism, etc. The PCR has also been used to study gene diversity, cloning, DNA sequencing, genetic fingerprinting, etc.

8. Real-time PCR

Real-time PCR is a relatively new molecular technique derived from conventional PCR. It is also known as real-time quantitative PCR or kinetic PCR because it allows simultaneous detection and quantification of target DNA molecules. When a standardized control is set, real-time PCR can be used to quantify target DNA and to estimate the absolute copy numbers of the targets in a sample. The basic procedures of real-time PCR are the same as regular PCR assays, but real time PCR includes a fluorescence reporter, which is excited during the PCR process and emits fluorescence signal corresponding to the amplification of target DNA. During the initial few cycles, the fluorescence signal is weak to be distinguished from the background noise. However, as the amount of the PCR products increase exponentially at the early stage of PCR, the signal strength increases accordingly. The fluorescence signal is then detected by computer software that allow for qualitative and quantitative measurements instantaneously.

During a real-time PCR, the computer program calculates the fluorescence signal changes (ΔRn) using the following equation: $\Delta Rn=(Rn^{+})-(Rn^{-})$, whereas (Rn^{+}) is the

emission density ratio at any give cycle, and *(Rn^-)* is the emission density ratio prior to the amplification in the same reaction tube. The amplification curve is generated by the software by plotting y-axis against the PCR cycles (x-axis).

In the early stage of the amplification curve, ΔRn remains low and not distinguishable from the baseline, the density of the fluorescence emission by the reporters increase as the PCR generates more products. The number of cycles at which fluorescence signal level reaches a certain threshold is defined as C_T value. The C_T values decrease linearly with increasing initial amount of the target DNA in a real time PCR (Heid et al., 1996). The mathematical relationship between C_T values and log concentration of the target DNA can be used in relative or absolute quantification. However, the C_T is a value and can be changed according to real time PCR conditions.

Absolute quantification can be achieved by measuring the relationships between the C_T values and their known initial concentration (or amount) of a set of serial diluted purified target DNA templates. The plot of regression relationship between C_T values and the log transferred initial amounts of the standard is called standard curve, with which a regression equation can be drawn. Most real-time PCR softwares are able to estimate the PCR efficiency automatically; therefore, the slope and the intercept are also readily quantifiable (Rutledge and Cote, 2003).

When designing real-time PCR protocol, the size of desired target amplicons may vary depending on the type of fluorescence reporters. For probe-based real time PCRs, it is recommended to limit the amplicons size to less than 200bp, although some studies involved amplicons as long as ~400bp (Bustin et al., 1999). Shorter amplicons have lower melting temperatures (T_m), which allow them to be fully denatured within a

relatively shorter time and bound with their complementary targets efficiently. Primers in real-time PCR are usually about 15 to 30 bases in length and 20~70% in G/C contents, with relatively higher and more restricted T_m (58~60 $^{\circ}$C) than conventional PCR. The two primers should have little difference (~1-2 $^{\circ}$C) in Tm. For the probe oligonucleotides used in the real time PCR, a length about 30 base pair is recommended, and they are designed to have a T_m 5~10 $^{\circ}$C higher than the primers to bind with the target prior to the primers and compete against primers in the reaction solution efficiently.

8.1 Fluorescent reporters

Fluorescent reporters in the real time PCR are distinguished by their chemistries: 1) probe based which is sequence –specific; and 2) intercalated-based DNA dye that is non-specific. TaqMan probes and hybridization probes are the most commonly used in sequence-specific probe group. SYBR-Green I is the most popular double stranded DNA binding dye used in DNA dye group. But more and more novel fluorescence reporter systems have been invented with the development of real-time PCR: probes with only one dye attached such as displacement probes (Li et al., 2002) and Light-Up probes (Svanvik et al., 2000); probes with modified chemistry to improve affinity with target DNA like Light-Up probes and MGB-probes (Kutyavin et al., 2000), which can be as short as 10~12 bases. Modified primers with fluorescence dye directly attached are Scorpion primers (Whitcombe et al., 1999); LUX primers (Nazarenko et al., 2002), and Ampliflour primers (Uehara et al., 1999). Even though these fluorescence reporters have different chemistries, the fundamental role of them in real time PCR is to emit fluorescence signal upon binding with target DNA templates.

a. TaqMan probe

The TaqMan probe is also known as the hydrolysis probe, because it utilizes the 5'to 3' exonuclease activity of DNA polymerase to hydrolyze the probe hybridized to the target amplicon. The TaqMan system consists of two sequence-specific primers and the added specificity of a dual-labeled sequence-specific probe, with a fluorescence report dye (fluoroescein) covalently linked at its 5'end and a second fluorescence dye (the quencher) at its 3'end. The quencher is able to absorb the emission spectrum from the first dye when they are at close vicinity through the fluorescence resonance energy transfer (FRET) mechanism. Because the effective exonuclease activity of the DNA polymerase is only effective on double-stranded DNA, the unbound, single-stranded TaqMan probe is intact and no fluorescence emission can be detected (Heid et al., 1996). After the denaturation step, the TaqMan probe binds to its complementary region on the target sequence and forms double-stranded probe-DNA hybrids until the extension step, when the DNA polymerase rapidly extends the sequence from the primers and reaches the hybridized probe. The hybridized probe is subject to the hydrolyzing and cleaving functions of the exonuclease because its double-stranded structure. The hybridization and cleavage of the probes results in the separation of the fluorescence reporter from the quencher. The emission from the reporter, no longer absorbed by the quencher, is accumulated and the signal is strong enough (above the threshold) to be detected by the real-time PCR instrument (Heid et al., 1996; Lyamichev et al., 1993). FAM (6-carboxyfluorescein) and TAMAR (6-carboxy-tetramethyl-rhodamine) are the first fluorescence dye and quencher pair and still the most common choice for the designing of

TaqMan real time PCR systems. Nowadays, there are much more selections for the 5' fluorescence reporter such as, FAM, HEX™, TET™, Cy3™, Cy5™, TEX™-163, JOE NHS Ester, or ROX NHS Ester. Similarly, for 3'end quencher, the selections can be Iowa black™ FQ, Black Hole Quencher®, TAMRA, or TAMRA NHS Ester (http://www.idtdna.com/Catalog/DualLabeledFluorescentProbes/Page1.aspx). The most important criteria for the fluorescence dye choice are the following: the quencher has to able to absorb the emission spectra from the fluorescence reported, and the wavelength of the emission from the reporter has to match the detection range of the real-time PCR instrument.

b. Hybridization probe

The hybridization probe system consists of two single-labeled probes to maximize specificity, and it was first developed for the Roche LightCycler (Wittwer et al., 1997b). One probe is located near the 5'-end, while its 3' end carries a fluorescein donor. Another probe is located near to 3'end, with the 5' end attached with a fluorophore acceptor (acceptor probe). The two fluorescence components are selected so that the emission spectrum of the donor overlaps with the excitation spectra of the acceptor. In addition, the 3' end of the acceptor probe is phosphorylated to prevent extension by DNA polymerase. These two sequence-specific probes are designed to bind to regions of the target amplicons complementarily in a head-to-toe formation arrangement. When both probes are hybridized to their target regions, they should be in close range, less than 5 bases, so that the emission from the fluorescein donor can be transferred to the fluorophore acceptor efficiently. Since two emissions from donor and acceptor have

different wavelengths, they can be detected by two different channels of the real-time PCR machine. In this system, the donor emission signal has shorter wavelength and is served as the background to compare density of the acceptor emission. As a result, the fluorescence density changes are measured as the change of the signal/background ratio.

The specificity is improved significantly because the fluorescent signal can only be detected when both probes are hybridized to the correct locations in the hybridization system. The probes are protected from exonuclease hydrolysis and a melting curve analysis can be applied.

c.SYBR-Green I dye

SYBR-Green I is an asymmetric cyanine dyes and one of the most popular dyes used in real-time PCR (Zipper et al., 2004). Asymmetric cyanine dyes emit minimum amount of fluorescence at the Free State due to its two nitrogen-containing aromatic components. But the dye bind to the minor groove of a double-stranded DNA, the rotation of the aromatic components and become restricted, as the result, the DNA-dye-complex start to emit bright green fluorescence light at the wavelength of 522nm (Nygren et al., 1998).

Like all the asymmetric cyanine dyes, SYBR-Green I is considered a sequence non-specific reporter in real-time PCR because it emits strong fluorescence signals by binding with any double-stranded DNA, including primer-dimers. Primer designing is crucial for a SYBR Green based real time PCR, with an important concern with minimized primer-dimer formation. SYBR-Green binding with double-stranded DNA is not at one to one ratio since multiple dye molecules can bind to a DNA molecule at the same time; so that

the fluorescence density of SYBR-Green is based on the mass of DNA. These are drawbacks for SYBR-Green based real-time PCR for absolute quantification. However, there are also advantages using SYBR-Green base systems, for it cost-effective characteristics with only primers needed in the system and the possibility of performing melting curve analysis.

8.2 Melting curve analysis

Melting curve analysis is usually performed after the completion of the PCR to determine the T_m profile of real-time PCR products, which are assessed by factors such as amplicon length, sequence, and G+C contents. By gradually increasing the temperature and continuing measuring the fluorescence, when the temperature reaches the T_m of the amplicon, the DNA strands separate and the SYBR-Green molecules dissociate. Since SYBR Green emits minimum amount of fluorescence at the free state, the fluorescence drops abruptly (Ririe et al., 1997). The fluorescence density is then plotted as a function of temperature as amplicons dissociate. The results can be converted to melting peaks, by plotting the negative derivative of fluorescence (-dF/dT) with respect to temperature. For each product from real time PCR amplification under optimal condition, the melting peak is represented by a single sharp. The Tm for primer-dimers can be readily differentiated from the distinctive sharp transition of the amplicons, with their lower melting curves. Melting curve analysis is highly sensitive, and can be used to differentiate amplicons with a few base pair differences (Espy et al., 2002).

8.3 Roche® LightCycler

The LightCycler is manufactured by Roche Molecular Biochemicals, Mannheim, Germany. Real-time PCR reactions are performed in micro-volume glass capillary tubes. The capillaries are then loaded into a rotor like carousel, which are heated or cooled efficiently by airstreams (Wittwer et al., 1989). The carousel rotates to past the individual capillary through the blue light source, and the fluorescence is measured by three or six (depending on the model) sensors, which are designed for the detection of different wavelengths. So it can also be used for multiplex PCR with multiple channels detecting different fluorescence reporters. Because of the efficient air-heated exchange system, the temperature changes of the reaction in the LightCycler can be up to 20˚C per sec; the rapid temperature transition also been improved with the glass capillary tubes to provide a high surface-to-volume ratio of glass to PCR mix (Wittwer et al., 1997a).

9. The detection of *C. jejuni*

To identify *Campylobacter* using PCR methods, scientists must perform intensive bioinformatic studies on the genome of the bacteria to search for the a specific region, which could be a gene, a fragment of a gene, and the coding or even non-coding region of the genome, that is unique to the desired bacteria. Primers are then designed to amplify this region, so that positive result can be determined if expected bands (traditional PCR) or amplification curves (real-time PCR) appear.

The first conventional PCR designed to amplify the 16s rRNA of *C. jejuni*, *C. coli* and *C. lari* resulted in the detection limits of 25 CFU per g of product and 500 CFU per ml in culture broth after 18 h enrichment, which was already a significant improvement

over conventional methods in terms of sensitivity and time (Giesendorf et al., 1992). Since the introduction of real-time PCR, there has been a lot of studies with this relatively new technique on the detection and quantification of *Campylobacter* spp. in foods (especially raw meat), milk, and water and feces samples (Abu-Halaweh et al., 2005; Best et al., 2003; Cheng and Griffiths, 2003; Krause et al., 2006; LaGier et al., 2004; Logan et al., 2001; Lund et al., 2004; Oliveira et al., 2005; Rudi et al., 2004; Sails et al., 2003; Wolffs et al., 2005; Yang et al., 2003). These assays demonstrated a wide dynamic range up to six logs, and the results are closely correlated with traditional culture methods (Moore et al., 2005).

9.1 Real-time PCR in the detection of C. jejuni

For detection, the 16s rRNA is one of the most important functional RNAs genes whose sequence is highly conserved across species. On one hand, PCRs using primers designed from this gene are mostly used for screening purpose (Krause et al., 2006; Lund et al., 2004; Perelle et al., 2004; Wolffs et al., 2005) or pre-selection for subsequent determination of specific (Abu-Halaweh et al., 2005; Logan et al., 2001). On the other hand, genes that are unique to *C. jejuni* can be used to design primers to identify the species in one PCR step. The genes that are commonly used for detection of *C. jejuni* are *hipO* (Abu-Halaweh et al., 2005; LaGier et al., 2004), *mapA* (Best et al., 2003), *ORF-C* (Sails et al., 2003), *cadF* (Cheng and Griffiths, 2003; Oliveira et al., 2005) and certain regions on the VS1 (Yang et al., 2003). The application of pre-selection (screening) does enhance the probability of identifying the pathogen in the following specific detection procedures (Abu-Halaweh et al., 2005; Logan et al., 2001).

The detection strategy is also determined by the objectives. In some cases, detecting positives and quantifying the numbers of *Campylobacter* is enough information to determine the safety of the food products. Thus, using *Campylobacter* genus-specific primers and probes can lower the detection limit (Krause et al., 2006; Lund et al., 2004; Perelle et al., 2004; Wolffs et al., 2005). Alternatively, if taxonomical differentiation is required, a multi-step detection or a combined analysis of multiple primer-probes sets is more desirable. Logan *et. al.* (2001) were able to differentiate multiple *Campylobacter* species using combinations of melting peak profiles of three different fluorescent dye and biotin labeled biprobes (Logan et al., 2001).

9.2 Types of Real-time PCRs and their Detection Efficiencies

Although SYBR-Green I based real-time PCR resulted in detection limits of 100 CFU per ml upon 14 hours enrichment (Cheng and Griffiths, 2003), the non-specificity related high background noise has limited its application in *C. jejuni* detection. Instead, fluorescence probe based real-time PCR systems are more popular choices for the rapid detection of *C. jejuni*.

Because of the inclusion of target-specific probe, TaqMan probe PCR is highly specific and relatively easy to design and perform. Therefore it has be extensively applied in the detection of *C. jejuni*, some of them have resulted in a detection limits ranging from 1 to 25 CFU per ml or 5 to 12 genome copies (Best et al., 2003; Krause et al., 2006; LaGier et al., 2004; Lund et al., 2004; Rudi et al., 2004; Sails et al., 2003; Yang et al., 2003).

Hybridization probe real-time PCR incorporates two single-fluorescent-dye-labeled oligonucleotides, which also anneal to the region inside of primers. Only when both probes are specifically bound with target DNA template, the probes start emitting fluorescent signal. The probes are displaced (not destroyed) by the polymerase during elongation phase, and will anneal to the DNA templates at the next amplification cycle. The applications of hybridization probes in the detection of *C. jejuni* does ensure the fulfillment of the requirement of high specificity, however, it did not improve the detection limit as compared to TaqMan probes (Abu-Halaweh et al., 2005; Perelle et al., 2004; Wolffs et al., 2005). Along with the difficulty and high cost involving probe designing, the application of hybridization probes real-time PCR in the detection of *C. jejuni* is limited.

9.3 Sample Preparation for Real-time PCR

In the development of a quantitative real-time PCR detection method, spiking or artificial contamination technique is commonly utilized. Since the distributions of *C. jejuni* are different with assorted type of samples, therefore quantitation analysis is required with different samples types. Hence, spiking samples with known concentrations of *C. jejuni* and subsequent cell count on media culture provides a relative quantitative measure for the determining the detection limit.

In PCR-based detection, DNA/RNA extraction processes are required in order to obtain DNA templates for the PCR amplification. Similar to other bacteria, *C. jejuni* DNA can be extracted by simply boiling the water solution at 100 ℃ for 10min and centrifuge at 16,000xg for 5min (Best et al., 2003). The addition of proteinase K and TE

buffer can help cell lyses and enhance the stability of DNA, thus extraction results in higher purity DNA templates (Abu-Halaweh et al., 2005). The convenience and efficiency of commercial DNA extraction kit has gained popularity in preparing DNA templates from bacterial samples. In fact, a comparative study by Cheng and Griffiths (2003) indicated that the simply boiling methods with 0.1% Triton X-100 solution resulted in the best recovery rate, which lowered the detection limit of C. *jejuni* with PCR by at least 10 fold comparing to other extraction methods (Cheng and Griffiths, 2003).

9.4 The Limitations of Real-time PCR Detection Method

Although real-time PCR technique is fast, sensitive, highly specific, and able to quantitatively detection pathogens, two major limitations hinder it applications. First, it is the weaknesses of PCR based detection methods; as long as the target DNA is present, they do not differentiate the original cell is dead or alive. Apparently, this may result in overestimation of the bacteria, thus provide false information for hazard analysis and decision-making. To address this conundrum, Wolffs *et al.* (2005) combined highly specific real-time PCR with a novel discontinuous buoyant density gradient method, which were designated as flotation technique, such that the viable, the viable non-culturable and the dead *Campylobacters* can be distinguished.

Real-time PCR is sensitive to the inhibitors presented in the samples, such as foods and feces, therefore it might underestimate the amount of target pathogen, or even display false negative. The remedy for this problem besides sample DNA/RNA purification procedures is to include internal controls (IC) in the solution. IC in contrast to a positive control is that IC is the non-target DNA sequence present in the same PCR reaction,

which can be amplified simultaneously as the target DNA. In a PCR reaction that does not contain an IC, the negative result could mean that, there is no target sequence in the reaction. However, it is also possible that there could be presence of inhibitor, malfunction of the PCR instrument, mistakes in making PCR mixture, or even low polymerase activity *etc.* Therefore, only when an IC present and normally amplified, can a negative result be interpreted as non-existence of target DNA (Hoorfar et al., 2004). Nevertheless, in the all the studies reviewed in this paper, only Perelle et al. (2004) and Lund et al. (2004) incorporated ICs in their real-time detection methods (Lund et al., 2004; Perelle et al., 2004).

CHAPTER III: THE DEVELOPMENT OF A SYBR-GREEN I BASED REAL-TIME PCR ASSAY FOR THE DETECTION AND QUANTITATION OF *CAMPYLBOACTER JEJUNI*

INTRODUCTION

Campylobacters are the most common pathogens that can cause human bacterial gastroenteritis, and they are the major cause of the food-borne illnesses in the developed countries (Altekruse et al., 1999; Coker et al., 2002; Moore et al., 2005; Tauxe, 2002). *Campylobacter jejuni and C. coli* together are identified in 95% of the reported cases of diarrhea caused by Campylobacters in the US, with *C. jejuni* having a higher prevalence than *C. coli* (LaGier et al., 2004). Because of the public health implications of these foodborne pathogens, studies are undergoing to develop methodologies for the early detection and identification of *C. jejuni* contaminations in foods, especially in poultry meat.

Because of the presence and the expression of the *C. jejuni*-specific *N*-benzoylglycine amidohydrolase (*HipO*) gene, a hippuricase test can be performed to differentiate *C. jejuni* from other Campylobacters, especially *C. coli* (Hani and Chan, 1995; Penner, 1988). Several PCR procedures have been developed based on primers designed from *C. jejuni HipO* gene (Abu-Halaweh et al., 2005; LaGier et al., 2004).

SYBR-Green chemistry is an alternative method used to perform real-time PCR analysis. SYBR Green is an asymmetrical cyanine dye that binds the minor groove of

double-stranded DNA. It can emit strong fluorescence signal upon binding to double-stranded DNA, and the intensity of the fluorescence emissions increases during PCR annealing and extension phases. As more double-stranded amplicons are produced during PCR processes, the fluorescent signal will also increase. SYBR-Green based real-time PCR has several advantages. First, compared to the probe-based real-time PCR, it is easy to design because it has no requirement for probe. Second, it can accommodate relatively long fragments, and most importantly, it allows melting peak analysis (Skeidsvoll and Magne Ueland, 1995), which can generate the melting peak profile for a specific PCR product. Since the length and the sequences composition of the specific PCR product determine the melting peak temperature, therefore, with the *C. jejuni* specific primers, the characteristic melting temperature of the target DNA (*HipO* gene) can be determined.

In the current study, a SYBR-Green based real-time PCR method was developed for the rapid detection and differentiation of *C. jejuni* based on a new set of primers designed from the *HipO* gene of *C. jejuni.*

MATERIALS AND METHODS

Bacteria strains and isolates

Eight *C. jejuni* isolates, eight *C. coli* isolates and one *C. lari* were used in the study (Table 3.1). All the isolates were stored at -80˚C in tryptic soy broth (Difco, Detroit, MI), which was supplement with 30% glycerol (v/v) and 5% (v/v) blood.

Media and culture conditions

Strains were recovered from the frozen storage and spread on the modified Campy-Cefex (mCC) plates (Oyarzabal et al., 2005). Plates were subsequently incubated for 24 h at 42˚C under microaerobic conditions (85% nitrogen, 10% carbon oxidize, 5% oxygen; Airgas, Radnor, PA) using anaerobic jars for 24h. Growth was mixed in PBS and then the suspension was adjusted to a 0.5 McFarland turbidity standard. One ml of the suspension of each strain was used to extract DNA. At the same time, the suspension was serially diluted in PBS, and 0.1ml from each dilution was spread-plated on mCC plates. Plates were incubated at at 42˚C under microaerobic conditions and counts determined after 48 h of incubation.

DNA extraction

DNA extractions were done using PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Froster City, CA) according to manufacturer's specifications.

Real-time primer and probe

Two oligonucleotide primers were selected from the *C. jejuni HipO* gene (GenBank Accession number Z36940). The forward primer: 5'-GGC TTC TTC GGA TAG TTA TAG CAT- 3' and reverse primer: 5'-ATG TCC TGC ATT AAA AGC TCC T- 3', generated an amplicon of 338 bp. The primers were synthesized by Sigma Genosis (Sigma-Genosys, The woodlands, TX). The target sequence for the SYBR Green assay was subjected to a BLAST search in nucleic acid database (NCBI BLAST service).

SYBR Green I real time PCR assay

The amplification reactions were conducted in a Roche LightCycler® System v1.5, and the data analyses were performed using LightCycler software® version 4.05. All the SYBR Green I assay reactions were performed in glass capillary tubes (Roche Applied Science, Indianapolis, IN). Each tube contains 20 µl of PCR reaction mixture with the ingredients as following: 2 µl of LightCycler® FastStart Master^{PLUS} SYBR Green I master mixture (Roche Applied Science, Indianapolis, IN), 0.8 µl of 10 µM forward primer, 0.8ul of 10um reverse primer, 2 µl of $MgCl_2$, 12.4 µl of PCR grade water and 2 µl of target DNA. LightCycler® FastStart Master^{PLUS} SYBR-Green I is a ready to use hot-start reaction mix for PCR on the LightCycler® carousel-based system with requirement of addition of only enzyme prior to use.

The program, temperature and hold time used for the real-time PCR profile are described in Table 3.1. A melting curve analysis was used to determining the melting temperature profile of the target DNA products generated from the amplification. This step is consisted of heating at 95˚C for 0s for denaturation of DNA with a temperature transition rate of 20˚C/s; and then hold at 65˚C for 15s for the sufficient binding with target DNA by the SYBR-Green molecules; the reaction was then heated to 95˚C with temperature transmission rate of 0.1˚C/s with a continuous acquisition.

Agarose gel electrophoresis

To verifying PCR result, amplicons were removed from the capillaries and examined by standard gel electrophoresis with a Bio-Rad gel electrophoresis machine. Gels were made with 1.5% agarose by mixing Ultra Pure DNA Grade Agarose (Bio-Rad Laboratories, Hercules, CA, USA) with 1xTris-borate-EDTA (TBE) buffer. A 100 bp

DNA ladder (Promega, Madison, WI) was used. Gels were stained in ethidium bromide for about 20 min and visualized using a Gel-Doc® UV ultra illuminator.

RESULTS

Primer testing

After selection, primers were checked for melting temperature, primer length (at least 18 to ensure sufficient hybridization to the target), and lack of repeat bases, especially G and C and G+ C content of the target sequences. The specificity of the target sequences and probe were also subject to a BLAST check (http://.ncbi.nlm.nih.gov/BLSAT). Non-*Campylobacter* bacteria or non-*jejuni Campylobacter* spp. did not have sequence similarities in the BLSTN databases.

Specificity testing

One commercially purchased pure *C. jejuni* genomic DNA standard, eight isolates of *C. jejuni*, eight isolates of *C. coli* and one isolate of *C. lari* were tested with the *C. jejuni* specific primers and the melting peak temperatures were also determined for the amplicons. As shown in Figure 3.1A and 3.2A, all *C. jejuni* isolates displayed a uniformed melting peak temperature at around 82.5˚C. All other strains and the negative control showed a lower peak temperature at around 78˚C (Fig. 3.1A and Fig. 3.2A), which was due to the unspecific binding of SYBR-Green dye to the primer dimmers. The results from the gel electrophoresis confirmed that all the isolates of *C. jejuni* displayed to targeted bands (Fig. 3.1B), while the other strains did not yield any band (Fig. 3.2B).

Sensitivity using pure bacteria culture

After growth for 24 h, bacteria were suspended in PBS buffer with an optical density value of 0.4 at wavelength of 600nm. One ml of the bacterial culture suspension was used to extract genomic DNA, and 10-fold dilutions of the DNA templates were examined by the current method. The results showed a linear logarithmic correlative curve over the dilution series (Fig 3.4A and Fig 3.4)

Quantitative analysis for SYBR Green I real time PCR

In quantitative analysis, serial dilutions were made for *C. jejuni* from pure culture. The starting concentration in colony forming unit (CFU) was determined using the spread plate methods. Two µl of the each dilution was subjected to the real-time PCR reaction. The standard curve of each serial dilution was constructed by regression analysis plotting log CFU against crossing threshold (Ct). The data were subsequently analyzed by the Minitab, by which regression equations were generated (Fig. 3.3B; Fig. 3.4B and Fig. 3.5B). For example, in the first run, the regression equation was Ct= 34.17-3.527 log CFU. A square regression coefficient of 0.999 indicated highly significant linear relationship between Ct value and log CFU. Therefore, with the regression equations, one can estimate bacteria concentration in the sample using the Ct value.

As shown in the Figure 3.3B, 3.4B and 3.5B, all regression relationships were significant, and they all have similar slopes. Based on the standard curves, the detection limit of the SYBR Green I based real-time PCR from pure culture was calculated to be approximately less than 10 CFU/ml.

DISCUSSIONS

Campylobacter jejuni has been recognized as a leading cause of human food-borne gastroenteritis. Moreover, the rapid detection, accurate, and identification of the organism is critical for controlling and prevention of an outbreak. In the current study, SYBR Green I based real time PCR assay was developed and tested for the qualitative and quantitative detection of *C. jejuni*. A primer set was designed from *C. jejuni*-specific *HipO* gene (Hani and Chan, 1995) using the Invitrogen Vector NTI® 10 (Carlsbad, CA). The target DNA sequence specificity was tested by homology search using BLAST with the nucleic acid database (NCBI BLAST service). The results indicated that all *HipO*-positive sequences were *C. jejuni* isolates, and non-*C. jejuni* organisms were absent of this gene. Thus, it demonstrated the high specificity of the current primers and the PCR amplicons.

During the preliminary experiments, the PCR products were further examined by agarose gel electrophoresis to verify PCR products and optimize the PCR conditions. Seventeen *Campylobacter* isolates and a pure *C. jejuni* genomic DNA were subject to the newly developed SYBR-Green based real-time PCR. Immediately after each PCR run, the melting temperature profile of each isolate was determined from the melting curves of the amplified products. The melting curves in Fig. 3.1A demonstrate a high peak for all *C. jejuni* isolates at 82.5˚C and a lower peak at 78˚C (Fig. 3.2A) for all non-*C. jejuni* isolates and negative control. Primer dimers displayed (Fig. 3.1 A and B) a low peak at 78˚C as well. The melting temperature profiles enhanced the specificity of the real-time PCR assay.

In quantitative analyses, three replicates of dilutions of *C. jejuni* DNA generated similar standard curves and the square regression coefficients indicated a significant correlation between Ct value and log bacteria concentration. Based on the regression equations, the detection limit of current real-time PCR procedure is approximately less than 10 CFU/ml. Additionally, the constant efficiency and the linearity of the standard curve suggest that current assay is suitable for rapid detection and quantification of *C. jejuni.*

Roche LightCycler based real-time PCR is performed in micro-volume glass capillary tubes, which are heated or cooled efficiently by airstreams (Wittwer et al., 1989). It provides a convenient instrument for the developing of a real-time PCR assay. The sealed capillary tubes significantly reduce the risk of contamination due to amplicon carry-over effect (Wittwer et al., 1997). Comparing to the probe-based real-time PCR assays, SYBR-Green assay is much more cost-effective (Silvano et al., 2001). After simple optimization, current experiment showed that, the relatively inexpensive SYBR-Green based real-time PCR assay can be used as an alternative for quantitative analyses.

In summary, based on the results listed above, the current SYBR-Green based real-time PCR is both highly specific and sensitive for the quantitative detection of *C. jejuni*.

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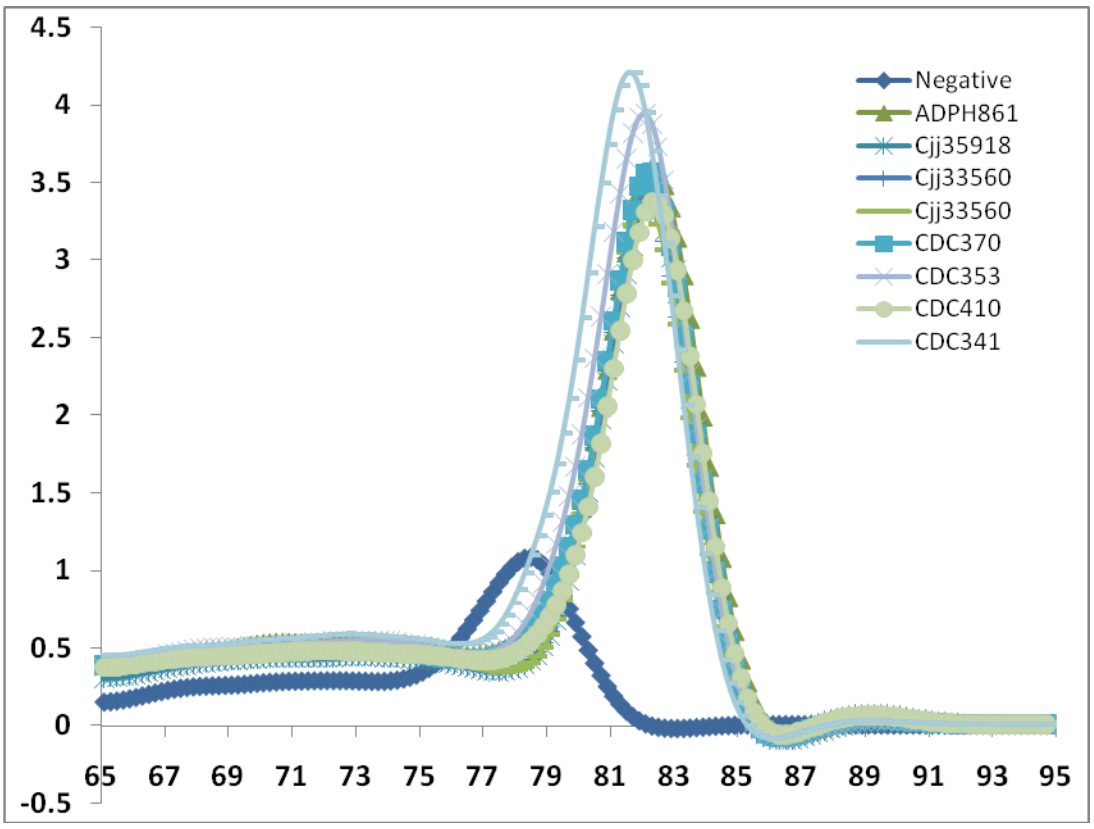
Table 3.1. *Campylobacter* strains used in SYBR Green I real time PCR assay

Species	**ATCC #**	**Other IDs**
C. jejuni	ATCC35918	CDC410
	ATCC33560	CDC370
	ATCC700819	CDC341
	ADPH861	CDC353
C. coli	ATCC BAA371	ATCC43484
	ATCC43481	ATCC51397
	ATCC43133	ATCC49941
	ATCC43471	MCC66
C. lari	ATCC35222	

Table 3.2. Profile of SYBR Green I Real-time PCR Assay

Program	**Temperature (℃)**	**Hold time**
Hot-Star & Denaturation	95	10 min
Amplification		
Denaturation	95	10 sec
Annealing	53 (0.4 ℃/ sec)	5sec
Extension	72	15sec
Melting Curve		
Denaturation	95	0 sec
Annealing	65	15sec
Melting	95	0 sec (0.1 ℃/ sec)
Cooling	40	30 sec



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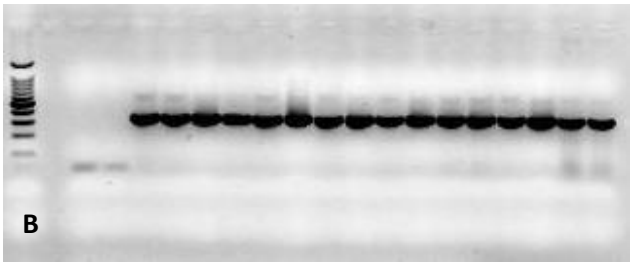
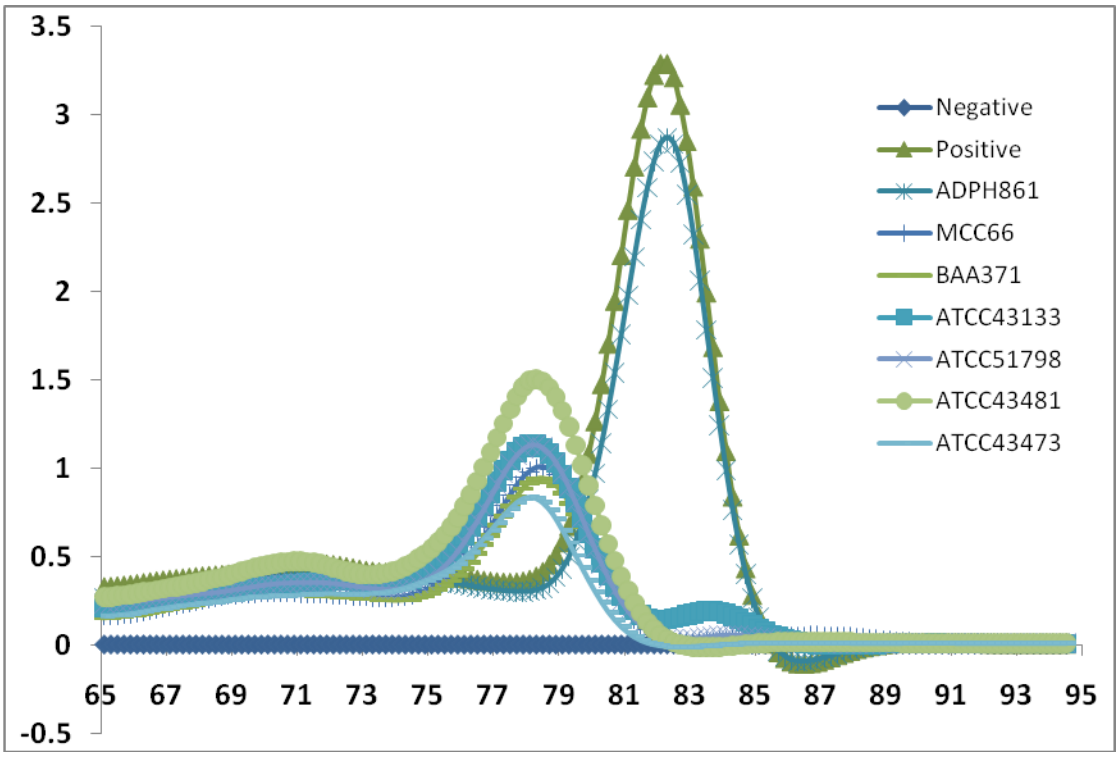


Figure 3.1 A). Specificity test for *C. jejuni* with SYBR Green I real-time PCR assay;
B). Agarose gel electrophoresis of real-time PCR products specificity test 1A



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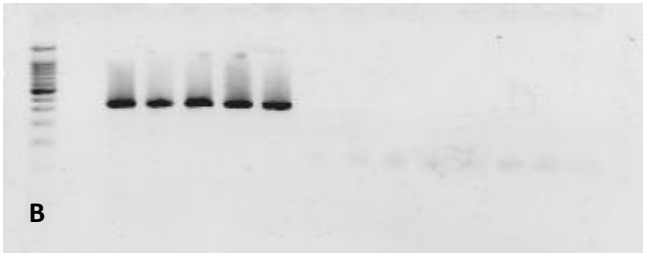
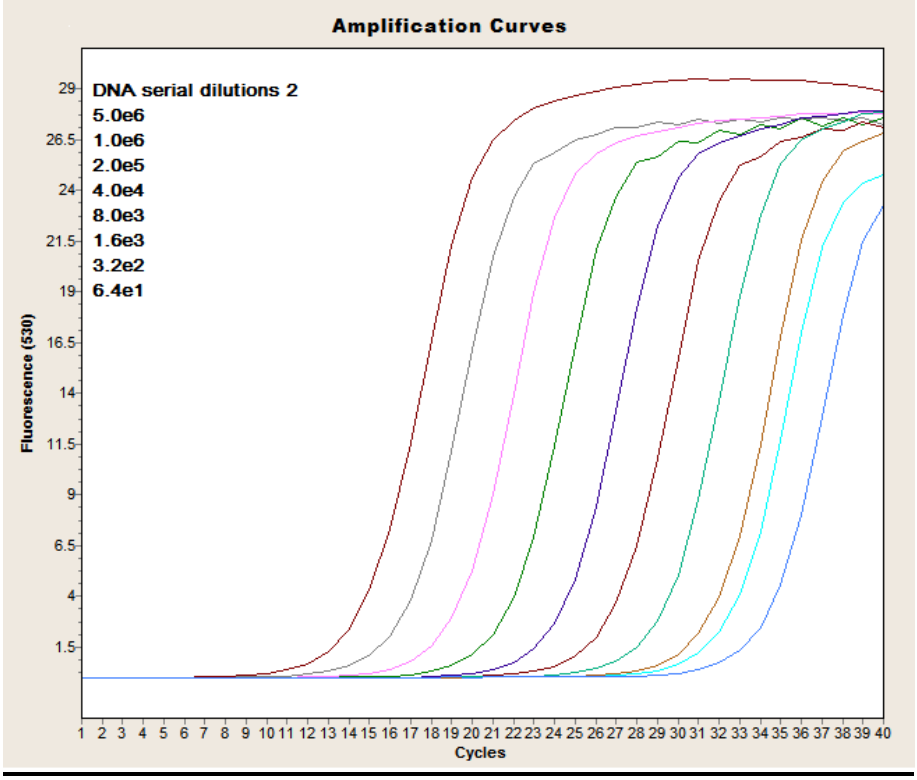


Figure 3.2 A). Specificity test for non-*C. jejuni* strains with SYBR Green I real-time PCR assay; B). Agarose gel electrophoresis of real-time PCR products



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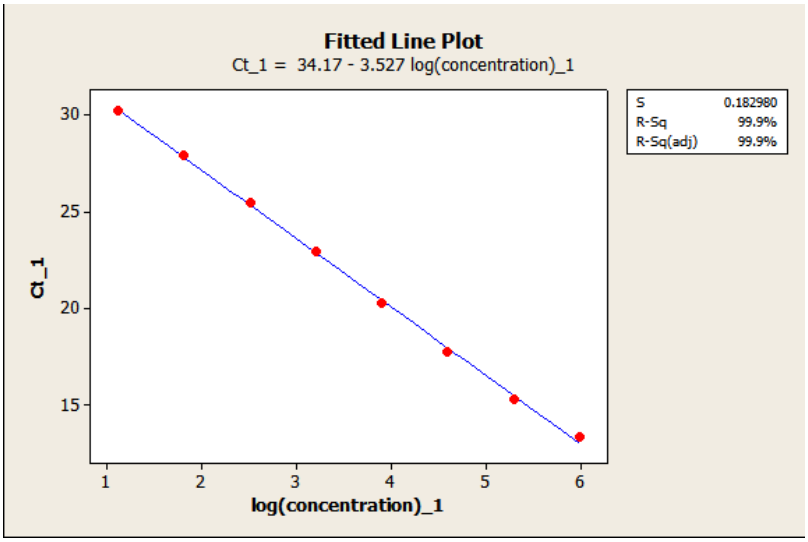
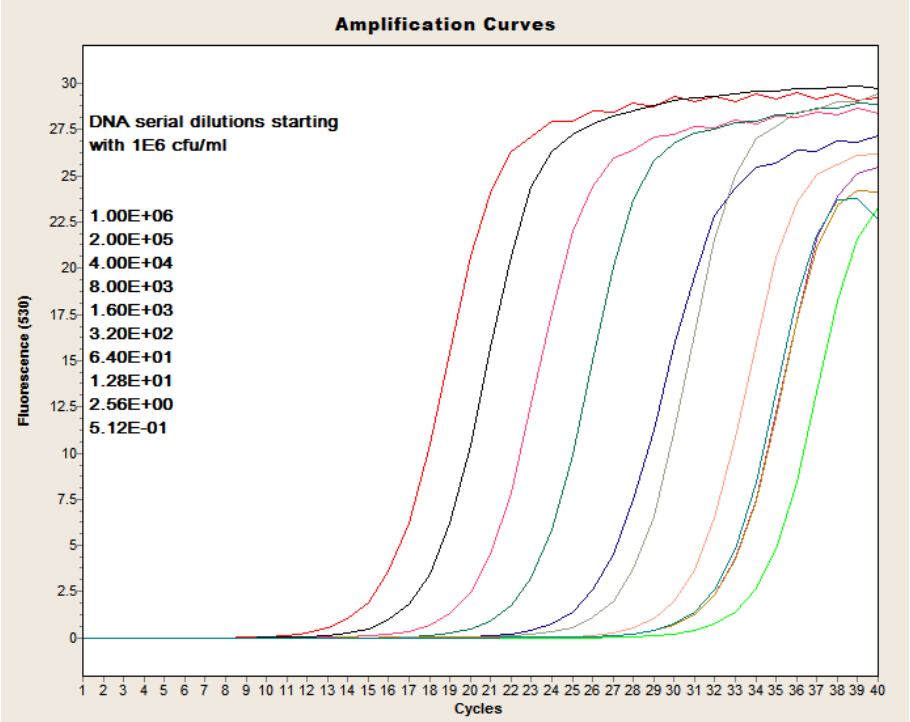


B

Figure 3.3 A). Quantitative analysis, the first run amplification curves of 5-fold serially diluted *C. jejuni* DNA (CFU/ml) from SYBR Green I real time PCR assay; B). Standard curve



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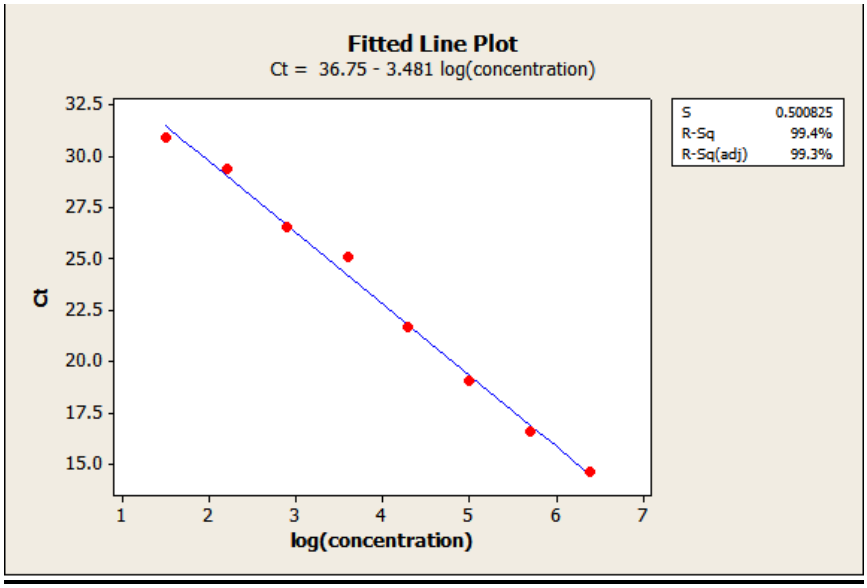
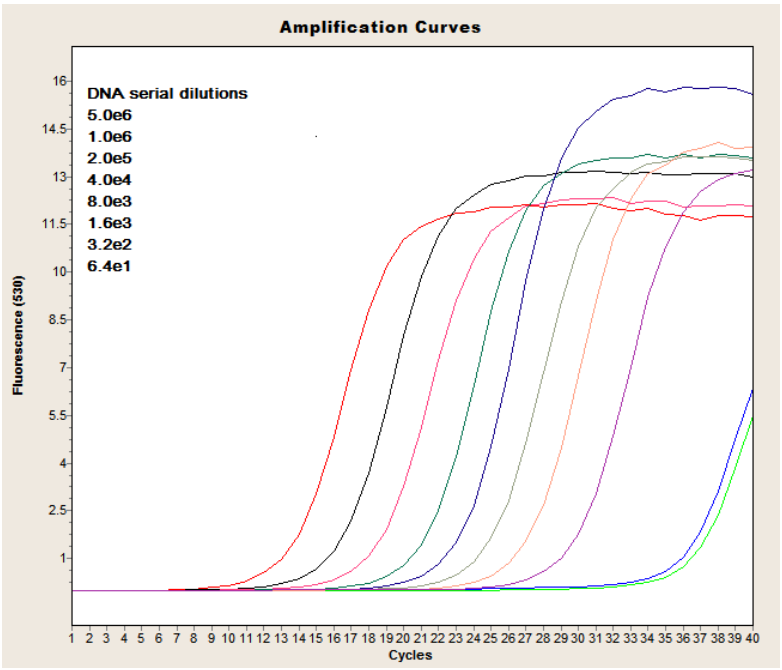


B

Figure 3.4. Quantitative analysis. A). The second run amplification curves of 5-fold serially diluted *C. jejuni* DNA (CFU/ml) from SYBR Green I real time PCR assay; B). Standard curve for constructed from the quantitative analysis in figure3.4A



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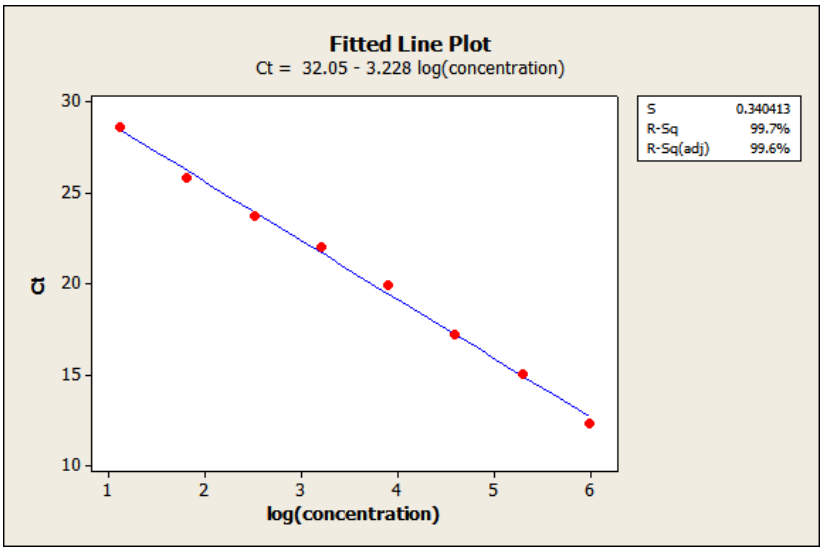

B

Figure 3.5. Quantitative analysis. A). the third run amplification curves of 5-fold serially diluted *C. jejuni* DNA (CFU/ml) from SYBR Green I real time PCR assay; B). The standard curve.

CHAPTER IV: THE DEVELOPMENT OF A TAQMAN® BASED REAL-TIME PCR ASSAY FOR THE RAPID DETECTION OF *CAMPYLOBACTER JEJUNI*

INTRODUCTION

Campylobacters are common members of intestinal microflora of a variety of domestic and wild animals, including cattle, swine, sheep, poultry, and wild birds and mammals (Friedman et al., 2000; Nielsen et al., 1997; Petersen et al., 2001). However, while they are commensal to most animal hosts, they are also the dominant food-borne pathogen worldwide that cause diarrhea and gastroenteritis (Allos and Blaser, 1995). Additionally, *Campylobacter* infections have also been associated with severe autoimmune disease – Guillian-Barré syndrome, which causes acute paralytic condition to the peripheral nervous systems (Nachamkin et al., 1998). Among all the *Campylobacter* spp., *C. jejuni* is the principal causes of human campylobacteriosis (LaGier et al., 2004).

Campylobacter jejuni may enter the environment, including water supplies via feces contamination from infected birds or animals, and human consumption of contaminated food, water and milk *etc*. can account for at least 70% of *C. jejuni* related disease annually (Fricker and Park, 1989; Klein et al., 1986; Mathewson et al., 1983). Improperly cooked food, especially poultry and poultry products have been associated with campylobacteriosis outbreaks (Pearson et al., 2000). Study indicated that chicken carcasses are most likely contaminated by fecal materials in the slaughterhouse and

during processing (Rivoal et al., 1999). As the result, a high percentage of retail broiler products are tested positive for *Campylobacter* contamination (Dickins et al., 2002; Kramer et al., 2000). During poultry processing, the average bacterial count of *C. jejuni* and *C. coli* in the carcass rinse can be as high as 3 ~ 3.7 logs CFU/ml immediately after evisceration (Oyarzabal, 2005; Yang et al., 2001). Therefore, in the industry, in order to detect *C. jejuni* contamination, an applicable detection method must have a sensitivity of 2 to 3 log CFU/ml.

The *HipO* gene encodes the hippuricase enzyme, which catalyzes the hydrolysis of hippuric acid to benzoic acid and glycine (Hani and Chan, 1995; Harvey, 1980). As the only reliable biochemical assay that can differentiate *C. jejuni* from other Campylobacters, especially the close related special *C. coli*. Therefore, *HipO* gene has become an attractive target for molecular test especially for the development of the PCR and real-time PCR assays for confirmation and differentiation of *C. jejuni*.

TaqMan® probe is also known as the hydrolysis probe, which is consisted of two sequence-specific primers and added specificity of a dual-labeled sequence-specific probe. The TaqMan® probe contains a fluorescence report dye (fluoroescein) at its 5'end and a second fluorescence dye, known as the quencher, at its 3'end, which is able to absorb the emission spectrum from the first dye when they are at close vicinity (Livak et al., 1995). TaqMan® probe binds to its complementary region on the target during PCR annealing step, and subsequently hydrolyzed by DNA polymerase exonuclease activity, which results in separation of the fluorescence reporter and the quencher. Therefore, the emission from the reporter is no longer absorbed by the quencher, and with the accumulation of emissions from the "freed" reporter, eventually, the signal is strong

enough (the threshold) to be detected by the real-time PCR instrument (Heid et al., 1996; Lyamichev et al., 1993).

In the current study, a TaqMan® based real-time PCR method was developed for the rapid detection and differentiation of *C. jejuni* using a new set of primers and a hydrolysis probe that was designed from *C. jejuni* specific *HipO* gene.

MATERIALS AND METHODS

Bacteria strains and isolates

Six *C. jejuni* strains, five *C. coli* strains, one *C. lari* strain, one *C. fetus* strain and 20 isolates from retail samples were used in the present study. All isolates except the 20 retail isolates were stored at -80˚C in tryptic soy broth (Difco, Detroit, MI), which was supplemented with 30% glycerol (v/v) and 5% (v/v) blood. The bacteria strains and isolates were listed in Table 4.1.

Media and culture conditions

All Six different strains of *C. jejuni*, five strains of *C. coli*, one *C. lari* strain, and one *C. fetus* strain were recovered from the froze storage. One small loopful of bacteria was took from stock culture and put on the Campy-Cefex (mCC) plates (Oyarzabal et al., 2005) and filtered through an 0.65 μm filter. The plates were then incubated for 24 h at 42˚C under microaerophilic gas conditions (85% nitrogen, 10% carbon oxidize, 5% oxygen; Airgas, Radnor, PA) using the anaerobic jars. If there were not enough bacteria to be collected upon the 24 h growth, the bacteria would be transferred to new plates by

streaking and incubated for another 24 h. The bacteria then were harvested using a sterile loop and mixed in sterile 1x phosphate buffer saline (PBS). In addition, the optical densities of the suspensions were adjusted to set a relatively constant value for all the strains used for specificity test using spectrophotometer at 600 nm.

One milliliter of each bacterial suspension of each strain was used to extract genomic DNA using the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Froster City, CA). For the strain used for the specificity test, the suspension 10-fold serial dilutions were made with PBS. Each dilution was spread (0.1 ml) on modified campy-Cefex (mCC) plates, which were then incubated at 42˚C under the same microaerobic gas conditions using the anaerobic jars. Colony forming units (CFU) were determined after 24 h and 48 h of incubation in the suspension.

The enrichment culture of retail isolates were prepared as the following: 25 gram boneless chicken meat from retailed stores were placed in Whirl-Pak g (Nasco), then 225 ml Bolton broth with antibodies were added at the ratio of 1:9 (weight: volume). Samples were stomached for 60 seconds and subsequently incubated at 42˚C for 48 h under microaerobic condition in sealed, anaerobic jars. After 24 h and 48 h of incubation, samples were examined under phase contrast microscopy for morphology and motility. All samples were then transferred to mCC agar plates and incubated under microaerobic condition. After the presumptive identification, bacterial DNA was extracted and subjected to a multiplex PCR for the identification of isolates (Oyarzabal et al., 2005). Twenty of the isolates were used for the specificity test in the present real-time PCR assay.

The artificially contaminated retail samples were treated by the following procedure: one strain (ATCC 35918) of *C. jejuni* was revived from frozen media as previously mentioned. The cells were prepared by transferring colonies from plates into phosphate buffer saline (PBS), and vortexing well to get suspension of an OD value 0.334 at 600 nm. The suspension was then used to spike pre-cut chicken pieces, which were put in Glad Fresh Protect Bag and kept at 4^{o}C for 24 hours. Subsequently, the spiked meat samples were processed as described as previously mentioned for retail samples.

DNA extraction

All the DNAs used for specificity, quantitation, and detection limit tests were extracted with PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Froster City, CA). Later, in examining the enrichments of spiked samples, PrepMan® Ultra Sample Preparation Reagent was compared to Qiagen DNeasy Blood & Tissue Kit (Valencia, CA, Qiagen). The procedures were performed according to the manufacturer's protocols.

Real-time primer and probe

Two oligonucleotide primers were selected from the *C. jejuni HipO* gene (GenBank Accession number Z36930). The primers were as follows: *HipO* forward, 5'-GGC TTC TTC GGA TAG TTA TAG CAT- 3', and *HipO* reverse, 5'-ATG TCC TGC ATT AAA AGC TCC T- 3', which encompass a amplicon of 187bp. The Taqman probe: 5'-{FAM}-ATG TCC TGC ATT AAA AGC TCC T-{TAMARA} - 3', which located between the two primers. All primers and probe were designed using the Invitrogen

Vector NTI® 10 (Carlsbad, CA). The target DNA sequence were subjected to a BLAST search in nucleic acid database (NCBI BLAST service), and there was no known non-*C. jejuni* organism that has the similar sequences in the BLSTN databases. All primers and probe were synthesized by Sigma Genosis (Sigma-Genosys, The woodlands, TX).

TaqMan Based Real-Time polymerase chain reaction

The amplification reactions were conducted on Roche LightCycler® System v1.5 (Roche Applied Science, Indianapolis, IN), and the data analyses were performed using LightCycler software® version 4.05. A total reaction volume of 20µl contains following ingredient: 4µl of LightCycler® TaqMan® Master Mix (Roche Applied Science, Indianapolis, IN), 0.6µl of forward primer (10µM) and reverse primer (10µM) (respectively), and 0.3µl of probe (10µM), 12.5µl of PCR grade water and 2µl of sample DNA

Real-time PCR conditions are set with a pre-incubation step at 95˚C for 10 min to activate the FastStart® DNA polymerase and denature the target DNA, and followed by 40 PCR cycles of 10s at 95˚C for denaturation, 30 s at 55˚C for annealing and 1s at 72˚C for extension. Capillaries and equipment were cooled down to 40˚C at the end of the PCR for 30s at (Table 4.2). A single acquisition mode was set at the end of extension stage of amplification.

Agarose gel electrophoresis

To verifying PCR result, amplicons were removed from the capillaries and examined by the standard gel electrophoresis. Stained gels were visualized using a UV ultra

illuminator (SYNGENE System, Cambridge, UK), and a picture created using a GeneSnap computer program (Cambridge, UK).

Sensitivity of the assay using pure bacteria culture

One *C. jejuni* strain (ADPH861) was used for the sensitivity analysis. After being recovered from frozen stock and grown on mCC plates for 24h, the bacteria were suspend in PBS buffer with an OD value of 1.0 at 600 nm. The suspension was serially diluted in pre-warmed 1x sterile PBS. Each dilution was spread-plated on mCC plates (100 µl) with three replicates made for each dilution. DNA was extracted using PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Froster City, CA), and were serially diluted (5-fold) in nuclease-free water.

RESULTS AND DISCUSSIONS

Specificity of the assay

Six different strains of *C. jejuni*, five strains *C. coli*, one strain of *C. lari* and one strain of *C. fetus* were included in the specificity test. All the *C. jejuni* strains were tested positive (Fig.4.1A) and all non-*C. jejuni* were negative. Twenty isolates from retail samples were also examined using the present method (Fig.4.2A). The results were in agreement with those from other typing methods (data not shown). Gel electrophoresis indicated that all positive samples have a band at187bp, no visible band observed for negative samples. (Fig.4.1B; Fig.4.2B). These results indicated that the primer-probe set was highly specific to *C. jejuni* and the current PCR assay was able to identify the *C. jejuni* from the enrichments of spiked chicken meat samples.

Sensitivity of the assay using pure bacteria culture

Nine dilutions of the DNA templates were subjected to the TaqMan real time PCR assay and a standard curve were constructed accordingly. The regression equation resulted from the standard curve had a Ct= 35.63- 3.197 log CFU per ml, which indicated a significant (R^2=0.998) linear relationship between the sample cross points and the log concentration of target DNA. (Fig 4.3A and B). Based the regression equations, the detection limit of current real-time PCR procedure can be estimated approximately as 4.6 CFU/ml from pure bacteria culture.

Sensitivity the assay using artificially contaminated samples

One strain of *C. jejuni* (ADPH861) was used to spike the retail boneless chicken pieces followed by storage at 4˚C for 24 hrs. Spiked samples were then enriched under standard microaerophilic condition for 24h. Subsequently, 500 µl of enrichment culture was diluted serially at 10-fold, then 1ml from both enrichment culture and each of the serial dilution were used to extract genomic DNA using PrepMan, respectively. Again, 100ul of each the dilution serials were transferred onto MCC plate for determining CFU count after incubation for 24 hrs and 48 hrs at 42˚C under microaerophilic condition. The DNA templates from the enrichment and dilutions were subject to real time PCR and two replicates were tested for each dilution. The results showed a detection limit of 2 logs CFU/ml from enrichment samples for the current real-time PCR detection method.

Comparison of extraction methods

Ten artificially contaminated samples were enriched using the same protocol as previously described. After 24 h enrichment, genomic DNA was extracted using PrepMan or Qiagen. Two initial sample volumes of 2 µl and 5 µl of DNAs extracted by both methods from all the 10 samples were subject to real-time PCR.

All samples extracted with Qiagen were tested positive by the real time PCR assay (Fig. 4.4, Table 4.3 and 4.4). The PCR amplification curves changed consistently as initial sample DNA concentrations increased from 2 µl to 5 µl. Higher initial DNA concentration has resulted in earlier (lower) Ct value indicating higher target DNA concentration (Fig.4.4 and 4.5). Qiagen extracted DNA samples also had relatively constant fluorescence signal levels at 7.2~7.9 and 7.5~9.0 for initial sample DNA volume of 2 µl and 5 µl, respectively.

On the other hand, sample DNAs extracted by the PrepMan at initial volume of 2 µl, all the samples showed Ct value earlier (lower) than the Qiagen, indicating a higher DNA concentration; however, the slopes of the amplification curves were smaller (flat), indicating less efficient PCR reaction or possible presence of inhibitors. Whereas, when 5 µl of initial sample was used, surprisingly, instead of and decrease in Ct value, the amplification curves appeared even later. Such results were consistent with the existence of PCR inhibitors in the reaction. As the increase of initial amount of DNA template, the concentration of the PCR inhibitor also increased thus further reduced the efficiency of the PCR assay (Fig. 4.4 and Fig 4.5).

Furthermore, in the specific tests, the concentrations of pure culture *C. jejuni* were adjusted to OD of 1.0, and subsequently diluted to 1:100, whereas, retail samples were

directly collected from MCC plates and both group were treated with PrepMan for total DNA extraction. The amplification curves displayed similar patterns that there were an averaged Ct value of 14.27 and fluorescence signal level between 9.2~9.8, and averaged Ct value of 12.67 and fluorescence signal level between 1.7~3.6 for pure culture and retail *C. jejuni* samples, respectively. There results again demonstrated that samples extracted with PrepMan containing large amount of PCR inhibitor, which resulted in more inhibition when initial sample concentration increased.

Campylobacters are fastidious bacteria that require special ingredients in the growth media, among which animal blood most commonly horse blood provides essential nutrients for the bacteria (Corry et al., 1995; Stern et al., 1985). However, blood and blood contents are strong inhibitors to PCR reactions and they may hinder PCR by several mechanisms. The hemoglobin and hemes themselves can affect *Taq* polymerase directly by regulating its activity (Byrnes et al., 1975). Studies indicated that PCR mixture containing as low as 0.004% ~1% (v/v) blood can completely inhibit the activity of *Taq* polymerase (Abu Al-Soud and Radstrom, 1998; Panaccio and Lew, 1991). Iron ions released from lactoferrin and hemoglobin can also interfere with DNA synthesis (Abu Al-Soud and Radstrom, 2001). Furthermore, animal serum immunoglobulin's (Gig) were capable of interacting with single-stranded DNA, and such function was enhanced when DNA was heated with the presence of Ig G (Abu Al-Soud et al., 2000).

In the current study, Qiagen is a filter-based extraction method, whereas is PrepMan is detergent-based and requires boiling and lysing the cells to release cellular contents. Because of the blood additive in *Campylobacter* growth media, some blood cells and blood contents may enters the extraction mix. While Qiagen can eliminate most of the

inhibitors by filtering, the blood origin inhibitors remained in the PrepMan extracted DNA samples. Additionally, the boiling step in PrepMan extraction method may also enhance the adverse effects of possible Gig contents in the blood to compete with DNA polymerase for single-stranded DNA molecules.

As an indicator of the template concentration in the reaction, the lower threshold cycle (Ct) value indicated a higher concentration. When applying the standard curve, the estimation of the concentrations for both of the extraction methods was obtained (Table 4.4). Comparing the estimated DNA concentrations between two extraction methods, the results clearly indicated that samples extract by Qiagen appears to have high purity than those by PrepMan. On the other hand, PrepMan may recover more target DNA templates than Qiagen, but it does not discriminate against the possible inhibitor within the sample. Over all, Qiagen provided consistent results in terms of PCR efficiency and sensitivity, whereas PrepMan failed to remove PCR inhibitors of blood contents from *Campylobacter* growth media, thus resulted in lower PCR efficiency. Since PrepMan extraction method is relatively simple and less time consuming than Qiagen, it is still a popular choice in extracting bacterial DNA. Slight modifications of PCR conditions by addition of bovine serum albumin or utilizing blood inhibitor resistant polymerase, such as, *Tth* DNA polymerase, may minimize the inhibitory effects of blood contents and provide satisfactory PCR efficiency (Abu Al-Soud and Radstrom, 2001; Panaccio and Lew, 1991).

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Table 4.1: *Campylobacter* strains used in the TaqMan® based real time PCR assay

Species	**ATCC #**	**Other IDs**
C. jejuni	33560	ADPH861
	35918	CDC410
	700819	CDC341
		CDC370
C. coli	BAA371	ATCC43484
	43481	ATCC51397
	43133	MCC66
C. lari	35222	
C. fetus	27374	
Retail	-	20 isolates

Table 4.2. Profile of TaqMan based Real-time PCR Assay

Program	**Temperature (℃)**	**Hold time**
Pre-incubation	95	10 min
Amplification		
Denaturation	95	10 sec
Annealing	55	30 sec
Extension	72	1sec
Cooling	40	30 sec

Table 4.3. Threshold cycles (Ct) Values for two extraction methods with initial volume of 2 µl of sample DNA

Spiked Sample Number	Counts on Spread Plates	Qiagen DNeasy Blood & Tissue Kit (Ct)	PrepMan Ct
1	6.00E+05	20.47	17.82
2	7.00E+05	20.22	18.31
3	8.00E+05	20.34	16.76
4	1.00E+06	20.94	16.31
5	1.00E+06	19.87	16.52
6	2.00E+06	18.27	15.25
7	1.70E+06	20.12	15.28
8	1.80E+06	19.08	14.7
9	2.20E+06	16.53	13.22
10	7.10E+06	16.02	10.09

Table 4.4. Estimated concentrations from two extraction methods using the standard curve

Spiked Sample Number	Counts on Spread Plates	Estimated Conc. of Qiagen Kit	Estimated Conc. of PrepMan
1	6.00E+05	5.54E+04	3.61E+05
2	7.00E+05	5.56E+04	2.54E+05
3	8.00E+05	5.05E+04	7.61E+05
4	1.00E+06	3.97E+04	1.05E+06
5	1.00E+06	8.49E+04	8.44E+05
6	2.00E+06	2.61E+05	2.22E+06
7	1.70E+06	7.08E+04	2.16E+06
8	1.80E+06	1.48E+05	3.26E+06
9	2.20E+06	8.95E+05	9.32E+06
10	7.10E+06	1.28E+06	8.50E+07

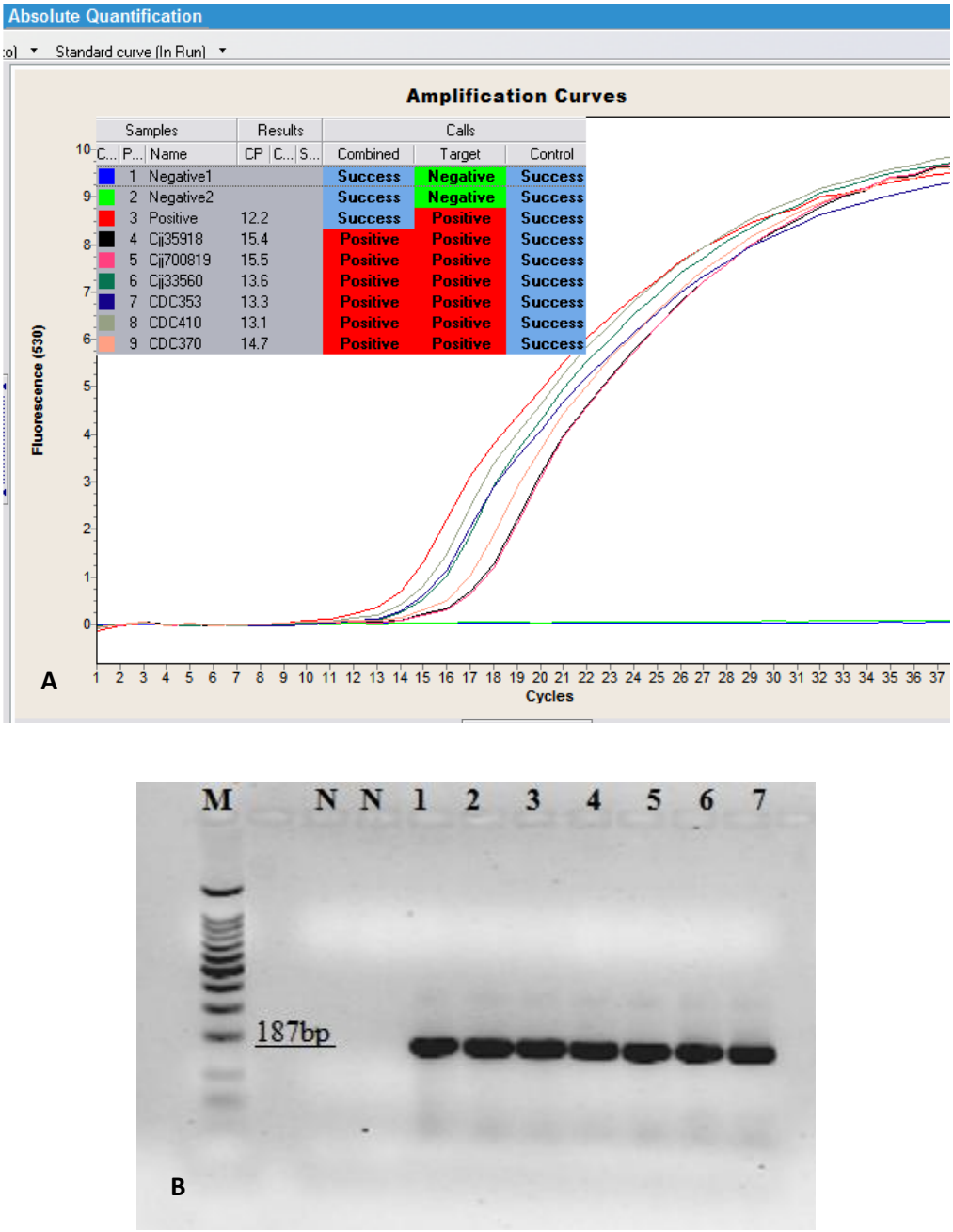
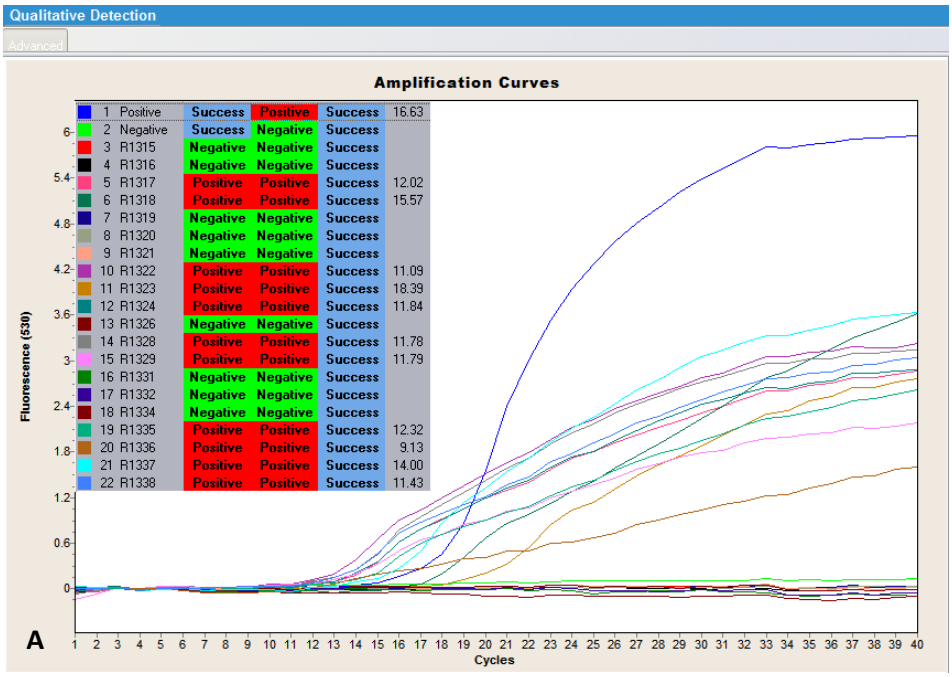


Figure 4.1. A). Specificity test for *C. jejuni* with TaqMan based real time PCR assay; B). Agarose gel electrophoresis of real-time PCR products specificity test figure4.1A. (M : DNA marker; N: negative control; 1: Positive control; 2: Cjj35918; 3: Cjj700819; 4: Cjj33560; 5: CDC353; 6: CDC410; 7:CDC370)



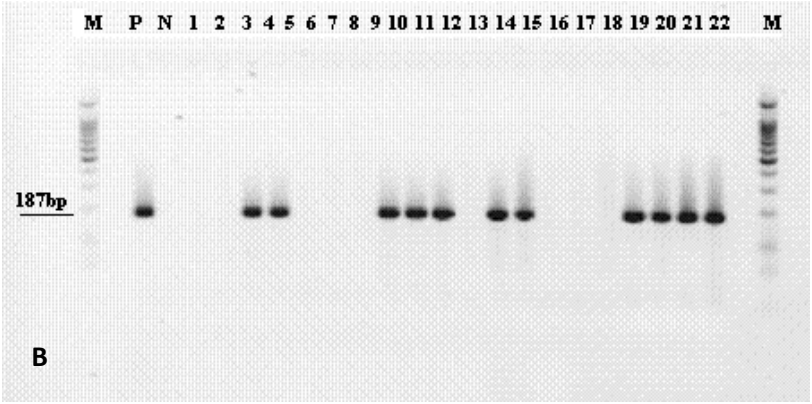


Figure 4.2 A). Specificity test for retail isolates with TaqMan based real time PCR assay; B). Agarose gel electrophoresis of real-time PCR products from 4.3A (M: DNA marker; P: postive control; N: negative control; 1: R1315; 2: R1316; 3: R1317; 4: R1318; 5: R1319; 6:R1320;7:R1321; 8:R1322; 9:R1323;10:R1324; 11:R1326; 12:R1327; 13:R1328; 14:R1329; 15:R1331; 16: R1332; 17:R1334; 18: R1335; 19:R1337; 20:R1338).

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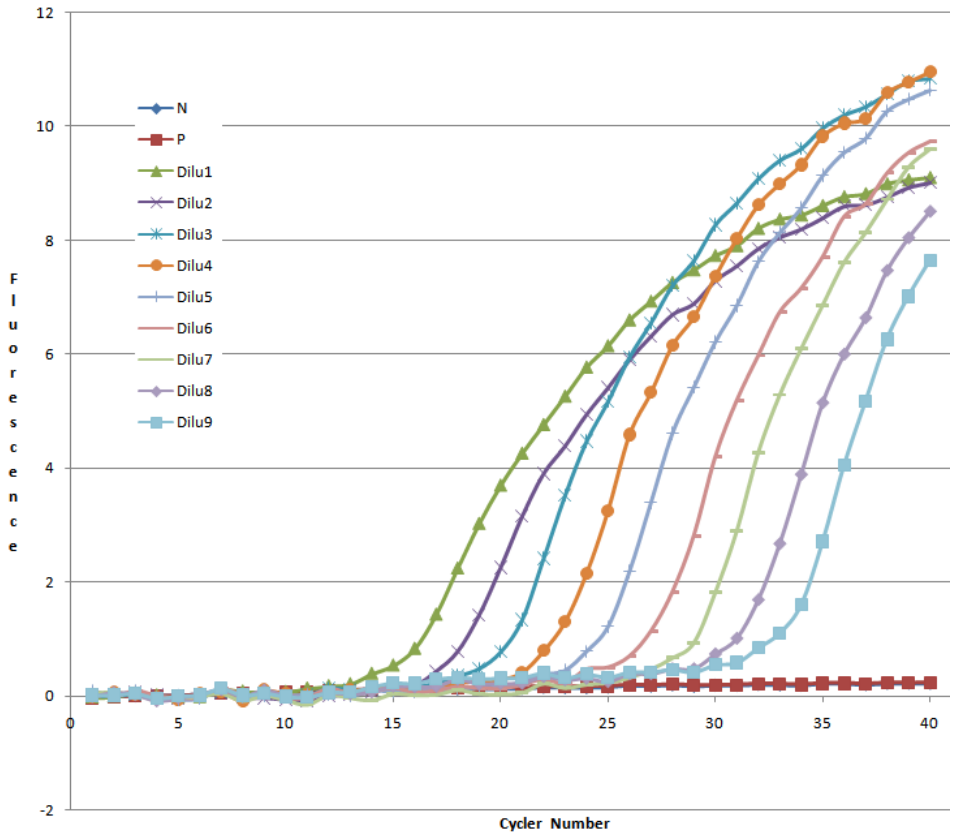

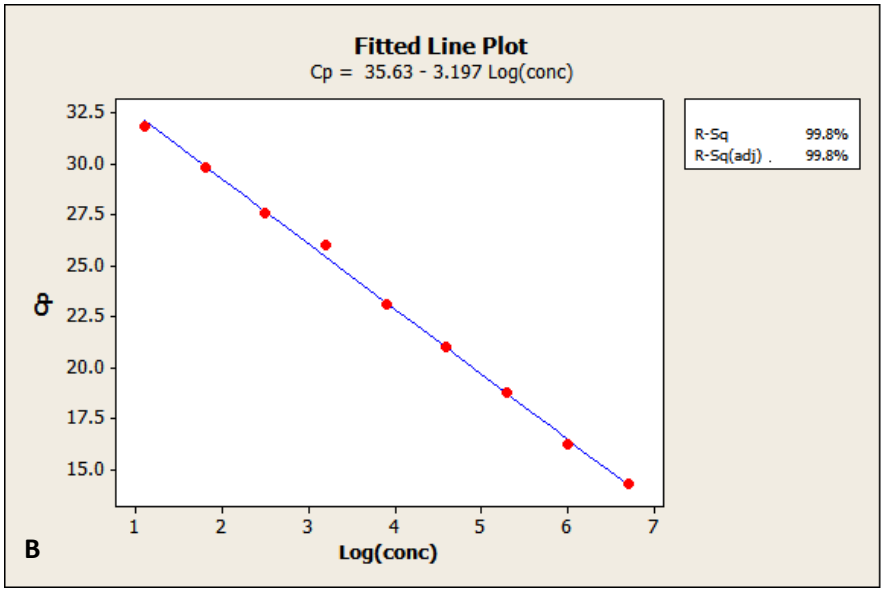

Figure 4.3. The sensitivity test. A). The amplification curves of serially diluted *C. jejuni*; B). The standard curve.

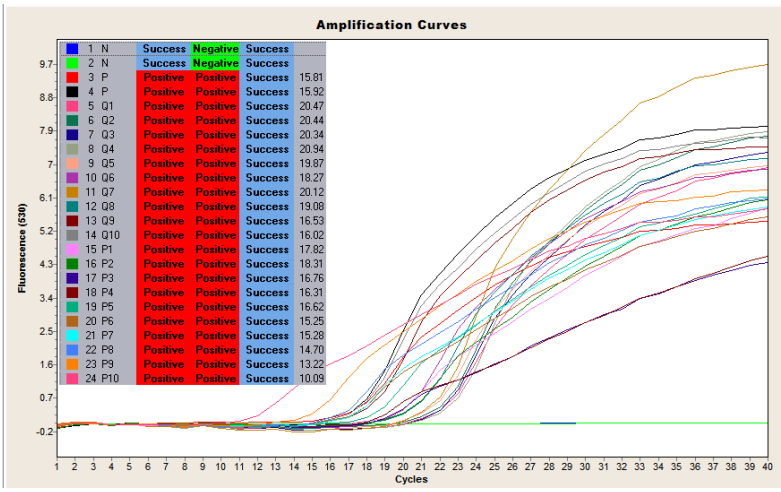

Figure 4.4. Comparison of two extraction methods: Qiagen Kit and PrepMan® Reagent for enrichment samples with TaqMan based real-time PCR assay using 2 µl sample DNA

(N: Negative control; P: Positive control; Q1 to Q10: Genomic DNA extracted from sample 1 to 10 respectively using Qiagen DNeasy Blood & Tissue Kit; P1 to P10: Genomic DNA extracted from sample 1 to 10 respectively using PrepMan® Ultra reagent.)

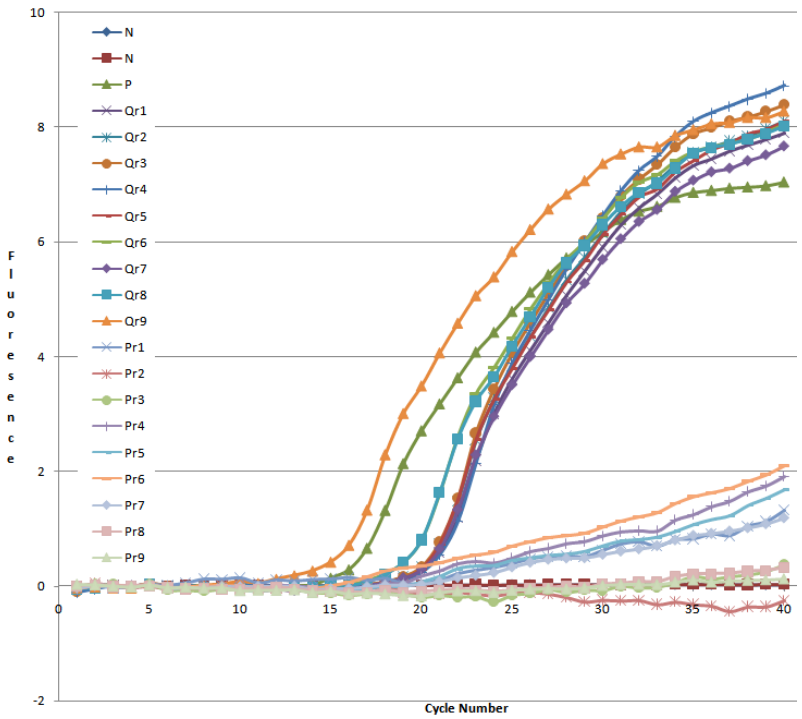

Figure 4.5. Comparison of two extraction methods using 5 µl DNA templates. (N: Negative control; P: positive control; Qr1 to Qr9: DNA templates from sample 1 to 9 respectively using Qiagen DNeasy Blood & Tissue Kit; Pr1 to Pr9: DNA templates from sample 1 to 9 respectively using PrepMan Ultra reagent)

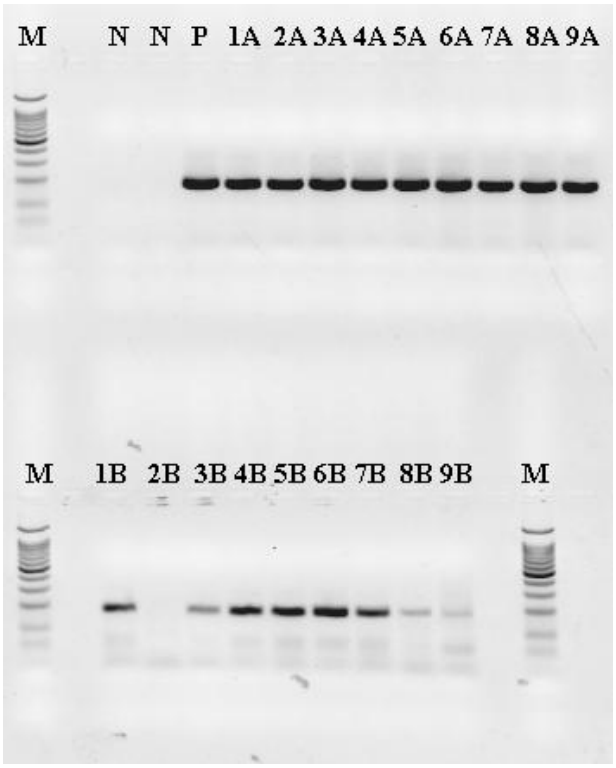

Figure 4.6. The comparison of two DNA extraction methods, agars gel electrophoresis of real-time PCR products. (M: DNA marker; P: Positive control; N: negative control; 1A to 9A: DNA extracted by Qiagen Uneasy Blood & Tissue Kit; 1B to 109 : DNA prepared using PrepMan® reagent).

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