University of Alberta

Development of a Single Nucleotide Polymorphism-Based Electronic DNA Microarray Technique for the Detection and Species Differentiation of Viable Campylobacter

by

Hai Zhang

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Your file Votre référence
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To my husband

Zhilong Gong

Without your unwavering love and understanding, support and encouragement, my thesis wouldn't be a reality. With all my love, I say thank you for always being there.

To my son

Ramon Gong

Thank you for coming into this world and fulfilling my life. Thank you for all the happiness and joy you bring to our family. Love you forever, my dearest elf.

To my parents and my sisters

Thank you for your forever love and encouragement from a long distance.

To all my teachers

Thank you for all the things I learned from you.

Abstract

Campylobacter jejuni, *C. coli*, and *C. lari* are the three major food-borne pathogenic *Campylobacter* species that cause the most frequent occurrences of acute bacterial gastroenteritis around the world. This thesis presents the successful development of a reverse transcription-polymerase chain reaction (RT-PCR) electronic DNA microarray approach for the simultaneous detection and species differentiation of viable *Campylobacter*. The mRNA of the 60-kDa heat shock protein gene (*hsp*60) was used as the viability marker, and two closely located single nucleotide polymorphisms (SNPs) within this gene were chosen as the species marker. A 200-bp fragment amplified from the *hsp*60 mRNA by RT-PCR was detected with species-specific fluorescently-labeled reporters using an electronic DNA microarray technique.

This technique can detect as few as two viable *Campylobacter* cells, and will not detect dead cells. The evaluation of 14 blind *Campylobacter* samples showed 100% agreement with their identities, demonstrating its high specificity. This technique was preliminarily applied to six authentic chicken samples as well.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

AFLP	Amplified Fragment Length Polymorphism
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
cDNA	complementary DNA
CFU	colony-forming unit
ddH_2O	distilled, deionized water (autoclaved before use)
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EHS	Environmental Health Sciences
fg	femtogram
*hsp*60	60 kiloDalton heat shock protein gene
HSP	Heat Shock Protein
kDa	kiloDalton
LB	Luria Broth
mRNA	messenger RNA
NAF	Nanogen Application File
nt	nucleotide
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
pg	picogram

RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Linked Polymorphism
RNA	ribonucleic acid
rpm	round per minute
rRNA,	ribosomal RNA
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
S/B	signal-to-background ratio
SNP	Single Nucleotide Polymorphism
spp.	species
TE buffer	Tris-EDTA buffer
TSB	Tryptic Soy Broth
UV	ultraviolet
VBNC	viable but non-culturable

CHAPTER 1. INTRODUCTION TO *CAMPYLOBACTER*

1.1 Biology of *Campylobacter*

1.1.1 Morphology

The *Campylobacter* organisms are a group of spiral-shaped bacteria that can cause disease in humans and animals. Organisms of the genus *Campylobacter* comprise 14 validly described species (Vandamme, 2000). Most of them appear to be pathogenic in humans and animals (On, 1996). *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* are five major clinically relevant species among the 12 *Campylobacter* species that have been isolated from humans (Lastovica and Skirrow, 2000). The microorganisms belonging to the genus *Campylobacter* have the following general characteristics: cells are slender curved, S-shaped, or spiral rods, 0.2 to 0.8 μm wide and 0.5 to 5 μm long. The cell length varies depending on the maturity of the culture. Actively dividing cells are short (1.5 μm), whereas cells in stationary phase are longer (5 μm). They are Gram-negative and do not form spores. Viable cells are motile with a characteristic corkscrew-like darting motion due to the presence of a single flagellum at one or both cell poles (Skirrow, 1990a). Figure 1.1 shows an image of *Campylobacter jejuni* under a scanning electron microscope. Its corkscrew appearance and bipolar flagella are displayed.

1.1.2 Metabolism

Members of the genus *Campylobacter* are biochemically less active in comparison with many other bacteria (Skirrow, 1990a). The main biochemical activities of *Campylobacter* include production of catalase, oxidase, urease, and H_2S; reduction of nitrate and nitrite; hydrolysis of hippurate and indoxyl acetate; and sensitivity to nalidixic acid and cephalothin. All species produce oxidase, but not all produce indole. Most species produce catalase and reduce nitrate to nitrite. *C. jejuni* is the only species able to hydrolyze sodium hippurate. *Campylobacter* species are unable to utilize carbohydrates. They obtain energy from the metabolism of amino acids or the intermediates of the tricarboxylic acid cycle.

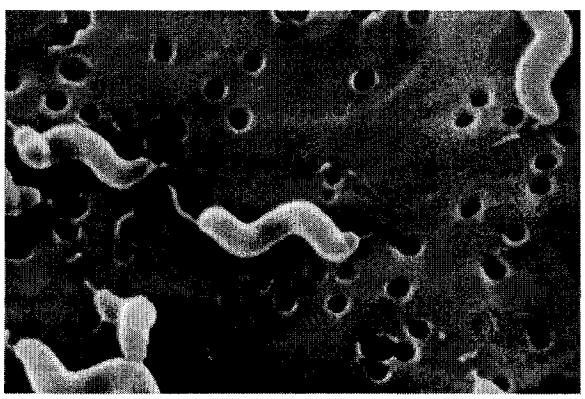


Figure 1.1 Scanning electron micrograph of *C. jejuni*
Source: http://chemweb.calpoly.edu/chem/bailey/377/PapersW03/Kileen/c*jejuni*.htm

1.1.3 Cultivation

Campylobacter spp. have fastidious growth requirements. The most critical factor in their cultivation is their sensitivity to oxygen. Except for *C. cryaerophila*, *Campylobacter* spp. are strictly microaerophilic and do not grow in air. They require an

oxygen concentration range of 5–10% and a 1–15% carbon dioxide concentration to grow; nitrogen is used as the remaining gas. The presence of hydrogen is also required for better growth, particularly for *C. jejuni* and *C. coli*. This oxygen sensitivity has been a major obstacle to the cultivation and preservation of *Campylobacter*, especially the catalase-positive species, such as *C. jejuni*, *C. coli*, *C. lari*, and *C. fetus*. The mechanism of this oxygen sensitivity is due to the excessive vulnerability of *Campylobacter* to superoxides and free radicals compared with aerotolerant bacteria (Skirrow, 1990a). *Campylobacter* species grow best on complex media with the addition of blood, serum, or activated charcoal to neutralize the offending superoxides and free radicals. Most *Campylobacter* species grow well at 37 °C, the human body temperature. However, the optimum temperature of growth for *C. jejuni*, *C. coli*, and *C. lari*, the genus members significantly associated with human gastrointestinal disease, is 42–43 °C, the approximate avian body temperature. For this reason, they are often referred to as the thermophilic or thermotolerant group. Although these three species grow well at 42 °C, they also grow at 37 °C, but not below 30 °C or above 47 °C. *Campylobacter* are slow-growing bacteria. Under ideal conditions, they produce visible growth after 24 hours, but colonies are not well formed until 48 hours.

1.2 Clinical aspects and epidemiology of *Campylobacter* infection

C. jejuni was first isolated from human diarrhea stools in 1972 (Friedman et al., 2000). Since then, with the development of selective growth media facilitating the stool specimens test for *Campylobacter*, *Campylobacter* spp. have been established as common human pathogens (Altekreuse et al., 1999). Now they are recognized as important

food-borne and water-borne pathogens (WHO, 2004; Altekreuse et al., 1999; Mead and Slutsker, 1999). They pose a significant threat to human health by causing a variety of diseases.

1.2.1 Campylobacter *enteritis*

Campylobacteriosis, the gastroenteritis caused by *Campylobacter* attacking the digestive system, is the most common form of acute infectious disease in humans. People get sick after 2–10 days of incubation from the initial exposure. The principal symptom is acute diarrhea that cannot be clinically distinguished from most other acute bacterial infections of the gut. Other typical symptoms include abdominal pain, fever, nausea, vomiting, headache, malaise, and muscle pain. Blood and mucus may be present in liquid stools. A majority of cases are mild, do not require hospitalization, and may be self-limited. However, the infection can be severe and life-threatening to people with other diseases, such as cancer, liver disease, and immunodeficiency diseases (Skirrow, 1990b).

Although chronic sequelae following *Campylobacter* infection do not usually occur, *Campylobacter* spp. do cause some complications, such as Guillain-Barré syndrome (GBS), reactive arthritis (ReA), Reiter's syndrome, abortion in pregnant women, meningitis (the bacteria infect the membrane that lines the surface of the brain), rheumatic manifestations, and Miller-Fischer syndrome (MF, another GBS variant) (Barros-Velazquez et al., 1999; Hannu et al., 2002). Among them, the most remarkable complication is Guillain-Barré syndrome. It is an illness that causes progressive weakness and paralysis; recovery is often slow and in some cases the condition may lead to death.

GBS, the most common cause of acute neuromuscular paralysis in the world (Nachamkin, 2002), is widely considered to be triggered by *C. jejuni* (Takahashi et al., 2005; Tam et al., 2003; Nachamkin, 2002; Fujimoto et al., 1997).

Infection routes include eating contaminated food, drinking contaminated water or unpasteurized milk, and close contact with pets, farm animals, or infected infants (Friedman et al., 2000). The main risk factors are eating undercooked poultry meat and drinking raw milk or untreated water (Koenraad et al., 1997).

1.2.2 Infective dose

The infectious dose of *Campylobacter* is quite low compared to many other enteric bacteria that need 10^7 to 10^8 cells to cause infection (Leclerc et al., 2004). *Campylobacter* infection was induced in a human volunteer when 500 organisms were taken in a glass of milk (Robinson, 1981). A similar infective dose was also observed in another study with young adult volunteers in Baltimore, MD, USA (Black et al., 1988), which showed that clinical symptoms developed in one person and that 50% of the volunteers were shedding the organisms (they had become infected and were excreting *C. jejuni*) when 800 cells of *C. jejuni* suspended in milk were ingested. As well, in this volunteer experiment the infection rates increased with increasing doses from 800 to 2×10^9 organisms, but symptoms were not related to the dose of pathogens administered, nor were the incubation period or severity of illness. This may be due to factors other than the pathogen dose, such as the pathogenicity of the specific strain, the host susceptibility, and the protective action of the infection vehicle (Thomas et al., 1999). The infective dose may be even lower when the buffering action of the infection vehicle and the rapid wash

of stomach contents is considered (Skirrow and Blaser, 2000). On the basis of the dose-response relationship, a probability of 7.0×10^{-3} was estimated by Rose and Gerba (1991) for people exposed to one *Campylobacter* organism to contract an infection.

1.2.3 Species characterization of enteritis

It is reported that clinical isolates are not routinely typed to species level, so in the majority of clinical cases, the species information is not known (Pebody et al., 1997). In situations in which typing has been applied, *C. jejuni* is the most frequent species isolated from humans. It has been reported that *C. jejuni* causes more than 80% of *Campylobacter* enteritis, followed by *C. coli* causing 7% (Steinhauserova et al., 2001). *C. lari* and *C. upsaliensis* are also considered emerging human pathogens even though they are less frequently associated with human enteritis (Klena et al., 2004). This could be due to the commonly used clinical laboratory isolation techniques which have difficulty in distinguishing between *Campylobacter* species based solely on phenotypic properties (Klena et al., 2004), or the selective isolation protocols which are highly specific toward *C. jejuni* (Thomas et al., 1999). When the diagnosis of infection was based exclusively upon culture on selective media, it appeared that >95% of *Campylobacter* infections were caused by *C. jejuni* and *C. coli*. However, with refinements in isolation and identification methods, other related species such as *C. lari*, have been isolated from human patients with diarrhea (Butzler, 2004).

1.2.4 Burden of disease

It is reported that *Campylobacter* causes approximately 5–14% of all diarrheal disease around the world (WHO, 2003). *Campylobacter* enteritis is the most common form of acute bacterial diarrhea in most developed countries, including the United States (Mead and Slutsker, 1999).

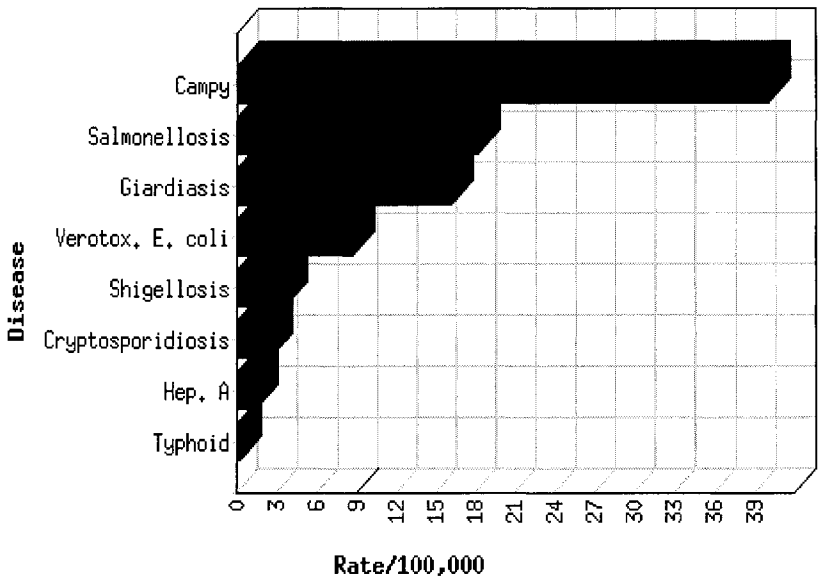


Figure 1.2 Incidence rates of common enteric, food- and water-borne diseases in Canada, 2000

Cases of both sexes combined for all ages were used to calculate incidence rate. Source: Notifiable Diseases on-line, Infectious Diseases, Public Health Agency of Canada. http://dsol-smed.phac-aspc.gc.ca/dsol-smed/ndis/c_dis_e.html, retrieved on 10/08/2004.

For example, in the United States, 46% of reported laboratory-confirmed cases of bacterial gastroenteritis were caused by *Campylobacter* species in 1996 (Altekreuse et al., 1999). Canada is not an exception. As shown in Figure 1.2, campylobacteriosis has the highest prevalence rate followed by salmonellosis among the common food- and water-borne enteric diseases. The same pattern was also observed in other years based on data from the Public Health Agency of Canada Notifiable Diseases On-Line.

Campylobacter was responsible for 2156 cases of infection in Canada from January to April of 2005, which corresponds to about 41% of all common food-borne and water-borne bacterial infection (PHAC, 2005).

Although the nature of *Campylobacter* infection is sporadic, outbreaks have been reported from time to time around the world. Outbreaks that happened in developed countries have been reviewed (Hrudey and Hrudey, 2004). Inadequately treated municipal water and untreated surface water are claimed to contribute to general outbreaks (Thomas et al., 1999; Hrudey and Hrudey, 2004). The economic impact of campylobacteriosis is significant in the form of medical visits, medication, hospitalization, and loss of productivity. It was estimated that in the United States the total cost of campylobacteriosis exceeds US$ 1.2 billion annually (Mixter et al., 2003).

The actual public health impact of *Campylobacter* infection may be greater because the true burden of disease caused by non-*C. jejuni* / *C. coli* *Campylobacter* spp. is difficult to measure (Butzler, 2004) and *Campylobacter* infection is usually under-reported. It has been estimated that only 1 in 38 cases of *Campylobacter* infection was reported in the United States (Mead and Slutsker, 1999). Better diagnostic methods may reveal the true burden of these organisms.

1.2.5 Treatment and drug resistance

Although most cases of *Campylobacter* infection are self-limiting and usually do not require further treatment apart from fluid and electrolyte restoration (Altekreuse et al., 1999), antimicrobial therapy is still required in severe cases and for patients with suppressed immune systems (Butzler, 2004). In such cases, erythromycin (a kind of

macrolide) and fluoroquinolones are often recommended. Erythromycin is the first drug of choice for treatment in humans (Piddock et al., 2000) because of its efficacy, low toxicity, and low cost (Butzler, 2004; Barros-Velazquez et al., 1999). Fluoroquinolones, which act against most enteric pathogens, offer an effective therapy, with ciprofloxacin being commonly prescribed for prophylaxis of enteric infections before travel (Moore et al., 2005; Gibreel et al., 2004).

However, resistance to these agents in *Campylobacter* spp. has been reported both in developing countries (Jain et al., 2005; Hoge et al., 1998; Li et al., 1998) and in industrialized countries (Butzler, 2004; Piddock et al., 2000; Gaunt and Piddock, 1996). Resistance rates show an increasing trend. For example, a study (Gaudreau and Gilbert, 2003) compared the annual rates of resistance to erythromycin and ciprofloxacin of *C. jejuni* strains isolated from patients in Montreal, Quebec, Canada, from 1998 to 2001 and found that the rates of resistance increased from 3% in 1998 to 12% in 2001 for erythromycin, and 10% to 47% for ciprofloxacin. Multidrug resistance of these *C. jejuni* strains to these antimicrobial agents was also observed in 2001, while multidrug resistance was absent in most cases in 1998.

While the emergence and dissemination of antibiotic resistance among *Campylobacter* spp. have been linked to improper treatment of human infections (Piddock et al., 2000; Jain et al., 2005), more studies have suggested that the use of antimicrobial agents in poultry results in the emergence and dissemination of drug-resistant *Campylobacter* species, which are then transmitted to humans through the food supply, and which provide a selective pressure for emergence of resistance to antimicrobial agents among human isolates (Pratt and Korolik, 2005; Nelson et al., 2004;

Gibreel et al., 2004; Gaunt and Piddock, 1996; Jain et al., 2005; Butzler, 2004; Li et al., 1998).

The increased frequency of antibiotic resistance and multidrug resistance among *Campylobacter* spp. is recognized as an emerging public health problem (Engberg et al., 2001) because antibiotic treatment of severe infections may be complicated and its effectiveness may be compromised by resistant isolates. The human health consequences of drug resistance in persons with *Campylobacter* infection have been investigated. In a case-control study (Nelson et al., 2004), the clinical outcomes of persons with fluoroquinolone-resistant infection were compared with those of persons with fluoroquinolone-susceptible infection. It was found that the persons who acquired ciprofloxacin-resistant *Campylobacter* infection experienced a longer diarrheal illness than did the persons with ciprofloxacin-susceptible *Campylobacter* infection. Another registry-based cohort study conducted in Denmark revealed that a >5-fold risk of invasive illness and death was associated with infection with quinolone- and erythromycin-resistant *Campylobacter* strains, compared with infection with drug-susceptible *Campylobacter* strains (Helms et al., 2005).

One aspect worthy of note in terms of drug resistance of *Campylobacter* is that different *Campylobacter* species may have different susceptibility to antimicrobial agents. *C. coli* is more resistant to erythromycin than *C. jejuni* (Engberg et al., 2001; Luber et al., 2003). For example, Li et al. (1998) observed that 83% of *C. coli* strains isolated from chicken samples, in contrast to only 17% of *C. jejuni* strains, were resistant to erythromycin. A higher resistance rate was also observed in *C. coli* strains (50%) isolated from patients than in *C. jejuni* strains (10%).

1.3 Distribution and survival in environment

1.3.1 Distribution in environment

Campylobacter species are commonly found in a wide range of domestic and wild animals, such as cattle, sheep, swine, horses, monkeys, birds (including poultry), dogs, and cats (On, 1996; Thomas et al., 1999; Hald and Madsen, 1997; Butzler, 2004), which serve as environmental reservoirs of infection. This pathogen has recently been detected in filth flies by polymerase chain reaction and culturing confirmation in Arkansas, USA (Szalanski et al., 2004). Flies were also identified as a potential source of *Campylobacter* infection in a study conducted by Nichols (2005) in London, UK.

Campylobacter are commensal microbes in avian species (the intestines are asymptomatically colonized by this bacterium), and birds are the major reservoir of the pathogen in nature (Butzler, 2004). In most industrialized countries, the prevalence of *Campylobacter* spp. in commercial broiler flocks is high (Newell and Wagenaar, 2000; Meldrum et al., 2005; Whyte et al., 2004) because poultry is likely to be highly contaminated at slaughter. In a cross-sectional survey conducted during a consecutive eight-week period in the spring of 2001 (VanderKop, 2003), a prevalence of 60.2% for *Campylobacter* spp. (*C. jejuni*, *C. coli*, and *C. lari*) cultured from whole body rinses of retail chicken samples was observed in Alberta. This result agrees with another study (Willis and Murray, 1997) in which 69% of chickens bought from a local supermarket in the US were found to be contaminated with *Campylobacter*. The levels of contamination may vary in the range of 10^2 to 10^5 CFU per carcass (Jacobs-Reitsam, 2000). In one study (Cason et al., 1997), the contamination level was reported to exceed 10^8 CFU of

Campylobacter per carcass. Though these bacteria do not multiply in food (Park, 2002) because they are unable to grow below 30 °C, cross-contamination with such high levels of *Campylobacter* prevalence could happen in kitchens following the preparation of raw chicken (Cogan et al., 1999; Humphrey et al., 2001).

Campylobacter from the intestines of animals and poultry may enter the wider environment through animal and avian feces, agricultural run-off, and sewage effluent (Jones, 2001). Thomas et al. (1999) reported that *Campylobacter* had been isolated from 82.1% of surface water samples with a most probable number (MPN) of less than 10 cells per 100 mL. Ground water, which is often but mistakenly assumed to be microbiologically clean, can also be contaminated by *Campylobacter*, as proven by the culturable evidence provided by Stanely et al. (1998). The survival of *Campylobacter* in ground water could be due to favorable environmental conditions, such as low redox potentials, the absence of molecular oxygen, year-round low temperatures, and the protection from UV and desiccation found underground (Jones, 2001). One important and noteworthy aspect is the correlation to contamination predictors. Fecal coliforms and fecal streptococci are the two generally accepted standard indicators for the presence of *Campylobacter* in surface water. However, poor correlation between the densities of the *Campylobacter* population and of the indicators has been reported (Arvanitidou et al., 1995; Obiri-danso and Jones, 1999). This phenomenon may further undermine the role of standard indicators in predicting pathogenic contamination in terms of water treatment (Thomas et al., 1999).

The intestines of many feral and commercially reared birds, livestock, domestic pets, and animals are asymptomatically colonized by this bacterium, which is widely

distributed in the environment and food. Transmission to humans is thought to occur through food, drinking water, and pets (Dingle and Colles, 2002).

1.3.2 Survival in environment

In general, *Campylobacter* species are less able to tolerate adverse environmental conditions than most bacteria (Skirrow, 1990a). They are susceptible to heat and cold, drying, ultraviolet radiation, disinfectants, pH, salt, and other environmental stresses. A number of studies have investigated the effect of different environmental conditions on the survival of *Campylobacter.* Fernandez et al. (1985) tested the desiccation resistance among thermotolerant *Campylobacter* species and found that they were very sensitive to desiccation and could not survive well on dry surfaces. Doyle and Roman (1982) found that they were sensitive to osmotic stress and could not grow in a sodium chloride concentration of 2%. Blaser et al. (1980) demonstrated that *Campylobacter* were incapable of growing below pH 4.9 and would be killed when the pH value was less than 4.9.

Several studies demonstrated that *Campylobacter* could survive for a long time at temperatures below room temperature in water systems (Rollins and Colwell, 1986; Buswell et al., 1998) and in culture media (Chan et al., 2001; Lázaro et al., 1999). For example, Rollins and Colwell (1986) showed that *C. jejuni* could survive for over 4 months in stream water held at 4 °C. Organisms were metabolically active at this temperature and demonstrated oxygen consumption, catalase activity, ATP generation, chemotaxis, and protein synthesis (Hazeleger et al., 1998). However, unlike many other bacteria that produce characteristic cold shock proteins, which are associated with their

ability to replicate at temperatures below the optimum growth temperature (Phadtare et al., 1999), *Campylobacter* species do not produce this type of cold shock protein and therefore are unable to grow below 30 °C, as indicated by an analysis of the *C. jejuni* genome sequence (Parkhill et al., 2000).

Campylobacter may also survive in the environment by sheltering in protozoa (Axelsson-Olsson et al., 2005). This phenomenon poses a threat to water treatment because *Campylobacter* may obtain some protection from protozoa and therefore gain resistance to free chlorine. In a study (King et al., 1988), *C. jejuni* could be cultured from chlorine-treated protozoa after a time long enough to inactivate 99% of free-living cells. Survival of *Campylobacter* in the environment may vary depending on the different species. One study (Danso et al., 2001) reported that *C. lari* survived for a longer time in surface water than did *C. jejuni* and *C. coli*.

The studies mentioned above demonstrated the ability of *Campylobacter* to survive in different environmental conditions. One thing to note is their ability to survive in chlorine-treated water by sheltering in protozoa, suggesting that additional measures are necessary for water treatment.

1.4 VBNC

As a consequence of environmental stress, certain bacteria are believed to enter a viable but non-culturable (VBNC) state. In this state, bacteria are not able to grow and form colonies on conventional culture media but still retain metabolic activity, cellular structures, and intact nucleic acid (Tholozan et al., 1999; Lázaro et al., 1999). Therefore,

they are still considered viable organisms and may revert to full viability under favorable conditions and may be able to grow again.

The VBNC state has been found in numerous bacteria of medical interest such as *Vibrio cholerae*, *Shigella dysenteriae*, *Escherichia coli* O157:H7, *Salmonella* Enteriditis, and *Legionella pneumophila* (Lleò et al., 2000; Tholozan et al., 1999). The VBNC state of *Campylobacter* was first described in 1986 (Rollins and Colwell, 1986). Organisms enter this state as a strategy for survival when they face adverse environmental stress conditions, such as limited nutrient availability for post-mature cultures with limited nutrients (Skirrow, 1990a), exposure to atmospheric oxygen (Skirrow, 1990a), and exposure to low temperature (Lázaro et al., 1999). It has been generally assumed that cells undergo a morphological change from the normal spiral shape to a non-motile coccal shape when they are in the VBNC state, and the coccoid form has been suggested to be a dormant state (Rollins and Colwell, 1986). Recently, one study observed that the transition to coccoid form was not always related to a decrease in culturability and suggested that non-culturable *Campylobacter* cells might still remain in spiral shape (Lázaro et al., 1999). Compared with other *Campylobacter* species, a rapid transformation was seen in *C. lari*, *C. jejuni*, and *C. coli* in decreasing degrees when they were exposed to air in culture media at room temperature (Skirrow, 1990a).

The epidemiological significance of VBNC *Campylobacter* is based upon their potential to resuscitate and give rise to infection. If these VBNC cells are still infective, they could possibly play a role in the infection cycle. The infectious capacity of *Campylobacter* in the VBNC state has been investigated by testing the ability of cells in that state to colonize animals and humans because colonization is an important step for

pathogens to remain, grow, and multiply in the host before they cause infection (Nicklin et al., 2002). The results revealed that the significance of VBNC cells in the infection of animals and as the cause of disease in humans still remains uncertain. Beumer et al. (1992) administered coccoid cells of *C. jejuni* to laboratory animals (rabbits, mice) and human volunteers, but no symptoms of campylobacteriosis or appearance of antibodies against *C. jejuni* in the serum was observed. Ziprin and Harvey (2004) reported that VBNC *C. jejuni* was not able to revert to the vibrionic form capable of colonizing the cecum in day-of-hatch leghorn and broiler chicks seven days after the chicks gavaged with VBNC cells suspension. However, Jones et al. (1991) reported that some strains of *C. jejuni*, which had become VBNC by storage in water for six weeks, could be recovered after being fed to suckling mice. Saha et al. (1991) observed that *C. jejuni* rendered non-culturable by freezing could be resuscitated by passage through the gastrointestinal tract of a rat. Stern et al. (1994) found that some isolates (four out of six) of VBNC *C. jejuni* were able to colonize 5 of 79 challenged one-week-old chicks. Cappelier et al. (1999) reported that VBNC cells of *C. jejuni* appeared capable of colonization in the yolk sacs of embryonated eggs. In their experiment, *C. jejuni* cells rendered VBNC by 30 days of starvation were injected into embryonated egg yolk sacs; after incubation at 37 °C for 12, 48, and 96 hours, the inoculated VBNC cells were recovered from a large portion (77.5% to 87.5%, depending on different strains) of the embryonated eggs. Another more important finding of this experiment was that the culturable *Campylobacter* cells recovered after passage through the embryonated eggs still maintained their adhesion properties for attachment with HeLa cells. So the authors claim that the VBNC state of *Campylobacter* is a public health concern.

These contradictory results may be explained by the differences in the reversion experiments of the different investigations, such as the different animal models used and the complexity of their gut flora. The differences in the conditions under which VBNC *Campylobacter* are formed may also contribute to these contrasting results. Thomas et al. (1999) stated that the physiological characteristics as well as the pathogenic potential of VBNC *Campylobacter* might be strongly influenced by the conditions under which VBNC *Campylobacter* are formed. A recent study (Gaynor et al., 2004) found that there was a marked difference between the abilities of a variant of *Campylobacter jejuni* NCTC 11168, whose genome had been sequenced, and its original clinical isolate in their abilities to colonize one-day-old chicks with the result that the original isolate colonized much more efficiently than the variant strain. This finding suggests that laboratory subculture and storage may reduce the ability to colonize. Recent research appears to favor the view that VBNC cells have an important role in the transmission of disease and constitute a public health concern (Cappelier et al., 1999; Lleò et al., 2000; Lázaro et al., 1999; Talibart et al., 2000). Thomas et al. (1999) stated: "The virulence of VBNC forms should be considered to be equivalent to that of the culturable forms, with the added risk that they are not detectable by conventional culturable methods."

1.5 Detection techniques for *Campylobacter*

A variety of different approaches have been developed and used to identify *Campylobacter* and/or differentiate them to the species level for clinical and epidemiological purposes, including diagnosis of diseases caused by *Campylobacter*, tracing sources and routes of transmission, identification and monitoring of

Campylobacter in the environment to fully understand the health risk, and development of effective disease-control measures to prevent human disease. This identification is also called typing. These typing methods vary in their complexity and ability to discriminate between species. Each method has strengths and weaknesses. They can be categorized into two major groups: phenotypic approaches and genotypic approaches. Particular methods within each group and the challenges they encounter for the identification and differentiation of *Campylobacter* will be described and discussed in detail in the following sections.

1.5.1 Phenotypic approaches

Typing methods in this category measure phenotypic properties of organisms, such as shape, size, staining properties, reactions to biochemical tests, and other essential properties that can be measured without reference to the genome. Usually, they are associated with cultivation. These conventional methods include typing by growth and morphologic characteristics (microscopic examination), typing based on biochemical characteristics (biotyping), typing by serologic characteristics (serotyping), typing by functional or physiologic characteristics (such as phage typing), and other different schemes.

1.5.1.1 Microscopic examination

Because *Campylobacter* enteritis cannot be distinguished clinically from other forms of acute diarrheal illness, a definitive diagnosis can only be made bacteriologically (Skirrow and Blaser, 2000). The microscopic examination of fresh liquid feces or fecal smears for *Campylobacter* can provide a rapid diagnosis. *Campylobacter* can be

recognized by microscopy. They can be seen and distinguished from other organisms by their spiral morphology and extremely rapid darting and spinning motion (characteristic corkscrew-like darting motion), either with a Gram stain or a phase-contrast optical system (Butzler, 2004). However, the sensitivity of microscopy is variable (36 to 90%) (Skirrow and Blaser, 2000). Sensitivity here is defined as the probability of testing positive if the disease is truly present. Under most conditions, microscopy probably detects no more than about 60% of acute cases (Skirrow, 1990b). Therefore, the traditional laboratory method for the detection of the enteric bacteria associated with invasive diarrhea has been the isolation of these organisms from stool specimens followed by phenotypic identification by biochemical and/or serological methods.

1.5.1.2 Biotyping

This method involves examination of the biochemical characteristics of the colonies appearing on selective media after cultivation under microaerophilic conditions. As summarized by Barros-Velazquez et al. (1999) and Butzler (2004), to find and identify *Campylobacter* species after primary isolation, suspicious colonies are selected to undergo at least three tests: (1) direct microscopic examination of motility and cell morphology; (2) Gram-staining, which should be negative; and (3) oxidase production, which should be positive. Other biochemical tests are also needed if classification of *Campylobacter* to the species level is necessary. Table 1.1 presents some features that are widely employed for the differentiation of *Campylobacter* species. The hippurate hydrolysis test differentiates most *C. jejuni* from other *Campylobacter* species. Skirrow and Benjamin (1980) introduced a biotyping scheme to distinguish *C. jejuni*, *C. coli*, and *C. lari* based on different growth temperatures and several biochemical tests.

	C. jejuni	*C. coli*	*C. lari*
Hippurate hydrolysis	+	-	-
Catalase production	+	+	+
Nitrate reduction	+	+	+
H_2S production	-	-/W	-
Nalidixic acid resistance	S	S	R
Cephalotin resistance	R	R	R

Table 1.1 Biochemical features employed in differentiation of *Campylobacter* spp.
Modified from (Barros-Velazquez et al., 1999) W: weak, S: sensitive, R: resistant

Biotyping has some disadvantages. One of them is due to the metabolic characteristics of the genus *Campylobacter*. As mentioned before, they are biochemically less active in comparison with many other bacteria and possess few biochemical characteristics that can be reliably used to distinguish between species. Another obvious weakness of this method is its complexity and the lack of application of highly standardized procedures since the discrimination among species or subspecies relies on only one or a few differential biochemical characteristics (Vandamme, 2000).

1.5.1.3 Serotyping

Serotyping is the most widely used phenotypic typing method for *Campylobacter*. This bacterial classification method is based on differences in the antigens that are present on the surface of *Campylobacter* cells. In practice, the organisms are serotyped by an agglutination test with a range of antigen-specific antisera (containing antibodies to recognize antigens). There are two generally accepted, well-established serologic typing

schemes that have been used since the 1980s to study species diversity, track epidemiological trends, and determine important epidemiological correlations (Dingle and Colles, 2002; Taboada et al., 2004). One is the Penner scheme, which is based on heat-stable (HS) antigens using a passive hemagglutination technique (Penner and Hennessy, 1980); the other is the Lior scheme which is based on heat-labile (HL) antigens and a bacterial agglutination method (Lior et al., 1982).

Even though agglutination assays are simple to perform, there are still some disadvantages associated with serotyping methods (Wassenaar and Newell, 2000; Riley, 2004; Taboada et al., 2004; Nachamkin et al., 2000; Nicholson and Patton, 1993). The major disadvantage of both of these techniques is the cross-reactions exhibited by cross-reacting antigens carried by different strains from the same species, or even strains from different species. This compromises the specificity of the test; it could be due to the culturing conditions, which can affect the expression of serotyping determinants. Another limitation of serotyping is that several strains are non-typeable because they do not express typing antigens on the surface of the organism at all. The third limitation associated with serotyping schemes is that high-quality antisera are not widely available due to the technical limitations of their production and quality control. So serotyping is performed in only a few reference laboratories because of the time and expense needed to maintain high-quality serotyping antisera.

1.5.1.4 Phage typing

Phage typing is usually required in epidemiological investigations of outbreaks as an extension to serotyping when large numbers of isolates belong to the most prevalent serotypes or when dealing with non-typeable isolates (Frost et al., 1999). Preston phage

typing combined with the Penner serotyping scheme and biotyping methods has been used to investigate diversity among *Campylobacter* isolates from sporadic cases of human enteritis in the United Kingdom (Wareing et al., 2002). The principle of this approach to *Campylobacter* classification is based on their susceptibility to lysis by a range of bacteriophages (phages for short). Different bacteria are susceptible to infection by different phages. A bacterium to be tested is spread on an agar plate surface and dried; then, a small drop of each bacteriophage suspension is placed on the plate, and the plate is incubated for a designated time period. If the test bacterium is susceptible to a phage, a zone of inhibition of growth or plaque is observed at the phage drop positions, indicating bacterial lysis. The pattern of plaque distribution on the plate is compared with a chart, and the phage type of the test bacterium is determined.

Except in major reference laboratories, this typing procedure is not widely available because of the need to maintain a stock of typing phages. Another shortcoming is the difficulty of standardizing testing because phage typing must be specifically developed and designed for each species or subspecies (Riley, 2004).

1.5.1.5 Common challenges with phenotypic approaches

Besides those specific disadvantages associated with each method discussed above, phenotypic approaches confront common challenges due to the cultivation procedure required to isolate *Campylobacter* from samples.

The first one is that the isolation and cultivation of *Campylobacter* is time-consuming and laborious because these microorganisms have fastidious nutritional and environmental requirements for growth on culture medium. Furthermore, to reduce the growth of background flora and enhance the recovery of *Campylobacter* from samples,

enrichment broth culture is usually required (Nachamkin et al., 2000). This requires a prolonged incubation period. For example, to detect *C. jejuni* and *C. coli* in water, the general procedure for the detection involves the following steps. First, water samples should be concentrated by filtration through a filter. Then the filter is placed in a nonselective broth and incubated at 42 °C for 4 hours (pre-enrichment culture), after which time antibiotics are added. Incubation is continued for 24 hours (selective enrichment culture); then the broth is plated onto selective media and incubated for 48 hours in a microaerobic environment. The isolates are then subjected to Gram stain examination and other tests (Fricker, 1999).

The second challenge, which is the major drawback of these culture-based methods, is that they may generate false negative results because they cannot take the VBNC state into consideration. As discussed above, organisms in this state cannot grow on media, so they cannot be detected by these methods. However, they still have the potential to cause infection, as claimed by some investigations. So in this sense, phenotypic approaches may not be accurate.

1.5.2 Genotypic approaches

Rapid and reliable identification of the different *Campylobacter* species is difficult to obtain solely by conventional procedures that are based on phenotypic properties of organisms. Therefore, molecular analyses based on differences at the nucleic acid level have been increasingly used to identify *Campylobacter* species for clinical and epidemiological purposes because these analyses are capable of providing a higher level of discrimination and reproducibility than the phenotypic techniques. These kinds of

typing systems are classified as genotypic approaches. They are divided into two general categories: those based on the analysis of extra-chromosomal DNA elements, such as plasmid-based typing methods, and those based on the analysis of the genome. Only genome-based typing methods are reviewed in this thesis, and the term "genotypic approaches" appearing in this thesis from now on refers to genome-based typing methods.

Genotypic approaches include a wide range of molecular typing methods and can be classified into three basic groups based on their analytical procedures: (i) hybridization, (ii) gel electrophoresis, and (iii) nucleic acid sequencing. Some common typing methods, such as pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP) analysis, ribotyping, amplified fragment length polymorphism (AFLP) analysis, and random amplified polymorphic DNA typing (RAPD) will be discussed in this section.

1.5.2.1 PFGE

Pulsed-field gel electrophoresis (PFGE) is a special gel electrophoresis technique providing higher resolution than traditional gel electrophoresis. It involves the digestion of whole-cell DNA with so-called "rare cutting" restriction enzymes, generating relatively few genomic fragments of comparatively large size (20–200 kb), which are poorly resolved by conventional agarose gel electrophoresis due to the small average pore size of the agarose gel matrix. The DNA fragments can be separated with a specialized gel apparatus under conditions in which the orientation of the electrical field across the gel is periodically changed. The genotypic profiles (or DNA fingerprints) resulting from

the presence of relevant restriction sites are compared by software to provide species information.

Executed properly, PFGE is a typing method of high reproducibility and high discrimination power (Riley, 2004) and is often considered as the "gold standard" in epidemiological studies of food-borne pathogens (WHO, 2004; Fitzgerald et al., 2001). It has been applied alone or combined with other typing methods to characterize *Campylobacter* spp. associated with water-borne outbreaks (Bopp et al., 2003; Clark et al., 2003; Hänninen et al., 2003).

One of the factors that have limited the use of PFGE is the time involved in completing the analysis. While the procedural steps are straightforward, the time needed to complete the procedure can be 3 to 4 days (Fitzgerald et al., 2001), so the laboratory's ability to analyze large numbers of samples would be limited. Another drawback of PFGE is that the expertise required for DNA preparation and interpretation of the data, and the special apparatus necessary for electrophoresis, place restrictions on this method for routine use (Champion et al., 2002).

1.5.2.2 RAPD analysis

The random amplified polymorphic DNA (RAPD) assay is a PCR technique that relies on randomly designed short primers that anneal to DNA templates of *Campylobacter* (Olive and Bean, 1999; Wassenaar and Newell, 2000). Nine- or 10-mer (9 to 10 nucleotides in length) primers are used to hybridize to chromosomal DNA sequences under low-stringency conditions (e.g., low-annealing temperature), which allow some mismatches to increase the number of primed sites. If a set of these randomly designed primers happens to anneal within the amplification distance (less than 5 kb) and

with the correct opposite orientation, PCR products of variable length will be generated. The lengths of these products vary for different strains of a bacterial species. Thus, following separation of the amplification products by agarose gel electrophoresis, a pattern of bands, which in theory is characteristic of each particular bacterial strain, is obtained.

This method can produce results rapidly and requires no prior knowledge of the target DNA sequences because the entire genome serves as the target for strain comparison (Riley, 2004). The discriminatory power and typeability of this technique were found to be as high as PFGE in a comparison study of several genotypic methods for subtyping *Campylobacter jejuni* (Nielsen et al., 2000). This could be because both techniques are based on the entire genome. *Campylobacter jejuni* isolated from patients with Guillain-Barré syndrome (Fujimoto et al., 1997) and from chicken litter (Payne et al., 1999) were genotyped by this technique combined with other typing methods in two separate studies.

The major drawback of RAPD is poor reproducibility, which outweighs its advantages of rapidity and cost-effectiveness (Olive and Bean, 1999; Wassenaar and Newell, 2000). This is because the random primers are not directed against any particular genetic locus, which results in imperfect hybridization between the primer and the target site. Thus, the amplification process is extremely sensitive to slight changes in the annealing temperature that can lead to variability in the banding patterns. The random sequences of primers also contribute to the susceptibility of this technique to the risk that contaminating DNA may become amplified (Riley, 2004).

1.5.2.3 RFLP analysis

RFLP is short for restriction fragment length polymorphism, which refers to the polymorphic nature of the locations of restriction enzyme sites within defined genetic regions. The specific locus to be examined is amplified with gene-specific primers by PCR. The PCR product is subsequently digested with restriction enzymes. The restriction site distribution within the specific gene can be polymorphic between strains; thus, DNA fragments of different sizes will be generated. The DNA fragments are then separated on an agarose gel, and the digestion patterns are visualized and interpreted.

A number of RFLP-based methods have been developed and applied for genotyping *Campylobacter* spp. (Shi et al., 2002; Steinhauserova et al., 2001; Clark et al., 2003). Among the genes involved, flagellin genes (*flaA* and *flaB*) are the genes most often used for RFLP analysis (Fujimoto et al., 1997; Fitzgerald et al., 2001). These typing techniques based on the PCR amplification of flagellin genes are also termed flatyping (Wassenaar and Newell, 2000). The flagellin genes, particularly the *flaA* gene, are at least partially conserved in *Campylobacter* species like *jejuni*, *coli*, *lari*, and *helveticus* while variable regions are also present. These make this gene suitable for RFLP analysis.

The discriminatory power of this method is not as good as that of the other typing methods mentioned above, primarily due to the limited region of the genome that can be examined (Olive and Bean, 1999). Another drawback of RFLP analysis is the difficulty of analyzing the data because of the large number of DNA fragments generated by restriction endonucleases cutting along the PCR products, which produce poorly resolved overlapping bands on gel electrophoresis (Riley, 2004).

1.5.2.4 Ribotyping

Ribotyping is a variation of RFLP-Southern blot hybridization. In this technique, genomic DNA is digested with restriction endonuclease(s) and the DNA fragments are resolved by agarose gel electrophoresis. Then the resolved DNA fragments are transferred onto a piece of nitrocellulose or nylon membrane by Southern blotting. The membrane-bound nucleic acid is then hybridized with labeled probes specific for rRNA genes (coding for 5S, 16S, and 23S rRNA, present at different positions on the *Campylobacter* chromosome).

This technique has been successfully used for subtyping *Campylobacter* spp. in several studies (Fitzgerald et al., 1996; Owen et al., 1993; Russell et al., 1994; Fayos et al., 1992). Most investigators used probes specific for 16S rRNA, while some used cDNA (complementary DNA) probes that were derived by reverse transcription of 16S rRNA and 23S rRNA sequences (Owen et al., 1993).

The interpretation of patterns generated by ribotyping is much easier compared with those from RFLP because only a few bands can be detected through the hybridization with ribosomal gene-specific probes (Riley, 2004). However, ribotyping is a less discriminatory method than PFGE since most *Campylobacter* spp. contains only three ribosomal gene copies (Lukinmaa et al., 2004). The relatively low discriminatory power along with the elaborate, low-throughput nature of the technique makes it a relatively unsuitable method for routine genotyping.

1.5.2.5 AFLP analysis

Amplified fragment length polymorphism (AFLP) analysis is a typing technique based on the selective amplification of a subset of DNA fragments generated by the

digestion of chromosomal DNA with restriction enzymes. In this technique, the total genomic DNA is digested with two types of restriction endonucleases, usually with one that cuts more frequently than the other, to produce DNA fragments with distinct overhanging sequences at each end. The generated DNA fragments are then ligated to linkers containing the restriction site sequences and further amplified with PCR primers that target the linkers. For comparison of the band patterns, the amplified fragments of different lengths are resolved electrophoretically and visualized either by staining the gel or by the use of radioactively or fluorescently labeled primers.

This technique has been used to subtype *C. jejuni* and *C. coli* derived from humans, chickens, and cattle (Duim et al., 1999; Schouls et al., 2003; Kokotovic and On, 1999). The major advantage of AFLP analysis is that it does not require knowledge of the target DNA sequences as a random portion of the whole genome is sampled. The major limitation is that this technique is complex and subject to experimental variability due to the many procedures required. Optimization of the enzymes is necessary since different combinations of restriction enzymes will generate different numbers of fragments of different sizes.

1.5.2.6 Other PCR-based typing methods

PCR assays are attractive for typing *Campylobacter* spp. due to their simplicity (a distinguishing feature of PCR technology), low cost, and potential application in large-scale screening programs (Riley, 2004). A wide range of PCR typing assays have been described. Some of them are conventional PCR assays incorporating a pair of oligonucleotide primers to amplify a specific gene, which is then detected using agarose gel electrophoresis combined with an intercalating dye (e.g., ethidium bromide) and UV

light (Josefsen et al., 2004; Dedieu et al., 2004), or is detected through hybridization with gene-specific probes (Moreno et al., 2003; Rashid et al., 2000; Konkel et al., 1999). Multiplex PCR assays use more than one set of primers in the PCR reaction to simultaneously detect several genes (LaGier et al., 2004; Wang et al., 2002; Denis et al., 1999; Harmon et al., 1997) or different regions of the same gene (Klena et al., 2004; Houng et al., 2001). Nested or semi-nested PCR assays were also developed for detecting *C. jejuni* and *C. coli* (Bang et al., 2002; Waage et al., 1999). Genes involved in such PCR assays include *flaA*, *flaB*, 16S rRNA, 23S rRNA, *lpxA*, *hipO*, *glyA*, *ceuE*, *cadF*, *sapB2*, *omp50*, and *mapA*.

The major limitation of PCR tests is the problem of DNA contamination. It may be accidental or it could be a constitutive part of the procedure. For example, if the DNA is extracted from *Campylobacter* obtained directly from a clinical specimen, mammalian cell DNA and other bacterial DNA will inevitably be mixed with the target organism DNA. In this case, interpreting the result from a PCR assay is more complicated than the simple presence or absence of a pathogen since the bands on the electrophoresis gel only serve as proxies for product identification.

1.5.2.7 Other genotypic methods

The techniques described above are the ones most widely investigated by researchers. Other than these, methods such as nucleotide sequencing (Meinersmann et al., 1997), microarray comparison (Keramas et al., 2003; Volokhov et al., 2003), MLST (multilocus sequence typing system) (Dingle et al., 2001; Sails et al., 2003), and others have been adopted for *Campylobacter* detection with or without differentiation.

1.5.2.8 Common disadvantages of genotypic approaches

As discussed above, a number of genotypic methods are based on PCR or are coupled with PCR. PCR has been proven to be highly sensitive and specific. However, one of the major limitations of PCR is that it cannot discriminate between viable and non-viable bacteria because it amplifies all DNA, whether from viable cells or dead ones, as long as the DNA material is intact. Josephson et al. (1993) demonstrated that positive PCR amplifications were obtained from boiled *E. coli* cells after 2 weeks of storage at 4 °C, although the dead bacteria are not of concern to public health. So, from this standpoint, genotypic methods associated with PCR may generate false positive results. Recently, a novel real-time PCR method was developed by Rudi et al. (2005) to quantify viable and dead *Campylobacter* cells in chicken meat samples. The viable/dead differentiation was obtained by using ethidium monoazide (EMA), which can penetrate compromised membrane/cell wall systems of dead cells and covalently bind to DNA in dead cells by photoactivation. DNA bound to EMA cannot be amplified by PCR. Unfortunately, this method does not provide the power to differentiate *Campylobacter* at the species level.

A cultivation step prior to PCR is usually included in many cases to avoid false positive results in a viability assay by diluting out any dead cells that may be present. This extra step increases the concentration of DNA produced from viable cells, and thereby decreases the potential for false positive results. However, the inclusion of the cultivation step can prolong the analysis time, eliminating many of PCR's advantages, such as simplicity, high throughput, and high sensitivity, as well as the detection power for VBNC cells.

Another drawback of genotypic methods is the difficulty of differentiating between closely related species or subtypes. Many of the molecular typing methods select highly conserved genes, such as the 16S rRNA gene, as a foundation. The percentage of DNA similarity between *C. jejuni*, *C. coli*, and *C. lari* is 96% or above for their 16S rRNA genes (Wong and Chow, 2002). Such highly conserved genes contain small or nonexistent variable sequences among different species and strains. This results in low discrimination power (Wang et al., 2004; Wong and Chow, 2002).

Interestingly, hypervariable sequences are often found in some genes encoding the biosynthesis or modification of surface structures, while the large regions of the genome are genetically stable (Taboada et al., 2004; Wren et al., 2001). These hypervariable regions include the lipooligosaccharide biosynthesis locus, the flagellar biosynthesis locus, the capsular polysaccharide biosynthesis locus, and the restriction-modification locus. These genes may play an important role in the survival strategy by helping *Campylobacter* adapt to new environments (Wren et al., 2001; Lázaro et al., 1999). The *flaA* gene was claimed to be an unreliable marker for species identification (Dingle et al., 2005). So those methods based on the *flaA* gene may generate inaccurate results.

Genomic variation either occurs spontaneously or is induced by (i) mobile elements, (ii) programmed DNA inversion, (iii) horizontal gene transfer, and (iv) natural transformation (Steinbrueckner, et al. 2001). Genomic plasticity may become visible by the typing methods involving restriction enzymes, such as PFGE (Boer et al., 2002; Wassenaar et al., 2000; Lázaro et al., 1999). The patterns generated by these methods may not be interpreted correctly in the sense of providing the species information.

So, due to the reasons mentioned above, genotyping results should be interpreted with caution.

1.6 Thesis objectives

The objective of this thesis is to develop a reliable and sensitive method that can accurately detect all viable *Campylobacter* cells, including those in the VBNC state, but not dead cells, and that can simultaneously differentiate *Campylobacter* at the species level.

In order to achieve this goal, the research of this thesis consists of the following steps:

(1) identification of unique viability markers for detection of viable *Campylobacter*;

(2) identification of unique SNP-based species-specific markers using cloning and sequencing techniques;

(3) development of a RT-PCR method for amplification of the targets for detection of a small number of *Campylobacter* cells;

(4) development and validation of an electronic microarray technique for accurate detection of the target bacteria; and

(5) application of this technique for analysis of *Campylobacter* species in food samples.

CHAPTER 2. PROJECT DESIGN — OVERVIEW

2.1 *hsp*60 mRNA AS VIABILITY MARKER

It is important to assess the viability status of pathogenic microorganisms when determining if the presence of these pathogens poses an actual threat to public health. Cell viability is difficult to define because there is no unique property common to all things normally regarded as living. Microbiologists usually define viable organisms as those that can multiply to form colonies on agar plates or visible turbidity in broth (Sheridan et al., 1998). However, bacteria may enter a dormant state called the viable but non-culturable (VBNC) state in order to survive under adverse conditions. In that state, cells will not grow on selective medium to form colonies, but they are still alive and, most importantly, they still have the potential to cause infection. Therefore, the reasonable definition of viable bacterial cells suggested by Keer and Birch (2003) and Sheridan et al. (1998) was adopted in this thesis. Viable bacterial cells are fefined as those maintaining membrane integrity and retaining some metabolic activity or responsiveness and possessing the potential to multiply under suitable conditions.

Because only viable pathogenic cells, including those in VBNC state, pose a threat to public health, the detection of viable but not dead cells is of practical significance. Those methods for the determination of bacterial viability that rely on cultivation are time-consuming, and often give false negative results due to VBNC cells, as discussed above. Alternatively, molecular methods offering speed, sensitivity, and specificity have been developed for determining cell viability. The presence of nucleic acid (DNA or RNA) in bacterial cells might serve as a useful indicator of viability. DNA has been used

as a cell viability marker (Nogva et al., 2000). However, it was found that DNA may persist in actively killed cells for a long period of time (up to 2 weeks), and can be detected by positive PCR amplification in culture-negative samples (boiled samples that could not grow in culture medium) (Josephson et al., 1993). RNA is more susceptible to hydrolysis than DNA because of the presence of the 2'-hydroxyl group of ribose (Turner et al., 2000). Therefore, RNA has been investigated as an indicator of viability. RNA molecules can be divided into two groups. Relatively long-lived stable RNA includes ribosomal RNA (rRNA) and transfer RNA (tRNA). Messenger RNA (mRNA) molecules have relatively short half-lives compared with the stable RNA molecules. Most bacterial mRNA exists for only short periods of time in viable cells with an average half-life of minutes (Klein and Juneja, 1997). This allows cells to rapidly adjust the synthesis of proteins to accommodate changes in the cellular environment. Among the different types of nucleic acids, mRNA is the most promising candidate for an indicator of viability. RT-PCR is one of the most commonly used amplification techniques for detecting mRNA (Bej et al., 1991; Patel et al., 1993; Sails et al., 1998). Klein and Juneja (1997) demonstrated the ability of the RT-PCR assay to distinguish between viable and nonviable *Listeria monocytogenes* cells by performing RT-PCR and PCR assays with RNA and DNA extracted from both live cells and dead cells killed by autoclaving. The results showed that the detection of mRNA by RT-PCR amplification provided a more sensitive indication of cell viability than the detection of gene sequences by DNA-based PCR amplification. Another study (Sheridan et al., 1998) also used RT-PCR to amplify mRNA of three different genes (*rpoH*, *groEL*, and *tufA*) and 16S rRNA in heat-killed

Escherichia coli cells and the results showed that mRNA is a much better indicator of viability than rRNA because of the rapid disappearance of mRNA from dead cells.

Messenger RNA of the gene encoding a heat shock protein with an approximate molecular weight of 60 kiloDaltons (*hsp*60 mRNA) was employed as a viability marker in my study to detect and distinguish "viable" *Campylobacter* cells from "dead" cells. Heat shock proteins (HSPs) are a group of universally conserved proteins that has evolved in all organisms, including bacteria. HSPs are not produced only in response to changes in temperature so that bacteria can adapt to this stress; a variety of environmental stress conditions, such as the addition of ethanol (Ashburner, 1982), oxidative shock (Gophna and Ron, 2003), and alkaline pH shock (Wu et al., 1994), can also induce heat shock proteins. The major function of HSPs is to promote the folding of most cellular proteins and the proteolysis of potentially deleterious, misfolded proteins (Lodish et al., 2000). HSPs may play other important roles in the host immunity and pathogenesis of *Campylobacter*, such as thermotolerance and colonization (Wu et al., 1994; Thies et al., 1999b; Konkel et al., 1998). In bacteria, the HSP60 family (also known as GroEL, 60-kDa HSP) is the most prominent group of HSPs (Villar, 2000) that have been intensively studied.

According to the central dogma of molecular biology, genetic information flows from DNA to RNA to proteins. So when living bacterial cells are exposed to a variety of stresses, before HSPs are synthesized, there must be an elevated level of corresponding mRNA accumulated in the cells. The single-copy *hsp*60 gene was claimed to be an alternate DNA target for species-specific identification of *Staphylococcus* and possibly other microorganisms as well due to the well-conserved DNA sequences within a given

species, but with sufficient sequence variation to allow for microbial identification at the species level (Goh et al., 1996). Another important reason for choosing the *hsp*60 gene as a starting point is its capacity to be rapidly induced in response to a broad spectrum of stimuli (Villar, 2000). In my study, heat shock treatment was applied to allow viable *Campylobacter* cells to induce *hsp*60 mRNA. The increased expression of *hsp*60 when *Campylobacter* was exposed to stresses was demonstrated by Thies et al. (1999a). Northern blot analysis was used to detect mRNA transcribed from the *Campylobacter GroEL* (*hsp*60) gene and it clearly showed that the *hsp*60 mRNA level increased rapidly after bacterial cells were shifted from 37 °C (growing temperature) to 48 °C (heat shock temperature). Due to the responsiveness to heat shock of only the viable cells, including those in the VBNC state, heat shock treatment would be a simple and direct measure to detect cell viability. As well, heat shock would induce and accumulate *hsp*60 mRNA, so that a higher sensitivity could be achieved. As discussed by Keer and Birch (2003), the detection of environmental responsiveness by *hsp*60 gene expression induced by heat shocking *Campylobacter* employed in my study provides a better, more direct indicator of cell viability because an active response is elicited and detected rather than relying on indirect analyses that detect existing mRNA for the assessment of bacterial viability. The last reason for choosing the *hsp*60 gene is that some sequence information of *hsp*60 of *Campylobacter* is available in the GenBank database (National Center for Biotechnology Information).

2.2 SNP as species marker

A single nucleotide polymorphism (SNP) is a single base change in a DNA sequence. SNPs are common DNA sequence variations among human individuals. They can be substitutions, deletions, or insertions in DNA, as shown in Table 2.1. If two human chromosomes are compared, SNPs can be encountered about every 1000-bp. SNPs can occur throughout the genome, in both coding and noncoding regions. Silent mutations create a new codon that still codes for the same amino acid. SNPs in intronic sequences may have no effect or may affect splicing, while SNPs in regulatory regions may affect translation. SNPs may occur between genes.

Table 2.1 Examples of single nucleotide polymorphisms

SNP type	Example of sequence
Wildtype DNA	3'-AAA GCT ACC TAT CGG TTA-5' 5'-TTT CGA TGG ATA GCC AAT-3'
Substitution	3'-AA**T** GCT ACC TAT CGG TTA-5' 5'-TT**A** CGA TGG ATA GCC AAT-3'
Insertion (frameshift)	3'-AAA GCT ACC **A**TA TCG GTT A-5' 5'-TTT CGA TGG **T**AT AGC CAA T-3'
Deletion (frameshift)	3'-AAA _CT ACC TAT CGG TTA-5' 5'-TTT _GA TGG ATA GCC AAT-3'

Similar to those in humans, a variety of SNPs have also been discovered in bacteria, such as the ones belonging to the category of pathogenicity-enhancing (or "pathoadaptive") mutations — genetic alterations in regulatory or structural genes that

provide a selective advantage during the course of a single infection, epidemic spread, or long-term evolution or virulence (Sokurenko et al., 1999; Weissman et al., 2003).

SNPs are utilized in diverse areas of research and the area receiving the most attention is their use as genetic markers (Vignal et al., 2002; Ellis, 2000). Regardless of the consequences caused by the presence of different SNPs, some SNPs in bacteria are carried by individual isolates, so that they may be isolate-specific. However, some SNPs have been found to be species-specific, carried by all members of the same species. This phenomenon provides a tool for SNP-based identification of bacteria at the species level.

A comparison of the partial coding sequences of the *hsp*60 gene of *C. jejuni* strain NCTC 11168, *C. coli* strain NCTC 11353, and *C. lari* strain ATCC 35221 obtained from GenBank revealed that 81 naturally occurring point variations exist within this region of the genome of *Campylobacter*. Some of them are species-specific and can be used as species markers for the differentiation of three species by hybridization with corresponding reporters that are specifically designed according to the chosen SNP.

2.3 Obtaining more *hsp*60 gene sequences from outsources

The information about the *Campylobacter hsp*60 gene in GenBank is very limited, and often the sequences have not been validated. This limited sequence information may have an impact on the correct detection of *Campylobacter* species if the species-specific SNPs were selected based solely on such limited information. To improve the accuracy of the identification of appropriate SNPs and the reliability of the assay, the *hsp*60 gene sequence information from a number of *Campylobacter* isolates were acquired and used in conjunction with the recorded information from GenBank.

2.4 Electronic DNA microarray as assay platform

DNA microarray is a relatively new technology that advances the detection of sequence-specific nucleic acid hybridization. The core principle of this technology is based on the fact that every nucleic acid strand carries the capacity to recognize complementary sequences through base pairing.

In essence, DNA microarrays are miniaturized reverse dot-blots, but in a high-throughput format (Call et al., 2003; Ye et al., 2001; Straub and Chandler, 2003; Theron and Cloete, 2004). Sequence-specific "probes" (genomic DNA, cDNA, or oligonucleotides) are immobilized either mechanically or by using photolithographic techniques on glass microscope slides or nylon membranes through covalent binding or electrostatic interaction between the phosphate backbone and the chemically treated substrate surface. Probes appear as "spots" in the final image in which each spot represents a unique probe sequence; spots are usually 100–200 μm in size and are located within 200–500 μm of each other. "Targets" are applied to the array and the hybridization of targets with complementary probes is detected by the radioactive or fluorescent tags with which the targets are labeled. Fluorescent labeling is more widely used now and usually employs the cyanine dyes Cy3 (green excitation) and Cy5 (red excitation) because of their high molar absorptivity, wavelength selectivity, and resistance to photobleaching.

General applications of DNA microarrays in prokaryotic systems include exploring genome-wide transcriptional activity, defining a regulon (commonly referred to as the set of genes controlled by a regulator), delineating operon structure, investigating unknown DNA regions, DNA-protein interactions, comparative genomics, and genotyping.

Specific applications include the determination of virulence factors of microbial pathogens and host responses to pathogens or resident microflora; gene expression profiles of drugs, inhibitors, and toxic compounds; and analyses of microbial evolution and epidemiology (Ye et al., 2001). Recently, researchers began to apply microarray technology to compare interstrain variations at the genomic level in bacteria (Dorrell et al., 2001; Behr et al., 1999) and to investigate its potential for genotypic characterization and identification of pathogenic bacteria (Keramas et al., 2003; Ahn and Walt, 2005).

However, for SNP-based detection, the conventional DNA microarray may not be a good choice. This is mainly because the conventional DNA microarray (1) is unable to specifically address different targets to different spots on the array, making multiple sample analysis unachievable; (2) often requires overnight passive hybridization, which greatly increases the analysis time; (3) cannot control the precise stringent conditions and processes for removing non-specific hybridization, and thus these assays may not be specific.

Therefore, an electronic DNA microarray system produced by Nanogen (San Diego, CA, USA) was chosen to facilitate my research project. This array system can produce a defined electric field to allow charged molecules, such as DNA, to be rapidly transported and concentrated to any electronically activated test site (called a "spot") on the microarray chip. This process is also called "addressing." In this way, the molecular binding onto the chip is accelerated and occurs faster than traditional passive methods. Furthermore, by applying thermal stringency, a process of controlling the

temperature of the electronic microarray fluid environment, non-specifically bound and mismatched bound DNA can be removed after hybridization.

Specifically for the SNP-based identification of *Campylobacter*, the electronic DNA microarray provides the following advantages: (1) by activating specific spots in different steps, different target molecules can be addressed to designated test sites, which makes it possible to address two different targets from two different sets of PCR reactions to different specific spots for their specific identification; (2) electronic addressing makes fast detection possible by accelerating the molecular binding onto the chip; (3) electronic addressing concentrates target molecules on test sites, which in turn improves the detection sensitivity; (4) thermal stringency minimizes cross and/or non-specific reporter binding, which greatly improves the identification accuracy, particularly for SNP-based assays; and (5) due to the high spot density (100 or 384 test sites/chip), a high throughput will be possible.

The overview of the strategy involved in this project is illustrated in the following block diagram (Figure 2.1).

2.5 Biosafety Requirements

Campylobacter jejuni, *C. coli*, *C. lari*, and some of the bacterial samples are pathogenic. Prior to the study, the persons involved in this study were trained and certified by the Biosafety Office at the University of Alberta. The handling and preparation of all cultures and samples was carried out strictly in a biohazard hood. The wastes from the experiments were autoclaved and disposed according to the biosafety procedures. The biosafety precautions were taken during the entire study.

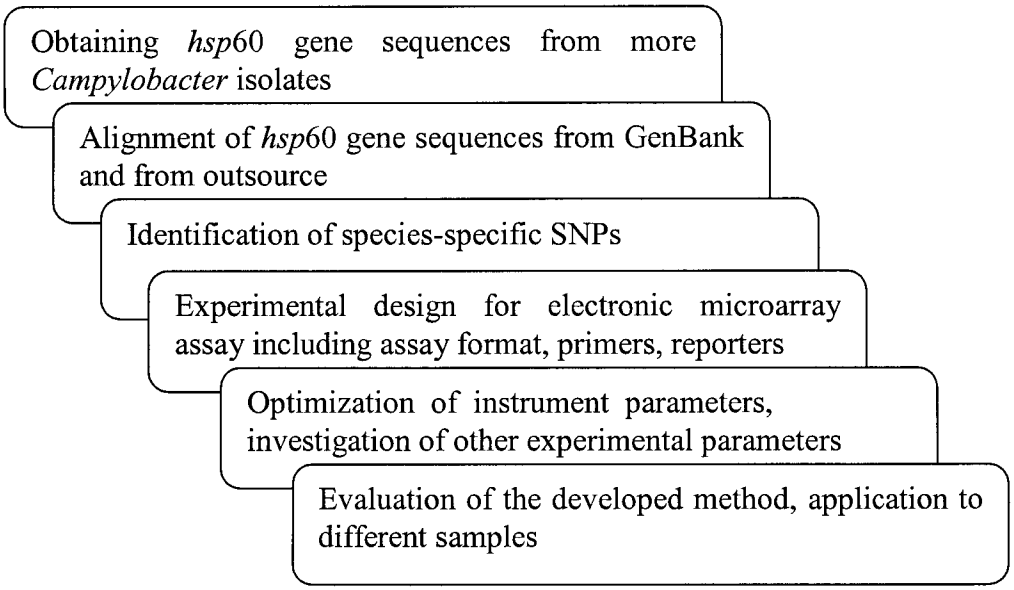


Figure 2.1 Block diagram showing the steps involved in this research project

CHAPTER 3. ACQUISITION OF *hsp*60 GENE SEQUENCE OF TWELVE ISOLATES OF *CAMPYLOBACTER JEJUNI*, *C. COLI*, AND *C. LARI*, AND IDENTIFICATION OF SPECIES-SPECIFIC SNP MARKERS

As discussed in the previous chapter, acquisition of more *hsp*60 gene sequences was required in order to improve the accuracy of the identification of species-specific SNPs. This chapter describes the methods and procedures for the acquisiotion of the *hsp*60 gene sequences of 12 *Campylobacter* isolates and the identification of the species-specific SNPs.

3.1 Materials

Campylobacter isolates were received as a generous gift from the Agri-Food Systems Branch of the Food Safety Division, Alberta Agriculture, Alberta, Canada. Primers and reporters were synthesized by Integrated DNA Technologies (IDT) (Coralville, IA, USA). The primers were ordered with standard desalting process while the reporters were ordered with HPLC purification. Both the primers and the reporters were prepared in sterilized ddH_2O and stored at -20 °C. The distilled deionized water was autoclaved before use throughout this project; it is referred to as ddH_2O in this thesis. Zero Blunt® PCR cloning kit, One Shot® *E. coli* TOP10 competent cells, Platinum® *Taq* DNA polymerase, Superscript^{III} RNase H^- reverse transcriptase, Platinum® *Pfx* DNA polymerase, UltraPure^{Tm} agarose powder, and other molecular biology reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA), and stored as

recommended by the manufacturer. QIAquick PCR Purification kit, QIAprep Spin Miniprep kit, and QIAquick Gel Extraction kit were purchased from Qiagen (Valencia, CA, USA), and stored at room temperature.

3.2 Experiments

3.2.1 Obtaining the hsp*60 gene sequence*

In order to identify with certainty the unique SNPs specific to *Campylobacter* species, *hsp*60 gene sequences of 12 *Campylobacter* isolates were analyzed. The processes of analysis included the heat shock induction of their *hsp*60 mRNA, extraction of total RNA, RT-PCR, cloning, and sequencing. These procedures are described below in detail.

3.2.1.1 Heat shock induction of *hsp*60 mRNA and total RNA extraction

Of the 12 *Campylobacter* isolates received, three isolates (797, 772, and 456) were *C. coli,* three (803, 796, and 787) were *C. lari*, and the other six (782, 783, 512, 542, 578, and 579) were *C. jejuni*. The *C. jejuni* isolates were categorized into three pairs based on patterns obtained using pulsed-field gel electrophoresis (PFGE). Isolate 782 paired with isolate 783, 512 with 542, and 578 with 579. Each pair shared similar electrophoresis behavior but was distinctive from the other pairs.

The cells from each isolate were inoculated in sterile tryptic soy broth (TSB) to a 3.0 McFarland standard, which gives a concentration of 9×10^8 CFU/mL. In microbiology, McFarland standards are used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range. Original

McFarland standards were made by mixing specified amounts of barium chloride and sulfuric acid together. Mixing the two compounds together forms a barium sulfate precipitate, which causes turbidity in the solution. The standard can be compared visually to a suspension of bacteria in sterile saline.

An aliquot of 5 μL of each isolate in TSB was transferred to a 1.5 mL centrifuge tube containing 1 mL of TSB, and incubated in a 48 °C water bath for 25 min to heat shock the organisms and allow them to induce *hsp*60 mRNA. The cell pellets were collected after centrifugation with 12,000 × g for 10 min at 4 °C. The supernatant was then removed, and 1 mL of Trizol reagent was added to each tube and mixed by pipetting up and down several times. The homogenized cells were then incubated at room temperature for 5 min to permit the complete dissociation of nucleoprotein complexes according to the protocol provided by the manufacturer. The samples were then stored at -80 °C before the total RNA was extracted. To investigate the effect of different amounts of starting cells, two separate extractions using 50 µL and 100 µL of the cell culture of each isolate were also treated following the same procedure described above, and stored at -80 °C. To clarify the the effect of culture medium on the extraction, 15 µL of each isolate in TSB was diluted in 1 mL of ddH_2O before heat shock, the other treatment procedures identical to those described above.

To extract total RNA, the homogenized samples stored at -80 °C were thawed at room temperature. After 0.2 mL of chloroform was added, the vial was capped securely and shaken vigorously by hand for 15 seconds, and then incubated at room temperature for 3 min, and centrifuged at 11,000 × g for 15 min at 4 °C. After centrifugation, the supernatant containing the total RNA was transferred carefully by pipetting into a new

tube. The total RNA solution was mixed with 0.5 mL of isopropyl alcohol and centrifuged at 11,000 × g for 10 min at 4 °C. The resulting RNA pellet was washed once with 1.1 mL of 75% ethanol, followed by vortexing for 5 seconds and centrifugation at 7200 × g for 5 min at 4 °C. The RNA pellet was briefly air-dried for 5–10 min, and was then dissolved in 50 μL of Rnase-free H_2O, mixed by pipetting up and down. The resulting solution was incubated at 55–60 °C for 10 min to let the RNA dissolve completely and was stored at -80 °C for future use.

To obtain the concentration and purity of the extracted RNA solution, the absorbance of the diluted RNA solution was measured at 260 nm and 280 nm using a SmartSpec™ 3000 spectrophotometer (Bio-Rad Latoratiories, Hercules, CA, USA). Here, the absorbance at 260 nm (A_{260}) was used to determine the RNA concentration, which was calculated by multiplying the reading at A_{260} by the conversion factor for RNA (1.0 unit of A_{260} = 40.0 μg/mL), and the absorbance at 280 nm (A_{280}) was used to determine the protein concentration in the solution. The ratio of A_{260} over A_{280} ($A_{260/280}$) was used to examine the purity of the extracted RNA samples. Table 3.1 provides the $A_{260/280}$ values of the extracted RNA samples.

As stated in the Trizol protocol, when the isolated RNA was diluted into TE buffer, a ratio of $A_{260/280} \geq 1.8$ suggests the least protein contamination in the extracted total RNA sample. However, due to the concern that the EDTA from the TE buffer might inhibit the subsequent PCR reactions, the isolated RNA was dissolved in RNase-free H_2O instead of TE buffer. As also mentioned in the protocol, the lower ionic strength and lower pH of the solution will increase the absorbance at 280 nm, so the $A_{260/280}$ ratio of RNA sample may drop under 1.65.

Table 3.1 Summary of total RNA extracted from different amounts of *Campylobacter* cells

Samples	15μL + 1mLH_2O	15μL	50μL	100μL	15μL + 1mLH_2O	15μL	50μL	100μL
Cell No. (million)	13.5	13.5	45	90	13.5	13.5	45	90
	***C. coli* 797**				***C. coli* 772**			
A260/280	1.7740	1.5626	1.2746	1.6943	1.5564	1.5980	1.7801	1.6705
Conc. (μg/mL)	15.04	19.17	72.87	17.59	18.29	18.84	17.86	31.50
	***C. coli* 456**				***C. lari* 787**			
A260/280	1.8516	1.6345	1.8395	1.6418	1.8286	1.5547	lost by	1.7638
Conc. (μg/mL)	20.36	20.42	17.54	49.02	13.42	18.14	mistake	30.96
	***C. lari* 796**				***C. lari* 803**			
A260/280	1.6168	1.5840	1.7267	1.5361	1.7981	1.7095	lost by	1.8105
Conc. (μg/mL)	17.01	13.44	97.41	24.06	15.31	29.50	mistake	31.08
	***C. jejuni* 542**				***C. jejuni* 512**			
A260/280	1.6465	1.5859	1.9587	1.7202	1.4651	1.7236	1.7365	1.6147
Conc. (μg/mL)	20.31	20.02	13.86	11.53	9.95	23.64	17.81	26.10
	***C. jejuni* 782**				***C. jejuni* 783**			
A260/280	1.5401	0.5227	0.8012	1.6339	1.5835	1.6841	1.7193	1.7250
Conc. (μg/mL)	152.05	63.62	242.91	12.21	17.55	24.47	23.72	20.92
	***C. jejuni* 578**				***C. jejuni* 579**			
A260/280	1.5479	1.6260	1.4689	1.6600	1.6836	1.8890	lost by	1.6881
Conc. (μg/mL)	17.14	18.31	20.59	22.29	19.18	15.28	mistake	40.93

From this table, a good correlation was not observed between the amount of starting cells and the amount of extracted total RNA from the comparison of the RNA concentrations obtained from 15 μL, 50 μL, and 100 μL cell cultures. No obvious effect of the medium (TSB) on the extraction was observed. This was concluded by the comparison of RNA concentrations obtained from 15 μL cell culture and 15 μL cell

culture diluted in ddH_2O before heat shock, which was shown as 15 μL and 15 μL+1 mL ddH_2O in Table 3.1, respectively. Variations in the extracted total RNA concentration are clearly shown in Table 3.1. The possible key contributory factor may be the variation from operation to operation.

The total RNA samples with higher $A_{260/280}$ ratios and higher concentrations were used for the method development.

3.2.1.2 Reverse transcription-polymerase chain reaction (RT-PCR)

After the total RNA extraction, *hsp*60 mRNA was reverse transcribed into corresponding cDNA, followed by polymerase chain reaction amplification.

3.2.1.2.1 RT-PCR primer design

Partial coding sequences (556-nt) from the *hsp*60 gene of *C. jejuni* strain NCTC 11168 (Accession # AY044099), *C. coli* strain NCTC 11353 (Accession # AY044098), and *C. lari* strain ATCC 35221 (Accession # AY044100) from GenBank (National Center for Biotechnology Information) were aligned and the corresponding primers were designed to be specific to each species to amplify a fragment of 460-bp for *C. jejuni* and *C. coli*, and a fragment of 446-bp for *C. lari*, as shown in Table 3.2.

Table 3.2 Designed RT-PCR primers

Primer		Sequence	Length (nt)	Tm (C)	Position
C. jejuni	F	5' - GCA GGT GCA AAT CCT ATC G - 3'	19	63.2	54→72
	R	5' - AAG TTG CAA GCG CTT CAC C -3'	19	65.4	495←513
C. coli	F	5' - GCT GGA GCA AAT CCT ATC G - 3'	19	62.3	54→72
	R	5' - AAG TTG CAA GTG CTT CAC C -3'	19	60.5	495←513
C. lari	F	5' - GCA GGT GCT AAT CCT ATC G -3'	19	59.8	54→72
	R	5' - TCA CCT TCA ATG TCT TCA GC - 3'	20	60.4	480←499

Notes: F: forward primer, R: reverse primer, nt: nucleotide, Tm: melting temperature.

All primers were analyzed for similarity with gene sequences recorded in GenBank using the Basic Local Alignment Search Tool (BLAST) at www.ncbi.nlm.nih.gov. The sequences of all organisms were analyzed with much more attention paid to the gene sequences of microorganisms. The BLAST results showed that the designed primers were specific to the target *Campylobacter* species. In addition, the secondary structures of the primers were assessed using the OligoAnalyzer 3.0 software available on-line at www.idtdna.com. The types of secondary structures included hairpins and primer-duplexes; the latter are further divided into homo-duplexes and hetero-duplexes. The results indicated that these secondary structures do not exist at the temperature of PCR reactions. All primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

3.2.1.2.2 DNase treatment

Prior to reverse transcription, the total RNA extracted from the *Campylobacter* cells was treated with DNase I to eliminate possible DNA contamination that may occur during the extraction. The detailed procedures of the DNase treatment are described as follows: 1 μg total RNA sample, 1 μL of 10× DNase I Reaction Buffer, 1 μL of DNase I (1 U/μL), and 7 μL of Rnase-free H_2O were added to an RNase-free micro-centrifuge tube (0.2 mL) placed on ice. After the mixture was incubated at room temperature for 15 min, the DNase I was inactivated by the addition of 1 μL of 25 mM EDTA solution to the reaction mixture and heated at 65 °C for 10 min. The DNase-treated RNA sample was then ready to use in reverse transcription. Duplicates of RNA samples were prepared in this step, one for regular RT and the other for parallel RT (designated as RT_0); RT_0 has no reverse transcriptase added during the preparation in the RT procedure described below, in order to check the efficiency of the DNase treatment's removal of the possible DNA contamination in the RNA sample.

3.2.1.2.3 RT

SuperScript RNase H^- Reverse Transcriptase was used for the first-strand cDNA synthesis from the DNase-treated total RNA solution. In a 0.2 mL nuclease-free micro-centrifuge tube, 20 μL of the RT reaction solution was prepared. The regular RT solution contained 11 μL of DNase-treated RNA solution for regular RT; 0.6 μL of Gene-specific Reverse Primer Mix (containing all three species-specific reverse primers, 5 μM for each); 0.4 μL of 25 mM dNTP; 4 μL of 5× First-Strand Buffer; 2 μL of 0.1 M DTT; and 1 μL of RNaseOUT™ Recombinant Ribonuclease Inhibitor (40 units/μL). The RT_0 solution was prepared in the same way as the regular RT solution. For the negative

control of the RT reaction, 11 μL of Rnase-free H2O was used instead of the RNA sample; all the other reagents were the same as for the regular RT solution. The contents were mixed gently and incubated at 42 °C for 2 min. Then 1 μL (200 units) of SuperScript Reverse Transcriptase was added to the regular RT solution and to the RT negative control solution. To the parallel RT (RT_0) solution, 1 μL of ddH_2O was added instead. Each solution was mixed by pipetting gently up and down, and was incubated at 42 °C. After 50 min incubation, the reverse transcriptase was inactivated by heating the reaction solutions at 70 °C for 15 min. Then, the cDNA was ready to be used as the template for PCR amplification.

3.2.1.2.4 PCR

Platinum® *Pfx* DNA polymerase was used to amplify the 460-bp targets for *C. jejuni* and *C. coli*, and the 446-bp target for *C. lari*. RT solution was used directly as the template, and the same amount of ddH_2O was used as the template for PCR negative control. Each 50 μL of PCR reaction contained 5 μL of 10× *Pfx* amplification buffer, 1 μL of 50 mM $MgSO_4$, 6 μL of dNTP mixture (2.5 mM of each), 1.5 μL of 10 μM forward primer, 1.5 μL of 10 μM reverse primer, 1.0 μL of Platinum® *Pfx* DNA polymerase (2.5 units), 2 μL of the template, and 32 μL of ddH_2O.

All the contents were mixed and incubated in a thermal cycler (either a PTC-100™ Programmable Thermal Controller (MJ Research, Waltham, MA, USA) or a GeneAmp® PCR System 2700 (Applied Biosystems, Foster City, CA, USA)) at 94 °C for 3 min to completely denature the template and restore enzyme activity by destroying the bound Platinum® antibody.

Forty cycles of PCR amplification were performed using the following temperature program: denaturation at 94 °C for 15 s; followed by 30 s annealing at 58.2 °C for *C. jejuni*, 50.5 °C for *C. coli*, and 54.8 °C for *C. lari*, and extension at 72 °C for 30 s. The cycling included a final extension step at 72 °C for 10 min to ensure full extension of the product. The PCR products were analyzed by 1% agarose gel electrophoresis and visualized on an ultraviolet (UV) illuminator (SYNGENE, Cambridge, UK).

To check the DNase treatment efficiency, the DNase-treated RT_0 solution, which did not contain the reverse transcriptase, was also used directly as the template in a PCR reaction. If DNA contamination was completely removed, there should be no amplification from this reaction.

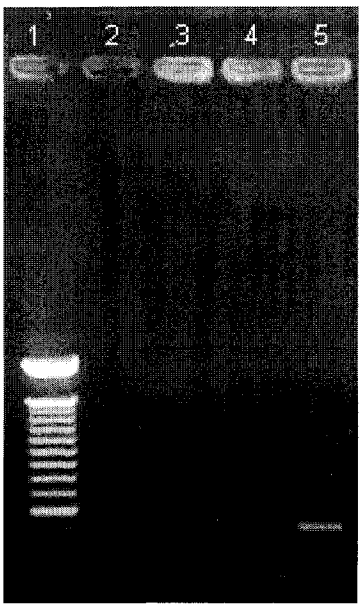


Figure 3.1 Agarose gel electrophoresis of PCR products

Lane assignment: Lane 1, 100 bp DNA ladder; Lane 2, PCR negative control; Lane 3, RT_0; Lanes 4-5, *C. jejuni* targets.

Figure 3.1 shows an example of the RT-PCR results. No band is visible in Lane 2, which indicates that there was no contamination during the PCR processes. No visible band in Lane 3 indicates that any possible DNA contamination was completely removed

from the total RNA sample. Lanes 4 and 5 show the *C. jejuni*-782 target bands amplified from 1 μL and 2 μL of RT solution, respectively. (In the original picture, the band in Lane 4 was weak due to the smaller amount of starting RT solution, but scanning the photograph rendered it invisible.) These results confirmed that the PCR products were generated from the RNA, but not from genomic DNA.

3.2.1.3 Cloning and sequencing

3.2.1.3.1 Gel extraction and purification

Prior to cloning, the PCR products were purified using agarose gel. The extraction of the purified DNA of the PCR products was conducted using the QIAquick Gel Extraction kit following the manufacturer-recommended protocol.

3.2.1.3.2 Cloning

Zero Blunt® PCR Cloning Kit (pCR®-Blunt Vector) was used to clone the desired DNA fragments. This kit was designed to clone blunt PCR fragments with a low background of non-recombinants. The pCR®-Blunt Vector allows direct selection of recombinants via disruption of a lethal *E. coli* gene, the *ccdB* gene, which is fused to the C-terminus of *LacZα*.

The *ccdB* gene is found in the *ccd* (control of cell death) locus on the F plasmid. This locus contains two genes, *ccdA* and *ccdB*, which encode proteins of 72 and 101 amino acids, respectively. The *ccd* locus participates in the stable maintenance of the F plasmid by post-segregational killing of cells that do not contain the F plasmid. The CcdA protein acts as an inhibitor of the CcdB protein. In the absence of the CcdA protein, the CcdB protein becomes toxic as it interferes with bacterial DNA gyrase (topoisomerase II), an essential enzyme that catalyzes the ATP-dependent negative

supercoiling of DNA. Inhibition of DNA gyrase results in DNA breakage and cell death (Invitrogen, 2004).

Ligation of a blunt PCR fragment disrupts the expression of the *lacZα-ccdB* gene fusion, permitting growth of only positive recombinants upon transformation. Cells that contain non-recombinant vectors are killed when the transformation mixture is plated. The vector also contains the kanamycin resistance gene for selection of *E. coli.* Blunt PCR products can be ligated directly without purification to achieve cloning efficiencies ranging from 80% to 95%. There is no need for primer modification or primer design constraints. The vector is supplied linearized and blunt-ended at a unique site in the polylinker. The map of the vector is shown in Figure 3.2.

To clone the obtained PCR products, the purified PCR products were ligated into a pCR-Blunt® vector by following the procedures described below. To obtain satisfactory ligation efficiency, an insert-to-vector molar ratio ranging from 10:1 to 100:1 was recommended by the manufacturer. Based on the estimated concentrations of the purified PCR products, 5 μL of the purified PCR products from each isolate and 1 μL of pCR®-Blunt vector (25 ng) were used to prepare 10 μL of ligation reaction with an insert-to-vector molar ratio of 14:1. The ligation reaction solution also contained 1 μl of 10× Ligation Buffer (with ATP), 2 μl of ddH_2O, and 1 μL of T_4 DNA Ligase (4 U/μl); it was incubated at 16 °C for 1 hour and then centrifuged briefly. The recombined vector was then ready to be transformed into One Shot® *E. coli* TOP10 competent cells. The transforming procedures are explained in detail below.

One 50 μl vial of One Shot® TOP10 cells for each ligation/transformation was thawed on ice. An aliquot of 2 μl of each ligation reaction was pipetted into the vial

containing the competent cells and mixed by stirring gently with the pipette tip. The remaining ligation mixtures were stored at -20 °C.

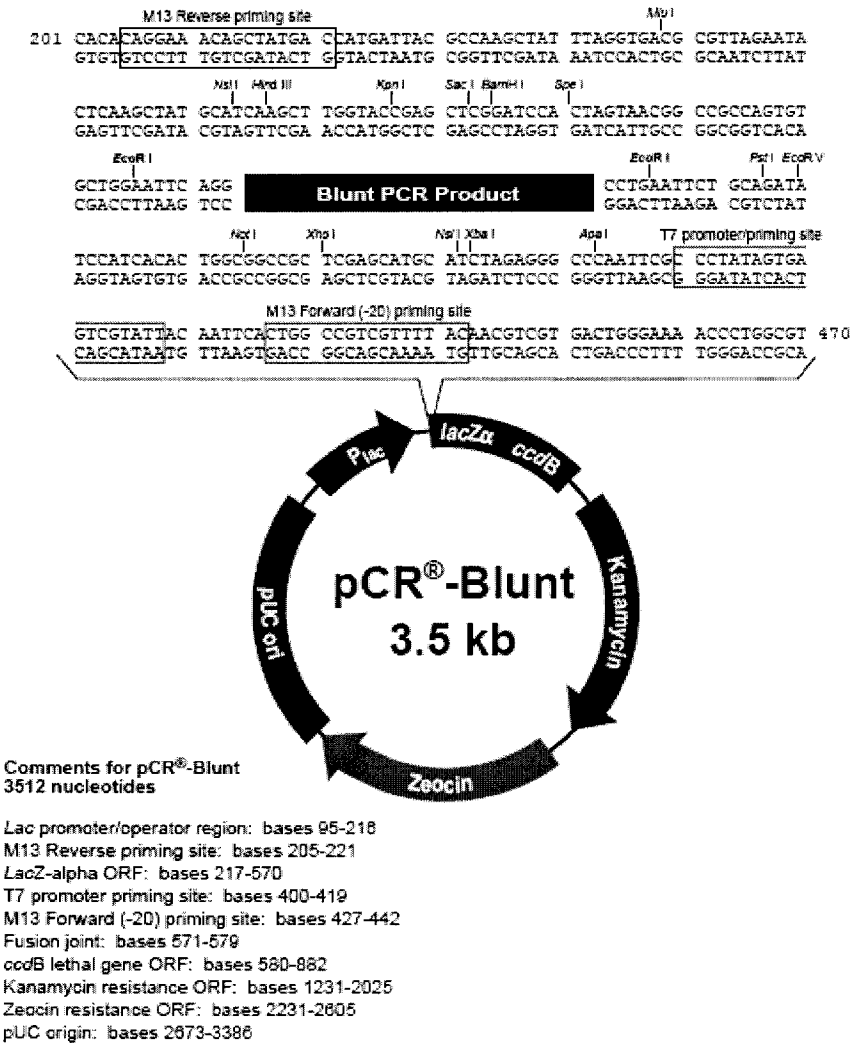


Figure 3.2 Map of pCR-blunt vector

The cells were incubated on ice for 30 min, followed by incubation at 42 °C in a water bath for exactly 45 s without mixing or shaking, and then the mixture was quickly placed on ice for 2 min. To each vial, an aliquot of 250 μl of pre-warmed (37 °C) rich

medium (containing 2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) was added. The vials were shaken at 225 rpm in a refrigerated incubator shaker (InnOva^{Tm} 4230, New Brunswick Scientific, Edison, NJ, USA) at 37 °C. After 1 hour, the vials with the transformed cells were placed on ice. Aliquots of 50 μl and 100 μl from each vial containing the transformation cells were spread individually on LB agar plates containing 50 μg/mL kanamycin. Two different volumes were plated to ensure well-spaced colonies. The plates were inverted and incubated at 37 °C overnight. The cells that contained recombinant vectors formed colonies on selective medium due to the disruption of the expression of the lethal gene by the insertion of blunt PCR fragments, while the cells that contained non-recombinant vectors were killed.

After incubation, 15 antibiotic-resistant single-colonies of transformants of each isolate were selected, and inoculated into 2 mL of LB containing 50 μg/mL kanamycin to grow at 37 °C overnight with shaking at 225 rpm. Plasmids containing the desired DNA fragments were isolated using the QIAprep Spin Miniprep Kit, and analyzed by restriction digestion with *EcoR* I to confirm the presence of cloned inserts of the desired DNA fragments. The inserts released from the plasmids were observed on 1% agarose gel, shown in Figure 3.3.

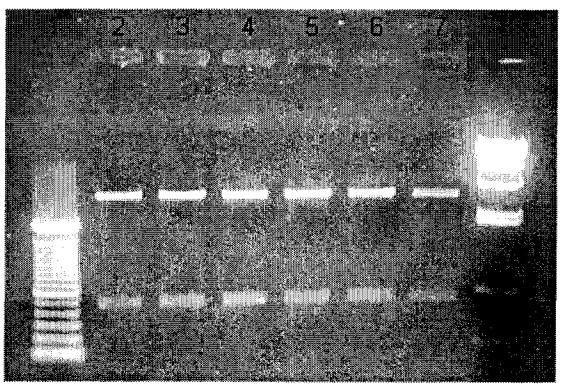


Figure 3.3 Agarose gel of digested plasmids

Lane assignment: Lane 1, 100 bp DNA ladder; Lane 8, λ DNA/HindIII ladder; Lanes 2-5, *C. coli* plasmid digested with *EcoR* I; Lanes 6-7, *C. jejuni* plasmid digested with *EcoR* I. Bands of ~450 bp are the inserts released from the plasmid which is 3.5 kb in length.

The isolated and purified plasmid DNA from the two colonies of each isolate was sent for sequencing separately.

3.2.1.3.3 Sequencing

Automated DNA sequencing was performed using a Beckman Coulter CEQ2000XL DNA sequencing system (Beckman Coulter, Fullerton, CA, USA). The Sanger dideoxynucleotide termination sequencing protocol was used. Thermo-cycle sequencing reactions were resolved and read in a single lane of capillary electrophoresis. Figure 3.4 shows a partial sequencing result for *C. coli*-456 as an example. The obtained *hsp*60 gene sequences of the 12 isolates are shown in the appendix.

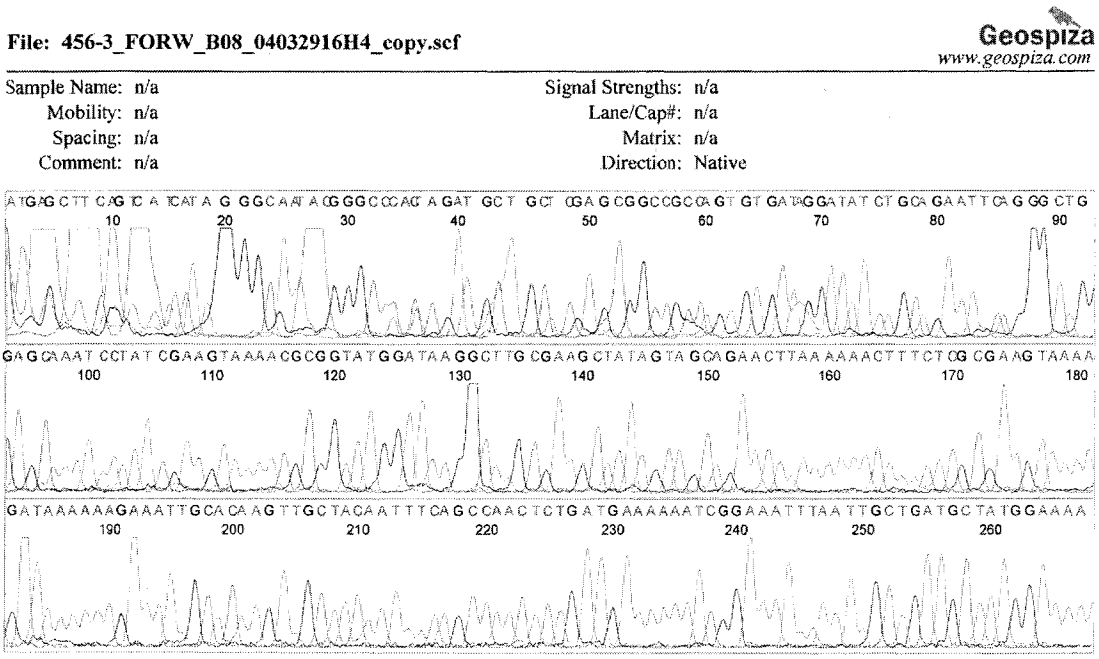


Figure 3.4 Partial sequencing result for *C. coli*-456

Two plasmid samples of each isolate were analyzed and sequenced at the DNA Core Services Lab at the Department of Biochemistry, University of Alberta. The parallel

sequencing results were compared and they were considered to be accurate when the two sequences were identical. The *hsp*60 gene sequences obtained from the 12 *Campylobacter* isolates were combined with the *hsp*60 gene sequences in GenBank for sequence alignment and SNPs identification, on this basis a further microarray method was developed.

3.3 Results and discussion

3.3.1 Alignment of sequences

All the sequencing results from the 12 isolates were aligned for comparison using the ClustaIW version 1.82 software (EMBL-EBI, European Bioinformatics Institute, online at www.ebi.ac.uk). The three *C. coli* isolates, 456, 772, and 797 have an identical sequence, and the three *C. lari* isolates, 787, 796, and 803 also have an identical sequence. The six isolates of *C. jejuni* showed two sequences. The first one was seen in isolates 512 and 542, while the other was seen in four isolates, 578, 579, 782, and 783.

The sequences of *C. jejuni*-512 and 782, *C. lari*-796, and *C. coli*-456 were used as representative to compare with the sequences of *C. jejuni* NCTC 11168, *C. coli* NCTC 11353, and *C. lari* ATCC 35221 in GenBank. The first group of *C. jejuni*, represented by *C. jejuni*-512, matches 99.6% with *C. jejuni* NCTC 11168; the second group, represented by *C. jejuni*-782, matches100% with *C. jejuni* NCTC 11168. The *C. coli* group has a 95.9% similarity with *C. coli* NCTC 11353 and the *C. lari* group has a 99.1% similarity with *C. lari* ATCC 35221.

3.3.2 Identification of species-specific SNPs

The comparison of sequencing data demonstrated the genetic variability among the isolates. Those isolate-specific variants were avoided when designing oligonucleotides for the SNP-based detection of microorganisms at the species level. Four SNPs were found to be appropriate to serve as species markers based on sequence alignment and comparison. They are located at positions 183, 291, 294, and 408. At these positions, the base compositions are exactly the same within each species but are distinct between species. Two closely located SNPs at 291 and 294 were chosen as species markers for developing the detection method on an electronic DNA microarray; a higher specificity was provided by using two close SNPs rather than one single SNP. Part of the sequence alignment of the *Campylobacter hsp*60 gene is shown in Figure 3.5 with the SNPs chosen as species markers displayed in frames.

Figure 3.5 Partial sequence alignment result of the *Campylobacter hsp*60 gene

Note: The sequences starting with the species names are from GenBank. The others starting with numbers represent the four types of sequencing information from the 12 isolates obtained through cloning and sequencing. They are arranged into three groups. The upper three are for *C. jejuni*, the lower two are for *C. lari*, and the two in the middle are for *C. coli*. The two SNPs shown in frames are chosen to serve as species markers because they are identical within the same species, but different between species.

In conclusion, *hsp*60 gene sequences from 12 *Campylobacter* isolates were successfully obtained through cloning and sequencing. Two species-specific SNPs were identified and chosen to serve as species markers through the alignment of *hsp*60 gene sequences from GenBank and the 12 obtained sequences.

CHAPTER 4. DEVELOPMENT OF A RT-PCR-ELECTRONIC DNA MICROARRAY TECHNIQUE FOR THE DETECTION AND SPECIES DIFFERENTIATION OF VIABLE *CAMPYLOBACTER JEJUNI*, *C. COLI*, AND *C. LARI*

This chapter describes the development and evaluation of a RT-PCR-electronic DNA microarray approach for the detection and differentiation of viable *Campylobacter jejuni*, *C. coli*, and *C. lari*. First, an introduction to the NanoChip® Molecular Biology Workstation used in this study is provided, followed by the detailed experimental design. The optimization of instrumental parameters, investigation of other experimental parameters, evaluation, and preliminary application of the developed method are also described.

4.1 Materials and instrumentation

4.1.1 Materials and reagent preparation

A high salt buffer (50 mM NaH_2PO_4-Na_2HPO_4, pH 7.4 with 500 mM NaCl) and a low salt buffer (50 mM NaH_2PO_4-Na_2HPO_4, pH 7.0) were prepared in ddH_2O, filtered through a 0.2 μm filter, and stored at room temperature. NaH_2PO_4, Na_2HPO_4, and NaCl were purchased in powder form from Fisher Scientific (Nepean, ON, Canada).

Solutions of 50 mM and 100 mM L-histidine were also prepared in ddH_2O and filtered through a 0.2 μm filter. The 100 mM L-histidine solution was divided into aliquots and stored at -20 °C for up to two months whereas the 50 mM L-histidine

solution was stored at 4 °C for up to one week. Prior to use, the conductivity of both solutions was measured using a Horiba® Twin Cond conductivity meter B-173 (Horiba, Irvine, CA, USA). The conductivity of the 50 mM L-histidine solution was kept at the optimum which was lower than 100 μS/cm, and that of the 100 mM L-histidine solution was kept at 100–200 μS/cm. L-histidine was purchased in powder form from MP Biomedicals (Aurora, OH, USA).

Target DNA was produced by RT-PCR as discussed in Chapter 3. Amplicons were purified using a QIAquick PCR purification kit following the protocol provided by the manufacturer. When needed, the purified PCR products were quantified using a lambda/Hind III DNA ladder (Invitrogen Life Technologies, Carlsbad, CA, USA) after agarose gel electrophoresis. Prior to use, target DNA was denatured to single strands by heating at 94 °C for 5 min, followed by a quick chill on ice for at least 1 min. Denatured target DNA was prepared in the 50 mM L-histidine solution. The 50 mM L-histidine solution was used as the blank. All solutions were prepared directly in designated wells on a 96-well plate (Nalgene, Rochester, NY, USA) to a final volume of 60 μL, sufficient volume to address up to 100 test sites on one cartridge.

Unless specified elsewhere, the procedures for solution preparation described above were used throughout the study. All chemicals were of analytical grade and the water used was autoclaved distilled deionized water.

4.1.2 Instrumentation, procedure, and data analysis

4.1.2.1 Instrumentation and calibration

A NanoChip® Molecular Biology Workstation (Nanogen, San Diego, CA, USA) was used as the platform for this study. The NanoChip® Workstation consists of a Loader

(Electronic Arrayer) and a Reader (Array Processor & Scanner). The Loader is used to electronically address target DNA molecules from a 96-well plate held in the Loader to designated sites on a cartridge according to a user-defined map, which tells the Loader to deliver specified sample wells to specified test sites. The Reader is used to detect the fluorescence signals generated from the reporters hybridized with the complementary target DNA fixed on the cartridge. The fluorescence detection is completed by using a two-laser system and a photomultiplier tube (PMT) as the detector. The excitation wavelength for Laser I (red laser) was 635 nm, and for Laser II (green laser) 532 nm.

NanoChip® Cartridges were used to develop microarray assays. Figure 4.1 shows the image of front and back views of the cartridge and the enlarged electronic microarray. The NanoChip® electronic microarray is the active surface where the electronically-driven addressing takes place. There are 100 individual test sites (also called spots) arrayed in a 10×10 grid on a 2 mm × 2 mm square semiconductor chip surface. Each test site is connected to an electrical contact on the bottom side of the cartridge. This contact makes electronic control possible by the Loader or the Reader. The test site size is 80 microns and the distance between the centers of two test sites is 200 microns. A thin, fully synthetic hydrogel permeation layer is the interface between the metal test sites and the solution on the electronic microarray. This layer helps to distance the DNA from damaging electrochemical reactions that occur on the chip surface. The permeation layer also contains streptavidin for the attachment of biotinylated DNA. Each test site can bind approximately 10^9 DNA fragments at a maximum.

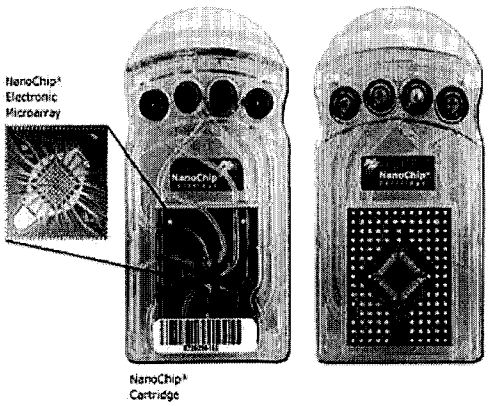


Figure 4.1 Front and back view of the electronic microchip

Source: http://www.nanogen.com/products/nanochip_mic

The performance of the NanoChip® Workstation was evaluated monthly with three verification cartridges. The Continuity cartridge was used to test the integrity of the electrical interface between the NanoChip® System and the cartridge. The specified conducting current range was 800–1200 nA. The Temperature cartridge examined the functionality of the environment process on the Reader. The accepted temperature variation was ±2 °C of the reported value. The Fluorescence cartridge was used to test the functionality of the optical detection platform (ODP) on the Reader. Low, medium, and high gain settings for the fluorescence detector's (PMT) sensitivity were tested. The monthly tests yielded results within normal specifications.

4.1.2.2 Procedure for microarray testing

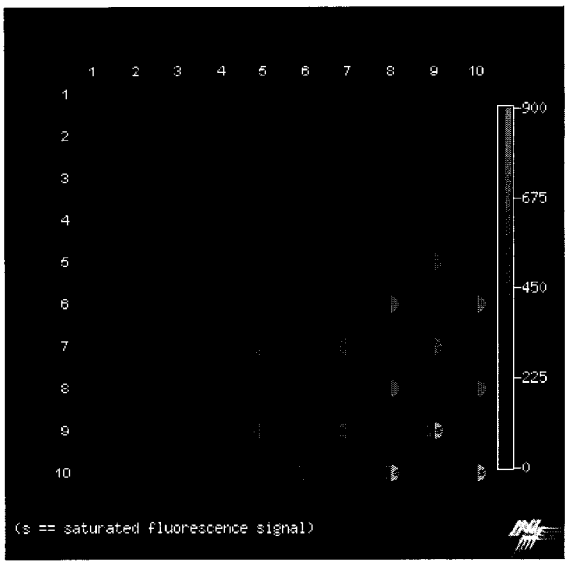
Prior to the target preparation in a 96-well plate, a file (called a Loader map file), instructing the Loader to address the contents of specific wells from the 96-well plate to the designated test sites, was created. The 50 mM L-histidine solution was included in each run to monitor background fluorescence. Once the targets were denatured and prepared in the designated wells, as described in Section 4.1.1, the target solutions and

L-histidine solution were all addressed in triplicate in a triangle pattern to account for variations in fluorescence intensity and to avoid potential interference from adjacent test sites. When the Loader map file was activated, the Loader performed a flushing step with 50 mM L-histidine to flush the chip and fill the whole chip with this solution. A conductivity test followed, which tested each spot designated for use according to the Loader map file to verify that they were properly electronically connected. After the addressing of the target molecules, another 50 mM L-histidine flushing step was included to wash away any unbound target molecules. When the addressing step was automatically completed in the Loader, the cartridge was removed from the Loader and rinsed with a high salt buffer twice in preparation for hybridization with the reporter(s).

Prior to use, the species-specific reporters were diluted to 1 μM in a high salt buffer to a final volume of 100 μL. The reporter solution was applied to the cartridge by pushing the solution through the cartridge by pipetting. The cartridge was left at room temperature for 3 min to allow the hybridization between reporters and the corresponding target strands to occur. After hybridization, the reporter solution was removed by pushing air through, and the cartridge was rinsed with a high salt buffer twice, 100 μL each time. The cartridge was then filled with a high salt buffer before being scanned for fluorescence signals in the Reader.

A Reader Nanogen Application File (NAF) was created to instruct the Reader – in practice, the fluorescence detector – to scan the test sites that had been addressed and to perform other processes, such as washing with reagents (e.g., a low salt buffer) and controlling the temperature of the assay environment on the cartridge. Once the cartridge was inserted, the Reader was calibrated by assigning coordinate points to the spots of the

four corners of the electronic microarray so that the optics could accurately locate the 100 individual spots on the microchip. Figure 4.2 shows a typical fluorescence view of the chip after scanning.



Pads are indicated according to their (X, Y) location.
(2, 8)(1, 9)(2, 10): 50 mM L-histidine;
(4, 8)(3, 9)(4, 10): 790#-1-t;
(6, 8)(5, 9)(6, 10): 790#-2-t;
(8, 8)(7, 9)(8, 10): 4#self-1-t;
(10, 8)(9, 9)(10, 10): 4#self-2-t;
(7, 5)(8, 6)(7, 7): C. coli-2-t;
(9, 5)(10, 6)(9, 7): E/C. coli mix-2-t.

Figure 4.2 View of a typical fluorescence scan result

For fluorescence scanning, its gain and duration were adjusted to achieve a fluorescence signal intensity ranging from 100 to 900 arbitrary units for all test sites except for blank and negative sites. In some cases, due to large differences in fluorescence intensity generated from testing samples, this was not achievable, but wherever possible, this guideline was observed. A fluorescence intensity over 900 indicates saturation. Due to variations from run-to-run, chip-to-chip, and spots-to-spots, the scanning parameters could not be kept constant. In most cases, they differ from one experiment to another.

4.1.2.3 Data analysis

Data analysis was performed using the built-in NanoChip® configuration software. For the analysis, fluorescence intensities from the spots addressed with 50 mM L-histidine were used as background. The fluorescence intensities of the targets were calculated from the spots addressed with the same targets. The Q-test was used to reject values that were not in agreement with the others in the set. The mean fluorescence intensity from target spots, from which the mean background was subtracted, was divided by the mean background value to calculate signal-to-background ratios (S/B). For target identification, the S/B ratio is the key factor to consider. As suggested by Nanogen and verified experimentally by us, a 3:1 ratio was considered the criterion for positive identification. Unless stated elsewhere, this criterion was used throughout the study.

4.2 Experiments

4.2.1 Dot-blot assay format and reporter design for detection and species differentiation using electronic DNA microarray

The electronic DNA microarray application has three different formats: the amplicon-down format, the capture-down format, and the dot-blot format. A brief introduction to the first two formats follows. For the amplicon-down format, a PCR-amplified biotinylated amplicon, a stabilizer (a short oligonucleotide complementary to a part of the amplified amplicon), and a fluorescently-labeled reporter are required. The biotinylated amplicon is addressed onto the chip test site first; then the stabilizer is loaded onto the site and binds to the amplicon to melt the secondary

structure, followed by passive hybridization with the reporter, which has a single SNP located at the 3' end. The capture-down format requires a biotinylated capture (also a short oligonucleotide complementary to the amplified amplicon), a PCR-amplified target, and a fluorescently-labeled reporter with a single SNP located at the 3' end. For this format, the biotinylated capture is addressed onto the chip test site first; then the target is loaded onto the site to facilitate binding and melting of the secondary structure, followed by passive hybridization with the reporter.

The dot-blot format requires only a PCR-amplified biotinylated target and fluorescently-labeled reporters. The SNP is located in the middle of the reporter. The dot-blot assay format adopted in this study is shown in Figure 4.3.

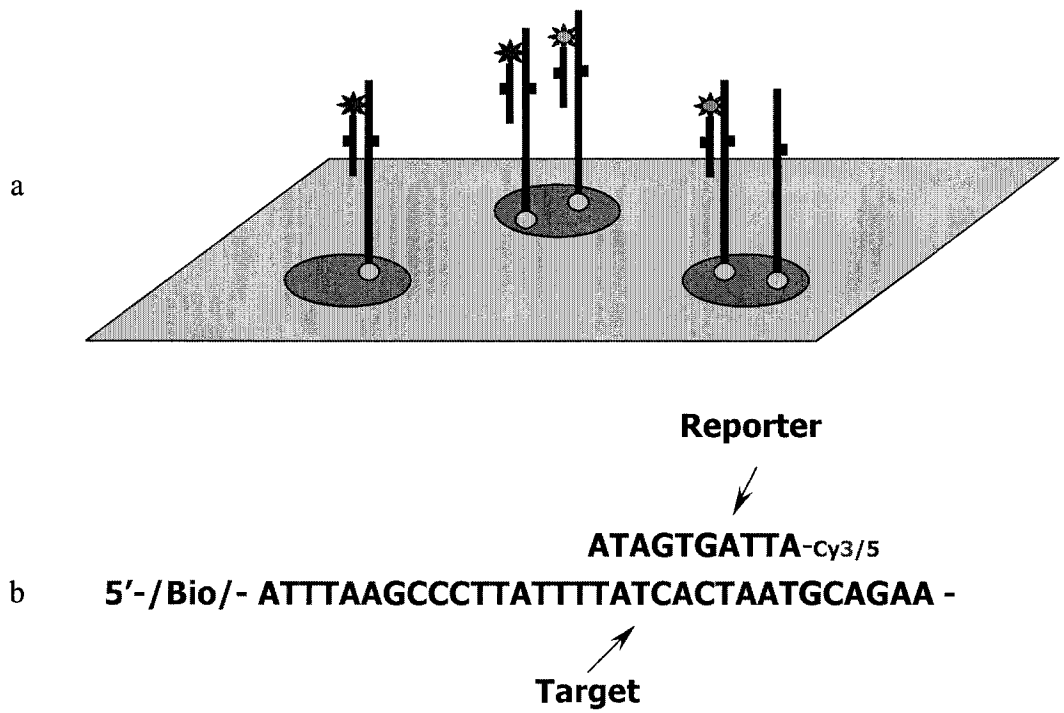


Figure 4.3 Scheme of assay format

(a) assay format on microarray chip (b) enlarged schematic of assay format showing molecules

Denatured target DNA molecules obtained through RT-PCR were addressed on a microarray chip. Biotinylated target strands were fixed onto the chip, and then incubated with species-specific fluorescently-labeled reporters so that the reporters hybridized with the corresponding targets. The target was detected and identified by analyzing the location, type, and intensity of the fluorescence signal generated.

Due to limitations of the instrument, only two fluorophores, Cy3 (green fluorescence emission) and Cy5 (red fluorescence emission), can be used to label the reporters. However, there were three species that were required to be detected simultaneously. To overcome this limitation, different strands of the *hsp*60 gene were chosen as the target strand to be amplified. One strand was chosen as the target strand and biotinylated for the detection of *C. jejuni* and *C. coli*; the other complementary strand was biotinylated as the target strand for the detection of *C. lari*. The detailed design is shown in Figure 4.4. The strand running from 3' to 5' was chosen as the target strand for the detection of *C. jejuni* and *C. coli*; the strand running in the opposite direction was used as the target strand for the detection of *C. lari*. Corresponding reporters were specifically designed according to the desired target strands' sequences. Cy3 was used to label reporters for *C. jejuni*; Cy5 was used to label reporters for *C. coli*. The reporters for the third species could be labeled with either Cy5 (generating red fluorescence) or Cy3 (generating green fluorescence). Here, for no particular reason, we used Cy3 labeling. The sequences of the reporters are summarized in Table 4.1. The bases shown in bold within the reporters are the SNPs chosen as species markers.

Table 4.1 Reporter design for microarray assay

Reporter	Sequence	Length (nt)	Tm (°C)
R-*jejuni*	5' - Cy3 - TAT CAC TAA TGC A - 3'	13	32.6
R-*coli*	5' - Cy5 - ATA ACC AAT GCA - 3'	12	32.4
R-*lari*	5' - Cy3 - ATT TGT AAT GAA ATA T - 3'	16	32.1

Notes: nt, nucleotide; Tm, melting temperature.

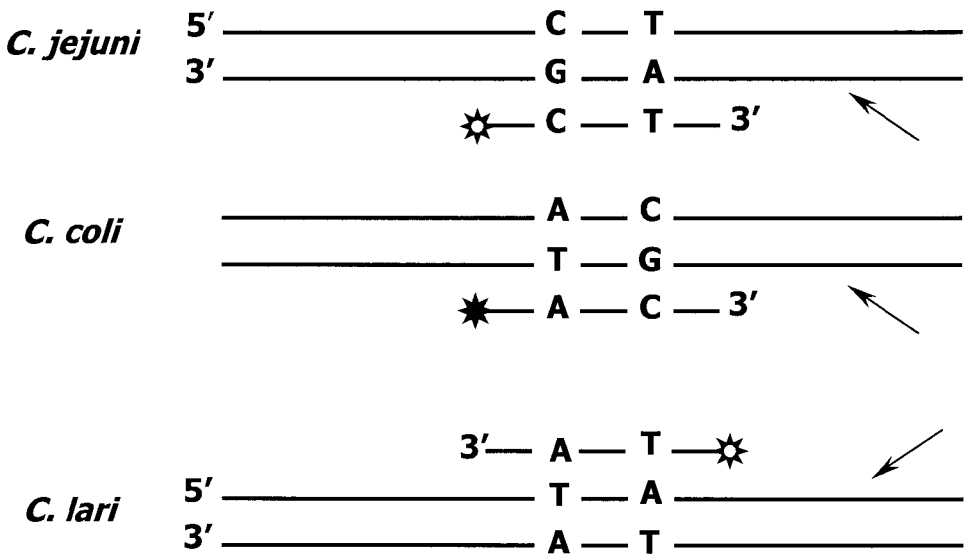


Figure 4.4 Simplified DNA sequences showing SNPs

Arrows point to the target strands chosen for detection and reporters are aligned close to corresponding target strands (hollow star indicates Cy3 labeling while solid star indicates Cy5 labeling).

4.2.2 PCR and primer design for simultaneous detection of three Campylobacter *species*

PCR amplification with biotin-labeled primers was used to facilitate the immobilization of the desired target strands on the chip. For a dot-blot assay, an amplicon

of approximately 200-bp in length is desirable. Therefore, new primers, other than the primers used for cloning, were designed for amplifying 200-bp targets. As mentioned in Section 4.2.1, two differently biotinylated targets will be required in order to simultaneously differentiate three species by one run on the microarray, and therefore two sets of PCR were employed. The only difference between these two parallel PCRs is the biotin-labeling position of the primer. In set 1, the forward primer has been biotin-labeled, while in set 2, the reverse primer has been biotin-labeled. The detailed PCR strategy is shown in Figure 4.5. The designed PCR primers are shown in Table 4.2. The sequence design of the two sets of primers and the PCR conditions were identical.

Table 4.2 Primer design for microarray

Primer		Sequence	Length (nt)	Tm (°C)	Position
Set - 1	F	5' - /Bio/ - GAA GGT ATG CAA TTT GAC AG - 3'	20	50.3	247→ 266
	R	5' - TCA CCT TCA ATA TCT TCA GC - 3'	20	50.7	427← 446
Set - 2	F	5' - GAA GGT ATG CAA TTT GAC AG - 3'	20	50.3	247→ 266
	R	5' - /Bio/ - TCA CCT TCA ATA TCT TCA GC – 3'	20	50.7	427← 446

Notes: Set-1, set-1 PCR reaction; Set-2, set-2 PCR reaction; F, forward primer; R, reverse primer; nt: nucleotide; Tm, melting temperature.

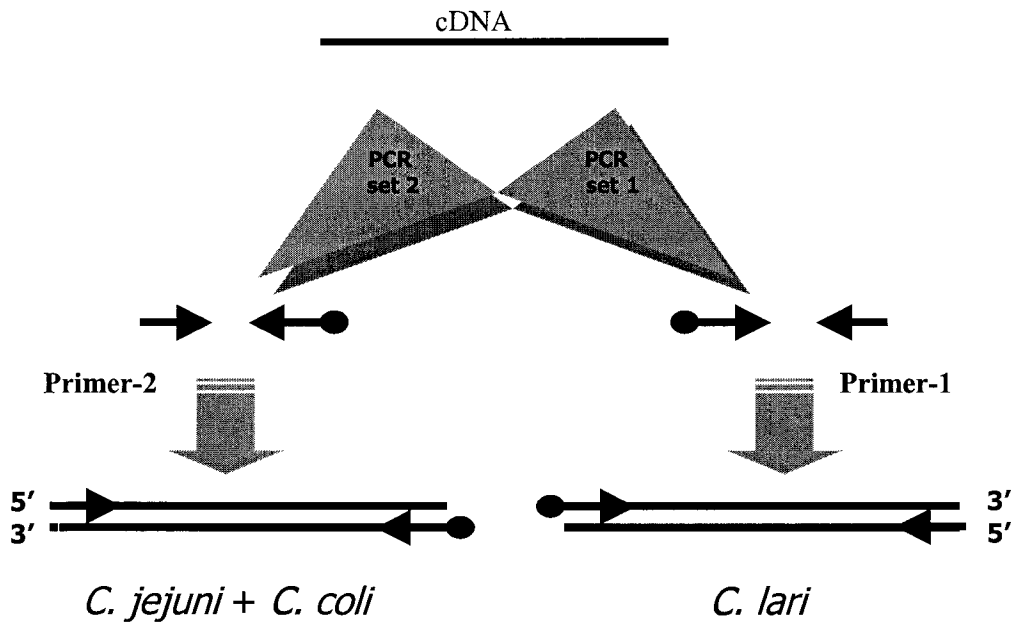


Figure 4.5 Schematic diagram showing two sets of PCR

The template was complementary DNA reverse-transcribed from *hsp*60 mRNA. After set-2 amplification, the strands running from 3' to 5' (the lower one) are labeled with biotin. The strands from *C. jejuni* and *C. coli* can be reported by the fluorescence from corresponding reporters. In set-1 amplification, the strands running from 5' to 3' (the upper one) are all biotin-labeled but only the strands amplified from *C. lari* can be detected (solid circle indicates biotin labeling).

The extracted and purified plasmids, which were obtained from the cloning step, were used as the template to amplify targets of 200-bp with the two sets of primers, for the investigation of experimental conditions. Complementary DNA, reverse-transcribed from *hsp*60 mRNA, was used as the template for PCR when testing the blind and real samples. Each PCR reaction contained 5 μL of 10× PCR buffer, 1.5 μL of 50 mM $MgCl_2$, 0.4 μL of 25 mM dNTP mixture (0.2 mM each), 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse primer, 0.2 μL of Platinum® Taq polymerase, 2 μL of template, and 38.9 μL of autoclaved distilled water to make a final volume of 50 μL. The contents were mixed and incubated in a thermal cycler at 94 °C for 3 min to completely denature the template and activate the enzyme. Forty cycles of PCR amplification were performed

with denaturation at 94 °C for 15 s, annealling at 45 °C for 30 s, and extension at 72 °C for 30 s. A final extension step at 72 °C for 10 min was also included to ensure full extension of the product. The PCR products were desalted, and visualized on 1.5% agarose gel.

Based on this design, the three *Campylobacter* species were simultaneously detected with no cross-hybridization. This was ensured by the following factors in the design:

(1) Even though the same target strand amplified from the set-2 PCR was used for the detection of *C. jejuni* and *C. coli*, their reporters had different fluorescence labeling. Combined with thermal stringency, a process to eliminate the mismatched reporter-target hybrids, these two species were distinguishable even when they were addressed onto the same spots. If the signal from this set of PCR products released green fluorescence emission, it indicated the presence of *C. jejuni*, while red fluorescence emission indicated the presence of *C. coli*.

(2) The target strands for the detection of *C. lari* were amplified from the set-1 PCR, and were addressed onto different spots. This ensured that the target strands for the detection of *C. lari* were not mixed with the target strands for the detection of *C. jejuni* and *C. coli*. In addition, since the *C. jejuni* and *C. coli* reporters had the same orientation as the *C. lari* target strand, and almost identical sequences except for the position of SNPs, the *C. jejuni* and *C. coli* reporters definitely did not bind to the *C. lari* target strand. Only the *C. lari* reporter, which has the opposite orientation and complementary sequence to the *C. lari* target strand, bound to the *C. lari* target strand, releasing green fluorescence

emission on the corresponding spots. So, the green fluorescence signal from the *C. lari* target spots showed the presence of *C. lari*.

(3) This information was the basis for correct and simultaneous identification of three *Campylobacter* species. From this explanation, it can be seen that the type of fluorescence signal and its corresponding location on the microarray chip are the two critical factors for the correct interpretation of the results generated from the microarray.

In addition, a BLAST search of both PCR primers and reporters revealed that only *Campylobacter* could be detected by combining RT-PCR amplification and specific reporting. No other microorganisms would affect the assay.

4.3 Results and Discussion

4.3.1 Effects of instrumental and experimental parameters

Because the electronic DNA microarray workstation consists of two key components, the Loader and the Reader, two sets of parameters were optimized, one for the Loader and the other for the Reader. The Loader delivers target DNA molecules onto the chip. For a specific length of target DNA, the delivery efficiency was determined by the addressing voltage and the addressing time. Target DNA concentration was another factor affecting the delivery efficiency.

When reporters hybridized to the target strands, they bound not only to perfectly matched target strands, but also to slightly mismatched strands. In order to improve the specific hybridization between the reporters and their corresponding target strands, a process known as thermal stringency was applied to the Reader to eliminate the

mismatched binding. This process included steps of low salt washing at certain degrees of temperature control. The reason for using thermal stringency to eliminate mismatched binding is that the binding stability of nucleic acids is greatly influenced by salt concentration and environmental temperature. Lower salt concentration and higher temperature created stringent conditions that caused some of the mismatched binding to melt and the reporters to be released and washed away, which reduced misreporting. The optimization of these parameters is described as follows.

4.3.1.1 Addressing voltage

Addressing voltage was used to activate the designated test sites, and to drive target DNA molecules to migrate from the solution into the gel and to bind to incorporated streptavidin. The magnitude of the voltage determined how fast the target DNA molecules migrated. Higher voltage would lead to faster migration of target DNA molecules, and thus a shorter addressing time would be required. One precaution should be taken here. Higher addressing voltage also produces higher addressing current, which may in turn generate more heat (Joule heating). When the voltage is up to a certain level, the accumulated heat may melt the gel and damage the test sites partially or completely. A proper addressing voltage should generate a current ranging from 300 nA to 900 nA.

The addressing voltage was optimized from 2.00 V to 2.06 V in increments of 0.01 V when amplicons of *C. coli* produced by set-2 PCR reaction with a final concentration of 16.4 nM were used as the target DNA. The addressing time was 120 s. The temperature for the Reader scan was 24 °C. Both the fluorescence intensity and the signal-to-background ratio were obtained, as shown in Figure 4.6.

The fluorescence intensity did not show a clear trend with the increase of the addressing voltage from 2.00 V to 2.06 V. This was probably because the target DNA molecule was short, and did not show significant migration change in this voltage range. The highest S/B ratio with average fluorescence intensity was obtained at 2.00 V. This voltage was used for the addressing of targets in subsequent studies.

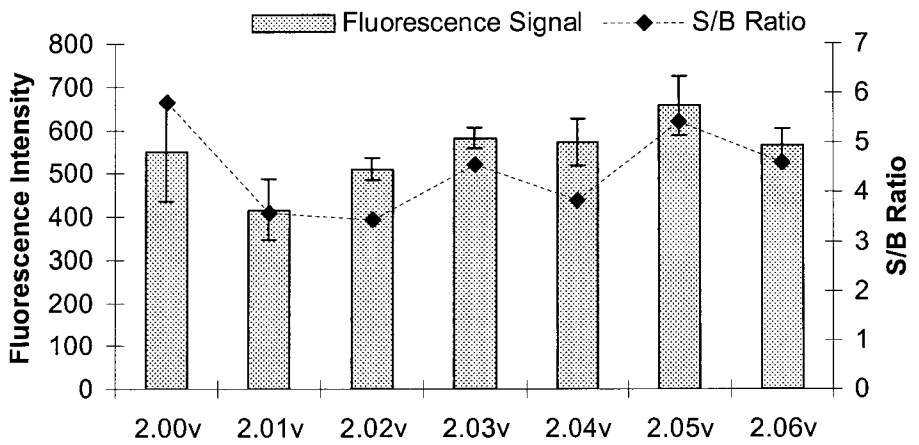


Figure 4.6 Effect of addressing voltage on fluorescence signal intensity and S/B ratio

Amplicons of *C. coli* generated by set-2 PCR reaction were addressed in triplicate onto the microarray chip with a final concentration of 16.4 nM in 60 μL of 50 mM L-histidine. This L-histidine solution was used as the background. The addressing time used was 120 s. Each column represents the mean value of the fluorescence intensity generated from the three test sites after the background signal was subtracted. The error bar represents the standard deviation of the three fluorescence signals for the targets addressed at each voltage. S/B ratios were obtained as described in Section 4.1.2.3.

4.3.1.2 Addressing time

After the addressing voltage was set, another key factor, the addressing time, was investigated. Similar to the optimization of addressing voltage, amplicons obtained from *C. coli* by set-2 PCR reaction with a final concentration of 16.4 nM in 50 mM L-histidine were used as the target. When the addressing time was changed from 120 s to 240 s in increments of 20 s, the fluorescence signals and S/B ratio were obtained, as shown in Figure 4.7. The signal intensity and the S/B ratio did not change significantly when the

target was delivered for a period of time ranging from 120 s to 160 s. However, as the delivery time was increased from 180 s to 240 s, both the fluorescence signals and the S/B ratios decreased. The reason for this decrease is not clear. It may be due to some damage to the spots caused by the accumulated Joule heating after a long period of time addressing. A higher S/B ratio was observed between 120 s and 160 s. The optimal addressing time of 140 s was chosen and used throughout the whole study because both a higher fluorescence signal intensity and a higher S/B ratio were observed at this point.

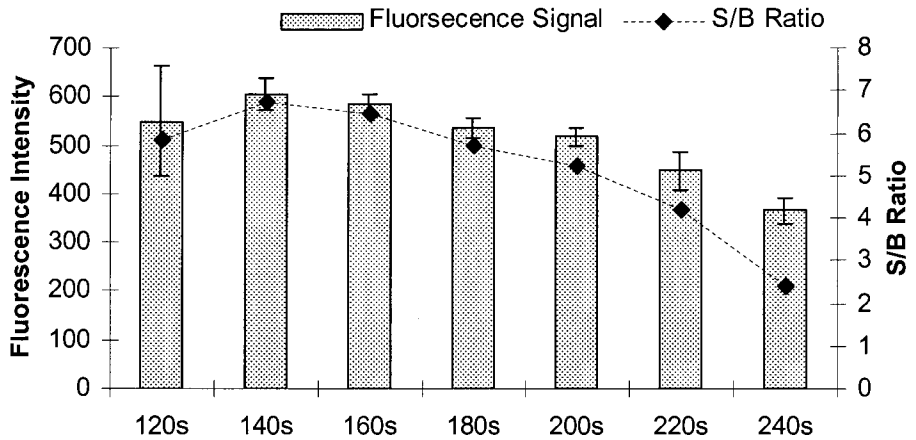


Figure 4.7 Effect of addressing time on fluorescence signal intensity and S/B ratio

Amplicons of *C. coli* generated by set-2 PCR reaction were addressed in triplicate onto the microarray chip with a final concentration of 16.4 nM in 60 μL of 50 mM L-histidine. This L-histidine was used as the background. The addressing voltage used was 2.00 V. Each column represents the mean value of the fluorescence intensity generated from the three test sites after the background signal was subtracted. The error bar represents the standard deviation of the three fluorescence signals for the targets addressed for each addressing time. S/B ratios were obtained as described in Section 4.1.2.3.

4.3.1.3 Target concentration

Using the optimized addressing voltage and addressing time, the effect of target DNA concentration on the fluorescence signal was investigated. This was carried out by changing the final concentration (60 nM to 0.14 nM) of amplicons of *C. coli* generated by

set-2 PCR reaction in 50 mM L-histidine solution under optimized addressing conditions (at 2.00 V for 140 s).

Two sets of experiments were carried out, one for the higher concentration group, and the other for the lower concentration group. Figure 4.8 shows the results obtained when the target concentration was changed from 60 nM to 5.5 nM. The fluorescence signals and S/B ratios changed slightly when the target concentration was in the range of 60 nM to 16.4 nM. When the target concentration was 5.5 nM, the fluorescence signal was low. Therefore, a separate experiment with the target concentration from 8.2 nM to 0.14 nM was further examined. Figure 4.9 shows that the fluorescence signals generally decreased when the target concentration decreased from 8.8 nM to 0.14 nM. The signal-to-background ratio also decreased in a similar fashion. The S/B ratios obtained from targets with concentrations above 1.4 nM were all greater than 3, supporting the positive identification. Therefore, the target concentration was always maintained above 1.4 nM in subsequent studies unless otherwise stated.

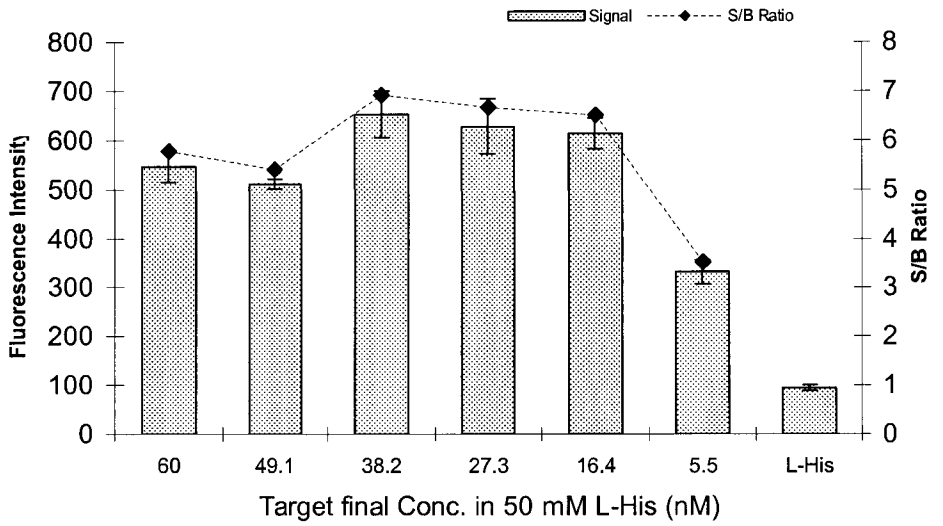


Figure 4.8 Effect of target concentration on fluorescence signal and S/B ratio (high range)

Amplicons of *C. coli* generated by set-2 PCR reaction were addressed in triplicate onto the microarray chip at 2.00 V for 140 s. The mean fluorescence signal of 50 mM L-histidine was used as the background. The solid columns represent the background-subtracted fluorescence intensity except for the column of L-histidine. The error bar represents the standard deviation of the three fluorescence signals from the targets. S/B ratios were obtained as described in Section 4.1.2.3.

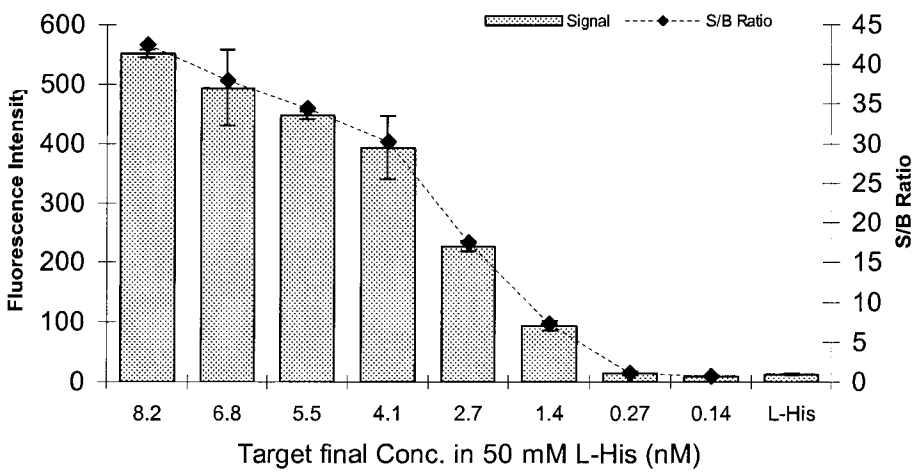


Figure 4.9 Effect of target concentration on fluorescence signal and S/B ratio (low range)

Amplicons of *C. coli* generated by set-2 PCR reaction were addressed in triplicate onto the microarray chip at 2.00 V for 140 s. The mean fluorescence signal of 50 mM L-histidine was used as the background. The solid columns represent the background-subtracted fluorescence intensity except for the column of L-histidine. The error bar represents the standard deviation of the three fluorescence signals from the targets. S/B ratios were obtained as described in Section 4.1.2.3.

As seen from these two figures, even for the same target concentration (e.g., 5.5 nM), different S/B ratios were obtained. This was due to the background variation reflected by the fluorescence intensity value for L-histidine. Regardless of the scanning gain and duration, variations in fluorescence signals from run-to-run and from chip-to-chip were always observed. There are three possible reasons: (1) the test sites on a chip may not be uniformly fabricated; (2) the test sites may be contaminated by salt accumulated when a chip is used more than once; and (3) chips may have been used after the expiration date, a problem because the permeation layer may have some degradation over time, particularly when it is a relatively long time after expiration. The salt accumulation was confirmed by the observation that the chips used for the first time generally produced lower background while the chips used for the second or more times produced higher background. To minimize this effect, expired chips were not used in later studies. However, due to the large number of test sites and the relatively small number of spots required for each experiment, the same chip was inevitably used repeatedly during the period of the method development to reduce the cost, which resulted in higher background and thus a lower S/B ratio. Even in these cases, a ratio of 3:1 S/B was still used as the criterion for positive identification.

4.3.1.4 Environmental temperature

The environmental temperature of the chip was optimized for thermal stringency to minimize the effect of mismatched target-reporter binding, and to obtain an optimal fluorescence signal-to-background ratio.

The temperature range for optimization is dependent on the melting temperature of the reporter. The starting temperature was usually about 2 degrees lower than the melting

temperature of the reporter. In this study, a wider range of temperature starting from room temperature (24 °C) was tested. Because different reporter-target hybrids may have different thermal stability, all three reporters were investigated at temperatures from 24 °C to 32 °C as shown in Figures 4.10, 4.11, and 4.12. After each temperature increase, a low salt washing step always followed.

Generally, with increasing temperature, the fluorescence signal intensity decreased. This may be due to (1) reporters that were unbound but had penetrated into the gel which were washed away; and/or (2) mismatched-bound reporters which were melted away. In terms of the signal-to-background ratio, the *C. lari* and *C. coli* reporters produced a relatively low ratio compared with the *C. jejuni* reporter. This is mainly due to the different background signals generated from the L-histidine spots in each separate test.

In the test for the *C. lari* reporter (shown in Figure 4.10), the signal-to-background ratios decreased slightly when the temperature increased from 24 °C to 30 °C. For the *C. jejuni* reporter, as shown in Figure 4.11, the signal-to-background ratios stayed relatively high and stable at temperature ranging from 27 °C to 30 °C. Figure 4.12 shows that the *C. coli* reporter produced quite stable signal-to-background ratios through the whole temperature range. Considering these results and the 2 °C temperature control accuracy on the Reader, 29 °C was finally chosen as the stringency temperature for removing all three imperfectly hybridized reporters throughout the subsequent study.

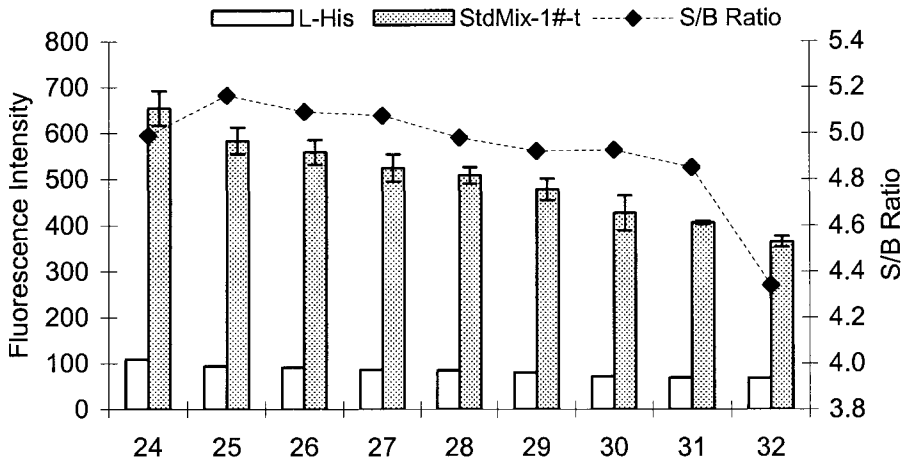


Figure 4.10 Effect of environment temperature on *C. lari* reporter-target hybrid

The solid columns (labeled as StdMix-1#-t in the legend) represent the background-subtracted fluoresence signals of the amplicons obtained from the mixture of the three *Campylobacter* species by set-1 PCR reaction. The amplicons were addressed in triplicate onto the microarray chip at 2.00 V for 140 s with a final concentration of 8.4 nM in 50 mM L-histidine, which was used as the background and represented as the hollow column. The error bar represents the standard deviation of the three fluorescence signals of the targets. S/B ratios were obtained as described in Section 4.1.2.3. The *C. lari* reporter solution was used at a concentration of 1 μM.

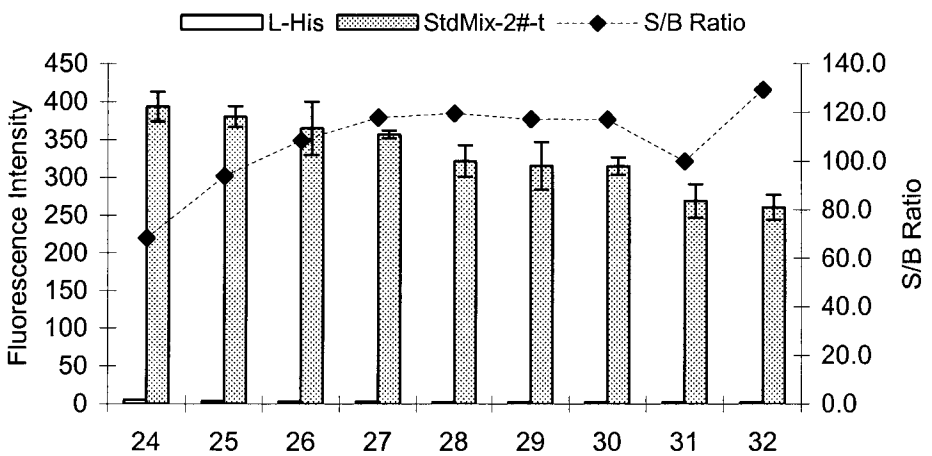


Figure 4.11 Effect of environment temperature on *C. coli* reporter-target hybrid

The solid columns (labeled as StdMix-2#-t in the legend) represent the background-subtracted fluoresence signals of the amplicons obtained from the mixture of the three *Campylobacter* species by set-2 PCR reaction. The amplicons were addressed in triplicate onto the microarray chip at 2.00 V for 140 s with a final concentration of 10 nM in 50 mM L-histidine, which was used as the background and represented as the hollow column. The error bar represents the standard deviation of the three fluorescence signals of the targets. S/B ratios were obtained as described in Section 4.1.2.3. The *C. coli* reporter solution was used at a concentration of 1 μM.

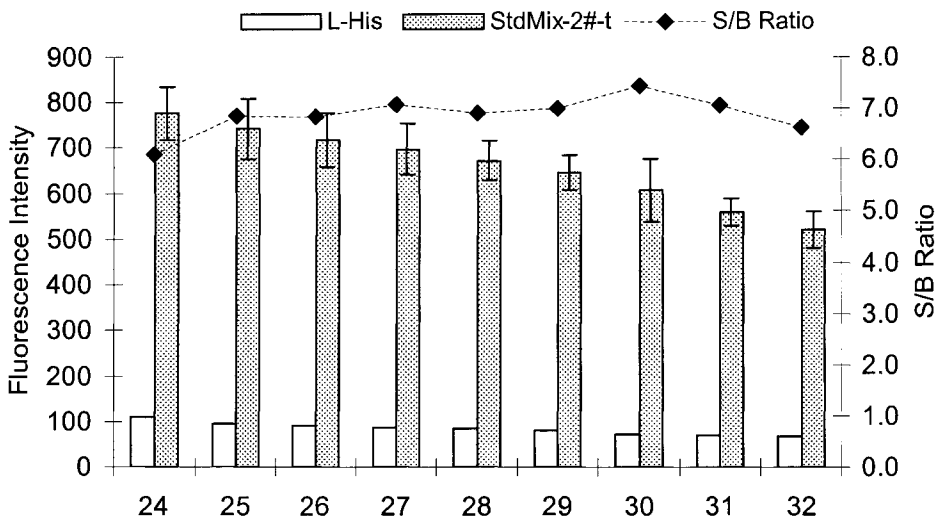


Figure 4.12 Effect of environment temperature on *C. jejuni* target-reporter hybrid

The solid columns (labeled as StdMix-2#-t in the legend) represent the background-subtracted fluoresence signals of the amplicons obtained from the mixture of the three *Campylobacter* species by set-2 PCR reaction. The amplicons were addressed in triplicate onto the microarray chip at 2.00 V for 140 s with a final concentration of 13.8 nM in 50 mM L-histidine, which was used as the background and represented as the hollow column. The error bar represents the standard deviation of the three fluorescence signals of the targets. S/B ratios were obtained as described in Section 4.1.2.3. The *C. jejuni* reporter solution was used at a concentration of 1 μM.

4.3.2 Specificity of designed reporters

The basis for the accurate identification of *Campylobacter* species was the specificity of the designed reporters. A number of factors were carefully considered during the design of the reporters to improve their specificity. First, using the two SNPs closely located in the middle of the reporter reduced the potential for mismatched hybridization between targets and reporters. Second, the stringency provided by combining increased environment temperature and low salt buffer washing, which utilizes the thermal and salt sensitivity of the nucleic acid pairing, greatly reduced undesired hybridization because perfectly matched hybrids are more stable than

mismatched ones. The following experiments demonstrated the high specificity of the designed reporters.

Experiment One: after the target strands amplified from each species were addressed to different spots on a chip, the chip was incubated with a solution of the reporters for *C. lari* only. After several washings and while using the optimized instrumental and experimental parameters, the spots addressed with target strands amplified from *C. lari* generated a positive signal with a S/B ratio of ~70, as shown in Figure 4.13. Other spots addressed with either *C. jejuni* or *C. coli* target strands did not generate a signal. This result demonstrates the high specificity of the *C. lari* reporter for *C. lari* targets.

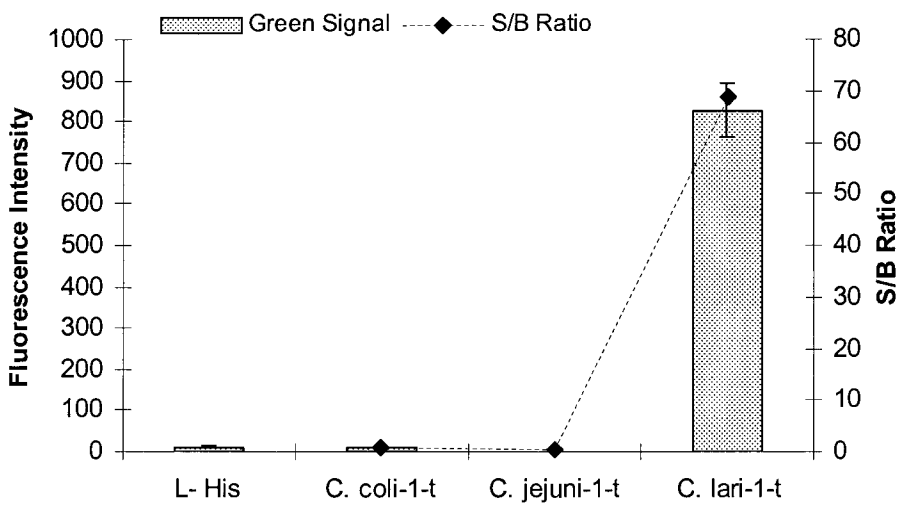


Figure 4.13 Specificity of *C. lari* reporter

Amplicons of the three *Campylobacter* species amplified by set-1 PCR reaction were separately addressed onto different test sites of the microarray chip at 2.00 V for 140 s. Each target was addressed onto 3 sites. Only the *C. lari* reporter with a concentration of 1 μM was used for hybridization. Target concentration was ~4 nM for each species. The solid columns represent the mean background-subtracted fluoresence signals from the triplicates with the error bar showing the standard deviation. L-histidine with a concentration of 50 mM was used as the background. S/B ratios were obtained as described in Section 4.1.2.3.

Experiment Two: the target strands amplified from the three species were addressed on a chip in the same way as described above, but the chip was incubated with a solution containing reporters for *C. jejuni* and *C. coli*. The result is shown in Figure 4.14. Clearly, only the spots addressed with *C. jejuni* and *C. coli* generated fluorescence signals by the corresponding reporters. No cross-identification was observed, which indicates that the specificity of these two reporters is high as well.

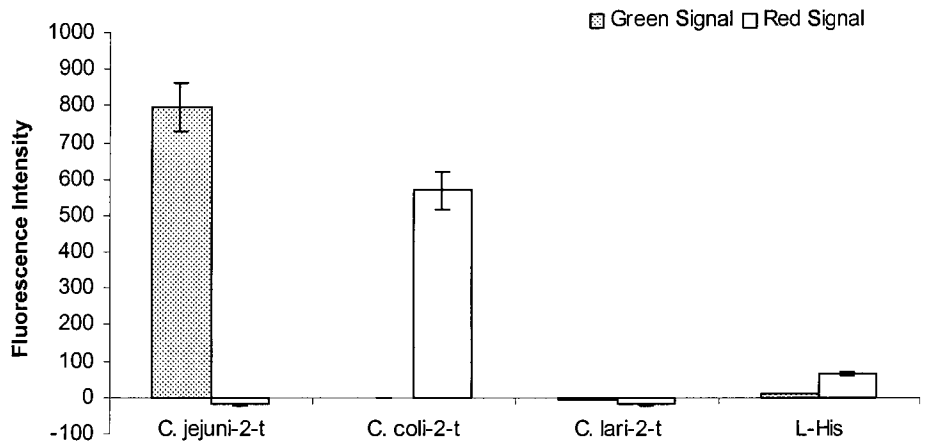


Figure 4.14 Specificity of *C. jejuni* and *C. coli* reporters

Amplicons of the three *Campylobacter* species amplified by set-2 PCR reaction were separately addressed onto different test sites on the microarray chip at 2.00 V for 140 s. Three sites were used for each species. A mixed reporter solution containing the *C. jejuni* and *C. coli* reporters with a concentration of 1 μM for each was applied for hybridization. The target concentration was ~27 nM for each species. The solid columns represent the mean background-subtracted green fluoresence signals from the triplicates; the hollow columns represent the mean background-subtracted red fluoresence signals from the triplicates. The error bars show the standard deviation of the triplicates. L-histidine with a concentration of 50 mM was used as the background.

4.3.3 Effects of different amounts of starting RNA and cDNA

The amount of starting material of total RNA – in essence mRNA, – directly affects the efficiency of reverse transcription, which in turn determines the amount of transcribed

cDNA, and further affects subsequent PCR amplification efficiency. Therefore, the effect of different amounts of starting material of total RNA was investigated. In addition, the effect of EDTA contained in the reverse transcription solution on PCR amplification was investigated because EDTA might inhibit the subsequent PCR enzyme reactions.

Experiments were carried out with serially diluted total RNA of *C. jejuni*-579 as the starting material; two different volumes of cDNA solution, 2 μL and 8 μL, were used for subsequent PCR amplification. The starting total RNA was treated with DNase I, reverse transcribed as described in Section 3.2.1.2, and then amplified by PCR as described in Section 4.2.2 with the set-2 primers. The amplicons were tested on the microarray. Figure 4.15 shows the results from using the total RNA amounts in the range of 0.2–24.6 pg and Figure 4.16 shows the results from using the total RNA amounts in the range of 22.9–245.6 fg. The S/B ratios obtained from most of these samples were all greater than 3 except for the last sample of 22.9 fg starting total RNA in Figure 4.16. This indicated the wide range of extracted total RNA sufficient for positive identification, even when the starting total RNA was as little as 49.1 fg. Therefore, the starting total RNA used was above 50 fg in subsequent studies unless otherwise stated.

Figures 4.15 and 4.16 present the S/B ratios obtained from the targets when 2 μL or 8 μL of cDNA solution was used as the template in the PCR amplification. These results demonstrated that no significant difference in the S/B ratios was observed between 2 μL or 8 μL of cDNA solution was used. This suggested that the EDTA in the reverse transcription solution did not show an apparent effect on PCR efficiency. Therefore, 2 μL of reverse transcribed cDNA solution was used in the subsequent experiments unless otherwise stated.

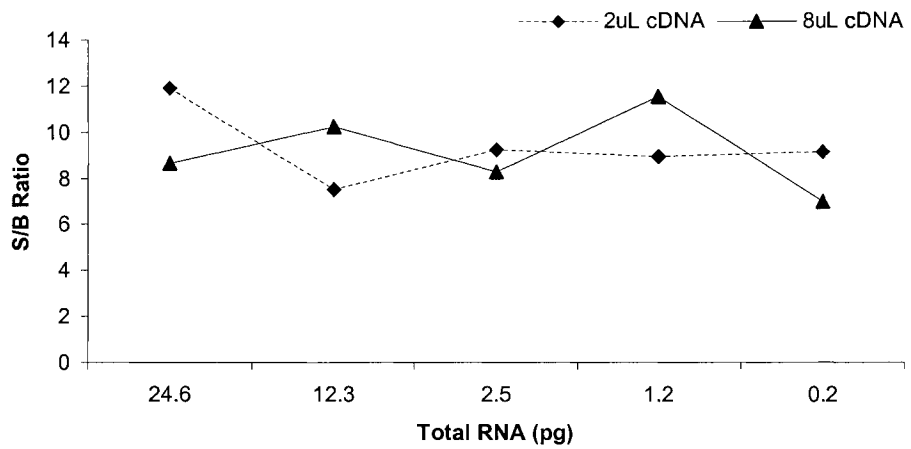


Figure 4.15 Diluted total RNA tested with different amounts of cDNA (high range)

Serially diluted total RNA of *C. jejuni* were used as the starting material; two different volumes of cDNA solution (2 μL and 8 μL) obtained as described in Section 3.2.1.2 were used as templates for the subsequent PCR amplifiction with set-2 primers. Amplicons were addressed onto the microarray chip in triplicate. The *C. jejuni* reporter (1 μM) was used for hybridization. The S/B ratios were obtained as described in Section 4.1.2.3.

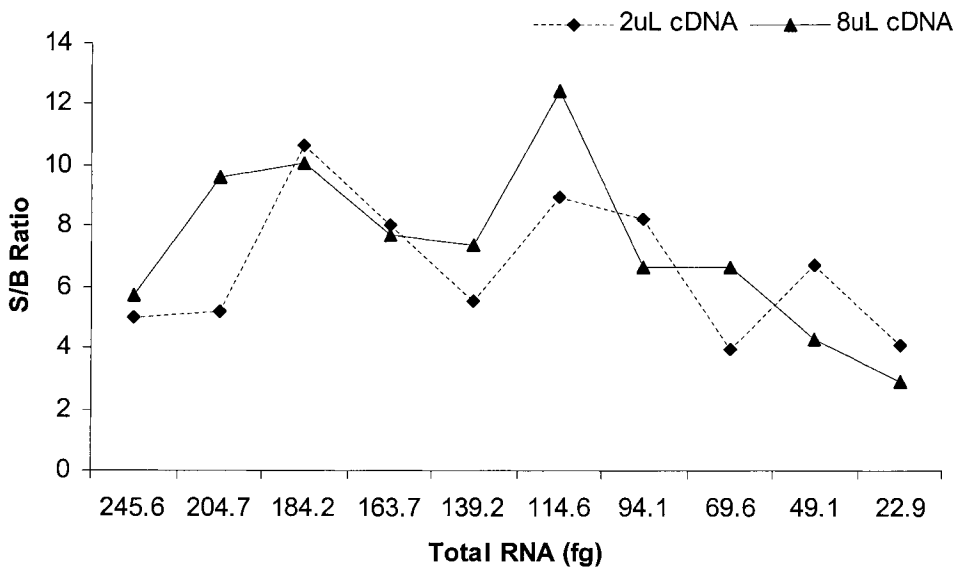


Figure 4.16 Diluted total RNA tested with different amounts of cDNA (low range)

The experimental procedures and conditions were the same as those described in Figure 4.15 except that smaller amounts of total RNA (in the range of fg) were used.

4.3.4 Sensitivity of the method

One of the goals of my study was to develop a sensitive method for *Campylobacter* detection. The sensitivity of the developed technique was first investigated when the amount of the total RNA of *C. jejuni* was serially diluted. The 2 μg of total RNA initially extracted from 90 million cultured cells of *C. jejuni*-579 was diluted serially with ddH_2O. The corresponding dilution factors were applied to the calculation of the cell number equivalents. For example, when the initial 2 μg of total RNA was diluted 10-fold, the dilution factor 10 would be used to calculate the cell number equivalents for the diluted total RNA solution. Dividing the initial cell number (90 million) by this dilution factor 10, we obtained 9 million. Therefore, 0.2 μg of total RNA is equivalent to 9 million cells. The same calculation was applied to further dilutions.

The sample preparation involved the following steps. The cells were heat shocked at 48 °C and the total RNA was extracted as described in Section 3.2.1.1. The RNA was serially diluted, and the diluted RNA samples were treated with DNase, reverse transcribed as described in Section 3.2.1.2, and followed by the PCR amplification as described in Section 4.2.2 with set-2 primers. The PCR products obtained were completely used for the subsequent microarray detection.

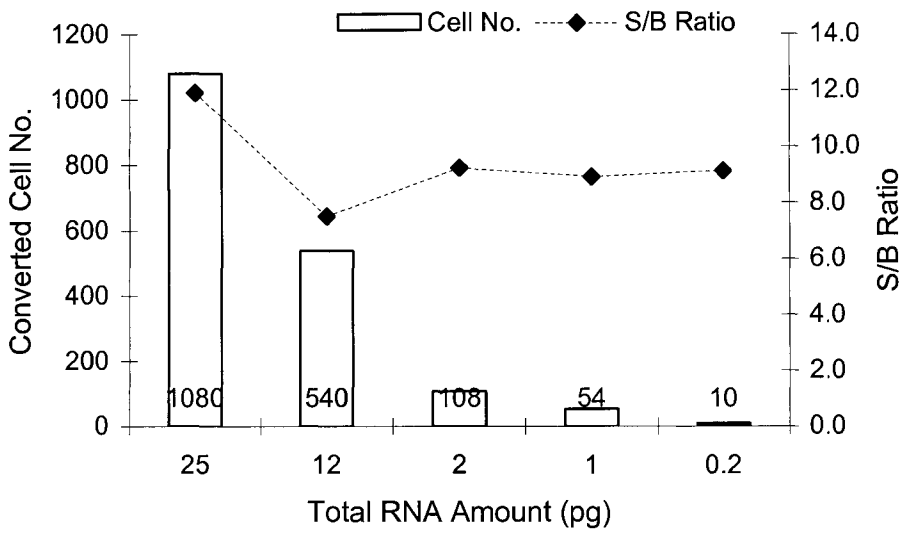


Figure 4.17 Cell numbers calculated based on dilution factors and the S/B ratios obtained using the microarray method with total RNA of 25–0.2 pg

Two μg of total RNA initially extracted from 90 million cultured cells of *C. jejuni* was diluted serially with sterilized ddH_2O. The corresponding dilution factors were applied to calculate the cell number equivalents, shwon as the hollow columns. Amplicons obtained by the set-2 PCR reaction were addressed onto the microarray chip in triplicate. The *C. jejuni* reporter (1 μM) was applied for hybridization. S/B ratios were obtained as described in Section 4.1.2.3.

As shown in Figure 4.17, the S/B ratios were obtained from various amounts of the diluted total RNA: 25, 12, 2, 1, and 0.2 pg. The amounts of the total RNA as the starting material were plotted on the X-axis. The cell number equivalents corresponding to different amounts of the total RNA were shown as open columns and their values were plotted on the left Y-axis. The S/B ratios were shown as dots-on-line and their values were plotted along the right Y-axis. This graph demonstrates that when the total RNA varied from 25 to 0.2 pg, the S/B ratios were relatively constant and all greater than 6, while the cell number equivalents decreased dramatically. The target pathogen was successfully detected with a S/B ratio of 9.1 when the RT-PCR started with as little as 0.2 pg of total RNA, which corresponds to 10 cell equivalents.

Further tests were carried out using smaller amounts of the total RNA (184–23 fg), and the results obtained are shown in Figure 4.18. The cell number equivalents ranged from 8 to 1, and the S/B ratios obtained were greater than or approximately equal to 4. The S/B ratios generally decreased with the decreasing amounts of the starting total RNA. Some observed variations could be due to the spots-to-spots variation on the microarray itself as discussed previously in Section 4.3.1.3. The results indicated that the developed method has the potential to detect less than 10 cells of *Campylobacter*.

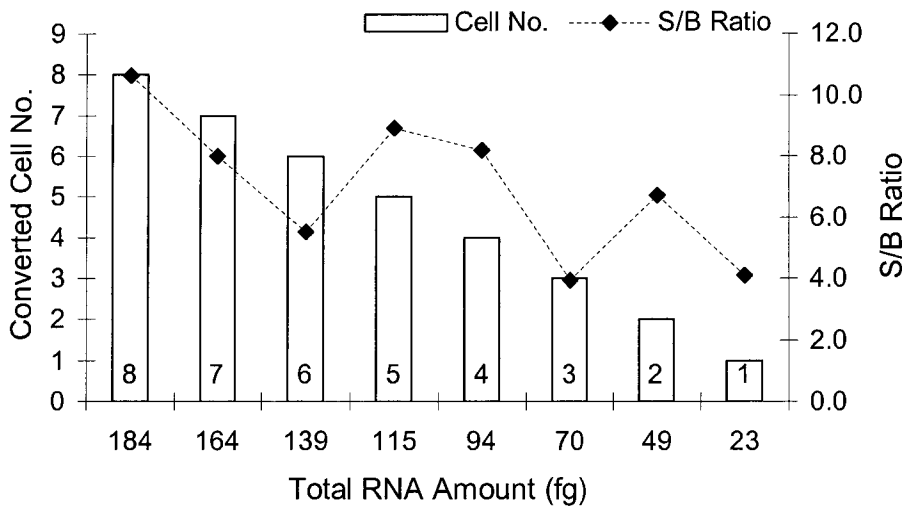


Figure 4.18 The S/B ratios obtained using the microarray method with the total RNA of 184–23 fg corresponding to 8–1 cells

Two μg of total RNA initially extracted from 90 million cultured cells of *C. jejuni* was diluted serially with sterilized ddH_2O. The corresponding dilution factors were applied to calculate the cell number equivalents, shown as the hollow columns. Amplicons obtained by the set-2 PCR reaction were addressed onto the microarray chip in triplicate. *C. jejuni* reporter (1 μM) was applied for hybridization. S/B ratios were obtained as described in Section 4.1.2.3.

The ability of this developed technique to detect a few cells was further examined using the cultured *C. jejuni*-782 cells. A culture containing *C. jejuni*-782 cells (9×10^8 CFU/mL) was serially diluted 10-fold using sterilized tryptic soy broth. To improve the accuracy of the cell number count in each dilution, the cell culture was

gently pipetted up and down several times to make the cell suspension as homogenized as possible. A certain volume of the culture was removed from the middle of the culture solution. Following the procedures described in Section 4.3.4, samples containing different cell numbers, 2×10^4, 2×10^3, 200, 100, 60, 30, 8, and 2, were prepared and heat shocked. The total RNA from these cell samples was extracted, treated with DNase, and amplified by RT-PCR. The amplicons were tested on the microarray. As shown in Table 4.3, the S/B ratios obtained from these samples were greater than or approximately equal to 4. Figure 4.19 shows the fluorescence signal and S/B ratios resulting from the two lowest cell number samples. These results were consistent with those obtained in the diluted total RNA test (Figure 4.18).

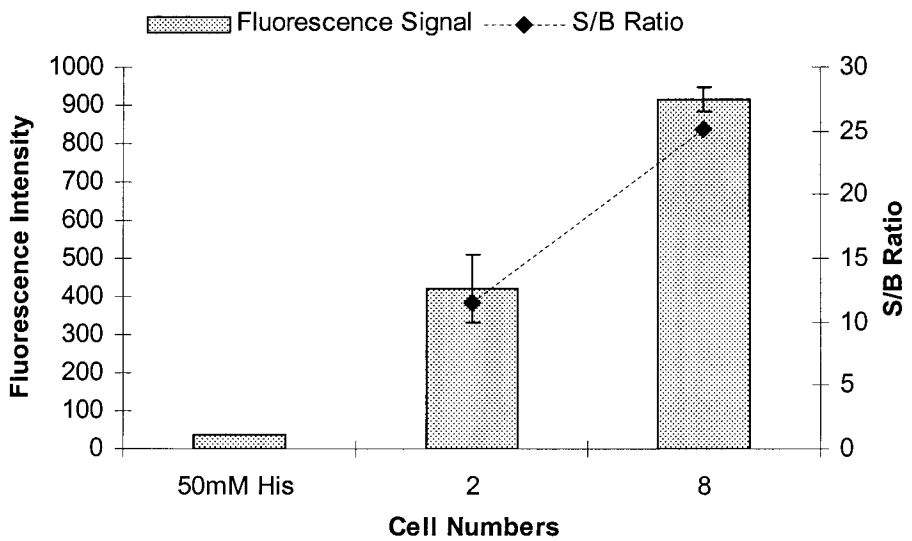


Figure 4.19 Fluorescence signal obtained from the two samples containing 2 and 8 cells

The cultured *C. jejuni* cells were serially diluted 10-fold with sterilized tryptic soy broth. The solid columns represent the background-subtracted fluorescence signals generated from the triplicates on the microarray for samples containing 2 and 8 cells. L-histidine with a concentration of 50 mM was used as the background. *C. jejuni* reporter (1 μM) was applied for hybridization. The error bar represents the standard deviation of the triplicates. S/B ratios were obtained as described in Section 4.1.2.3.

Table 4.3 The S/B ratios obtained from the diluted cell samples

Cell Number	2×10^4	2×10^3	200	100	60	30	8	2
S/B	23.7	4.7	3.7	14.3	10.1	3.6	25.2	11.5

Note: these results were obtained from different microarray chips.

As discussed in Section 1.2.2, the minimum infective dose of *Campylobacter* is approximately 500 cells. The detection sensitivity of fewer than 10 cells demonstrated above suggests that the sensitivity of the developed technique is more than sufficient to satisfy the demands of different applications.

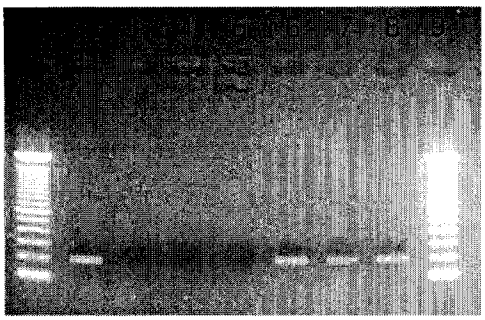
It is worth mentioning that the S/B ratios are not linearly proportional to cell numbers in the diluted cell culture test. A number of factors may contribute to this, including the variations in (1) total RNA extraction efficiency; (2) reverse transcription efficiency; (3) PCR amplification efficiency; (4) recovery in the desalting step after PCR amplification; and (5) run-to-run and chip-to-chip performance on the microarray as mentioned in Section 4.3.1.3. Among them, the total RNA extraction efficiency might be the key reason, because the culture medium must be centrifuged to collect the cells. After centrifugation, visible cell pellets were formed for samples containing 10^6 or more cells. For samples containing 10^5 or fewer cells, no visible cell pellets were observed. This makes the removal of supernatant and pellet washing step difficult. A similar situation was encountered when precipitating the extracted RNA by centrifugation. No visible gel-like RNA pellets formed for samples containing a lower number of cells. The other contributing factor is the inevitable loss of RNA during the phase separation step of the extraction. RNA remaining in the aqueous phase could not be completely recovered in

order to avoid DNA and protein contamination from the organic phase. These unpredictable variables resulted in variation in subsequent steps.

4.3.5 Interference of other bacteria

Even though no interference was observed from other microorganisms when searching GenBank, some commonly found food- and water-borne enteric pathogens, including *E. coli* O157:H7, non-pathogenic *E. coli* (ATCC 25955), *Salmonella* Typhi (R704101), *Vibrio cholerae* O1, *Listeria monocytogenes* (R1694101), *Yersinia enterocolitica* (K1332/03), and *Cryptosporidium parvum*, were nonetheless tested to investigate possible interference with the detection of target pathogens. Except for *Cryptosporidium parvum* and *Vibrio cholerae* O1, whose total RNA samples were obtained from our colleagues, the other five bacteria were obtained from patient stool samples and serotyped by the Provincial Laboratory of Public Health (Microbiology). One mL of each of the five bacterial cells was heat shocked and the total RNA was extracted as discussed before. The extracted total RNA, along with the total RNA of *Cryptosporidium parvum* and *Vibrio cholerae* O1, were amplified by RT-PCR and the amplicons were tested on the microarray.

After the PCR amplification, the results from the agarose gel picture showed potential interference from *Vibrio cholerae* O1 and *Salmonella* Typhi as bands visualized on the gel (Figure 4.20). The other five microorganisms did not generate any interference because no visible bands were observed on the gel (Figure 4.21).



Lane assignment: Lane 1, 100 bp DNA ladder; Lane 2, PCR positive control; Lane 3, PCR negative control; Lane 4, RT negative control; Lane 5, *Salmonella*-RT_0; Lane 6, *Salmonella*-RT; Lane 7, *Vibrio*-RT_0; Lane 8, *Vibrio*-RT; Lane 9, DNA ladder.

Figure 4.20 Agarose gel electrophoresis of RT-PCR products of *Vibrio* and *Salmonella*

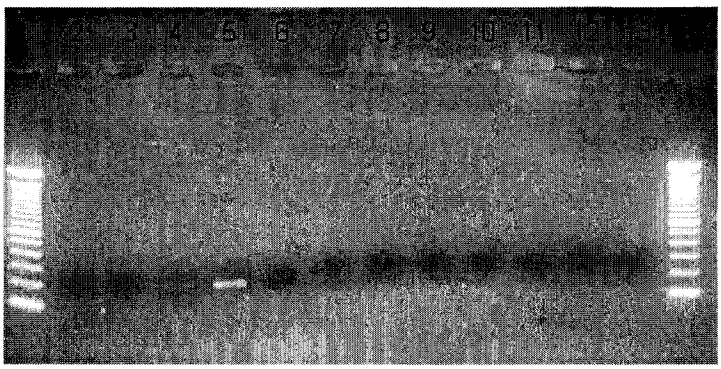


Figure 4.21 Agarose gel electrophoresis of RT-PCR products of other microbes

Lane assignment: Lane 1, 100 bp DNA ladder; Lane 2, PCR negative control; Lane 3, RT negative control; Lane 4, *C. lari*-RT_0; Lane 5, RT-*C. lari*; Lane 6, non-pathogenic *E. coli*-RT_0; Lane 7, non-pathogenic *E. coli*-RT; Lane 8, *E. coli* O157:H7-RT_0; Lane 9, *E. coli* O157:H7-RT; Lane 10, *Listeria*-RT_0; Lane 11, *Listeria*-RT; Lane 12, *Yersinia*-RT_0; Lane 13, *Yersinia*-RT; Lane 14, 100 bp DNA ladder.

As shown in Figures 4.22 and 4.23, only the positive control spots that were addressed with target strands generated fluorescence signals. No signals were obtained from all the test sites addressed with amplicons of other microorganisms, demonstrating that there is no interference from these microbes with the detection of target pathogens by the developed SNP-based electronic DNA microarray method.

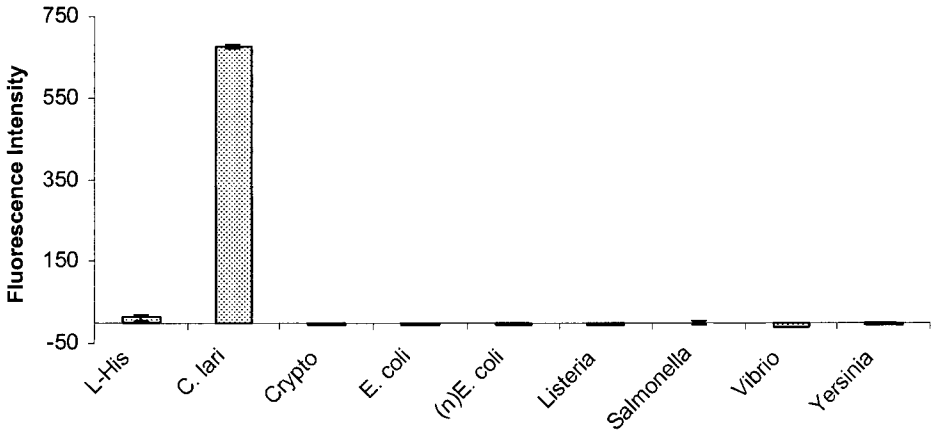


Figure 4.22 Microarray test results showing no interference from other seven microbes

The solid columns represent the mean background-subtracted fluorescence signals of the triplicates addressed onto the microarrray chip for each microbe. L-histidine with a concentration of 50 mM was used as the background. The error bar represents the standard deviation. Amplicons obtained from set-1 PCR reactions were used as the targets. The *C. lari* reporter (1 μM) was used for hybridization. The final concentration of the *C. lari* target was 8.5 nM; *Salmonella* Typhi, 10 nM; and of *Vibrio cholerae* O1, 7.7 nM. Target concentrations for the other microbes were not available because there was no amplicon generated by PCR (Figure 4. 21).
Crypto: *Cryptosporidium parvum*; E. coli: *E. coli* O157:H7; (n)E. coli: non-pathogenic *E. coli*; Listeria: *Listeria monocytogenes*; Salmonella: *Salmonella* Typhi; Vibrio: *Vibrio cholerae* O1Yers inia: *Yersinia enterocolitica*.

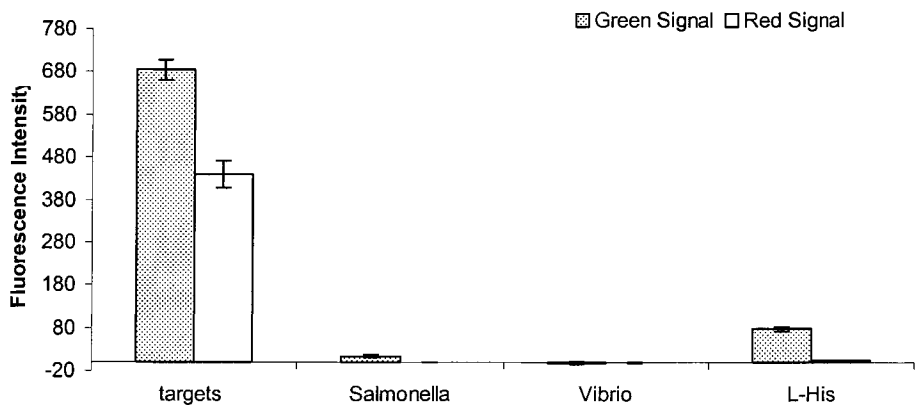


Figure 4.23 Microarray analysis showing no interference from *Salmonella* and *Vibrio*

The columns represent the mean background-subtracted fluorescence signals of the triplicates addressed onto the microarrray chip for each microbe. L-histidine with a concentration of 50 mM was used as the background. The error bar represents the standard deviation. Amplicons obtained from the set-2 PCR reactions were used as the targets. The reporter solution containing the *C. jejuni* and *C. coli* reporters (1 μM for each) was used for the hybridization. The *C. jejuni* target (29.7 nM) and the *C. coli* target (40.9 nM) were separately addressed as positive controls, shown as the columns labeled as targets. The final target concentration for *Salmonella* Typhi and *Vibrio cholerae* O1 were ~6.2 nM, respectively.

In real-life situations, however, different bacteria may not co-exist in equal numbers in authentic samples. To evaluate the interference from other microbes present in large numbers relative to the small numbers of target pathogens, an experiment was carried out testing the combined total RNA of a non-target pathogen (*E. coli* O157:H7 with a final concentration of 54.4 ng/μL in the mixture) with the total RNA of the target pathogen (*C. coli*-456 with a final concentration of 0.049 ng/μL in the mixture). The RNA concentration of the non-target was 1100-fold higher than that of the target in this combined RNA solution. Two μL of this RNA combination solution was used as the starting total RNA for testing. Total RNA of *E. coli* O157:H7 with a concentration of 108.8 ng/μL was also tested, and total RNA of *C. coli*-456 with a concentration of ~0.1 ng/μL was used as the positive control.

The result is shown in Figure 4.24. The *C. coli* column represents the signal generated from ~0.1 ng/μL total RNA of *C. coli*-456; the *E. coli* column represents the signal generated from 108.8 ng/μL total RNA of *E. coli* O157:H7; the *E/C. coli* column represents the signal of the combined RNA. Only the spots addressed with *C. coli* alone and the spots addressed with the combination of *E. coli* and *C. coli* generated positive identification. This demonstrates that the target pathogen can be successfully detected even when it co-exists with a non-target pathogen a thousand times more abundant.

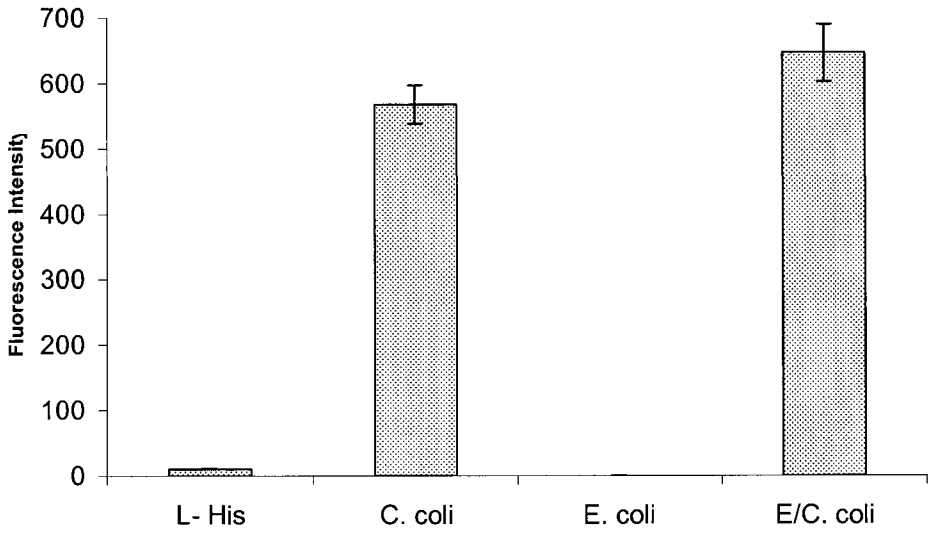


Figure 4.24 Microarray analysis of a sample containing the target pathogen *C. coli* mixed with 1000-fold higher amount of the non-target *E. coli*

By the set-2 PCR reactions, amplicons obtained from the total RNA of *C. coli*, *E. coli* O157:H7, and the mixture of these two were addressed in triplicate onto the microarray chip. The columns represent the mean background-subtracted fluorescence signals of the triplicates with error bars showing the standard deviation. L-histidine with a concentration of 50 mM was used as the background. The reporter solution containing the *C. jejuni* and *C. coli* reporters (1 μM for each) was used for the hybridization. The *C. coli* column represents the signal generated from ~0.1 ng/μL total RNA of *C. coli*; the *E. coli* column represents the signal generated from 108.8 ng/μL total RNA of *E. coli* O157:H7; the *E/C. coli* column represents the signal of the total RNA mixture with concentration of 54.4 ng/μL for *E. coli* O157:H7 and 0.049 ng/μL for *C. coli*. The RNA concentration of the non-target pathogen was 1100-fold higher than that of the target pathogen in this RNA mixture.

4.3.6 Dead cell controls

As discussed previously, the developed method should only detect viable cells, and not dead cells. This was demonstrated in the dead cell control test that used heat-killed cells. In this test, cells in 0.5 mL of *C. coli*-456 isolate (9×10^8 CFU/mL) were killed at 100 °C in a water bath for 15 min. The heat-killed cells were kept at room temperature for 2 days before the total RNA was extracted for further experiments based on an experiment conducted by Sheridan et al. (1998) in which the *groEL* mRNA (*hsp*60

mRNA) of *E. coli* cells became undetectable after 2 h incubation at room temperature following the heat killing. Dead cells would give positive signals only if their *hsp*60 mRNA still existed. However, as shown in Figure 4.25, no signal was observed from the spots addressed with the sample of dead cells while signals were obtained from all positive controls. This indicates that dead cells will not generate a false positive signal. This result confirmed that the developed method is unique for viable cell detection.

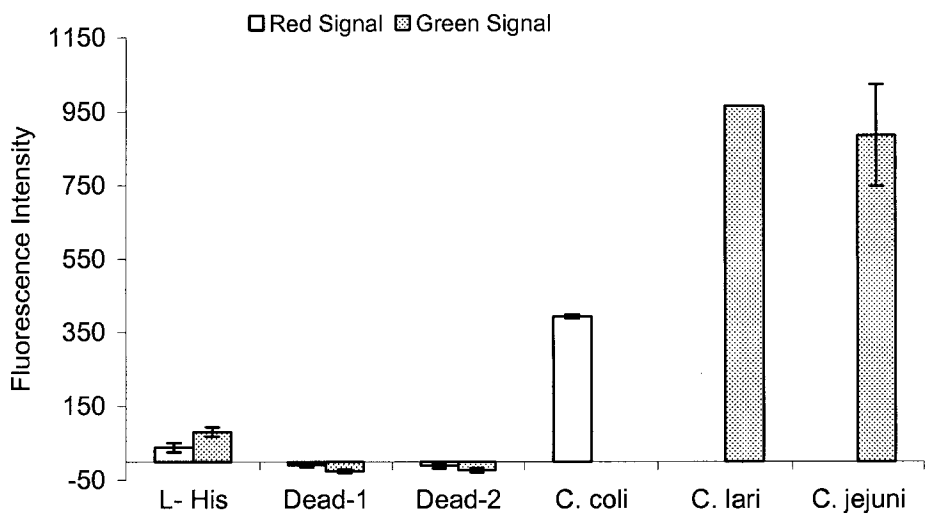


Figure 4.25 Dead cell control test

Total RNA extracted from heat-killed *C. coli* cells was amplified by set-1 and set-2 PCR. The amplicons were addressed onto the microarray chip in triplicate and their fluorescence signals were shown as columns labeled as Dead-1 and Dead-2, respectively. Targets of *C. coli*, *C. lari*, and *C. jejuni* (50 nM for each) were in triplicate addressed as positive controls, shown as the columns labeled with each species name. The columns represent the mean background-subtracted fluorescence signals of the triplicates with error bars showing the standard deviation. L-histidine with a concentration of 50 mM was used as the background. Reporter solution containing the three species reporters (1 μM for each) was applied for the hybridization.

4.3.7 Evaluation with blind samples

Fourteen blind cell samples prepared from cultured cells were tested to evaluate the developed method. Seven of them were independently prepared in another lab from that

lab's *Campylobacter* cultures. The other 7 were prepared with cultured *C. jejuni*-782, *C. lari*-803, and *C. coli*-456 by a colleague in the EHS lab.

Table 4.4 Test results of unknown samples

Sample Type	Sample No.	Signal-to-Background Ratio				Tested Results	Sample Identity	Accuracy
		Set 1 amplicons		Set 2 amplicons				
		Green	Red	Green	Red			
Third Party Blind	61	0.03	0.11	5.41	0.63	*C. jejuni*	*C. jejuni*	√
	98	0.31	-0.32	20.03	0.42	*C. jejuni*	*C. jejuni*	√
	123	0.62	0.26	5.37	0.26	*C. jejuni*	*C. jejuni*	√
	143	0.25	0.74	0.03	17.63	*C. coli*	*C. coli*	√
	181	0.66	0.00	5.72	0.05	*C. jejuni*	*C. jejuni*	√
	11y	0.37	1.05	-0.06	91.95	*C. coli*	*C. coli*	√
	790	38.37	-0.11	4.72	1.32	*C. lari*	*C. lari*	√
EHS-prepared Blind	1	6.34	-0.21	1.56	59.00	*C. lari & C. coli*	*C. lari & C. coli*	√
	2	-0.08	0.11	-0.14	18.33	*C. coli*	*C. coli*	√
	3	10.81	-0.68	17.56	-0.58	*C. lari & C. jejuni*	*C. lari & C. jejuni*	√
	4	8.33	1.41	30.89	69.15	*C.lari, jejuni & coli*	*C.lari, jejuni & coli*	√
	5	53.78	-0.68	1.16	-0.05	*C. lari*	*C. lari*	√
	6	-0.50	-0.42	7.37	87.68	*C. jejuni & C. coli*	*C. jejuni & C. coli*	√
	7	-0.16	-0.68	22.59	3.95	*C. ejuni*	*C. jejuni*	√
Notes	Set 1: Green – *C. lari* Set 2: Green – *C. jejuni* Set 2: Red – *C. coli* Target concentration: ~17 nM for all samples							

The original concentration of each isolate in tryptic soy broth was 9×10^8 CFU/mL. The original medium was diluted 10-fold before being used to prepare the blind samples, which were designated as samples 1, 2, 3, 4, 5, 6, and 7. Each blind sample was prepared to a volume of 1 mL, containing either one species or more than one species to a total concentration of 10^7 CFU/mL. Table 4.4 summarizes the results obtained from the

microarray. The S/B ratio of 5 was used as the criterion for positive identification. A comparison of the tested results with the sample identities resulted in 100% accuracy.

4.3.8 Preliminary application of the developed method to chicken samples

The developed method was also applied to preliminary testing of some real samples. The prevalence of *Campylobacter* in a sampling of Edmonton retail chicken is around 58% (VanderKop, 2003). Based on the suggestion by Messer et al. (1992) on the required sample size (the number of samples needed to detect) for at least one positive result, six samples must be obtained in order to find a positive unit with probability 0.95 for the 58% prevalence.

Six retail air-chilled whole broil chickens were randomly purchased from three grocery stores in Edmonton. Two chickens were randomly selected from all the whole broiler chickens on display at each store when the last digit of the price of each chicken matched the sequential numbers in a random number table. As soon as these chickens were transported on ice to the EHS lab, *Campylobacter*, along with other bacteria if present, were collected according to USDA/FSIS Microbiology Laboratory Guidebook (USDA/FSIS, 1998). The procedure is explained in detail as follows. A whole raw chicken was placed in a large sterile plastic bag (Stomacher^{TM} 3500), and 200 mL 0.1% peptone water was added. The bag was twisted to seal it and the contents were shaken for 2 min. Then the chicken was held while tilting the bag to allow the rinse solution to flow to one corner. The corner was cut in an aseptic manner after it had been sanitized with 70% ethanol and rinsed with sterile distilled water. The rinse solution was poured through sterile double-layered cheesecloth and collected into a sterile 250 mL container. After

incubation in a 48 °C water bath for 25 min, the rinse solution was centrifuged at 16,000 × g for 15 min at 4 °C to concentrate the bacteria into a pellet. The supernatant was discarded and the pellet was suspended as much as possible in 5 mL of Trizol reagent by vortexing. For each homogenized sample, triplates of 1 mL were saved for the test. Products from one PCR reaction were totally addressed and detected on the microarray. The results were interpreted and are summarized in Table 4.5. The criterion used for positive identification was an S/B of 5. The samples with S/B value less than 5 were considered non-detectable. One sample was detected positive for *C. coli*.

Table 4.5 Test Results of Real Samples

Sample No.	Signal-to-Background Ratio				Tested Results
	Set-1 PCR amplicons		Set-2 PCR amplicons		
	Green Signal	Red Signal	Green Signal	Red Signal	
1	-0.30	-0.34	-0.36	-0.04	ND
2	-0.39	0.00	-0.49	-0.11	ND
3	-0.42	-0.13	-0.52	-0.15	ND
4	-0.40	0.72	-0.52	-0.13	ND
5	1.05	0.69	1.27	5.99	*C. coli*
6	1.04	0.41	1.97	0.75	ND
Notes	ND: not detected Set 1: Green – *C. lari* Set 2: Green – *C. jejuni*; Red – *C. coli*				

This preliminary experiment demonstrates the potential of applying the developed method to real samples. Further experiments on a large scale need to be done.

4.4 Concluding remarks

This chapter describes the development of a RT-PCR electronic DNA microarray technique for detection and identification of *Campylobacter jejuni*, *C. coli*, and *C. lari*. The developed method can simultaneously detect the three *Campylobacter* species with high accuracy, and can detect as few as 2 *Campylobacter* cells. In addition, the developed method can differentiate between viable cells, including those in the VBNC state, and dead cells, and provide high throughput. Evaluation of the developed method showed 100% agreement with blind sample identities, demonstrating the high specificity of the developed technique. Preliminary application to real samples showed the potential of the developed method for application in areas including clinical diagnosis, molecular epidemiological studies, and water quality monitoring.

CHAPTER 5. SUMMARY AND FUTURE RESEARCH

In this thesis, a reverse transcriptase-polymerase chain reaction electronic DNA microarray technique was successfully developed for detection and differentiation of three major *Campylobacter* species, *C. jejuni*, *C. coli*, and *C. lari*.

Some of the major developments of this technique are briefly summarized below:

(1) To detect only viable cells and not dead cells, mRNA of the 60-kiloDalton heat shock gene (*hsp*60), a gene universally present in all microorganisms, was selected as the viability marker. This choice was made because of the responsiveness to heat shock that occurs only in viable cells, including those in the VBNC state. Heat shock treatment is not only a simple and direct measure allowing detection of cell viability, it is also a measure to induce and accumulate *hsp*60 mRNA, which improves the sensitivity of the detection. The ability to detect only viable cells by this approach was confirmed with dead cell control experiments.

(2) To detect *Campylobacter* at the species level, single nucleotide polymorphisms (SNPs) were selected as the species markers. A comparison of the *hsp*60 gene sequences in GenBank from *C. jejuni*, *C. coli*, and *C. lari* shows that naturally occurring variations exist in the genome of *Campylobacter*. Some of them are species-specific and can be used for the differentiation of the three species. To ensure the identified SNPs are truly species markers, 12 more *Campylobacter* isolates of these three different species were heat shock-treated, their *hsp*60 RNA was extracted, a ~450-bp fragment within their *hsp*60 gene was RT-PCR amplified, and the amplified products were cloned and

sequenced. Together with the sequences from the database, species-specific SNPs were identified.

(3) To develop a technique that can rapidly, accurately, and sensitively detect *Campylobacter* samples on a large scale, an electronic DNA microarray method was developed. In this method, a fragment of 200-bp within *hsp*60 mRNA was RT-PCR amplified. Two closely located SNPs within this 200-bp fragment were chosen as the species marker. After the analysis of the secondary structure of this fragment, a dot-blot format was adopted for the DNA microarray assay. Corresponding reporters specific to each of the three *Campylobacter* species were designed. After optimization of instrumental and experimental parameters such as addressing voltage, addressing time, target concentration, and environmental temperature, some key aspects of the technique were investigated.

a. Specificity of the designed reporters. This was investigated by the hybridization between the targets of the three species and the corresponding reporter(s). The results showed that each reporter specifically recognized its corresponding target. No cross-identification was observed, demonstrating the high accuracy of this technique.
b. Dead cell control. One of the advantages of the developed technique is its ability to detect only viable cells and not dead cells. To demonstrate this, *C. coli* cells were killed at 100 °C in a water bath for 15 min and kept at room temperature for 2 days before the total RNA was extracted. The results showed no interference from the dead *C. coli* cells.
c. Interference from other microorganisms. Although the database search did not show any significant interference, the microorganisms *E. coli* O157:H7, non-pathogenic

E. coli (ATCC 25955), *Salmonella* Typhi (R704101), *Vibrio cholerae* O1, *Listeria monocytogenes* (R1694101), *Yersinia enterocolitica* (K1332/03), and *Cryptosporidium parvum* were tested. After PCR amplification, only *Vibrio cholerae* O1 and *Salmonella* Typhi showed potential interference as bands observed from gel electrophoresis. In order to confirm whether *Vibrio cholerae* O1 and *Salmonella Typhi* truly interfere with the identification of target bacteria, DNA microarray analysis was carried out. Only *Campylobacter* was correctly detected, and not other microorganisms. Therefore, non-interference from other microorganisms tested was confirmed on the DNA microarray.

d. Sensitivity. To demonstrate the high sensitivity of this technique, experiments with serially diluted total RNA and cells were carried out. The results showed that the developed technique can detect as few as 50 fg of total RNA or only a few cells.

e. Evaluation and application. To evaluate the developed method, 14 blind samples prepared from cultured cells were analyzed. The results showed 100% agreement with their identities, demonstrating the accuracy of the identification and the usefulness of the developed technique for identification of unknown samples. Furthermore, the developed method was successfully applied to retail chicken rinse samples. Among the six samples tested, one showed positive results for *Campylobacter coli.*

Future research can be carried out to improve the total RNA extraction efficiency, to test cells in the VBNC state, and to apply the developed technique to more samples from the real world, as discussed below.

(1) RNA extraction loss. In this thesis, RNA was extracted using the traditional technique. The experimental results showed that the extraction efficiency varied, which greatly affects the subsequent steps. One suggestion for future work is to find a better method to extract RNA. Using a single column-based method could be a solution.

(2) Detection of cells in the VBNC state. *Campylobacter* should be induced somehow into the VBNC state and detected directly to demonstrate one of the advantages of the developed approach. However, this was not done in this thesis due to the difficulty of confirming cells in this state. In the future, if possible, this should be demonstrated.

(3) Applications to samples from the real world. Some preliminary experiments of the six chicken samples demonstrated the capability of the developed technique to detect *Campylobacter* at the species level in real samples. More chicken samples and samples from other sources such as water and feces should also be tested.

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Appendix

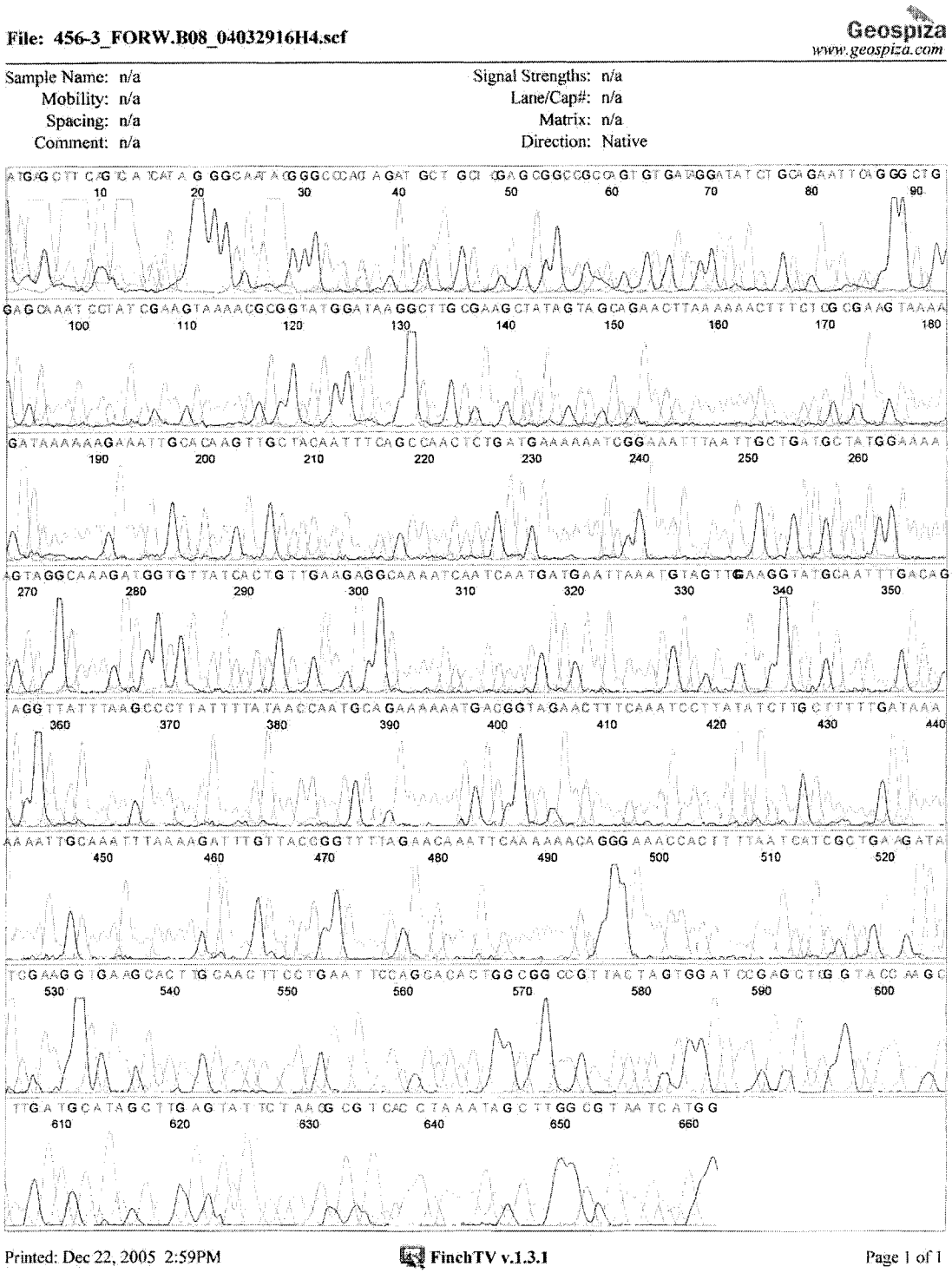


Figure A1 *C. coli*-456 sequencing result

Sample Name: n/a
Mobility: n/a
Spacing: n/a
Comment: n/a

Signal Strengths: n/a
Lane/Cap#: n/a
Matrix: n/a
Direction: Native

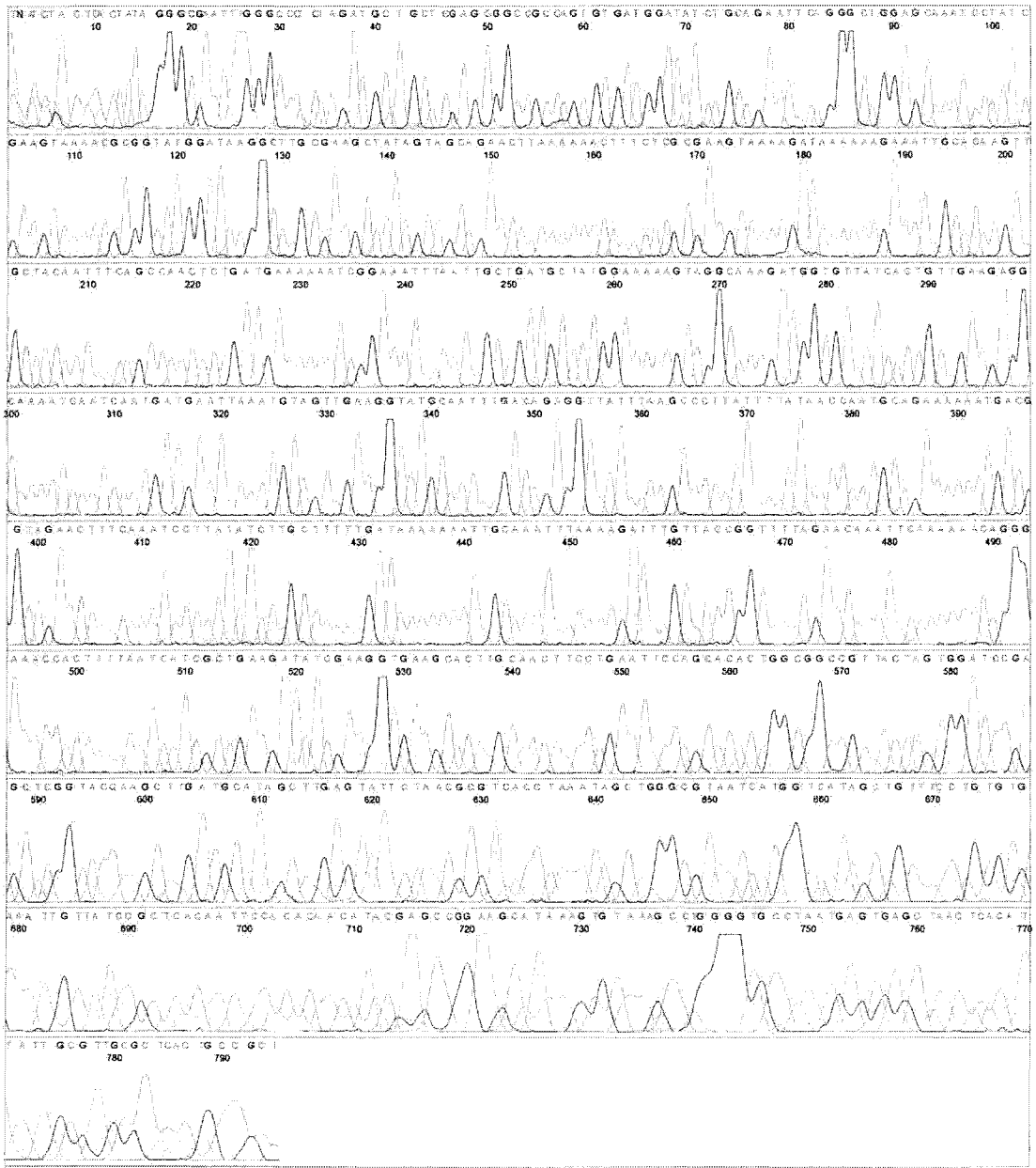


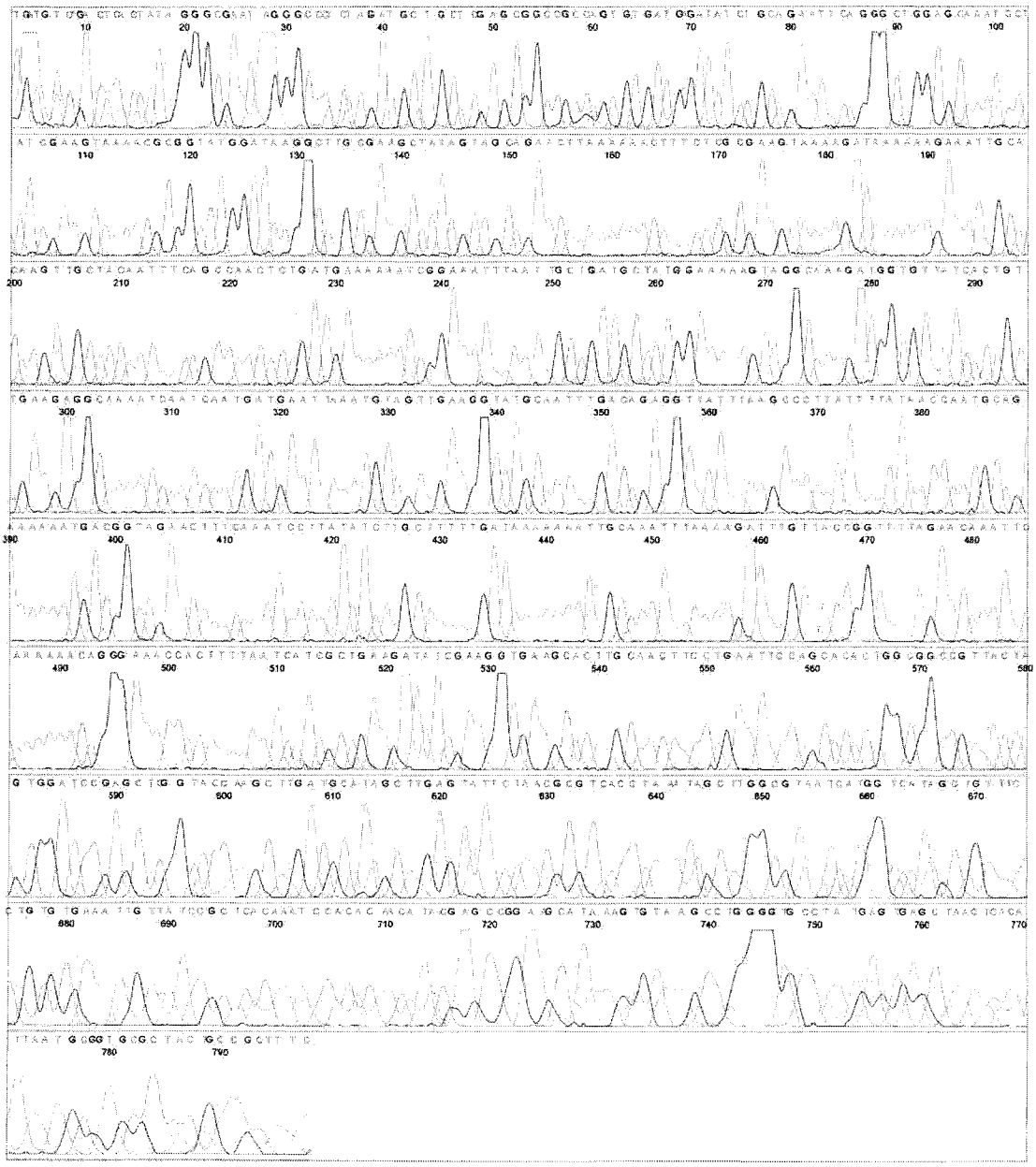
Figure A2 *C. coli*-772 sequencing result

File: 79709_FOR.E02_04040717FJ.scf

Geospiza
www.geospiza.com

Sample Name: n/a
Mobility: n/a
Spacing: n/a
Comment: n/a

Signal Strengths: n/a
Lane/Cap#: n/a
Matrix: n/a
Direction: Native



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FinchTV v.1.3.1

Page 1 of 1

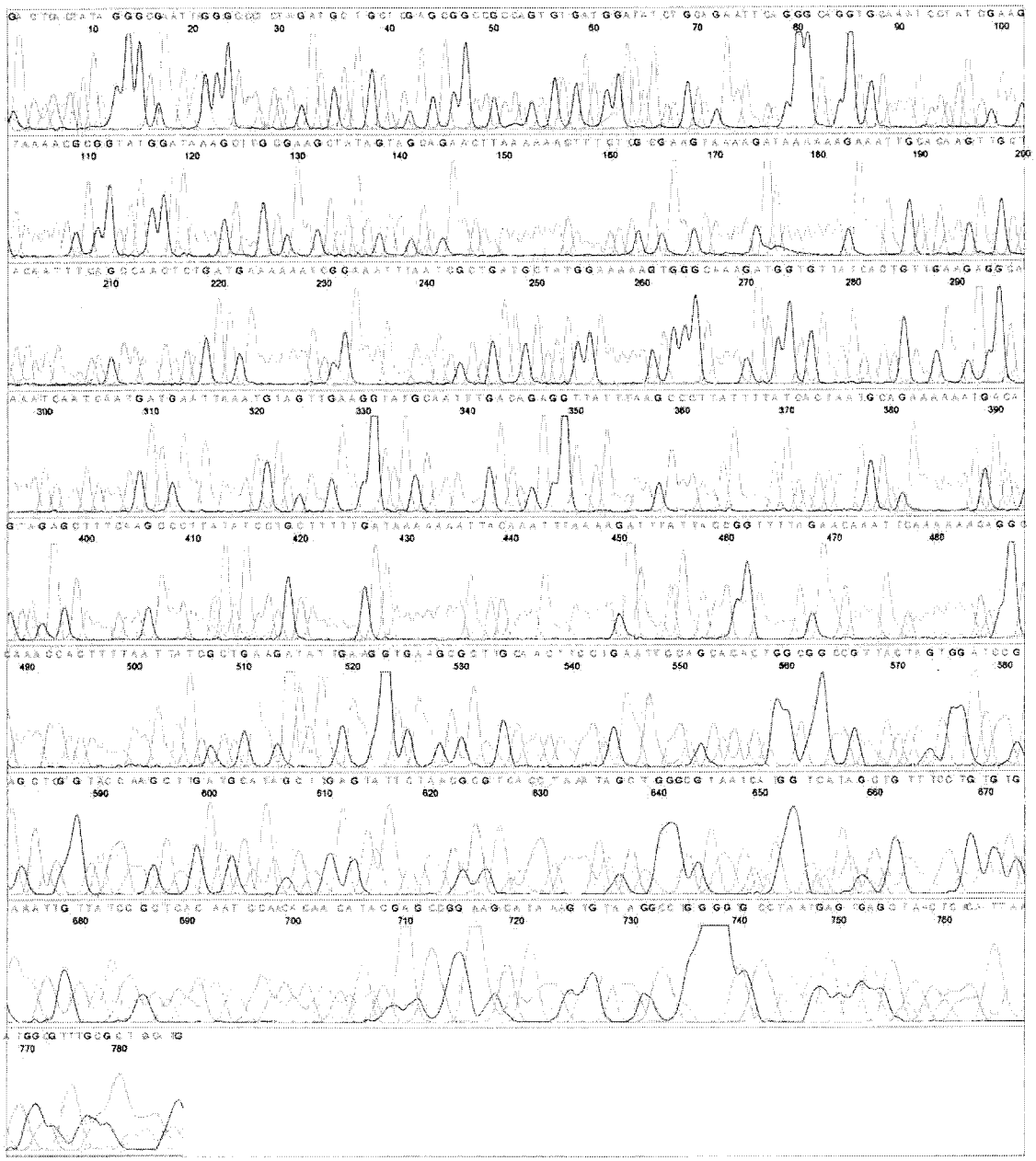
Figure A3 *C. coli*-797 sequencing result

File: 51212_FOR.B03_0404071929.scf

Geospiza
www.geospiza.com

Sample Name: n/a
Mobility: n/a
Spacing: n/a
Comment: n/a

Signal Strengths: n/a
Lane/Cap#: n/a
Matrix: n/a
Direction: Native



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FinchTV v.1.3.1

Page 1 of 1

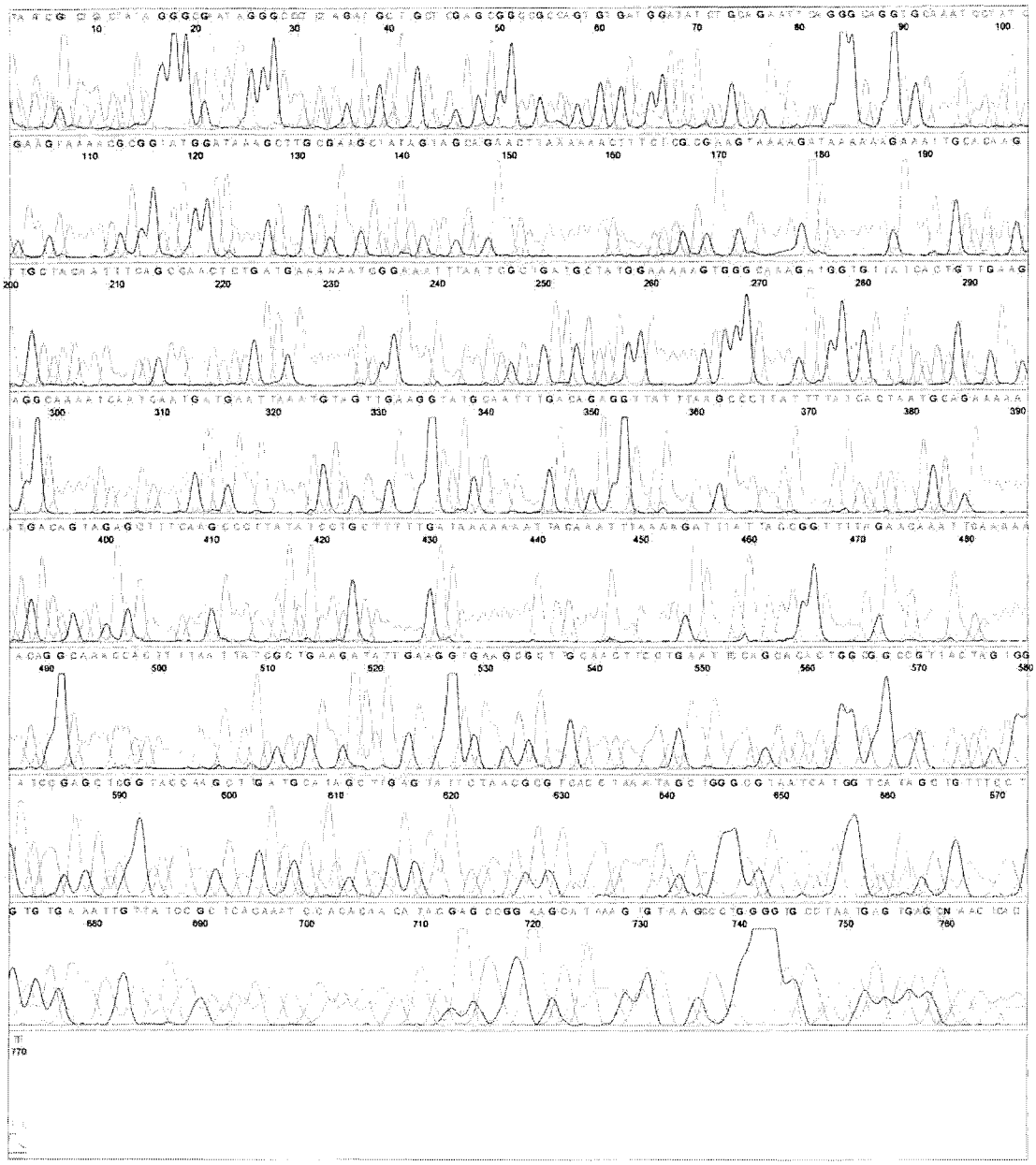
Figure A4 *C. jejuni*-512 sequencing result

File: 54210_FOR.C02_04040717FJ.scf

Geospiza
www.geospiza.com

Sample Name: n/a
Mobility: n/a
Spacing: n/a
Comment: n/a

Signal Strengths: n/a
Lane/Cap#: n/a
Matrix: n/a
Direction: Native



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FinchTV v.1.3.1

Page 1 of 1

Figure A5 *C. jejuni*-542 sequencing result

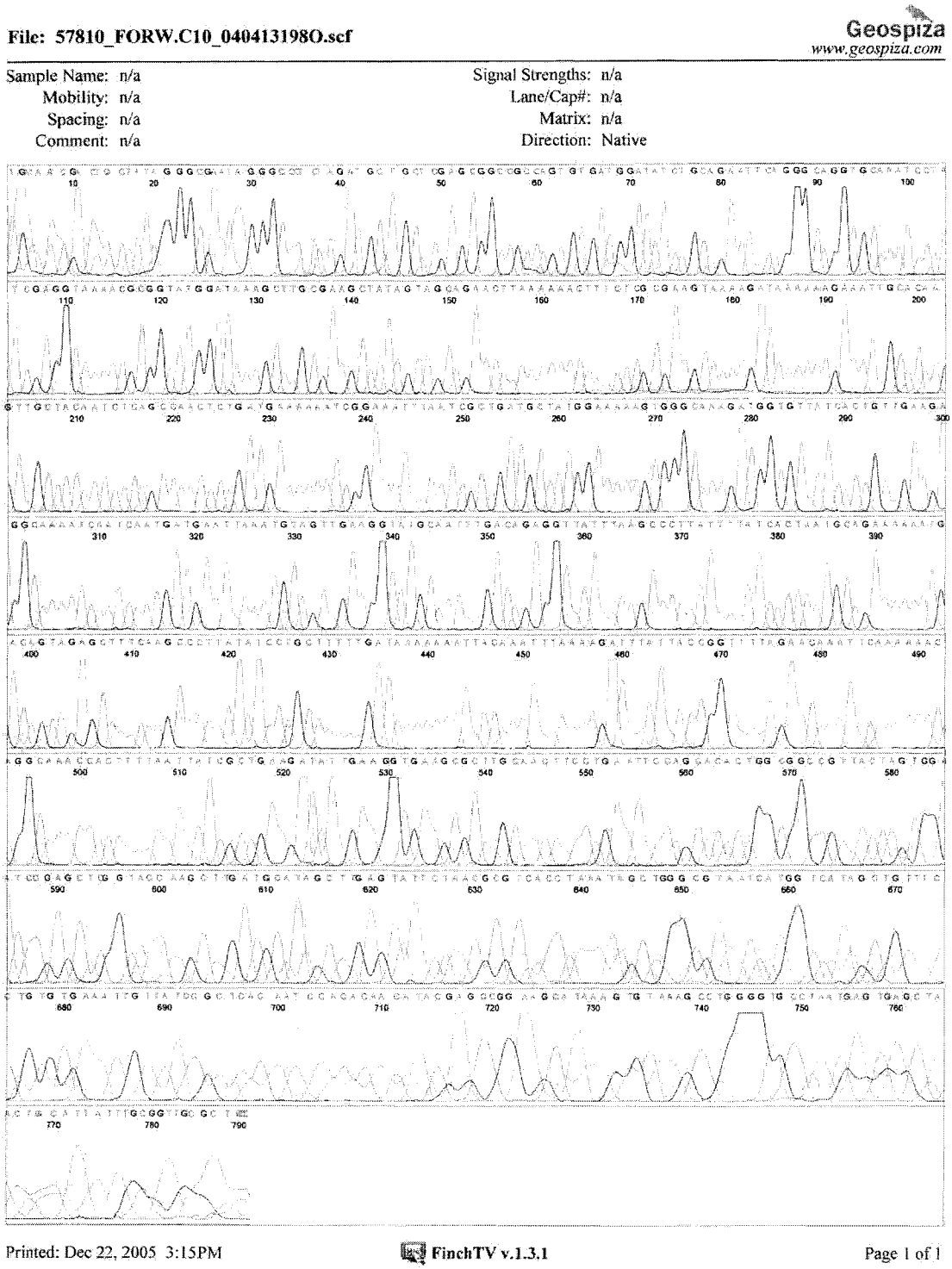
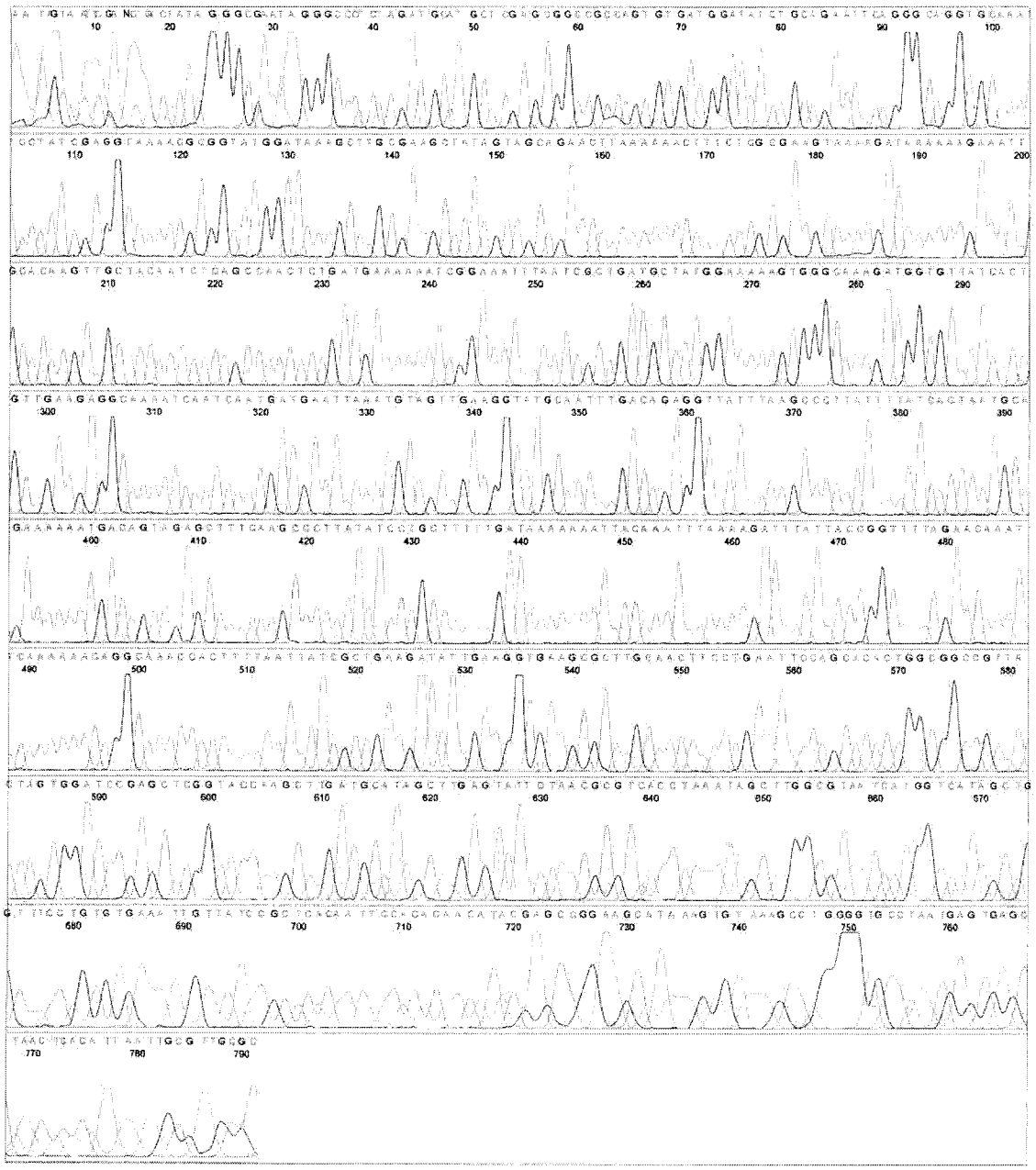


Figure A6 *C. jejuni*-578 sequencing result

File: 57912_FORW.F10_040413198O.scf

Geospiza
www.geospiza.com

Sample Name:	n/a	Signal Strengths:	n/a
Mobility:	n/a	Lane/Cap#:	n/a
Spacing:	n/a	Matrix:	n/a
Comment:	n/a	Direction:	Native



Printed: Dec 22, 2005 3:16PM FinchTV v.1.3.1 Page 1 of 1

Figure A7 *C. jejuni*-579 sequencing result

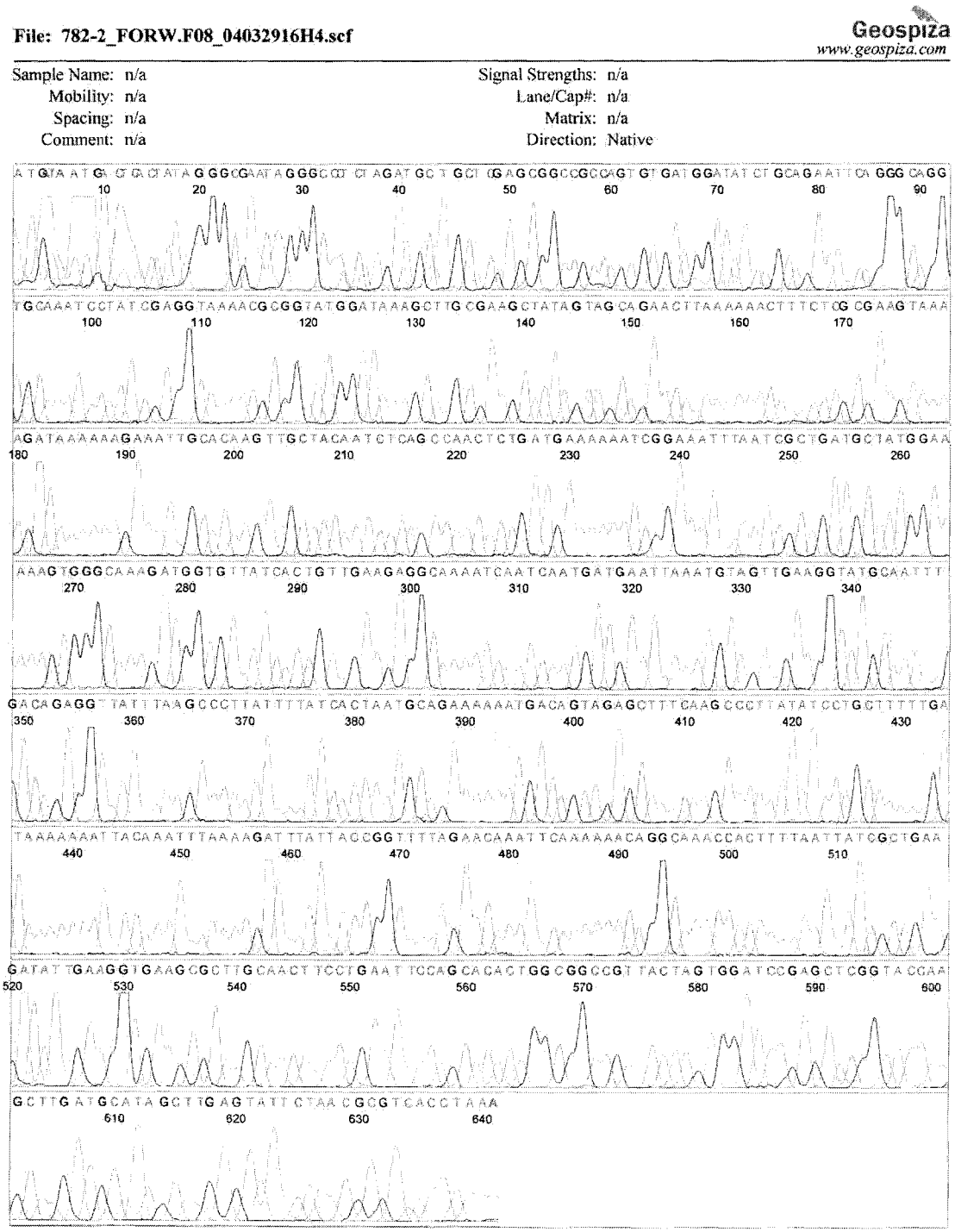


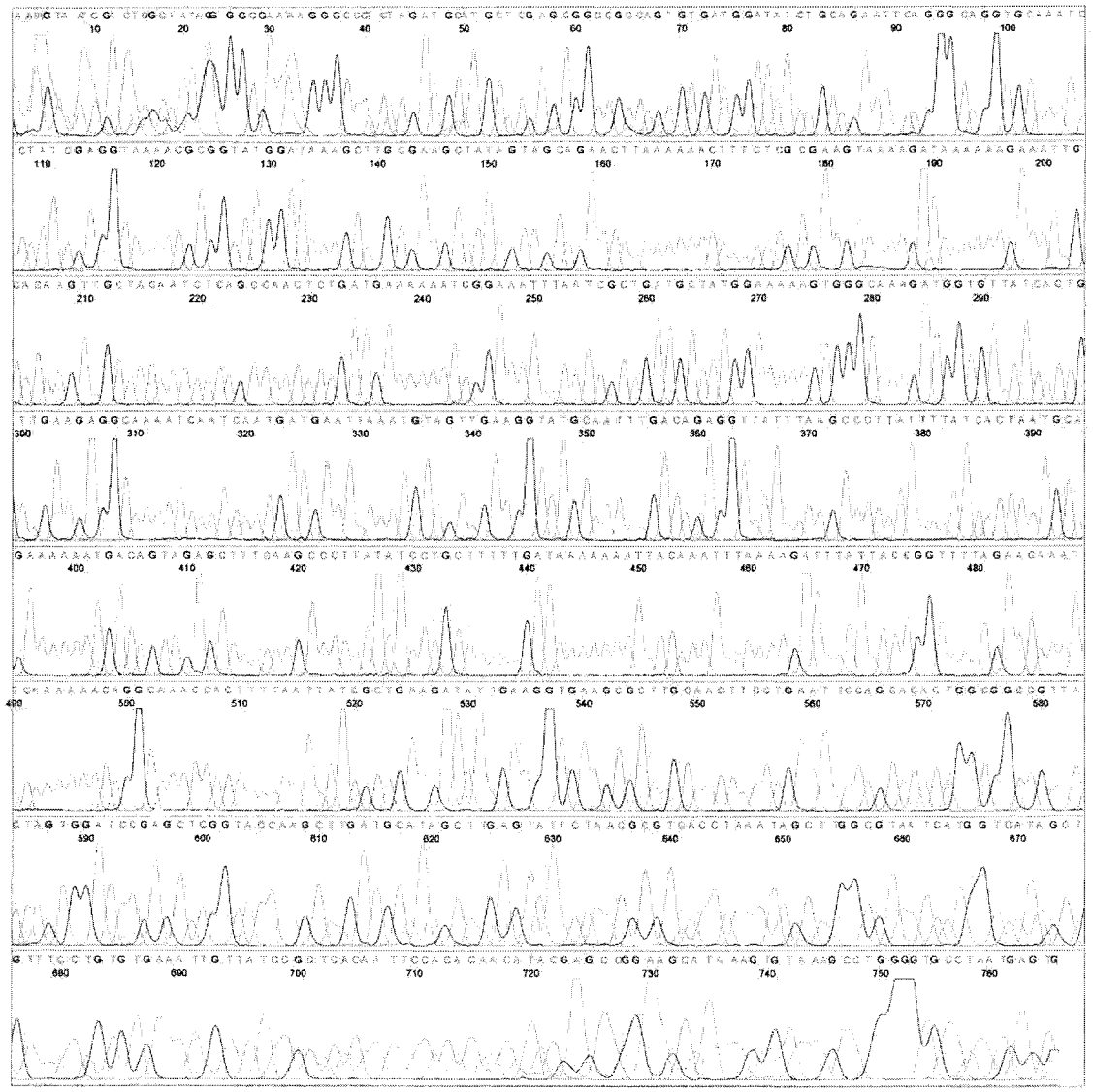
Figure A8 *C. jejuni*-782 sequencing result

File: 78301_FORW.G10_040413198O.scf

Geospiza
www.geospiza.com

Sample Name: n/a
Mobility: n/a
Spacing: n/a
Comment: n/a

Signal Strengths: n/a
Lane/Cap#: n/a
Matrix: n/a
Direction: Native



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FinchTV v.1.3.1

Page 1 of 1

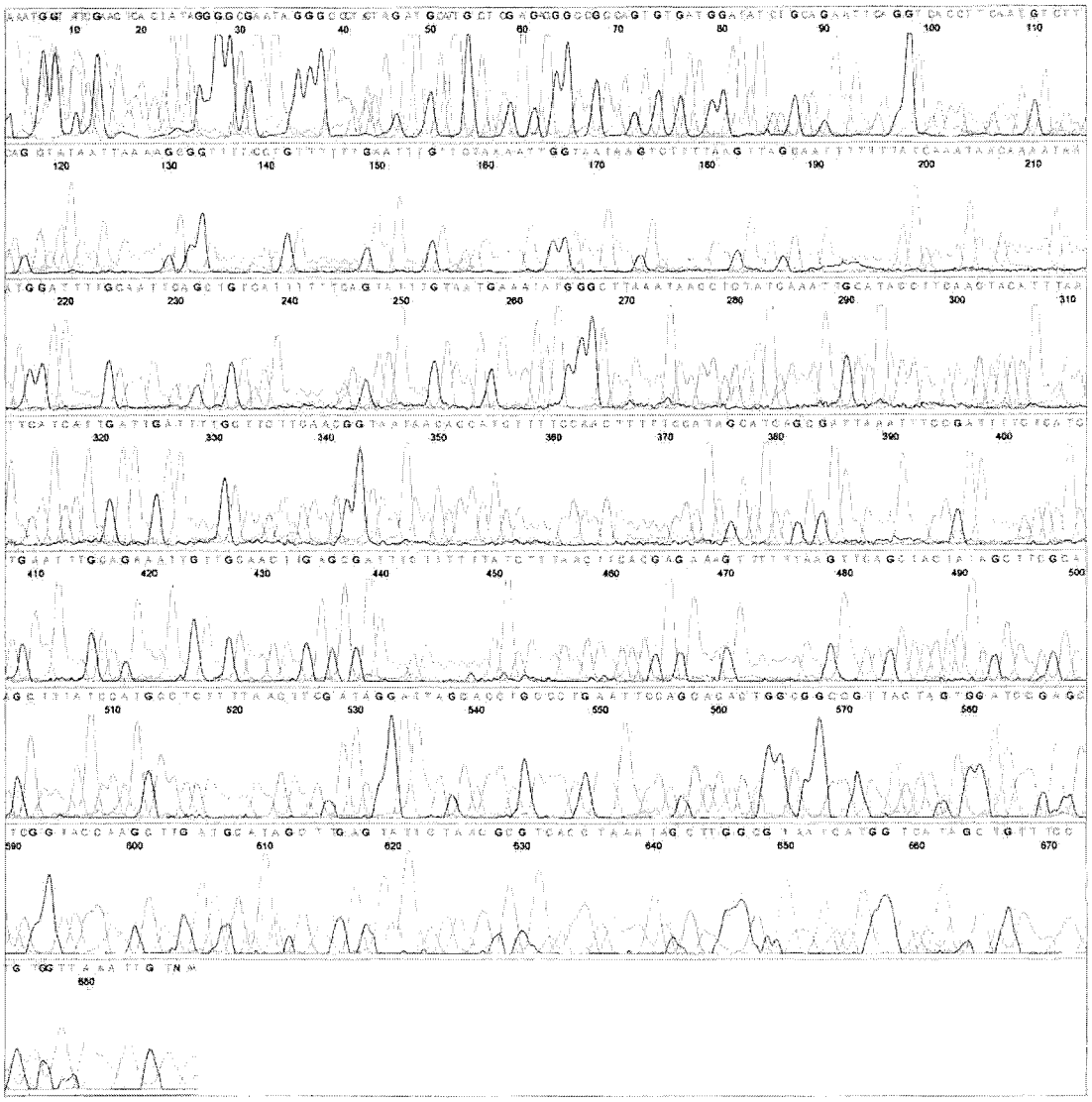
Figure A9 *C. jejuni*-783 sequencing result

File: 787-9_FORW.E08_04032916H4.scf

Geospiza
www.geospiza.com

Sample Name: n/a
Mobility: n/a
Spacing: n/a
Comment: n/a

Signal Strengths: n/a
Lane/Cap#: n/a
Matrix: n/a
Direction: Native



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FinchTV v.1.3.1

Page 1 of 1

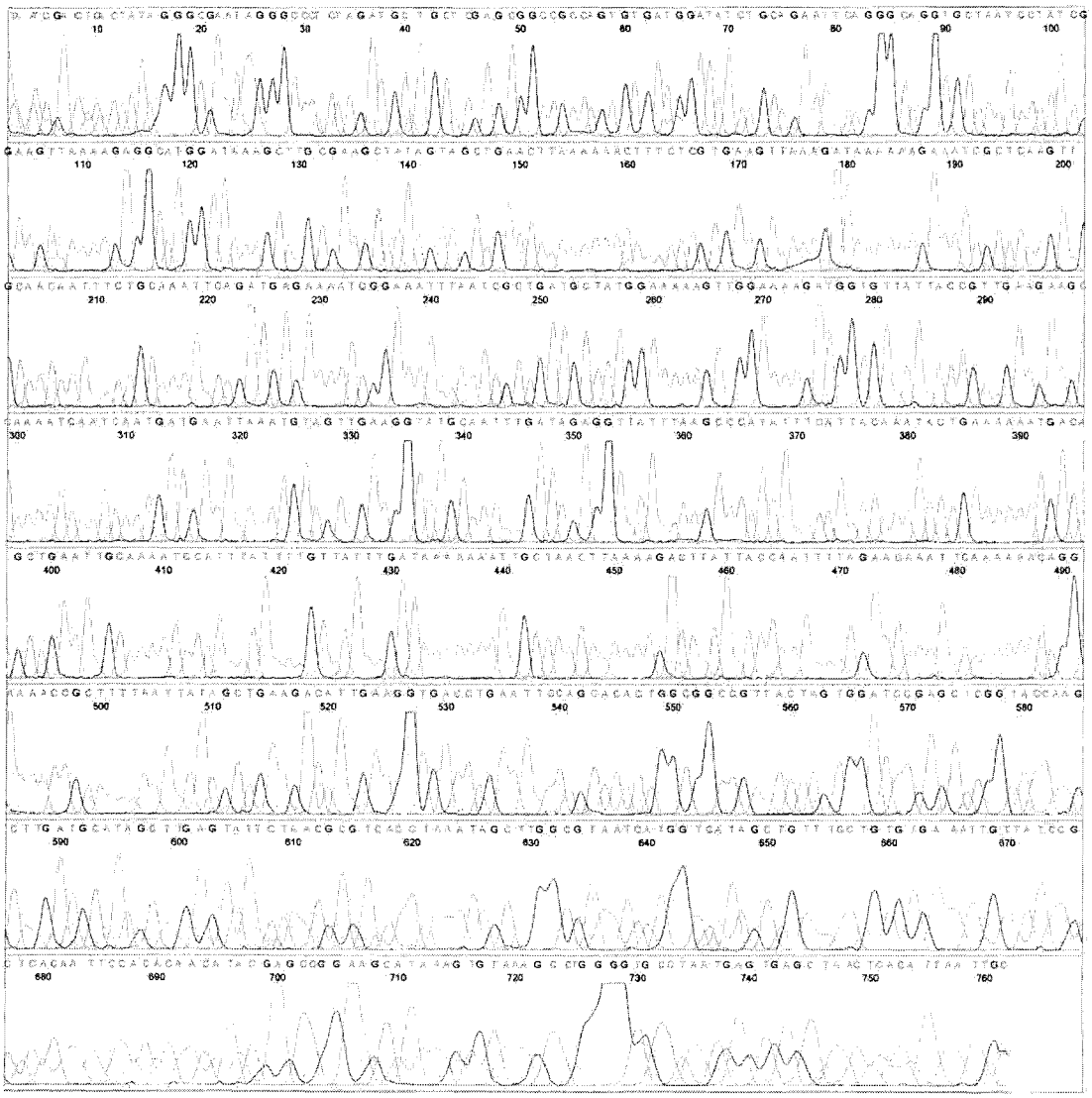
Figure A10 *C. lari*-787 sequencing result

File: 79609_FOR.C03_0404071929.scf

Geospiza
www.geospiza.com

Sample Name: n/a
Mobility: n/a
Spacing: n/a
Comment: n/a

Signal Strengths: n/a
Lane/Cap#: n/a
Matrix: n/a
Direction: Native



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FinchTV v.1.3.1

Page 1 of 1

Figure A11 *C. lari*-796 sequencing result

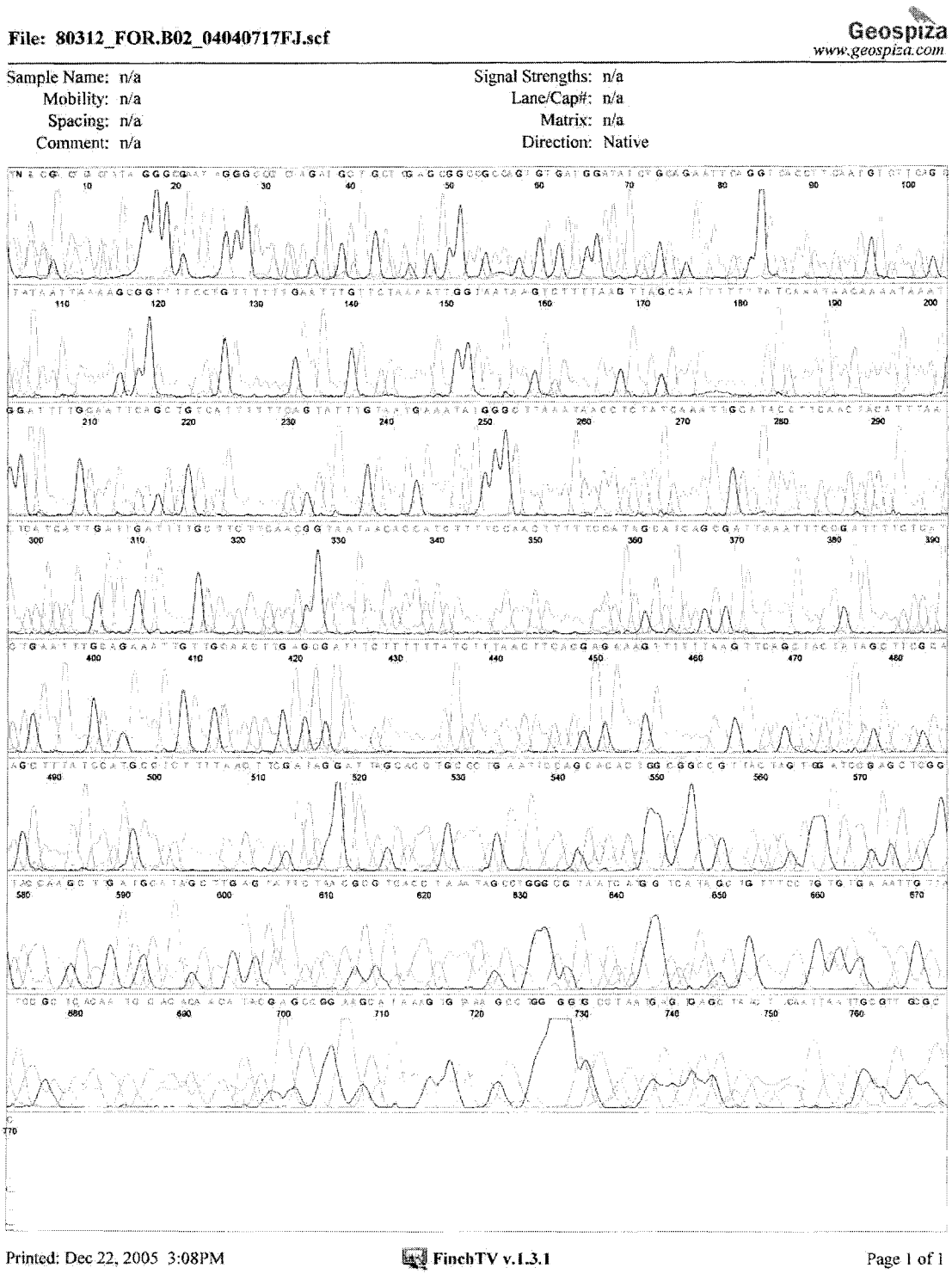

Figure A12 *C. lari*-803 sequencing result