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Design and validation of oligonucleotide primers suitable for waterborne bacterial pathogen detection via real-time qPCR

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DESIGN AND VALIDATION OF OLIGONUCLEOTIDE PRIMERS SUITABLE FOR WATERBORNE BACTERIAL PATHOGEN DETECTION VIA REAL-TIME qPCR

by

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ABSTRACT

Fecal coliforms have been used as indicators to evaluate health risks associated with the microbiological quality of water for many years. Recent studies have challenged their ability to accurately predict bacterial numbers in the natural environment. DNA-based assays are proposed candidates to replace existing methods, but protocols suited for standardized direct-use have not yet been sufficiently developed. The objective of this study was to examine the feasibility of using real-time quantitative PCR (qPCR) to detect contamination from five waterborne bacterial pathogens in surface and treated drinking waters. Robust oligonucleotide primers were assembled to target virulence-associated genes. Primers were found to have high specificity and increased sensitivity for low pathogen loads of 10 cells/mL, as determined experimentally via qPCR. Detection of pathogenic cells directly from an environmental matrix has also been demonstrated using a filtration-extraction procedure. The developed protocols have shown their potential for use in conjunction with traditional indicator techniques.

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LIST OF ABBREVIATIONS

C. jejuni	*Campylobacter jejuni*
C. parvum	*Cryptosporidium parvum*
C. perfringens	*Clostridium perfringens*
CDW	Federal-Territorial Committee on Drinking Water
C_P	Crossing point
CRPG	Chlorophenol red β-galactosidase
DNA	Deoxyribonucleic acid
DST	Defined substrate test
E. coli	*Escherichia coli*
EU	European Union
G. lamblia	*Giardia lamblia*
K. pneumoniae	*Klebsiella pneumoniae*
MAC	Maximum acceptable concentration
MF	Membrane filtration
MPN	Most probable number
MTF	Multiple tube fermentation
MUG	4-methylumbelliferyl-β-D-glucoronide
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
P. aeruginosa	*Pseudomonas aeruginosa*
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction

S. flexneri	*Shigella flexneri*
S. Typhimurium	*Salmonella enterica* serovar Typhimurium
US EPA	United States Environmental Protection Agency
UV	Ultraviolet
WHO	World Health Organization
VBNC	Viable but non-culturable

CHAPTER 1: INTRODUCTION

1.1. Background

Each year, the ingestion of water contaminated by pathogens such as *Escherichia coli* (*E. coli*) O157:H7 accounts for over 2 million deaths worldwide (World Health Organization, 2003). The actual number of fatalities attributed to waterborne diseases is currently underestimated, due to prevalent underreporting of gastroenteritis and asymptomatic infections amongst some infected individuals. In developed nations, advanced water treatment technologies operate on the belief that water consumed by the general public is potable and pathogen-free following such processes. To strengthen this claim, regular monitoring and control of microbial growth is essential in preventing illness since millions of gallons of source water are purified and consumed on a daily basis (Berry *et al.*, 2006).

The microbiological quality of water is currently monitored using techniques reliant on indicator organisms to identify recent fecal contamination and the possible presence of pathogens. Nonetheless, outbreaks of waterborne disease still occur in developed nations since such techniques lack perfect efficiency and vigilance in water quality monitoring by treatment operators is often less than routine. Limited correlation between pathogens and indicators has been recently documented (Polo *et al.*, 1998; Lemarchand *et al.*, 2004; Dorner *et al.*, 2007, Ahmed *et al.*, 2008; Wilkes *et al.*, 2009), and therefore these methods may not be entirely reflective of actual pathogen loads. After the Walkerton outbreak in 2000, where isolates of *E. coli* O157:H7 and *Campylobacter jejuni* (*C. jejuni*) were found in a rural groundwater supply, Canada has attempted to implement a multiple-barrier "source-to-tap" approach to drinking

water safety. This includes placing barriers at key points within the water collection, treatment and distribution processes to prevent any pathogens in the influent from passing to the finished water. There are currently no maximum acceptable concentrations (MAC) for pathogens in water and guidelines are only voluntarily enforced (Health Canada, 2006). Two important factors with regards to current guidelines can be questioned, specifically; the effectiveness of the disinfection methods to inactivate waterborne pathogens and the validity of the current techniques used for their identification.

Recent threats of bioterrorism highlight the need to protect Canada's water resources. The development of standardized, real-time, automated testing systems, with short analysis times and increased specificity for the detection of particular contaminants is desirable. In the last decade, much of the literature available on waterborne pathogen detection focuses on the potential application of DNA-based techniques to satisfy the necessity for more rapid detection technologies and act as supplements to current testing measures. The ability to limit the potential health risks associated with water is critical in maintaining healthy societies and preventing unnecessary deaths.

1.2. Objectives

The objective of this study was to examine the feasibility of applying real-time qPCR as a rapid technique for standardized pathogen detection, and a possible supplement for traditional indicator-reliant methods. This would be determined by satisfying several goals; (i) to compile a robust set of oligonucleotide primers having virulence-associated gene targets, (ii) to develop a rapid filtration-extraction procedure to isolate DNA from water, and (iii) to use one of the selected pathogens as a model to test detection limits with seeded environmental samples.

The importance of such a test can come from an examination of current water quality monitoring technologies. It is known that there is a lack of standardization with treatment techniques between the public and private spheres. Issues regarding drinking water quality need to be addressed with high priority.

The success of such a system is dependent on the robustness and sensitivity of the technique. The use of qPCR as a detection platform would contribute to the development of an early warning pathogen detection system since sensitive, quantifiable results would be generated much sooner and with higher accuracy than current protocols. This would enhance the ability of treatment facilities to reduce and prevent illnesses associated with waterborne pathogens and protect vital drinking water resources.

1.3. Expectations

It is expected that the selected amplification targets and the customized oligonucleotide primers will be robust, highly specific and sensitive for their intended pathogen sequences. Combined with a membrane filtration isolation method to reduce false positives due to exogenous DNA, it is expected that detection of the chosen pathogens will be possible in artificially contaminated water matrices. The detection limits are predicted to be at or below the minimum infectious doses of each of the pathogens, relative to the amount of water sampled. In the case of the environmental samples, although sensitivity may be reduced due to PCR-inhibitory substances, sensitivity levels are still predicted to detect minimum infectious doses. Overall, it is believed that the qPCR technique will show potential for application in early warning pathogen detection systems.

CHAPTER 2: LITERATURE REVIEW

2.1. Waterborne disease and its origins

Disease-causing microorganisms, otherwise known as pathogens, are of great concern to humans since they are able to cause potentially life-threatening infections and death in cases where an infection is sufficiently aggressive. Water can be a reservoir for a host of pathogens and chemical contaminants. It has been suggested that there is a greater risk to humans from exposure to these organisms in drinking water than chemical and radiological contaminants because of the implication of immediate and easily transmissible health effects (Boyd, 2006). There are several exposure pathways which allow these organisms to gain entry into the human body, including; ingestion, inhalation, dermal contact or direct passage through the mucoid membranes of bodily orifices such as eyes and ears (Arnone and Walling, 2007; Clesceri *et al*., 1999). The fecal-oral route is the most common method of enteric pathogen transmission, whereby a fecally contaminated substance, such as water, is ingested by an individual.

The World Health Organization (WHO), the international agency concerned with public health and safety, emphasizes that water-related diseases currently remain a high priority because the number of cases continues to increase annually, partially due to the constant increase in numbers and diversity of causative agents (World Health Organization, 2003). There is a great deal of initiative to provide systems to accurately monitor the potability of drinking water and the presence of these harmful microorganisms not only to developing nations but also industrialized countries (Hunter *et al*., 2009).

2.1.1. Entry and survival of pathogens in aquatic environments

Surface water and watersheds are considered by many to play important roles in the transport of pathogens and the transmission of waterborne disease. Water bodies can act as reservoirs for large numbers of these organisms, which are not members of the normal heterotrophic microbiota. Bacteria, protozoa, viruses and eukaryotic parasites known as helminths are the predominant pathogenic microorganisms (Canepari and Pruzzo, 2008). Surface water is subject to contamination by what is referred to as "pathogen pollution" (Edge *et al*., 2001), since they are unsecured and directly exposed to their surroundings.

Pathogens generally enter bodies of water from two possible outlets referred to as either point or non-point discharges. Point discharges are considered as specific contamination routes such as the seepage of raw sewage, wastewater effluents and storm water (Savichtcheva and Okabe, 2006). Non-point sources in comparison are those that are diffuse, occur over a broad geographical region and can be attributed to run-off originating from agricultural or urban areas (Lemarchand *et al*., 2004; Savichtcheva and Okabe, 2006). Under ideal water conditions, pathogen contamination cannot be observed with the human eye. As many as 10^9 bacterial cells can go unnoticed in as little as a single glass of water, leading to the consumption of contaminated water without prior knowledge of its potability. In the natural environment however, the concentration of pathogens and indicators that are present in a body of water at any given time is usually low, which complicates their detection and enumeration (Lemarchand *et al*., 2004).

Outbreaks of waterborne disease have been known to follow seasonal trends, with the number of observed cases being directly related to the temperature increases in the spring and summer months (Charron *et al*., 2004; Schuster *et al*., 2005). These warmer temperatures are similar to that of the human body and thus provide ideal conditions for bacterial pathogens to survive outside of hosts. Many species have adapted to lower temperatures to increase their survival rates by reducing their cellular metabolism (Arnone and Walling, 2007). A 2004 review by Charron *et al*. has also predicted that there may be an even greater increase in the number of waterborne outbreaks in the near future as a result of global warming. Weather is a key factor in compromising water quality by increasing microbial loads following intense precipitation events (Edge *et al*., 2001; Charron *et al*., 2004). To support this claim, a 2001 study by Lipp *et al*. found that concentrations of fecal indicators and enteric viruses were higher in both sediment and water samples collected from an estuary following intense precipitation events caused by El Niño. An influx of pathogens may enter a water body through agricultural or urban runoff during an intense rain event since there is an increased likelihood of transporting fecal particles containing pathogenic cells by flowing rainwater or seepage into groundwater. Cellular distribution within source water is non-uniform, since clustering of the pathogenic cells is favoured for survival in the natural environment (Dorner *et al*., 2007).

The survival of pathogenic microbes is further enhanced by cellular adsorption to particulate matter as either suspended, free-floating, flocculated particles in turbid water or bottom sediments. They are able to withstand environmental pressures and extend their life cycle since they are not directly exposed to abiotic stressors such as sunlight (Arnone and Walling, 2007). Several studies have focused on examining the effect of re-suspension on free-floating

pathogen numbers by using lab-scale mechanisms to model these environments. It has been suggested that when adsorbed to floc, the pathogens pose no significant threat to humans (Ferguson *et al*., 2003). In shallower nearshore waters however, disturbances such as forceful water currents can cause the water and sediment layers to mix, releasing the pathogens into the surrounding water and thus increasing the risk of ingestion and subsequent infection (Ferguson *et al*., 2003).

2.1.1. Waterborne disease in Canada

Illnesses associated with waterborne outbreaks are not exclusive to economically-strained, developing countries despite their higher disease rates from low levels of sanitation and water quality (Toze, 1999). The efficiency of water and wastewater treatment processes in industrialized nations is not always ideal and as a result, sporadic outbreaks of waterborne disease have been reported and documented throughout North America, Europe and Australia in recent years, including several which affected large percentages of the overall population (Clesceri *et al*., 1999).

In Canada specifically, a total of 288 outbreaks and over 8,000 cases of drinking water-related illness were confirmed between 1974 and 2001, when Health Canada first began monitoring the status of waterborne outbreaks (Edge *et al*., 2001; Schuster *et al*., 2005). It has been estimated that as many as 90 Canadians die each year from water-related illnesses (Eggertson, 2008). A 2006 analysis by Thomas *et al*. indicated that there are approximately 20,000 yearly cases of enteric illness reported to the Canadian National Notifiable Disease registry. In comparison, the information presented in an earlier 2001 review by Edge *et al*.

estimated that as many as 90,000 cases of waterborne illness occur annually in Canada, based on statistical data gathered within the US, indicating that actual and reported case numbers differ significantly.

All of the major classes of pathogens have been previously linked to waterborne disease in Canada. The majority of illnesses have been predominantly associated with the bacterial pathogens *E. coli* O157:H7 and *Campylobacter jejuni* and protozoan pathogens *Giardia lamblia* and *Cryptosporidium parvum*. These organisms have either evaded chemical disinfection, infiltrated groundwater systems or were capable of survival in recreational water such as public beaches. In as many as 43 of the drinking water-related outbreaks, the specific pathogens causing the disease remained unidentified and were likely linked to emerging waterborne organisms (Edge *et al*., 2001; Schuster *et al.*, 2005). The data observed in etiological studies such as these is, however, only a relative estimate of the total disease state, since enteric diseases are commonly under-reported due to the sometimes mild gastroenteritis and flu-like symptoms which go undiagnosed.

The most highly publicized Canadian drinking water-related outbreak occurred in Walkerton, Ontario, where intense precipitation caused a concentrated mixture of *E. coli* O157:H7 and *C. jejuni*, originating from cattle feces, to contaminate the town's groundwater supply, killing 7 and causing illness in more than 2,000 residents (Ibekwe and Grieve, 2003). Following the Walkerton outbreak in May 2000, public concern over water quality and preventative measures for future outbreaks sharply increased. Many small-town municipalities, whose primary water supplies were unsecured from the elements and not subject to disinfection,

began to implement basic disinfection technologies such as chemical chlorination in an attempt to secure their water supplies. Prior to these events, several other threats to water quality occurred in Canada, including outbreaks of the protozoan species *Toxoplasmosis gondii* in British Columbia and viral Hepatitis A in Québec, both in 1995, and the discovery of *C. parvum* in North Battleford, Saskatchewan in 2001 (Edge *et al*., 2001). Ten years later some remote areas in Canada, relying on groundwater or those who do not have adequately trained treatment technicians still have issues with the availability of potable drinking water (Charron *et al*., 2004). Evidence was seen with the 2005 evacuation of the Kashechewan community in Northern Ontario, following the discovery of elevated levels of *E. coli* in the water supply system (Boyd, 2006). In instances where high coliform counts are observed or chlorination treatments at distribution facilities have failed, many municipalities throughout the country issue boil-water advisories in an attempt to inactivate any fecal coliforms and pathogens which may be present (Eggertson, 2008). As of April 2008, the province of Ontario had the largest number of cumulative advisories since 2006, with an estimated 679 instances where the potability of drinking water was questioned (Eggertson, 2008).

2.1.2. Microbiological parameters in Canadian drinking water guidelines

Increasing concern over the spread of waterborne disease highlights the need for augmented regulatory standards for drinking water. In Canada, guidelines for water quality have been established by the Federal Provincial-Territorial Committee on Drinking Water (CDW) for both chemical and microbiological parameters. A complete set of legally-binding federal standards as seen in both the United States and Europe has not yet been implemented. Such guidelines imply that the pathogens detailed within are only voluntarily regulated and routine

testing remains unenforced by the Canadian government (Boyd, 2006).

The microbiological parameters for drinking water outlined by the CDW suggest that only heterotrophic organisms as well as both total and fecal coliforms be part of routine testing (Health Canada 2006a). Heterotrophs are bacteria which comprise the normal microbiota of aquatic environments and are used to measure changes in water quality (Boulos *et al*., 1999; Bitton, 2005). Members of the coliform group are gram negative, non-spore formers that have the ability to ferment lactose at 35°C. Total coliforms refer to the entire coliform population present, those of both fecal and non-fecal origin (e.g. *Enterobacter, Klebsiella* and *Citrobacter*), while fecal coliforms are those which are indigenous to the digestive tract, shed in feces, and grow at elevated temperatures (44.5°C) (e.g. *E. coli*). When compared with water quality regulations from both the US and EU, Canada is visibly lacking in dedication to safe drinking water practices as a result of the lack of enforcement of the suggested guidelines. The current maximum acceptable concentrations (MAC) for microbiological parameters in Canadian drinking water relative to the US EPA and EU standards can be seen in Table 2.1.

For source and groundwaters where chemical disinfection and other treatment technologies have not been applied, such as cottages and rural areas, Health Canada recommends that water from visibly polluted turbid streams should not be ingested and all untreated water should be boiled for one minute prior to use (Health Canada 2008). Several methods to reduce pathogens in these waters are suggested, including; chlorination, iodination and ceramic filtration, however these methods are impractical to perform on a regular basis (Health Canada, 2008).

TABLE 2.1. Comparison of CDW guidelines for microbiological parameters in drinking water to US and EU regulations (Adapted from Rompré *et al*., 2002 and Health Canada 2006 a,b,c,)

Tested Parameter	Canadian Guidelines	US EPA Standards	EU Standards
E. coli	0 in 100 mL; 100%	0 in 100 mL; 100%	0 in 250 mL
Total coliforms	0 in 100 mL; 90%, none above 10 CFU/ 100 mL and 0 in consecutive sample	0 in 100 mL; 95% consecutive sample must be pathogen-free	0 in 100 mL
Heterotrophic bacteria	No MAC outlined	< 500 organisms / mL	20 / mL (37°C) 100 / mL (22°C) 0 in 250 mL (*P. aeruginosa*)

2.1.2. Bacterial pathogens

Many of the disease-causing organisms found in water are classified as bacterial pathogens of enteric origin and are predominantly found within the intestinal tract of mammals. Entry into the natural environment occurs when they are shed within the feces of warm-blooded animals. As many as 10^{14} enteric pathogens can be released into the water column per 100 g of feces (Gerba, 2000). The detection of enteric pathogens is crucial, since they have been previously linked to gastrointestinal illnesses of varying degrees. Host-infection is initiated when a set of genes are expressed which encode for the production of toxins, adhesins and invasins known as virulence factors (Mekalanos, 1992). Expression is influenced by host-specific environmental cues including optimal temperatures, iron levels, pH and osmolarity (Mekalanos, 1992). These genes are highly regulated because continual expression is both a survival disadvantage and metabolic burden to the cells. Once inside the host, these proteins inflict damage on host cell tissues, with the majority targeting the epithelial lining of the intestinal tract and colon.

The CDW views enteric organisms such as *Campylobacter* spp., *E. coli* O157:H7 and *Salmonella* spp. as pathogens which warrant status monitoring in Canadian drinking water, because of the potential high risk factors, including; (i) the low minimum infectious doses required to become harmful to humans, (ii) the ability to multiply outside of a host and (iii) long-term survival in the natural environment (Arnone and Walling, 2007; Health Canada, 2006). An analysis of waterborne outbreaks by Schuster *et al*. in 2005 revealed that the largest percentage of illnesses caused solely by bacterial pathogens were from members of the *Campylobacter* genus. Other bacteria such as *Pseudomonas aeruginosa* (*P. aeruginosa*), *Legionella pneumophila* and *Aeromonas* spp. have been deemed emerging pathogens by Health Canada, since they had not been previously classified as waterborne and pose health risks to the immuno-compromised. Several of the most prominent species of disease-causing microbes frequently associated with human infection are *E. coli* O157:H7, *Salmonella enterica* serovar Typhimurium, *C. jejuni*, *P. aeruginosa*, *Shigella flexneri* and *Klebsiella pneumoniae*.

2.1.2.1. Escherichia coli O157:H7

One of the most frequently encountered human pathogens is *E. coli*, more specifically, the serotype O157:H7, which has been associated with many instances of waterborne and foodborne disease outbreaks because of its highly virulent attributes. The non-pathogenic *E. coli* variants are found within the lower intestinal tract of all warm-blooded mammals, a trait which allows for its use as an indicator of recent fecal contamination (Health Canada, 2006).

The pathogenic variants of *E. coli* are classified into six subgroups based on their virulence and serological properties; enterohaemorrhagic (EHEC), enteropathogenic (EPEC),

enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAEC) and diffuse adherent (DAEC) (Health Canada, 2006). Each strain is generally associated with diarrheal-related diseases, causing acute gastroenteritis and renal failure in 2-7% of the cases where the progression to haemolytic-uremic syndrome had occurred (Health Canada, 2006). Cattle are said to be the primary reservoir harbouring the pathogen, which has no ill effects upon its bovine host (Ibekwe and Grieve, 2002). It has been recognized that the EHEC form of the bacterium is more frequently detected in developed countries, whereas the ETEC form is more commonly encountered in food and water from the developing world (Hunter *et al*., 2009).

As few as 10 cells of EHEC are sufficient to cause human illness, indicating that the bacterium has an extremely low infectious dose (Liu *et al*., 2008; Ram *et al*., 2008a). The EHEC form of the bacterium is able to infect humans by producing toxic proteins, predominantly the shiga-like verotoxins, whose functions are to inhibit protein synthesis in the intestine, kidneys, and central nervous system of infected hosts (Ibekwe *et al*., 2002; Smith *et al*., 2009; Ram *et al*., 2008a). Such toxins play major roles in the pathogenesis of haemolytic-uremic syndrome and hemorrhagic colitis, two complications of infection by the organism (Ibekwe *et al*., 2002). Other genes associated with the virulence of the organism are the alpha hemolysin (*hlyA*) gene, which is involved in the lysis of eukaryotic cells via pore formation (Ram *et al*., 2008a), *eae*, which encodes for the production of intimin, a protein that aids the attachment to intestinal epithelial cells (Ibekwe and Grieve, 2003) and *tir*, the single-copy chromosomally-integrated gene which acts as a receptor for intimin and strengthens bacterial attachment to epithelial cells (Goosney *et al*., 2000).

Once shed in feces, *E. coli* O157:H7 encounters difficulty surviving in non-host environments over extended time periods because of fluctuations in nutrient concentrations and temperature (Winfield and Groisman, 2003). The continual transfer of cells between human and animal reservoirs stabilizes its survival rate when outside of a suitable host (Winfield and Groisman, 2003). Variation in survival has been documented from approximately 2 to 90 days at 8°C in studies by Wang and Doyle (1998), McGee *et al*. (2002) and Czajkowska *et al*. (2005), where titer decreases in artificially contaminated surface water and distilled water have been examined over time. Environmental variability also plays a significant role in the lifespan of the non-pathogenic variant, and several studies have indicated that it can survive from a range of 30 to 260 days in river water (Edberg *et al*., 2000).

2.1.2.2. Salmonella enterica

Members of the *Salmonella* genus are known to cause disease in both humans and animals, with currently over 2,500 serovars capable of causing an estimated 3 million infections annually (Maki and Hicks, 2002; Grassi and Finlay, 2008). All gram-negative *Salmonella* serotypes are classified under a single species, *enterica* and are part of the *Enterobacteriaceae* family (Mølbak *et al*., 2006). Serotypes such as *typhi* and *gallinarium* have become adapted to infect a specific type of host, whereas Typhimurium, *paratyphi* and *enteridis* have a wider array of potential targets, demonstrating the broad host range of the microorganism (Galán *et al*., 1992). As with other enteric pathogens, the fecal-oral route is the primary mode of transmission. Rapid invasion and colonization of the intestine can occur within as little as 15 minutes following ingestion (Mølbak *et al*., 2006). The mechanism behind *Salmonella* infections is well understood, since the organism (*S. typhi*) has been used as a pathogenicity model in ruminants

for many years. It is believed that once inside the host via ingestion, colonization and penetration of epithelial cells occurs, and the release of enterotoxins by the bacterial cells causes tissue inflammation and a buildup of diarrheagenic fluid to occur in the intestine (Ohl and Miller, 2001; D'Aoust, 1999).

The most frequent illness caused by the Typhimurium serotype of *S. enterica* is mild gastroenteritis referred to as salmonellosis noted by symptoms of fever, abdominal pain and diarrhea; while gastroenteritis and extremely high fever commonly encountered with typhoid fever are associated with the *typhi* serotype (Ohl and Miller, 2001). It has been estimated from epidemiological studies in the 1990s that only 10 to 45 cells are required for infection by *S. typhi* (Mølbak *et al*., 2006) and 10^4 to 10^7 cells for *S.* Typhimurium (Maki and Hicks, 2002). Without treatment, the mortality rate of such infections is approximately 10-15% (Ohl and Miller, 2001). Water samples have been shown to remain culture positive for *S.* Typhimurium for up to 54 days (Moore *et al.*, 2003), demonstrating that the microorganism can be persistent.

Several important genes associated with the organism's virulence are located within one of two virulence-blocks, or pathogenicity islands (SPI-I and SPI-II) on the chromosome. Expression amongst all pathogenic *Salmonella* isolates from food animals has been documented using DNA microarray technology (Chen *et al*., 2005). Chromosomally integrated virulence genes include members of the *sip* gene family (A, B and C) as well as both the *sop* and *invA* genes. All of the aforementioned genes are transported into the host via a type III secretion pathway and function in the initial host invasion (D'Aoust, 1999; Chen *et al*., 2005).

The *Salmonella* outer protein (*sop*) genes are associated with the uptake of pathogenic invader cells through rearrangement of the actin cytoskeleton of host cells (Hirsch, 2004). A common characteristic of all *Salmonella* species is that they carry chromosomally integrated invasion-associated (*inv*) genes, which are involved in initial host invasion and release of entero- and cyto- toxins (Chiu and Ou, 1996). In particular, the *invA* gene is a single-copy gene and is said to be the genetic determinant involved in the release of these toxins (Fey *et al*., 2004). The *sip* genes, encoding the *Salmonella* invasion protein are also involved with entry into the host cell, creating transmembrane channels within the cells (D'Aoust, 1999). These are included within a selection of approximately 28 genes which ensure the successful invasion, colonization and growth of *Salmonella* within infected hosts (D'Aoust, 1999).

2.1.2.3. Campylobacter jejuni

It has been suggested that the gram negative, microaerophillic *Campylobacter* species are the leading cause of gastroenteritis in humans, ahead of both *E. coli* O157:H7 and *Salmonella* spp. (Abulreesh *et al*., 2006). There are three *Campylobacter* species which have been detected within the natural environment and can be linked to human illness; *C. jejuni*, *C. lari* and *C. coli*. *Campylobacters* are known to exist within the intestines of nearly all bird species alongside many wild and domesticated animals, including pigs and dogs (Szewzyk *et al*., 2000; Fricker, 2006). These organisms are found in most surface waters at concentrations ranging from 10^1 to 10^2 CFU per 100 mL (Stelzer and Jacob, 1991) as a result of recent and recurring fecal contamination from avian sources (Jones, 2001; Fricker, 2006). As with *E. coli* O157:H7, *Campylobacter* species do not illicit harmful effects on their primary hosts, but rather maintain a commensal relationship (Abulreesh *et al*., 2006).

The transmission of *C. jejuni* occurs indirectly by the fecal-oral route, through the ingestion of substances contaminated with the pathogen (Fricker, 2006). Generally a condition known as campylobacteriosis results from *Campylobacter* infections, causing bloody diarrhea. In more extreme cases, arthritis, meningitis, pneumonia and muscular paralysis as a result of Guillain-Barré syndrome may occur (Levin, 2007). Infection from water is possible for some time after the initial contamination, since the bacterium is able to survive well over extended periods in aquatic environments, with survival rates peaking in the winter months (Abulreesh *et al*., 2006). In a study by Thomas *et al*. (1999), only a 2-3 log reduction was observed when *Campylobacter* spp. were placed in a modeled aquatic microcosms and were culturable for 60 days at 5°C and 40 days at 15°C. The trend of low infectious doses amongst the most pathogenic enteric bacteria is also observed with this organism. It has been estimated that a minimum of 500 cells are required to illicit infection (Yang *et al*., 2004).

The pathogenesis of *Campylobacter* infections can be attributed to several virulence genes, however little is known regarding the actual cellular mechanisms behind these processes. The *flaA* and *B* genes, coding for flagellin are considered important virulence factors and are involved in the organism's flagellar motility and cellular adhesion (Wassenaar *et al*., 1994; Levin, 2007). Also of significant importance are the *cdtA*, *B* and *C* genes which encode the cytolethal-distending toxin, responsible for cellular distension and death (Levin, 2007). Of recent interest is a novel chromosomal gene encoding an unknown protein product, referred to as the VS1 gene, discovered by Stonnet and Guesdon (1993). Sequence information specific to *C. jejuni* is contained within this gene, allowing for precise detection of only *C. jejuni* in DNA-based techniques. This is beneficial in differentiating between and preventing cross-hybridization

of *C. jejuni* and *C. lari*, which have 40% sequence homology (Stonnet and Guesdon, 1993).

2.1.2.4. Pseudomonas aeruginosa

Pseudomonas aeruginosa is an opportunistic pathogen, which can cause infantile diarrhea, eye infections and mild skin rashes referred to as foliculitis (Geldreich, 2006b). It is ubiquitous in the natural environment and has been frequently isolated from fecally contaminated surface water as well as both bottled and distilled water because of its ability to adapt well to nutrient-limited environments (Hardalo and Edberg, 1997; Geldreich, 2006b). Several other species of *Pseudomonas* have also been isolated from bottled water, including *P. putida* and *P. stutzeri* (Geldreich, 2006b). The organism is also said to be shed in the feces of approximately 2-3% of healthy humans (Szewzyk *et al*., 2000).

The infectious dose of the organism is extremely high in relation to other enteric pathogens, with anywhere from 10^8 to 10^9 cells required for infection (Fok, 2005). The natural environment harbours approximately 10^1 to 10^4 cells per 100 mL (Fok, 2005), indicating that the ingestion of a significant amount of a contaminated substance would be necessary for illness in non-immuno-compromised individuals. The opportunistic nature of *P. aeruginosa* brings about a debate as to whether the organism is of high concern to the general population (Hardalo and Edberg, 1997). Health Canada removed the pathogen from its recent guidelines since the organism tends to become infectious primarily within those who are immuno-compromised and has a low risk of infection in healthy individuals (Fok, 2005). Secondary human infection has been documented mainly within hospitalized patients, where bacteremia and wound colonization is a major concern in previously damaged tissues (Geldreich, 2006b). There have, however, been

documented cases of acute gastrointestinal disease in healthy children.

To cause disease, *P. aeruginosa* produces many extracellular and cell-associated virulence factors which cause extensive tissue damage and invasion of the bloodstream following colonization (Feltman *et al*., 2001; van Delden, 2004). The exotoxin A protein, which is lethal in animals, is encoded for by the *toxA* gene and is involved in the inhibition of protein synthesis and apoptosis in different eukaryotic cell types (Hardalo and Edberg, 1997; van Delden, 2004). In severe cases, several effector proteins known as *exoS*, *T*, *U* and *Y* are injected into the cytoplasm of eukaryotic hosts by means of a type III secretion system (Shaver and Hauser, 2004). The *exoS* and *exoT* genes share similar functions and inhibit the internalization of bacterial cells in eukaryotes (Feltman *et al*., 2001). Shaver and Hauser (2004) determined that the *exoT* gene in particular was found in 89% of clinical isolates and it is required for the virulence of *P. aeruginosa* (Garrity-Ryan *et al*., 2004). The remaining effector proteins *U* and *Y* are associated with epithelial cell death and the rounding of specific eukaryotic cell types, respectively (Feltman *et al*., 2001). There is also no known *Pseudomonas* strain which encodes and secretes all four exotoxin genes at the same time (Garrity-Ryan *et al*., 2004).

Pseudomonads are of particular interest since they have been detected within pipe lines of drinking water distribution facilities and are known to be resistant to chemical chlorination (Szewzyk *et al*., 2000; Fok, 2005). Proliferation is possible because of growth in an aggregated biofilm form, which offers protection from such disinfectants. These biofilms can become a reservoir for the spread of other pathogens which may colonize the biofilm and be later released into the bulk water (September *et al*., 2007; Szewzyk *et al*., 2000). A 2007 study by September *et*

al. looked to confirm this hypothesis, and determined that *Salmonella* spp. and high numbers of *Shigella* spp. were found in some biofilm coupons along with *P. aeruginosa*. The majority of enteric bacteria are not seen following the water treatment process unless there has been an extreme failure of disinfection; however because of the resistance of biofilm-associated *Pseudomonas* to treatment, it is commonly detected in the final finished water (Bartram *et al*., 2003). Lee *et al*. (2006) reported detecting the organism at four separate sampling sites in proximity to a drinking water distribution system in Korea both prior to and following treatment.

2.1.2.5. Shigella flexneri

The *Shigella* genus can be divided into four different serotypes; *flexneri*, *dysenteriae*, *sonnei* and *boydii*. Shigellosis, the disease caused by *S. flexneri* and other *Shigella* serotypes, is associated with watery, sometimes bloody diarrhea from impaired water absorption, resulting in intense abdominal pain (Lampel *et al*., 1999). Worldwide, approximately 164.7 million cases of shigellosis occur annually, with fatalities in many as 1.1 million individuals (Thiem *et al*., 2004). The infective dose is very low, between approximately 10 to 10^4 cells, depending on the immune status of the infected individual (Theron *et al*., 2001). Similar characteristics to fecal coliforms have been suggested for the survival of the organisms in water and their susceptibility to chemical disinfection methods (Health Canada, 2006).

The virulence characteristics of the microorganism are temperature dependent, whereby growth at 37°C is required in order for traits associated with its pathogenicity to become activated (Lampel *et al*., 1999). This indicates that the organism is not virulent until it has been ingested by a warm-blooded mammal. Virulence genes can be found both chromosomally and on

a large plasmid which is maintained within all four pathogenic variants of *Shigella* (Lampel *et al*., 1999; Theron *et al*., 2001). The invasion plasmid antigen H (*ipaH*) gene is carried amongst all four *Shigella* serotypes, and has been used as a target for detection in nearly all DNA-based assays associated with the organism, including those involving both clinical and water samples (Lampel *et al*., 1999; Theron *et al*., 2001; Thiem *et al*., 2004). It is secreted extracellularly and is an effector protein associated with the invasion of colonic epithelial cells (Ashida *et al*., 2007). The gene is found in low copy, with approximately 7 copies contained both chromosomally and plasmid borne as determined in a 1989 study by Venkateson *et al*. Also, of importance are the chromosomal shiga toxin (*stx*) genes, which are have similar modes of action to those from EIEC *E. coli* O157:H7, affecting the synthesis of proteins within the host cell (Lampel *et al*., 1999).

2.1.2.6. Klebsiella pneumoniae

As with *Pseudomonads*, members of the *Klebsiella* genus are opportunistic pathogens known to cause pneumonia, urinary tract infections and liver abcesses, the mechanisms behind which are currently not understood (Struve *et al*., 2005). *Klebsiella pneumoniae* is frequently encountered in the natural environment in soil and vegetation and has been detected in quantities of 10^4 to 10^6 per milliliter within the effluent from pulp and paper mills, wastewater and urban run-off (Geldreich, 2006a). The infectious dose of the organism has been estimated to be 10^5 cells; however the ingestion of drinking water with as few as 35 cells per millilitre is sufficient to cause infection (Geldreich, 2006a). Mortality rates from pneumonia-related *Klebsiella* infections are as high as 60% while only 12% amongst liver abscess infections (Yu *et al*., 2006). In addition, it has been estimated that as many as 40% of mammals have around 10^8 *Klebsiella* per gram of feces in their intestinal tract which is then shed into the environment (Geldreich, 2006a).

A novel low-copy gene referred to as the mucoviscosity-associated (*magA*) gene, has been determined amongst 98% of clinical isolates of K1 serotype *Klebsiella* in studies by both Struve *et al*. (2005) and Yeh *et al*. (2006), suggesting that it is specific only to this serotype. It is responsible for the hypermucoviscosity phenotype, whereby the organism secretes a hypermucoid outer capsule that is considered to be a virulence factor (Yu *et al*., 2006). Also associated with the mucoid phenotype is the plasmid-integrated regulator of mucoid phenotype (*rmpA*) gene, which has control over the amount of capsular polysaccharide material released by the organism during infection (Yu *et al*., 2006).

2.2. Monitoring the microbiological quality of water

There are three main standardized techniques which have been applied to monitor the microbiological quality of water for many years. These techniques rely on the use of fecal indicators in media-based assays such as; (i) membrane filtration (MF) and (ii) multiple tube fermentation (MTF) as well as in (iii) defined substrate technologies (DST) (Griffin *et al*., 2001). As previously stated, there are currently no standardized methods to routinely monitor pathogens in Canadian drinking water; however the assays which will be discussed are currently regarded as the "gold standard" by both the Federal-Territorial Committee on Drinking Water (CDW) and the United States Environmental Protection Agency (US EPA) as being representative enough to give adequate assessments of potential health risks (Schuster *et al*., 2005).

2.2.1. Indicators of fecal contamination

Operators at drinking water treatment facilities are unable to detect the presence of all possible pathogens because of their fastidious growth characteristics and low environmental

abundance (Lemarchand *et al*., 2004). Several enteric bacteria, including *E. coli*, *Klebsiella* and some species of *Enterococcus*, are therefore used to serve as indicators of recent fecal contamination. Fecal indicators and coliforms are lactose-fermenting organisms (mostly non-pathogenic) which can grow at elevated temperatures (44.5°C), and are indigenous to the mammalian intestinal tract and feces (Griffin *et al*., 2001; Lemarchand *et al*., 2004). It is because of this that their presence is assumed to directly correlate with the probable presence of enteric pathogens in water bodies since both their time of entry and point of origin should be identical (Szewzyk *et al*., 2000; Lemarchand *et al*., 2004). With indicators, it is assumed that there is increased probability of detecting pathogens with increasing concentration of fecal indicator (Savichtcheva and Okabe, 2006). The use of fecal coliform indicators is a cost-effective and easy-to-use detection format that gives an indicative assessment of water quality since routine monitoring of a much larger number of samples is possible (Health Canada, 2006b).

In general, organisms selected to act as indicators must satisfy several different criteria in order to be considered effective for water quality analyses. It is required that the organisms be: (i) unable to multiply within the natural environment once shed by a host, (ii) present when pathogens are present in fecally-relevant concentrations and absent in uncontaminated waters, (iii) detectable by simple, reliable methods, and (iv) should have similar resistance properties (both disinfection and environmental) to the pathogens of interest (Griffin *et al*., 2001; Lemarchand *et al*., 2004; Health Canada, 2006b; Savichtcheva and Okabe, 2006). Currently, *E. coli* is considered to be the most effective biological indicator of water quality; however it still does not satisfy all of the aforementioned criteria. There is as of yet no "ideal" universal fecal indicator to account for bacteria, viral and protozoan pathogens (Edberg *et al*., 2000).

2.2.1.1. Alternative indicators of fecal contamination

It has been acknowledged that the presence of indicator organisms may not be directly correlated to the presence of all pathogens by many recent studies, because of the increasing diversity of environmental pathogens (Polo *et al*., 1998; Lemarchand *et al*., 2004; Health Canada, 2006b, Dorner *et al*., 2007, Ahmed *et al*., 2008). The introduction of alternative fecal indicators may solve some of the problems associated with traditional fecal indicators when they are dually applied. Members of this alternative group are generally characterized as either fecal anaerobes such as *Bacteroides fragilis (B. fragilis)* and *Clostridium perfringens (C. perfringens)*, viruses such as male-specific RNA coliphages or fecal organic compounds such as sterols (Savichtcheva and Okabe, 2006). As with the traditional indicators, all of the aforementioned organisms are also of fecal origin. A 2004 study by Horman *et al*. examined the application of *C. perfringens* and F^- specific RNA coliphages in combination with traditional coliform indicators and reported good correlation and reliability with the presence of enteric pathogens. Significant laboratory scale studies must be performed to achieve more accurate estimations of the effectiveness of applying such alternative indicators in the future. Efforts are underway by the US EPA to introduce several of the above alternative indicators into standard practices by the year 2012 (US EPA, 2007).

2.2.2. Traditional biochemical and culture-based detection methods

2.2.2.1. Membrane filtration (MF)

The standard membrane filtration technique involves the passage of a water sample, usually 100 mL, over a sterile membrane filter which has pore sizes (generally 0.45 µm)

specifically designed to trap bacteria and allow most exogenous DNA, viruses and bacteriophages to pass through (Health Canada, 2006b). The filter is then placed onto selective microbiological media such as enriched lactose formulations known as m-FC or m-ENDO to detect fecal and total coliforms, respectively. Following incubation at 35 or 44.5°C for 24 hours, growth of *E. coli* or total coliforms will be enhanced while non-coliforms will be selectively eliminated (Rompré *et al*., 2002).

Direct cell counts are recorded as CFU per 100 mL of sample and are obtained by enumerating the number of colonies that have grown on the filter that have characteristic appearances of the total and fecal coliforms under specific conditions. With m-ENDO media, colonies having a red appearance and exhibiting a metallic green sheen are identified as coliforms. Fecal coliforms appear blue on m-FC agar. In some instances, *E .coli* isolates have appeared as yellow on m-FC agar, and are denoted as atypical, but still considered as *E. coli* (Rychert and Stephenson, 1981; Csuros and Csuros, 1999). Additional confirmation with MUG media is often required since false positives from selected non-coliform bacteria may occur (Rompré *et al*., 2002; Health Canada, 2006b). Standardized techniques for membrane filtration have been designed by the CDW and US EPA and are common practice in water quality analyses (Clesceri *et al*., 1999).

Measures of the heterotrophic microflora must also be obtained through viable heterotrophic plate counts to give estimates of the aerobic and facultative anaerobic populations within a water sample (Bitton, 2005). This information can then be used to determine the microbiological quality of water (Health Canada, 2006c). Several opportunistic pathogens of the

genera *Pseudomonas*, *Aeromonas*, *Enterobacter*, and *Citrobacter* among others form this group of bacteria (Bitton, 2005). Media with low nutrient concentrations such as R2A agar (Health Canada, 2006c) are used, to mimic conditions within the natural environment. As with the aforementioned selective media, membrane filters are placed onto R2A agar, and grown at 35°C. It is expected that drinking water will contain between 0 to 10 CFU/mL of heterotrophic bacteria in the final finished water, while much higher counts will be observed with environmental samples (Health Canada, 2006c). An increase in bacterial density following treatment would relate back to problems with the efficiency of the disinfection strategy at the treatment facility (Bitton, 2005).

2.2.2.2. Multiple tube fermentation (MTF) / Most probable number (MPN)

The MTF technique involves preparing replicate tubes of a serially diluted water sample to estimate the mean coliform density (Rompré *et al*., 2002; Health Canada, 2006b). Following incubation for 24 hours at 35°C, the production of acid, gas, or abundant bacterial growth indicated by increased turbidity may indicate a positive reaction. In theory, they are presumptive since the acid/gas production observed may not be caused entirely by coliforms; therefore additional biochemical testing on coliform-specific media is required. Increased precision requires that the number of tubes is increased; for example it is suggested that either 1 (100mL), 5 (20mL) or 10 replicate tubes (10mL) are used to analyze drinking water samples (Clesceri *et al*., 1999).

The MTF technique relies on reporting the most probable number (MPN) of microorganisms present in an unknown sample. The MPN designation utilizes statistical analysis

to measure the most probable number of bacterial cells (coliforms) based on turbidimetric estimation. Samples with greater turbidity are believed to contain higher coliform concentrations than those with only slight turbidity. Despite the MF technique overshadowing the MTF technique in terms of widespread use, this technology is still useful for samples with high turbidity or flocculated particles which cannot be filtered using MF (Rompré *et al*., 2002). The MTF technique is time consuming in nature because of the large number of dilutions that need to be prepared for each successive water sample. There are also differences in sensitivity when compared to the MF method (Rompré *et al*., 2002).

2.2.2.3. Presence-Absence / Defined substrate testing (DST)

The need for rapid, easy-to-use detection formats for fecal coliforms led to the formulation of presence-absence tests, or defined substrate tests, in the early 1990s. DSTs are biochemical assays which exploit the metabolic abilities of coliforms (both total and fecal) to hydrolyze known substrates in order to detect their presence or absence in unknown water samples. Such tests are designed to specifically allow only *E. coli*, the target microbe, to use nutrients from the media (Rompré *et al*., 2002). They allow for routine analysis of large volume samples by using a single 100 mL sample that is representative of the contaminated status of the water in question (Clesceri *et al*., 1999; Health Canada 2006b). Of the DST, MF and MTF/MPN procedures, DST is the sole test which occurs in one step through a direct colorimetric reaction, and does not require an additional confirmatory procedure for *E. coli* (Edberg *et al*., 2000).

The technique involves the cleavage of the substrate o-nitrophenyl-β-D-galactopyranoside (ONPG) by the coliform and *E. coli*-specific β-D-galactosidase enzyme, to produce a yellow

o-nitrophenol product following incubation at 35°C (Clark *et al*., 1991; Eckner, 1998; Rompré *et al*., 2002). The formation of this yellow product can be visualized with the human eye and is indicative of the presence of both *E. coli* and total coliforms. Additionally, the glucoronidase enzyme from *E. coli* possesses the ability to cleave a second substrate, 4-methylumbelliferyl-β-D-glucoronide (MUG) to 4-methylumbelliferone, which fluoresces under long-wavelength ultraviolet (UV) light (Clark *et al*., 1991; Rompré *et al*., 2002). Samples containing *E. coli* are detected by observing blue-white fluorescence upon placing tubes positive for ONPG hydrolysis under UV light at 365 nm (Eckner, 1998; Rompré *et al*., 2002).

Sensitive biochemical detection techniques have become commercially available, with testing kits such as the Colilert®, Colilert®-18 and Colisure® formats, marketed by IDEXX Laboratories (Maine, USA), having detection limits as low as 1 organism per 100 mL sample and considered efficient enough for use as standard practice by the US EPA (Edberg *et al*., 2000). The Colilert® and Colilert®-18 systems provide simultaneous detection of coliforms and *E. coli* within 24 and 18 hours, respectively, using the aforementioned properties for identification. With the Colisure® test, a negative result is indicated by a yellow colour, and the hydrolysis of chlorophenol red β-galactosidase (CPRG) to produce a magenta/red colour is used as a positive test for coliforms and *E. coli*, to reduce the incidence of false-positive results. Many studies have compared the effectiveness of test kits relative to the MF and MTF technologies, including those by Clark *et al*. (1991), Eckner (1998) and Chao *et al*. (2004). Each study compared or evaluated the incorporation of Colilert® systems to detect *E. coli* and coliforms and determined they exhibited comparable sensitivity and accuracy to both the MF and MTF assays.

The ability of such techniques to detect total coliforms in a sample was challenged by Lifshitz and Joshi (1998) with a comparison between the ColiPlate™ kit by Bluewater Biosciences (Mississauga, Canada), which has identical functionality to the Colilert® systems, and the MF technique. It was determined that higher counts (38%) of highly stressed, freeze-dried *E. coli* cells were detected using the ColiPlate™ kit, while the MF technique alone failed to detect over 48% of stressed cells (Lifshitz and Joshi, 1998). False negatives produced by the Colilert® system were examined by Clark *et al*. (1991) and were found to be prevalent in 19% of untreated and 81% of treated water samples that were positive for *E. coli*.

2.2.3. Limitations of traditional detection methods

Although traditional methods are currently the "gold standard" for pathogen detection, there are many limitations associated with their use, which have been noted in many studies (Gerba, 1996; Tryland and Fiksdal, 1998; Boulos *et al*., 1999; Griffin *et al*., 2001; Rompré *et al*., 2002; Khan *et al*., 2007; Shaban, 2007; Walters *et al*., 2007). It has been stated that there is sufficient evidence to indicate that lack of knowledge of how to adequately assess the health risks associated with water exists. The correlation of indicators to the potential presence of pathogens may not be as accurate as once believed. Recently, several studies have documented that indicator concentrations are not reflective of actual pathogen loads (Polo *et al*., 1998; Lemarchand and Lebaron, 2003; Horman *et al*., 2004; Savichtcheva and Okabe, 2006; Dorner *et al*., 2007; Ahmed *et al*., 2008). Ahmed *et al*., (2008) observed poor correlation between fecal indicators and the potential pathogens used in their study of roof harvested rainwater, including *S.* Typhimurium and *C. jejuni*. A study by Dorner *et al*. (2007) determined that the correlations between peak concentrations of indicator organisms, turbidity and pathogen content in a subset

of water samples from the Grand River watershed in Ontario was significantly weak. This was explained by the suggestion that following precipitation events, peak pathogen concentrations and the peak turbidity levels occur at different times as a result of cell clustering. Coliform loads were also found to be more closely related to turbidity (Dorner *et al*., 2007). No clear correlations between pathogens and fecal indicators were observed by Lemarchand and Lebaron (2003) whose examination of the incidence of *Salmonella* in French coastal waters showed no direct relationship between the presence of the pathogen and the presence of fecal coliforms. In addition, protozoan and viral pathogens have been found in the absence of coliform indicators, further suggesting that predicting pathogen presence via indicators is insufficient to assess the true microbiological quality of water (Straub and Chandler, 2003; Gerba, 1996).

The detection of pathogens in environmental samples is primarily restricted by the ability to culture them *ex-situ* (Gilbride *et al*., 2006). The majority of environmental isolates (including those which are stressed and injured) are unculturable on commercially formulated media, because of their fastidious nature (Abulreesh *et al*., 2006). It has been estimated that only 0.1 – 15% of all environmental bacteria are able to be cultured (Rompré *et al*., 2002). Such methods do not allow for the detection of all possible microorganisms which may be present in an unidentified water sample, lowering their effectiveness and increasing the potential risk of infection from unknown pathogens (Health Canada, 2006). Of additional concern is the fact that these techniques do not allow for detection on a taxonomic level, and generally differentiation among species is nearly impossible without further identification (Gilbride *et al.*, 2006; Shaban, 2007).

It has been suggested that false positives may be generated by bacterial species such as *Pseudomonas* which may mimic the physical traits of fecal coliforms in morphology-based assays (Griffin *et al*., 2001). In addition, the results obtained by some commercial biochemical detection kits such as the Colilert™ detection format (IDEXX Laboratories Inc., Maine, USA) are also open to user interpretation, with the potential for results to vary among treatment personnel. Also with such systems, approximately 50% of *E. coli* O157:H7 strains (including all EHEC strains) are unable to cleave MUG, since they lack the β-glucoronidase enzyme (Momba *et al*., 2006; Khan *et al*., 2007, Walters *et al*., 2007). Methods relying on enzymatic activity will therefore underestimate fecal coliform, and potentially pathogen loads (Walters *et al*., 2007). In addition, Tryland and Fiksdal (1998) have discovered that interference may also occur from non-target bacteria (e.g., Enterococci and *Aeromonas* spp.) that carry high levels of glucoronidase activity, when these organisms are in equivalent concentration to indicators.

Finally, the laborious, time-consuming procedures from the application of selective growth media are the major obstacles preventing rapid identification by traditional methods since many bacteria are sensitive to culture conditions (Boulos *et al*., 1999). Extended incubation periods (between 72 hours to 1 week) may be required to grow organisms isolated from the environment on their respective selective media and for the battery of confirmatory tests to be fully completed. Culture enrichment may also be necessary when dealing with low pathogen concentrations to resuscitate injured and weakened cells, and biochemical identification may be required to differentiate species and genera on the basis of metabolic activity. An additional time period is also needed for an advisory to be issued following serological identification and genetic fingerprinting of the specific pathogenic strain. As a result, individuals may have already

ingested the questionable water and advisories may come too late.

2.2.3.1. Viable but nonculturable organisms

Underestimates of true indicator loads plague traditional detection techniques, since many organisms can exist in viable states over extended periods, while evading detection (Walters *et al*., 2007). Bacteria can enter periods of dormancy, known as viable but nonculturable (VBNC) states, where the organism is viable and metabolically active, however they cannot be cultured on growth medium (Abulreesh *et al*., 2006). Unfavourable environmental conditions including starvation, temperature shifts and exposure to radiation from sunlight are believed to be some of the causes of these stress responses (Winfield and Groisman, 2003; Khan *et al*., 2007). Under these conditions, a completely different set of genes are transcribed to enhance survival, at the expense of virulence genes. These include those corresponding to morphological and physiological changes (Touron *et al*., 2005). An example of such characteristics has been seen with *C. jejuni*, which has the ability to form spiral-shaped morphologies and alter intracellular ATP concentrations in response to harsh conditions (Abulreesh *et al*., 2006).

It has been suggested that when VBNC bacteria enter a mammalian host they are able to become resuscitated and re-express their virulence factors. There is however some uncertainty as to whether the bacteria return to their fully functional pathogenic state, or whether the stability of toxins contained within non-viable bacterial cells plays a role in the re-emergence of their pathogenic potential following revival (Keer and Birch, 2003; Winfield and Groisman, 2003). Enteric bacteria in the VBNC state have been detected using molecular techniques, including;

E. coli O157:H7 (Liu *et al*., 2008), *C. jejuni* (Yang *et al*., 2003; Alexandrino *et al*., 2004) and *Salmonella enterica* (Kapley *et al*., 2001; Touron *et al*., 2005), among others. The ability of pathogenic bacteria to exist in the VNBC form has presented an additional challenge in assessing the health risks associated with water supplies (McKay, 1992).

The necessity of having reliable and convenient methods that are able to differentiate between viable, dead cells or cellular debris is unquestionable (McKay, 1992). Several alternatives including flow cytometry, fluorescent staining and nucleic acid amplification in the presence of chemical modification have been used to determine bacterial viability (Keer and Birch, 2003; Nocker and Camper, 2006). In one such instance, Boulos *et al*. (1999) were able to differentiate between viable and dead organisms in water samples using laser microscopy; however they were unable to successfully identify any of these organisms as pathogens since stains are not pathogen-specific. Quantitative molecular techniques appear to be the most promising advancements in water quality monitoring, giving results reflective of total pathogen loads.

2.3. Molecular techniques for waterborne pathogen detection

The quantification of nucleic acids using *in vitro* DNA-based techniques (commonly referred to as molecular methods) from clinical and environmental samples has been in development for some time (Toze, 1999). Variants of the polymerase chain reaction (PCR) including real-time quantitative PCR (qPCR), multiplex and nested formats as well as microarray technology have been tested against waterborne targets (Table 2.2) in an attempt to alleviate problems with traditional detection formats.

DNA-based techniques carry the robustness of ideal detection methods since they are rapid, sensitive, specific and allow for the simultaneous analysis of many samples (Toze, 1999). In addition, the quantification results are equivalent in sensitivity to selective plating techniques and do not always require pre-enrichment to resuscitate damaged cells (Abulreesh *et al*., 2006). Many scientific studies have shown the high sensitivity of such techniques for their intended targets in water environments, which are summarized in Table 2.2. The uniqueness of these methods can be identified by the low detection limits which have been achieved thus far in literature. Single cells or genome equivalents that correspond to the minimum infective dose of the pathogens have been detected in matrices such as raw foods, source water and wastewater.

2.3.1. Overview of PCR-based detection assays

The PCR technique has been applied within many biological disciplines. Protocols for the detection of microbial pathogens from a variety of sources, environmental monitoring of genetically modified organisms, analysis of gene expression and diagnostic applications in clinical studies have been created (Bitton, 2004; Lo *et al*., 2006). With this technology, template DNA or RNA/cDNA can be amplified exponentially starting from very low concentrations in the initial sample (Lemarchand *et al*., 2004).

Amplification of target DNA by PCR functions in a series of three stages; denaturation, annealing and elongation/extension. In the initial denaturation stage, reaction temperatures are increased to 95°C, causing disruption of the hydrogen bonds holding the complementary single-strands of DNA together (McPherson and Møller, 2000). Secondly, the annealing stage allows oligonucleotide primers of 18 to 22 bases to bind to their complementary regions on the now

TABLE 2.2. Detection of selected waterborne bacterial pathogens using molecular techniques

Pathogen	Sample Type	Detection Technique	Target Genes	Detection Limit	Reference
***E. coli* O157:H7**	Source water	qPCR	*stx1*, *stx2*, *rfbE*	50 CFU / 40 L	Mull and Hill, 2009
	Agricultural water	qPCR	16S-23S ITS	10 CFU / mL	Khan *et al*., 2007
	River and tap water	RT-PCR & Microarray	*rfbE*, *fliC*	3-4 CFU / L tap water 7 CFU / L river water	Liu *et al*., 2008
	River water	qPCR	*LTI*	2 CFU / mL	Ram *et al*., 2008b
	Drinking water	Multiplex PCR	*stx1*, *stx2*, *eae*	1 CFU / mL	Campbell *et al*., 2001
***S.* Typhimurium**	River water	Nested PCR	Unknown fragment	10 CFU / 100mL	Waage *et al*., 1999
	Estuarine water	Nested-multiplex PCR	*fliC*	360 CFU / 100mL	Touron *et al*., 2005
	River water	Magnetic capture & qPCR	*invA*	30 CFU / 50mL 5 CFU / 50mL	Thompson *et al*., 2006
	Irrigation water	qPCR		3 CFU / 100mL	Wolffs *et al*., 2006
C. jejuni	Surface and ground water	qPCR	VS1	6-15 CFU / PCR	Yang *et al*., 2003
	Drinking and lake water		*flaA*, *flaB*	10-20 CFU / mL	Moore *et al*., 2001
	Surface water		16S rRNA	1 CFU / PCR	Purdy *et al*., 1996
	Lake and coastal water	PCR-ELISA	*hipO*	20 CFU / mL	Sails *et al*., 2002
	Groundwater leachate	qPCR	Unknown fragment	60 CFU/mL	Rothrock *et al*., 2009
P. aeruginosa	Drinking water	PCR	*toxA*	5-10 CFU / 10mL	Khan and Cerniglia, 1994
		PCR/Probe Capture	23S rRNA	20-30 CFU / mL	Frahm *et al*., 2001
S. flexneri	Sea and drinking water	Multiplex PCR	*ipaH*	10^1 CFU / Reaction	Fan *et al*., 2008
	Sea water			10^2 CFU / 100mL	Kong *et al*., 2002
	Drinking and ground water	Semi-nested PCR		11 CFU / mL $2x10^3$ CFU / mL	Theron *et al*., 2001
	Drinking and source water			25 CFU / 100mL	du Preez *et al*., 2003
K. pneumoniae	Currently no molecular experiments have focused on the quantification of this microorganism in water bodies				

single-stranded DNA, in the 5′ to 3′ direction. Finally, in the elongation stage, the polymerase enzyme extends the region adjacent to the forward and reverse primers, by incorporating single dideoxynucleotides to synthesize a complementary copy of the original DNA strand (McPherson and Møller, 2000). This continues in an exponential manner for around 40 cycles, until a detectable amount of target has been produced.

PCR amplifications follow an exponential trend, whereby the amount of target is assumed to double with each successive cycle, until billions of amplicons are present at the completion of the reaction, assuming perfect efficiency. The reaction itself is limited by the kinetics of the polymerase enzyme used to replicate the template. The amplification process will continue exponentially to the plateau phase, or endpoint of the reaction, where the ratio of enzyme to primer-template complexes shifts in favour of the products (Lo *et al*., 2006). In its conventional form, PCR is better suited for qualitative, presence-absence detection. With gel electrophoresis of the resulting amplicons a single band is visualized to indicate the successful amplification of the desired gene. Quantitative information relating to the original target concentration is therefore not obtained (McPherson and Møller, 2000).

Incorporating PCR-based techniques into water quality monitoring practices would be an ideal scenario, because direct detection of pathogens themselves would be possible, rather than relying solely upon indicator organisms. Oligonucleotide primers can be designed against any bacterial genome. This means that every pathogen of known sequence could be detected; however this is not viewed as a practical approach (Brettar and Höfle, 2008). One variant of the PCR technique that has been in development for waterborne detection has been the real-time

qPCR technique; alongside technology such as DNA microarray and biosensors.

2.3.2. Real-time qPCR detection technology

The introduction of real-time monitoring for PCR reactions has greatly enhanced the field of molecular biology. The accumulation of the desired amplification products can be monitored in "real time" following each successive cycle of the reaction (McPherson and Møller, 2000; Logan *et al.*, 2009). The theory behind the amplification process is very similar to that of conventional PCR, however there are some additional adjustments. Amplicons are detected within the early exponential phase of amplification, the portion of the reaction where theoretical doubling occurs (Fig. 2.1), as opposed to the endpoint of the reaction (Gilbride *et al.*, 2006). This allows for a quantitative relationship to exist between the starting concentration of a target nucleic acid and the quantity of product during the exponential phase of the reaction (Bustin, 2004).

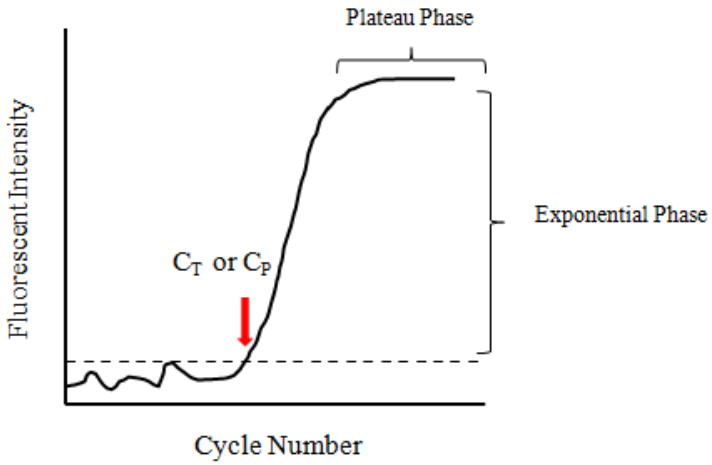

FIG. 2.1. Typical amplification plot obtained during the qPCR cycling process

The copy number of the target gene is determined by the threshold cycle (C_T) or crossing point (C_P), which is the point in the reaction when a sufficient amount of amplicons have been generated. When the C_P is met, the fluorescence of the sample rises above the background and can be registered by the instrument's detector (Fig 2.1) (Mackay, 2004; Bustin, 2005). As the fluorescence increases, a sigmoidal curve characteristic of a typical PCR is produced, marking each phase in the reaction (Yang *et al*., 2004). It is generally believed that the more copies present at the beginning of the reaction, the fewer cycles required for the signal to pass the background fluorescence and produce enough amplicons for detection (Bustin, 2005).

2.3.2.1. Quantification techniques

Two methods of quantification can be applied to any qPCR reaction, which are denoted as either absolute or relative. Absolute quantification is applied to scenarios where exact copy numbers and therefore the exact number of a particular microbial particle are required (Mackay, 2004). Serially-diluted reference strains carrying defined copy numbers of the target are used to construct standard curves (Monis and Giglio, 2006; Sun *et al*., 2010). The copy number (and number of pathogenic cells) is then determined by a comparison of the C_P of the unknown samples to those of defined standards (Monis and Giglio, 2006). In general, the linear dynamic range for detection extends from as many as 10^{10} to as little as 1 copy of the target with reference strains (Logan *et al*., 2009).

Relative quantification in contrast compares gene expression levels, denoted by changes in the signal generated by a target gene, to that of a reference gene in the same or similar sample matrix (Mackay, 2004). In this format, no exact or absolute values can be extrapolated, only an

estimate of the number of genes expressed at a given time. External controls are used for relative quantification experiments, with housekeeping genes, those which are required for cellular metabolism and viability, being used since they are expressed continuously and at constant levels intracellularly. Several commonly used housekeeping genes in relative quantification studies include the *gapA*, *groEL* and *gyrA* genes (Wertz *et al*., 2003) as well as the 16S rRNA gene, which has been shown to be stably expressed across several induced-stress scenarios (Tasara and Stephan, 2007).

It has been demonstrated that in some instances the linear range of detection may be extended to reach a much smaller spectrum. Work by Purdy *et al*. (1996) showed that a single genome equivalent of *C. jejuni* can be detected in surface water by qPCR. Similarly, Thompson *et al*., (2006) demonstrated that 5 CFU/mL of *S.* Typhimurium can be detected in river water using magnetic capture combined with qPCR. Detection limits obtained by qPCR are dependent on several factors, including the efficiency of nucleic acid purification and inhibitor removal, and the robustness of the oligonucleotide primers designs (Straub and Chandler, 2003). Robust in the PCR refers to the ability of the primer to sufficiently anneal to the intended target with a significantly high degree of efficiency and effectively amplify the desired target with minimal dimerization and mispriming.

With environmental water samples, specific concerns pertaining to detection limits are humic acids, free cations and related organic compounds, which are known to interfere with cell lysis, DNA capture and polymerase activity (Wilson, 1997; Stevens and Jaykus, 2004). DNA extraction protocols must therefore be optimized in order to minimize reaction inhibition when

low detection limits are desired. Hänninen *et al*. (2003) have also shown that detection sensitivity can rely heavily on sample volume, since as much as 10 L was required to obtain a detectable signal from drinking water contaminated by *C. jejuni*. With more turbid samples, free-floating inhibitors would be co-concentrated alongside these cells, stressing the importance of producing purified products following extraction. Traditionally, organic extractions such as phenol-chloroform treatments have been used to purify environmental DNA; however the process adds additional time and hazardous reagents that are problematic for the development of rapid technologies. There are many commercially available DNA isolation kits including the UltraClean™ Water DNA Purification kit (MO-BIO Laboratories Inc., Carlsbad, CA, USA) which are said to be specifically designed to minimize sample inhibition and maintain quick turnover rates. In addition, they are able to handle many litres of sample by means of a MF process. Regardless of sample volume, a method is considered successful if a unit reflecting the detection of a cell number corresponding to the minimum infectious dose of a pathogen per volume analyzed is achieved.

2.3.2.2. Detection chemistries

The incorporation of molecules which exhibit fluorescence under certain conditions allows the quantity of a target nucleic acid in an unknown sample to be determined experimentally (MacKay, 2004). In order for quantification to occur, a fluorescent signal is released by dyes or probes incorporated into the reactions. The binding of dyes and probes is directly proportional to the number of double-stranded amplicons generated during each cycle in the reaction (Mackay, 2004). Changes in fluorescence levels within a reaction capillary are monitored, and at any given time the amount of signal produced correlates to the amount of

amplicons present (MacKay, 2004).

Fluorescent dyes such as SYBR Green are generally the most widely used detection chemistry, since they are less costly than other formats and can be applied to any amplification reaction (Malinen *et al*., 2003; Monis and Giglio, 2006). They function by recognizing and intercalating with any double stranded DNA in the reaction. Once bound, a conformational change occurs in the dye molecule, which then emits fluorescence which is measured by the detector at a wavelength of 520 nm (Logan *et al*., 2009). Dyes are seen as the more generic option for detection, since they bind non-specifically to all nucleic acids present in the reaction, including non-specific reaction artifacts such as primer dimers. The accumulation of primer dimers, duplexes formed from an excess of primer, will ultimately lower the efficiency of the PCR reaction and have a negative effect on the accuracy of quantification (Monis and Giglio, 2006).

Water-related studies where SYBR Green has been incorporated in qPCR include those by Nam *et al*. (2005) and Wolffs *et al*. (2006) for the investigation of *Salmonella* spp. in surface and lagoon water and Khan *et al*. (2007) whose study focused on detecting viable and non-viable *E. coli* in naturally and artificially contaminated agricultural water from watersheds. Adequate sensitivities and detection limits were achieved by each study (Table 2.2), and the desired amplicons were distinguished from artifacts by the use of melting curve analysis. Melting curves accurately identify the desired amplicon and distinguish it from reaction artifacts such as primer dimers, since each double stranded nucleic acid will have a temperature at which denaturation occurs (Monis and Giglio, 2006). In order to generate these curves, PCR reactions are slowly and

continuously heated over a specified temperature range (usually 50°C to 95°C) to dissociate the DNA duplexes. This process causes a decrease in fluorescence from the release of dye molecules (Logan *et al.*, 2009). Peaks are computer-generated at a specific temperature for each product by plotting the first derivative of the fluorescence with respect to temperature against the temperature (Logan *et al.*, 2009). These maxima correspond to the points at which the maximum rate of change in fluorescence occurs for each product (Logan *et al.*, 2009).

Fluorescently labeled probes can also be used as a detection approach in qPCR studies. Several different probe formats are available, including the most popular technology, the TaqMan® probes alongside others such as molecular beacons and Scorpion probes. With the TaqMan® format, the 5′ exonuclease activity of the polymerase enzyme is used to cleave a labeled probe during the elongation phase of PCR. Probes are labeled at the 5′ end with a fluorescent reporter molecule, usually a tetramethylrhodamine derivative such as carboxyfluorescin (FAM), and a quencher on the 3′ end such as tetramethyl-6-carboxyrhodamine (TAMRA) (Ram and Shanker, 2005; Monis and Giglio, 2006). Once the probe has bound to the target, fluorescence is released and is detected when the quencher molecules are separated from the reporter (Ram and Shanker, 2005; Monis and Giglio, 2006). Molecular beacons in comparison are stem-loop structures which, when bound to a target, unwind to separate the reporter from the quencher to produce a signal (Monis and Giglio, 2006). Scorpion probes are similar to molecular beacons, however once incorporated into the amplicon, binding of the loop to the complementary sequence causes the structure to open and emit fluorescence (Monis and Giglio, 2006). Fluorescent probes have been applied in numerous qPCR studies on waterborne pathogens, because of their high degree of specificity when applied to complex and inhibitory

sample matrices. These studies include the detection of the *stx* and *eae* genes of *E. coli* O157:H7 by Mull and Hill (2009), Smith *et al*., (2009), Ibekwe and Grieve (2003) and Ibekwe *et al*. (2002), the *VS1* gene of *C. jejuni* by Yang *et al*. (2003) and the *invA* gene of *S.* Typhimurium by Thompson *et al*., (2006).

2.3.2.3. Advantages and limitations of qPCR

There are numerous advantages of applying qPCR to microbial analyses, which are summarized in Table 2.3. Firstly, the real-time system integrates amplification and detection, eliminating final analysis by gel electrophoresis and the use of the carcinogenic ethidium bromide used for visualization in agarose gel electrophoresis. With qPCR, melting curve generation is the confirmatory phase and occurs following the amplification cycling, as previously stated. Taking advantage of the integrated nature of such systems, Higgins *et al*. (2003) have attempted to make use of a handheld advanced nucleic acid analyzer thermal cycler for qPCR studies which may give an indication to the future of real time qPCR technology.

The use of fluorescent dyes and probes allows for constant reaction monitoring in real-time, through the consistent increase in fluorescent signal. This strengthens the potential for using these techniques in health and quality related studies, such as waterborne pathogen detection, since new technologies attempt to present more rapid reaction times and shorten the time-frame for confirmation. This in turn allows for a faster assessment of water quality and potential threats to human health. The need for such rapid, real time systems has been suggested by both Pickup *et al*. (2003) and Straub and Chandler (2003).

TABLE 2.3. Summary of the benefits and limitations of the application of qPCR in pathogen detection (Adapted from Logan *et al*., 2006 and Bustin *et al*., 2005).

Advantages	Disadvantages
- Integrated system for amplification and detection, eliminates gel electrophoresis	- Quantification of low abundance targets (< 1,000 copies) problematic without enrichment
- Constant reaction monitoring in "real time" with the use of fluorescent dyes and probes	- Possible inhibition from a variety of sample types (i.e. humic acids from water)
- Rapid cycling and analysis times and high sample throughput (200-5,000 per day)	- Development of standardized protocols, primer design and operation of instrumentation and analysis require high degree of skill
- Increased sensitivity (As little as 1 genome equivalent of DNA can be detected)	- Limited capacity for multiplex applications
- Detection across a broad dynamic range (10 – 10^{10} copies)	- Cannot differentiate between viable/non-viable cells

An added advantage is that since reaction times are shortened, a larger number of samples can be screened within a single day. For example, up to 1,000 potentially different samples can be cycled, since either 32 or 96-well blocks are present in most commercially available thermal cyclers (Ibekwe and Grieve, 2003; Logan *et al*., 2009). In comparison, DNA microarray technology, a molecular technique which allows for analysis of gene expression, permits detection of up to as many as 1,000 target sequences on a single chip, at the same time, which can be seen as an advantage over qPCR (Lemarchand *et al*., 2004). This technology has however not been greatly developed for waterborne pathogen detection, because of high costs and inadequate sensitivity levels (Call *et al*., 2003). A 2005 study by Maynard *et al*. attempted to develop a microarray for waterborne targets, however detection limits of 10^4 *S*. Typhimurium cell equivalents were considered above the desirable limits.

The main benefit to using oligonucleotide probes rather than fluorescent dyes is that specificity for the intended target is greatly increased, since a fluorescent signal is only generated upon direct binding between probe and target. As previously stated cyanine dyes in comparison are non-specific and bind to any double-stranded nucleic acid present within the reaction tube. These reactions must be stringently optimized to prevent mispriming and nonspecific amplification. Multiplexing can also be performed with probes, since each primer pair can be labelled with a different fluorochrome, allowing for each to be differentiated in the same reaction capillary due to their different emission wavelengths. Multiplexing abilities are however limited by the instrumentation itself, since some qPCR instruments only allow for the detection of fluorescence emitted at 3 or 6 different wavelengths such as the LightCycler® by Roche Diagnostics (Monis and Giglio, 2006).

With respect to reaction sensitivity and detection limits, as previously mentioned, the technique has the ability to detect as few as 1 cell or genome equivalent per unit analyzed, which is significantly lower than most other molecular-based techniques used as a standalone detection platform. Targets which are present in environmental samples at low abundance, for example single-copy genes or genes which are present in less than 1,000 copies are difficult to detect (Logan *et al.*, 2009). Various inhibitors can be present in environmental samples, including humic acids, salts, divalent cations and various organic compounds in water (Toze, 1999). Such compounds drastically inhibit polymerase activity, resulting in the generation of false negatives. The method is also prone to false positives resulting from nucleic acid-contaminated laboratory equipment (Toze, 1999). Also, for low abundance targets, concentration is required in order to achieve detection, which lengthens analysis times since additional steps must be taken before

analysis can be performed (Straub and Chandler, 2003). Straub and Chandler (2003) have suggested that when environmental water samples are concentrated, inhibitors are also co-concentrated. To individually detect some pathogens according to *Standard Methods* protocols (Clesceri *et al*., 1999), large volumes of water (2 to 10 L) must be concentrated. As a result, the efficiency of molecular detection would decrease as a result of increasing amounts of inhibitory substances preventing the polymerase enzyme from replicating the target.

PCR techniques are non-discriminatory in nature, which can be seen as both an advantage and a limitation unto itself. They are unable to provide information on the physiological status of target bacterial cells since all DNA, including that from both viable and non-viable cells is amplified (Rompré *et al*., 2002). Generally in these scenarios, an additional pre-enrichment stage is employed in order to maintain cell viability and resuscitate those that were previously damaged or stressed. It can be said that DNA testing alone therefore does not accurately assess the potential risk, since viable and non-viable cells cannot be differentiated and thus actual pathogen loads at a specific time may not be entirely reflected. The technique does however have an advantage over culture-based methods in that amplification of DNA from viable but non-culturable cells can be achieved. This has been proven in studies by Khan *et al*. (2007), *et al*. Ram *et al*., (2008b) and Yang *et al*. (2003). The study by Yang *et al*. (2003) specifically indicated that a significant difference of several orders of magnitude was observed between the numbers of *C. jejuni* cells detected solely by selective plating ($4.3x10^3$ CFU/mL) relative to those detected using molecular methods ($6.4x10^6$ CFU/mL).

The development of protocols and validated methods is time consuming since several years may be required before they are implemented and finalized. A high degree of skill with oligonucleotide primer design as well as the theory and instrumentation behind real-time technology is also required for success of such systems. Knowledge of primer design is essential in order to use design software to select primers with adequate characteristics and specificity for the intended targets. Optimization takes time and expertise to identify where problems may lie within a reaction. Proper training is also required on both the instrument itself and on the software in order for it to be used to its maximum potential since several parameters are user-specified. Training is also required for the analysis of qPCR results (C_T and melting curve data), since they are not necessarily straightforward depending on the quantification method used.

2.3.3. Use of qPCR as a standardized detection platform

In order to examine the feasibility of applying qPCR for water quality monitoring, protocols must be properly validated to ensure accuracy and reproducibility, as well as sensitivity and specificity for the intended target, while minimizing interlaboratory variations. Hoorfar and Cook (2003) have outlined a series of practical steps which have been used by the European Commission to validate and standardize PCR for use with foodborne pathogens, and could also be applied to waterborne targets. In the first phase, oligonucleotide primers are designed for the intended target genes and a battery of tests are performed. Some of these include optimization of reaction conditions with different enzymes/reagents, primer specificity assays and the determination of appropriate detection limits in order to adopt the "ideal" set of primers for each type of analysis (Hoorfar and Cook, 2003).

The specificity of the primers is determined by several laboratories using nucleic acids from an array of reference microorganisms. These normally include strains both closely related and unrelated to the target species (Hoorfar and Cook, 2003). A strong specificity for the target is expected with no other non-specific binding as well as the ability of the primers to be used with several different enzymes and/or reagents to further demonstrate the robustness of the primers. In the final phase of validation, primers are tested using samples artificially contaminated with known amounts of the target (high, medium and low concentrations) in trials by approximately 10-12 different laboratories using the standard optimized protocol developed by the original laboratory (Hoorfar and Cook, 2003). Results are then compared to traditional detection methods to determine whether the same degree of accuracy is achieved.

Several assays have been designed using the above criteria to target foodborne pathogens such as *Campylobacter* spp., including those by Josefson *et al*., 2004 and Lübeck *et al*., 2003. The overall diagnostic sensitivities obtained from these interlaboratory validation studies were 96.7% and 93.7% respectively, indicating that there was a high degree of similarity in detection limits between laboratories. There are currently no similar standardized methods designed specifically for waterborne pathogen analysis. The development of which will be the basis for this study.

CHAPTER 3: MATERIALS AND METHODS

3.1. Bacterial cultivation and DNA extraction

Cultures of *E. coli* O157:H7 ATCC 700927 and *S.* Typhimurium ATCC 14028 were obtained from Dr. Dae-Young Lee (University of Guelph, Ontario), *C. jejuni* NCTC 11168 was obtained from Dr. Eytan Wine (University of Alberta, Alberta) and *K. pneumoniae* ATCC 13882, *S. flexneri* ATCC 12022 and *P. aeruginosa* ATCC 27853 were obtained from Liberty Victorio-Walz (Ryerson University, Ontario) and were used as positive controls in this study. All bacterial strains with the exception of *C. jejuni*, were cultured overnight from frozen glycerol stocks (30% v/v) to a cell density of approximately 10^9 CFU/mL in 10 mL of 3 g/L Tryptic Soy (CASO) Broth (TSB) (EMD Chemicals Inc., Mississauga, ON, Canada) prior to DNA extraction. Optimal incubation temperatures were 37°C for all organisms with the exception of 30°C for *P. aeruginosa* and 42°C for *C. jejuni*. The *C. jejuni* strain was grown on 5% anti-coagulated sheep's blood agar (5% sheep's blood in Tryptic Soy Agar) under microaerophilic conditions using the BBL™ CampyPak™ Plus system (Beckton, Dickinson and Company, Franklin Lakes, NJ, USA) and incubated at 42°C for 5 days as per manufacturer's recommendation.

3.2. PCR primer design

Oligonucleotide primers were designed using the LightCycler® Probe Design2 software (Roche Diagnostics, Laval, QC, Canada) and Primer3 (http://frodo.wi.mit.edu/primer3/) (Rozen and Skaletsky, 2000). The most commonly encountered low-copy virulence related genes from each of the aforementioned reference strains were used as targets (Table 3.1). Sequence information for each gene that was selected was obtained from the previously deposited sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html). Targets

and descriptions can be seen in Table 3.1. A series of criteria for primer design from Dieffenbach *et al*. (1993) and Apte and Daniel (2003) were followed during the design process, in order to select the most suitable primer sets from those generated by the computerized software. Design parameters included analysis of characteristics such as; similar T_m, comparable G and C content between each set of forward and reverse primers, and small amplicon length. A schematic diagram of the selection process and criteria used for primer design can be seen in Fig. 3.1.

TABLE 3.1. Waterborne bacterial pathogens and gene targets selected for this study

Pathogen	Gene Target	GenBank Accession No.	Gene Target Information
E. coli O157:H7	*tir*	AF125993	- Translocated intimin receptor; strengthens attachment to intestinal epithelial cells (Goosney *et al*., 2000)
S .Typhimurium	*invA*	M90846	- Invasion associated protein A; involved in the initial invasion of host epithelial cells (Fey *et al*., 2004)
S. flexneri	*ipaH*	M32063	- Invasion plasmid antigen H; associated with the invasion of colonic epithelial cells (Ashida *et al*., 2007)
P. aeruginosa	*exoT*	L46800	- Exotoxin T; effector protein which inhibits bacterial cells internalization in eukaryotes (Feltman *et al*., 2001)
C. jejuni	*VS1*	X71603	- Variable sequence region 1; produces an unknown protein product, but contains genetic regions specific to *C. jejuni* (Stonnet and Guesdon, 1993)
K. pneumoniae	*magA*	AB085741	- Mucoviscosity associated gene A; produces a hypermucoviscous extracellular capsule, allowing the evasion of the human immune system (Struve *et al*., 2005)

Predictions of the formation of hairpins, self-dimers and hetero-dimers were examined using the IDT SciTools OligoAnalyzer 3.1 program (http://www.idtdna.com/SCITOOLS) (Integrated DNA Technologies Inc., USA) and Gene Runner (http://www.generunner.net/). A Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was also performed on each primer set to determine theoretical primer specificity and any non-target sequence homology prior to testing the parameter experimentally. Primers were then synthesized by the DNA Synthesis Facility at the MaRs Centre (Hospital for Sick Children, Toronto, ON, Canada), maintained at a working concentration of 5 µM and stored at -20°C in sterile MilliQ water. The validation process outlined by Hoorfar and Cook (2003) was followed for the remainder of the experiment.

3.3. DNA extraction from reference strains

Prior to DNA extraction, 1 mL aliquots of overnight cultures grown to a cell density of approximately 10^9 CFU/mL in 3 g/L TSB were concentrated by centrifugation at 13,000 x *g* for 15 min. Pellets were then re-suspended in 200 µL of 1 x PBS solution. For *C. jejuni*, colonies were aseptically removed from the culture medium and re-suspended as described. Genomic DNA was extracted from reference strains using the High Pure PCR Template Purification Kit (Roche Diagnostics, Laval, QC, Canada) according to the manufacturer's protocol. An additional RNAse treatment (1.5 µg/µL) (Sigma-Aldrich, Oakville, ON, Canada) was performed during the lysis stage of the extraction for 10 minutes at 37°C in combination with lysozyme treatment (10 µg/µL). Purified DNA was eluted in sterile Tris-EDTA buffer (pH 8.0) in a final volume of 200 µL, and stored at -20°C. DNA concentration and purity ($A_{260/280}$) were determined spectrophotometrically with the Eppendorf® BioPhotometer (Eppendorf AG, Hamburg,

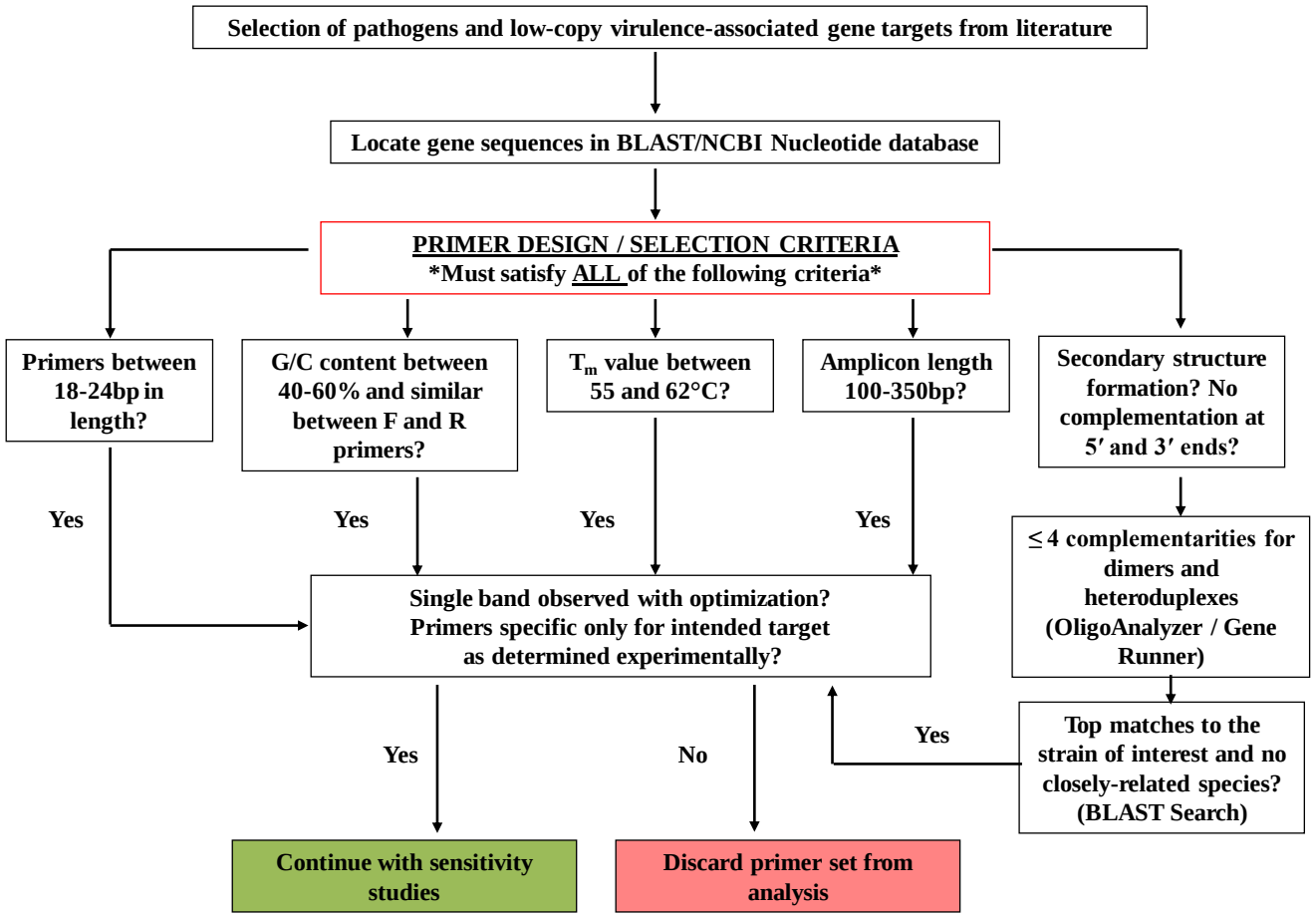

FIG. 3.1. Strategy and criteria for the primer design/selection process adapted from Dieffenbach *et al* (1993) and Apte and Daniel (2003).

Germany) prior to use in PCR. Only pure DNA with an $A_{260/280}$ (DNA: protein ratio) value larger than 1.8 (Maniatis *et al*., 1982) was used in PCR. All DNA was used at a final working concentration of 20 ng/µL.

3.4. Protocol optimization and primer evaluation

To validate the usefulness of the newly designed primers, optimal annealing conditions were determined in endpoint PCR, using the MyCycler™ Thermal cycler (BioRad Laboratories Inc., Mississauga, ON, Canada). Reaction mixtures (25 µL) contained 0.5 µL of 50 x TITANIUM™ Taq, 2.5 µL of 10 x TITANIUM™ Taq PCR buffer and 0.2 mM dNTP mixture from the TITANIUM™ Taq PCR kit (Clontech Laboratories Inc., Mountainview, CA, USA), in addition to 0.2 mM of the exoT, magA and ipaH2 primer pairs (in their respective experiments), 2 µg/µL BSA (Roche Diagnostics, Laval, QC, Canada) and 2 µL of template DNA (20 ng/µL). Volumes were adjusted to 25 µL with sterile MilliQ water. Negative controls used sterile MilliQ water (18.2 MΩ/cm^2) in place of template. Temperature profiles consisted of denaturation at 95ºC (2 min) followed by of 35 cycles of 95ºC (1 min), primer annealing between 49-65ºC (30 s) with a temperature gradient, extension at 72ºC (1 min), followed by a second 1 min extension at 72ºC.

For a qualitative examination of the desired amplification products, samples were subjected to 2% agarose gel electrophoresis following PCR cycling, for 35 minutes at 120 V using the iMupid 2-Plus Mini Agarose Gel Electrophoresis System (Helixx Technologies Inc., Scarborough, ON, Canada), with the GeneRuler™ 100bp DNA Ladder (Fermentas Life Sciences, Burlington, ON, Canada). Ethidium bromide stained gels (30 µg/µL) were then visualized using

the BioDoc-It Imaging System (UVP, Upland, CA, USA). Temperature profiles were then modified as necessary, according to observations of non-specific binding. As noted in the selection criteria outlined in Fig 3.1, primers were deemed suitable for further use upon visualization of a single band of the expected size corresponding to the gene of interest. Observations of extreme amounts of non-specific binding following protocol adjustments indicated that the primer set was not suitable for further analysis. The remaining 3 primer sets (ETIR, SINV and VS1) were also checked in the same manner.

3.5. Primer specificity tests

Specificity assays were performed to ensure whether primers were accurately designed and specific for their intended targets. A list of strains used for specificity purposes can be seen in Table 3.2. For each primer set, PCR reactions containing target or non-target DNA were set up to verify that no false-positives were generated with DNA from non-target organisms. Reactions (25 µL) were prepared using 2 µL of the various template DNA (20 ng/µL). Amplification was performed using the previously optimized endpoint PCR protocols for each primer set. The PCR products were then visualized by 2% agarose gel electrophoresis (See section 3.4).

3.6. QPCR sensitivity assays in pure culture

Standard curves were generated by qPCR for each selected primer set that was successfully validated in endpoint PCR to be used to quantify detection limits. Curves were constructed in the LightCycler® 2.0 system (Roche Diagnostics, Laval, QC, Canada) by amplifying genomic DNA extracted from 10-fold serial cell dilutions in 0.9% NaCl from each reference strain (ranging from 10^8 to 1 cell) using the LightCycler® Software 4.0 (v.4.0.0.23).

TABLE 3.2. Reference strains used for PCR optimization and primer specificity studies

Bacterial Species	Type Strain No.
Campylobacter jejuni	NCTC 11168
Enterobacter aerogenes	ATCC 13048
Enterococcus faecalis	ATCC 19433
Escherichia coli	ATCC 11229
Escherichia coli	ATCC 23723
Escherichia coli	ATCC 23725
Escherichia coli	ATCC 25922
Escherichia coli O157:H7	ATCC 700927
Klebsiella pneumoniae	ATCC 13882
Klebsiella pneumoniae	ATCC 13887
Pseudomonas aeruginosa	ATCC 27853
Salmonella enterica	ATCC 13314
Salmonella Typhimurium	ATCC 14028
Shigella flexneri	ATCC 12022
Streptococcus faecalis	ATCC 19432

Cell concentrations were determined spectrophotometrically by OD_{600} and confirmed by triplicate spread plating of the appropriate dilution onto 3 g/L TSA. Amplification reactions (20 µL) consisted of 4 µL of 5 x Reaction Mix from the LightCycler® FastStart DNA Master^{PLUS} SYBR Green I kit (Roche Diagnostics, Laval, QC, Canada), 0.2 µg/µL BSA, 0.6 mM $MgCl_2$, 0.25 mM of each primer, 2 µL of template (ranging from 20 ng/µL to 2 fg/µL) and the remaining volume with PCR grade H_2O.

Detection limits were then determined by creating standard plots of crossing point (C_P) relative to the logarithm of bacterial cell numbers. Reaction efficiencies were then computer generated from the slope of each plot by using a formula of: Efficiency = $10^{-1/slope}$. Melting peaks were generated following amplification to confirm desired end products and consisted of an initial heating at 95°C (0 s) followed by a 0.1°C increase from 65 to 95°C every second for 1 min. Plots were constructed by comparing the derivative of the fluorescence at 530 nm relative to the temperature at which the end product denatured.

3.7. Membrane filtration and optimization of DNA recovery

In an attempt to remove any extracellular nucleic acids from environmental water samples and reduce false positives in a complex environmental matrix, a membrane filtration step was introduced prior to DNA extraction by the HighPure PCR Template Preparation Kit (Roche Diagnostics, Laval, QC, Canada). Either a 0.45 µm GN-6 membrane filter, 25 mm in diameter (Pall Corporation, Port Washington, NY, USA), 0.22 µm GSWP Durapore filter, 25 mm in diameter (Millipore Canada Ltd., Etobicoke, ON, Canada) or 0.22 µm GVWP filter, 25 mm in diameter (Millipore Canada Ltd., Etobicoke, ON, Canada) was applied to an autoclaved

(121°C, 15 min), UV-sterilized vacuum filtration apparatus (VWR International, Mississauga, ON, Canada). For these experiments, *S*. Typhimurium was used as a representative pathogen, similar to the method described by Ahmed *et al*. (2009). Cells of known concentration (10^7 cells) were inoculated into individual 100 mL aliquots of autoclaved, UV-sterilized, low-turbidity source water obtained from DeCew Falls (Thorold, Canada) to simulate a range of contaminated environmental source water samples, an approach similar to that described by Khan *et al*. (2007, 2009). An un-inoculated environmental water sample was also used as a control and treated as described.

To test the efficiency of recovery and purification of DNA from target cells retrieved from the different filter types, combinations of mechanical, enzymatic and chemical lysis methods were tested with seeded lake water in duplicate (Table 3.3). Following extraction and centrifugation, cell pellets were resuspended in 1 x PBS solution (200 µL), and DNA extracted as previously described using the HighPure PCR Template Preparation Kit (Roche Diagnostics, Canada). DNA was then quantified spectrophotometrically and purity was also confirmed by PCR amplification with the SINV primers. The recovery efficiency/percent recovered DNA was calculated using the following equation: % DNA Recovered = (Concentration of DNA from $1x10^7$ cells with filtration / Concentration of DNA from $1x10^7$ cells without filtration) x 100%. The method with the highest recovery and lowest standard deviation was selected for further analysis.

TABLE 3.3. Methods used to extract cells and DNA from each type of membrane filter used in the study.

Method	Filter Size (µm) & Type	Description
1	0.45	Filters placed aseptically into 15 mL screwcap tubes containing 5 mL of sterile STE buffer (0.1M NaCl, 10 mM Tris, 1 mM EDTA [pH 7.6]) (Ahmed *et al*., 2008, 2009) and bead solution (2 mL) from the UltraClean™ Soil DNA Isolation Kit (MO-BIO Laboratories Inc., Carlsbad, CA, USA), vortexed vigorously (10 min) and centrifuged at 10,000 x g (20 min).
2	0.22 GSWP	Treated as per Method 1
3		DNA extracted using the UltraClean™ Water DNA Isolation kit (MO-BIO Laboratories Inc., Carlsbad, CA, USA), as per manufacter's protocol.
4		Filters were placed into a 2 mL screwcap tube containing a lysis/binding buffer [250 mM Tris-HCl [pH 8.0], STE buffer and 0.2 g Zirconia/Silica beads (BioSpec Products Inc., Bartlesville, OK, USA)] and vortexed (20 min).
5		Treated as per Method 4, however 20% SDS solution replaced the STE buffer. Following vortexing, supernatants were transferred to a sterile 1.5 mL microcentrifuge tube, while 300 µL of Tris-HCl solution was put back into the screwcap tube. A secondary bead beating step (10 min) was then performed, the supernatants pooled and centrifuged at 10,000 *x g* (20 min).
6		Treated as per Method 5, however beads from the UltraClean™ Soil DNA Isolation kit replaced the Zirconia/Silica beads.
7	0.45	Treated as per Method 5
8	0.22 GSWP	Treated as per Method 5, however prior to bead beating, 5 µL of lysozyme (10 mg/mL) was added and samples incubated at 37°C (15 min). Proteinase K treatment (40 µL) was also performed, with samples incubated at 70°C (10 min) followed by bead beating (10 min). No secondary bead beating was performed
9		Treated as per Method 5, however the 20% SDS solution was added following lysozyme and proteinase K treatment and no secondary bead beating was performed
10	0.22 Durapore	Treated as per Method 8

3.8. Lakewater sensitivity assays with nested and qPCRs

A newly designed nested PCR primer set was constructed using Primer3 to flank the original SINV primers to qualitatively ensure the successful isolation of DNA from all possible cell concentrations, using the selected method. The F and R primers (5′→3′) were; invAnested-F: TGTCACCGTGGTCCAGTTTA and invAnested-R: CTCGCCTTTGCTGGTTTTAG, amplifying a 640bp region of the *invA* gene. Initial PCR reactions (25 µL) were prepared as previously described, incorporating the new primers with an amplification profile of denaturation at 95°C (2 min), followed by 25 cycles of 95°C (1 min), 66°C (30s), 72°C (30s) and 72°C (30s). Template DNA from 10^7 *S.* Typhimurium cells isolated using extraction Method 8 was diluted to extinction to give an estimated detection limit prior to qPCR analysis. The nested PCR was processed with the SINV primers using 2 µL of the previous reaction and the optimal thermocycling program for the primer set.

Real-time qPCR reactions with the SINV primers were prepared using template DNA recovered from 10^9 cells/100mL to 10 cells/100mL dilutions of *S.* Typhimurium in seeded source water. Samples were filtered and isolated by Method 8. A total of 50 cycles of amplification were repeated for each reaction, and melting curves were generated following each trial. Detection limits were determined following the creation of a second standard plot of C_P against the logarithm of cell number. Crossing points were then compared with those generated in pure culture.

3.9. Microscopic examination of membrane filters

To qualitatively examine whether cells (both viable and non-viable) were adhering to and being removed from each type of membrane filter, a viability assay was used. Cells of the *S.* Typhimurium strain were first washed three times with 1 mL of 0.9% NaCl (10,000 x *g* for 10 min) to remove any residual media which may interfere with the staining process. Cell staining (25°C for 15 min) with 3 µL of a 1:1 mixture of the DNA-binding dyes SYTO 9 and propidium iodide (0.3% DMSO) using the LIVE/DEAD® *Bac*Light™ Bacterial Viability kit for microscopy and quantitative assays (Molecular Probes Inc., Willow Creek, OR, USA) was performed as per manufacturer's recommendation. Stained cells were then inoculated into duplicate 100 mL aliquots of distilled water and filtered through each of the 3 filter types tested in this study. Controls consisted of a 1 mL aliquot of *S.* Typhimurium cells prior to filtration (positive) and 1 mL of stained distilled water (negative control). One of the duplicate filters was then removed aseptically, cut in half, and placed onto a clean microscope slide with one drop each of *Bac*Light™ mounting oil (Molecular Probes Inc., Willow Creek, OR, USA), and CITIFLUOR Anti-Fading Agent AF2 (Citifluor Ltd., London, England, UK) and kept in the dark until analyzed. This was then repeated for each filter type.

Filters were examined using a Leica Model DM5000B upright microscope (Leica Microsystems, Richmond Hill, ON, Canada) at 400x to 630x magnification under epifluorescence using either GFP (500-550 nm) or dsRed filters (588 nm). Viable cells were noted by the presence of rod-shaped bacterial cells emitting green fluorescence, while red cells indicated those which were assumed dead. For the second of the duplicate filters, filters were treated with lysozyme, proteinase K and bead beat prior to examination, as per Method 8.

Following the bead beating, filters were placed onto microscope slides and examined as previously described.

In addition, to account for any cell/DNA losses as a result of passage through the pores of the membrane filters or through the filtration unit itself, filtrates were analyzed after being subjected to Method 8. Triplicate aliquots (100 µL) of filtrate were first spread plated unto 3 g/L TSA and incubated overnight at 37°C to check for viability. The remaining filtrate (~ 99 mL) was then concentrated (15 min, 10,000 *x g*) and resuspended in 1 mL of 1 x PBS solution. DNA was extracted for PCR analysis as previously described as per reference strains (See section 3.3).

CHAPTER 4: RESULTS

4.1. PCR primer design

The final primers can be seen in Table 4.1 while the remaining sets tested but not further explored can be found in Appendix B. Each individual primer set satisfies the characteristics required for adequate primer design outlined by both Dieffenbach *et al*. (1993) and Apte and Daniel (2003), which were used to create the original testing criteria from Fig. 3.1. In Table 4.1, it can be seen that each of the primers were within the 18 to 22 bp range, had similar T_m within a 5°C threshold between forward and reverse primers as well as similar % GC (31.8 to 55.0% range). Ideally, the G and C content should fall within 45 to 50%. An exception can be seen, however, with the VS1 primer set, which had lower than expected G and C contents of 31.8 and 33% respectively for the forward and reverse primers. Despite this fact, the primers were still able to perform effectively in the study. Three primer sets were novel designs to the study, the exoT and magA forward and reverse primers, designed using the LightCycler® Probe Design 2.0 software and and the ipaH2 primer set designed with Primer3. The ETIR and SINV primer sets were previously designed in the laboratory (Haffar and Gilbride, 2010); however they had not been fully optimized nor applied to environmental water samples. The VS1 primers, as designed by Stonnet and Guesdon (1993), were selected since their reported high specificity and detection as low as 1 cell/mL in a study by Yang *et al*. (2004), made them applicable to this study. Gene alignments for each primer set can be seen in Appendix A.

4.2. Primer validation by protocol optimization

Optimal amplification conditions for each newly designed primer set were determined by

a combination of temperature profile alterations and the use of the gradient feature on the MyCycler™ instrument (BioRad Laboratories Inc., USA). The point at which a single band was observed via 2% agarose gel electrophoresis following adjustments of elongation times (between 30 s and 5 min), and annealing temperatures (ranging from 50 to 65°C) meant that the reaction conditions were optimal. The ipaH2 and exoT primers were found to have annealing temperatures of 66°C and 65°C respectively, with endpoint PCR protocols of 35 cycles of 95°C (2 min), 95°C (1 min), annealing (30s), 72°C (1 min), 72°C (1 min). Temperature gradients for both primer sets can be seen in Figs. 4.1 and 4.2, where the amount of non-specific amplification products decreases as the annealing temperature increases, to a point where only the desired amplicon is visualized. Temperatures above 66°C were not analyzed, since it has been suggested that higher annealing temperatures are not recommended for use in PCR-related studies.

In an attempt to further reduce the non-specific binding by a means other than protocol adjustment, 2 µg/µL of BSA was added to all reactions, since BSA is known to function as a PCR enhancer (Kreader, 1996). For the remaining 3 primer sets (ETIR, SINV and VS1), temperature gradients were unnecessary since previously optimized PCR protocols were available (Yang *et al*., 2003; Haffar and Gilbride, 2010).

Each primer set also produced amplicons of differing sizes, ranging from 207 to 358bp to allow visual differentiation through 2% agarose gel electrophoresis. The expected PCR products can be seen in Fig. 4.3, indicating the successful amplification of the expected 358 bp product of the VS1 primer set (*C. jejuni*); the 285 bp product of the exoT primer set (*P. aeruginosa*); the 252 bp product of the SINV primer set (*S.* Typhimurium) the 247 bp product of the ipaH2 primer

TABLE 4.1. Oligonucleotide primers selected to target waterborne bacterial pathogens in this study.

Primer Name	Target Organism	Nucleotide Sequence (5′ → 3′)	Primer Length (bp)	5′ Position	3′ Position	% GC	T_m (°C)	Amplicon Size (bp)
KmagA	*K. pneumoniae*	F: ACGGAGCAATATGGCCAGTC	20	995	1014	55.0	63.9	164
		R: GAATCTGCAGCAGAAACGGG	19	1158	1139	55.0	62.9	
ETIR[a]	*E. coli* O157:H7	F: GTCAGCTCATTAACTCTACGGG	22	98	119	50.0	60.2	207
		R: GCCTGTTAAGAGTATCGAGCG	21	304	284	52.4	61.1	
ipaH2	*S. flexneri*	F: ATAATGATACCGGCGCTCTG	20	1299	1318	50.0	61.6	247
		R: CGGCTTCTGACCATAGCTTC	20	1545	1526	55.0	60.2	
SINV[a]	*S.* Typhimurium	F: TATGCCCGGTAAACAGATGAG	21	525	545	47.6	60.5	252
		R: GTATAAGTAGACAGAGCGGAGG	22	776	755	54.5	61.7	
exoT	*P. aeruginosa*	F: GGTCTCTATACCAACGGCGA	20	1017	1036	55.0	59.6	285
		R: GAACAGGGTGGTTATCGTGC	20	1301	1282	55.0	60.6	
VS15 & 6[b]	*C. jejuni*	F: GAATGAAATTTTAGAATGGGG	21	93	113	33.3	54.2	358
		R: GATATGTATGATTTTATCCTGC	22	450	429	31.8	53.3	

[a] designed by Haffar and Gilbride (2010); [b] designed by Stonnet and Guesdon (1993)

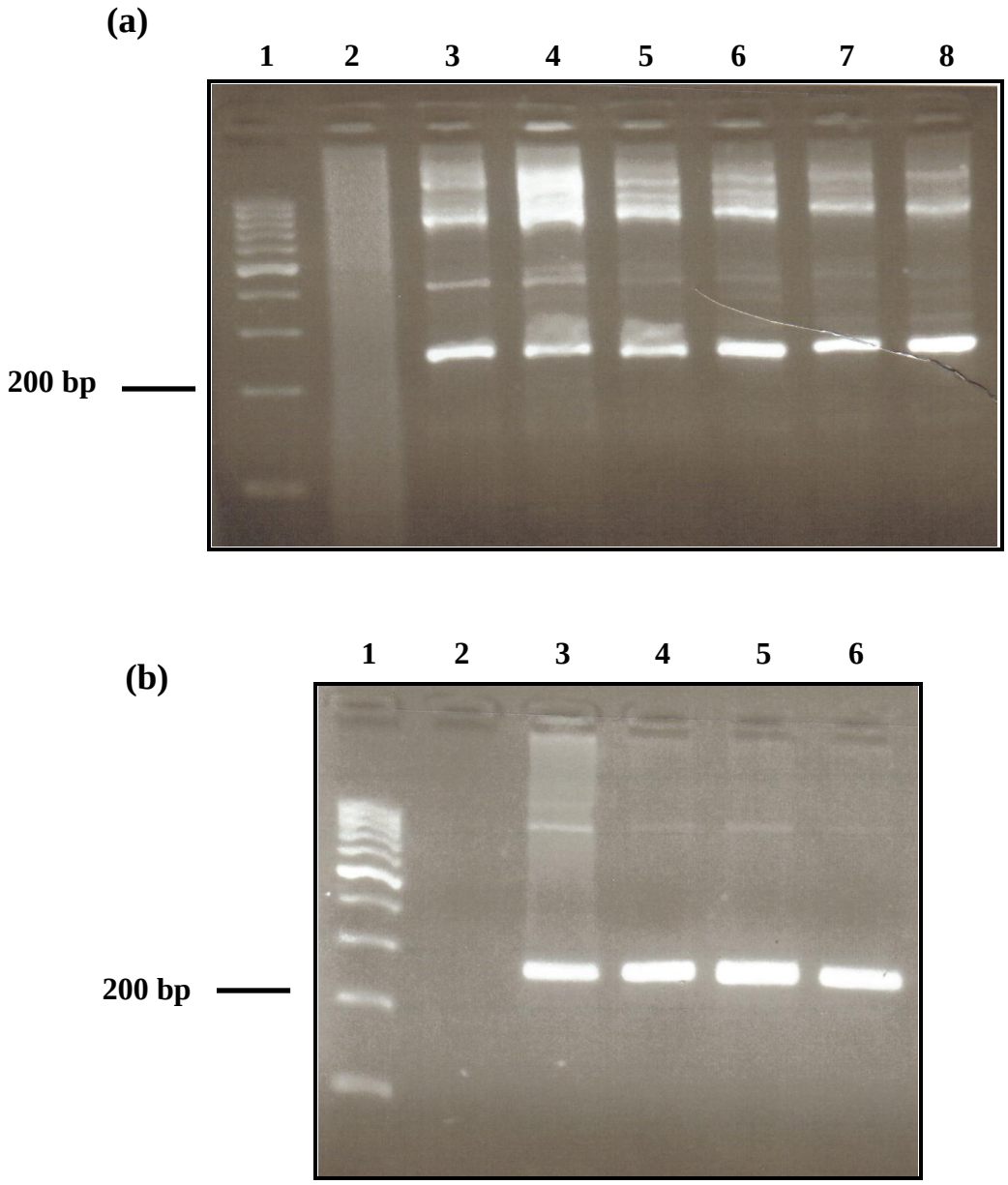

FIG. 4.1. Electrophoresis (2% agarose, 120V) of amplicons generated in endpoint PCR for the ipaH2 primer set (specific for *S. flexneri*) following annealing temperature optimization. **(a)** (1) 100 bp M.M.; (2) NTC; (3) 57°C; (4) 58°C; (5) 59°C; (6) 60°C; (7) 61°C; (8) 62°C. **(b)** (1) 100 bp M.M.; (2) NTC; (3) 63°C; (4) 64°C; (5) 65°C; (6) 66°C. The optimal annealing temperature was determined to be 66°C for this primer set.

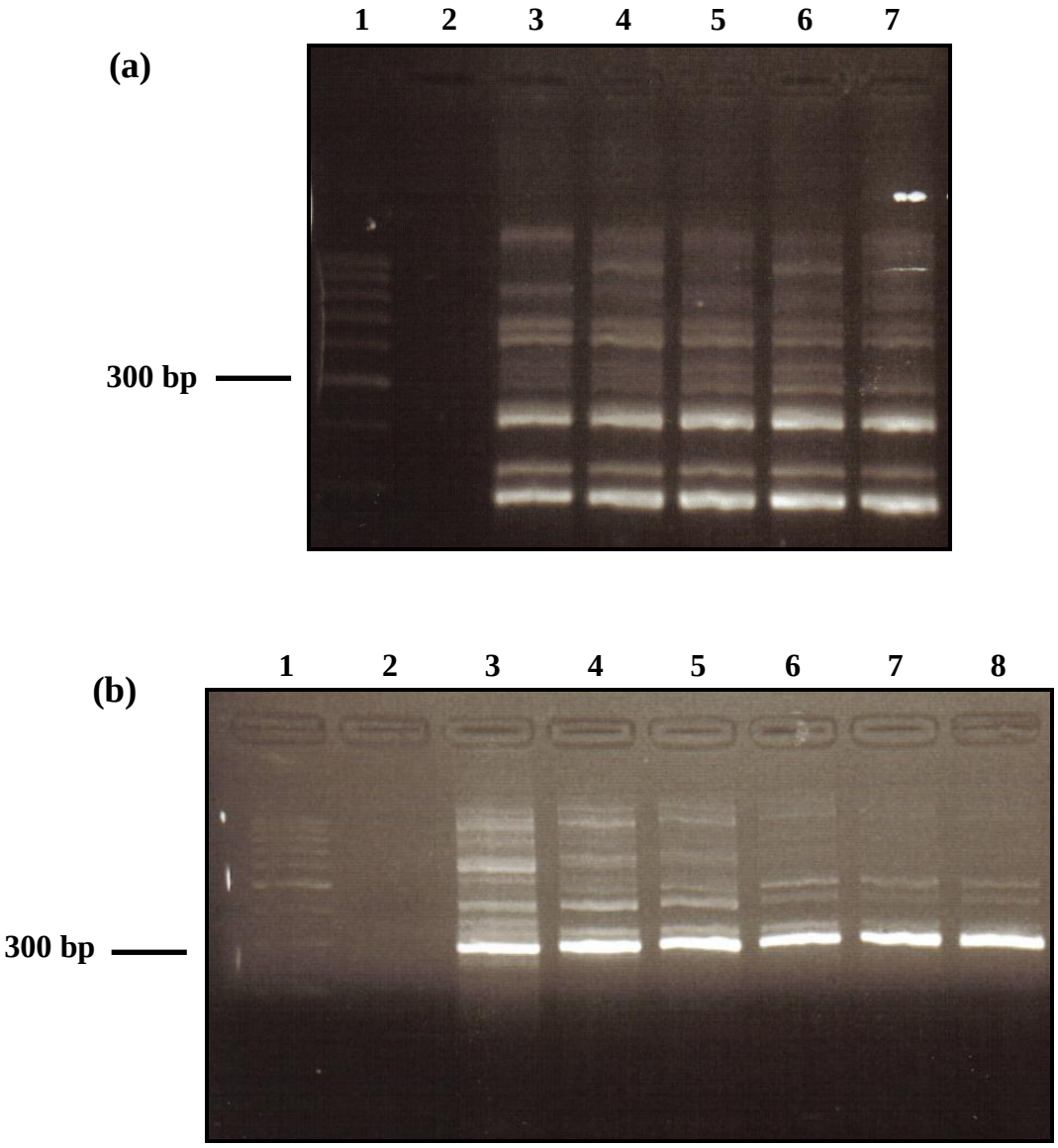

FIG. 4.2. Electrophoresis (2% agarose, 120V) of amplicons generated in endpoint PCR for the exoT primer set (specific for *P. aeruginosa*) following annealing temperature optimization. **(a)** (1) 100 bp M.M.; (2) NTC; (3) 55°C; (4) 56°C; (5) 57°C; (6) 58°C; (7) 59°C. **(b)** (1) 100 bp M.M.; (2) NTC; (3) 60°C; (4) 61°C; (5) 62°C; (6) 63°C; (7) 64°C; (8) 65°C. The optimal annealing temperature was determined to be 65°C for this primer set.

set (*S. flexneri*) as well as the 205 bp product of the ETIR primer set (*E. coli* O157:H7). The magA primer set (*K. pneumoniae*) was eliminated from further analysis, because the expected product of 164 bp was not observed and only a significant amount of non-specific binding was seen upon use of temperature gradient (Fig. 4.4).

4.3. Determination of primer specificity

As a means of testing the accuracy of the primer designs for their intended targets, specificity assays were performed for all primer sets. During the design stage, a Primer-BLAST search was performed with each primer set in the NR database of NCBI. Sequence homology results revealed that each primer set was specific for the target organisms at the species level, with the most common alignments being that of the respective organism from the 5 target species used in this study (Appendix C). Different serotypes of the same pathogenic species also known to harbour the gene targets were also visible in the search results. Specificity tests confirmed that each set was highly specific for their intended targets, as seen in Figs 4.5 to 4.9. These findings were supported by the earlier BLAST results. Each agarose gel image indicates that reactions between the primers and non-target microorganisms did not produce amplicons of the expected size. Most importantly, this trend continued between each of the 5 pathogens selected for the study, indicating their selectivity. There was however a slight degree of non-specific binding in the region above 400 bp, which was observed amongst all of the primer sets and non-target strains. This was later reduced and differentiated during qPCR cycling. The ETIR and exoT designs were found to produce only a single band each with the respective positive controls (*E. coli* O157:H7 ATCC 700927, *P. aeruginosa* ATCC 27853) and had no non-specific reactions

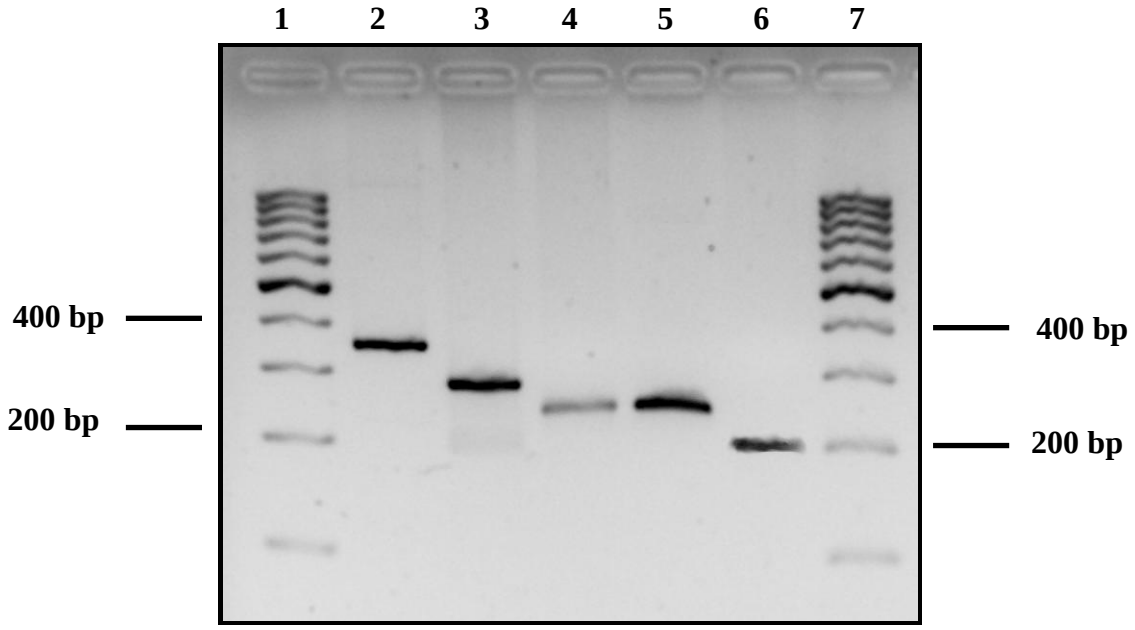

FIG. 4.3. Amplicons obtained following endpoint PCR amplification of each primer set and their respective positive controls (2% agarose gel, 120V). L to R: (1) 100bp M.M.; (2) *C. jejuni* NCTC 11168 (358bp); (3) *P. aeruginosa* ATCC 27853 (285bp); (4) *S.* Typhimurium ATCC 14028 (252bp); (5) *S. flexneri* ATCC 12022 (247bp); (6) *E. coli* O157:H7 ATCC 700927 (207bp); (7) 100bp M.M.

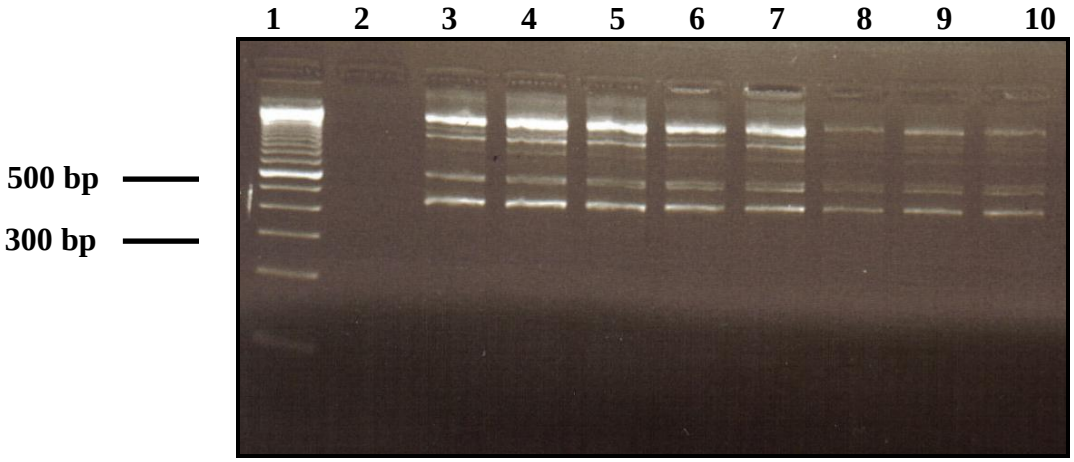

FIG. 4.4. Electrophoresis (2% agarose, 120V) of amplicons generated in endpoint PCR for the magA primer set (specific for *K. pneumoniae*) following annealing temperature optimization. (1) 100 bp M.M.; (2) NTC; (3) 58°C; (4) 59°C; (5) 60°C; (6) 61°C; (7) 62°C; (8) 63°C; (9) 64°C; (10) 65°C. The expected product of 164 bp was not observed.

with the non-target strains. The VS1 primers had the most non-specific binding, with several non-specific amplicons observed in PCR reactions of *E. coli* ATCC 23723, *E. coli* ATCC 23739 and *S. faecalis* ATCC 19432; however the 358bp fragment was not generated in the non-target bacterial reactions.

4.4. Determination of detection limits under reference conditions

For detection limit determinations, PCR protocols were first re-optimized for rapid qPCR cycling conditions. As seen in Table 4.2, the cycling lengths were much shorter than those needed previously for endpoint PCR. The overall amplification process was reduced by a minimum of at least 1 hour, depending on the primer set. The shortest total cycling time was around 50 minutes for the ETIR primers, which included a 10 minute pre-incubation prior to cycling and an additional melting curve generation. Annealing temperatures were also found to be slightly altered relative to the original endpoint determinations. The ipaH and exoT primers were those with the largest changes, with a 4 to 5°C decrease for each (68°C to 64°C and 65°C to 60°C, respectively). The remaining primers varied by ±1°C, in order to limit the production of non-specific amplicons. Standard curves were produced using DNA isolated from 10-fold serial cell dilutions of each reference strain. Correlation coefficients (R^2) were determined to range from 0.938 to 0.995 depending on the primer set (Figs 4.10 a to e). A linear relationship between the C_p and cell concentrations was observed, with the ETIR, ipaH2 and VS1 primers producing curves with the highest degree of linearity. Each primer set was determined to be suitable for quantitative assays, since relatively reliable reproducibility was achieved.

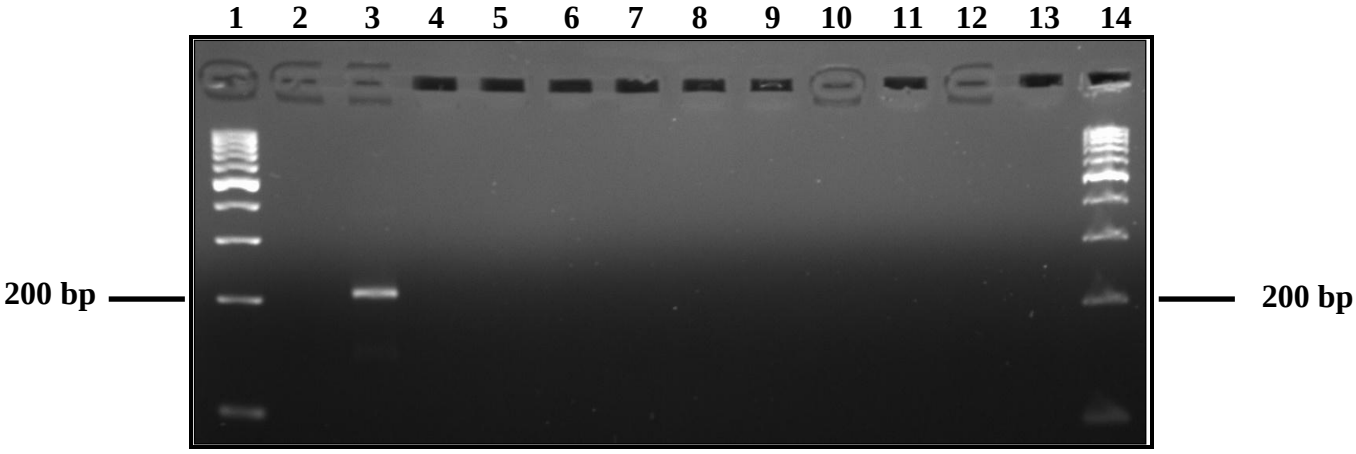

FIG. 4.5. Specificity assay for primers targeting the *tir* gene (*E. coli* O157:H7). L to R: (1) 100bp M.M.; (2) NTC; (3) *E. coli* O157:H7 ATCC 700927 (+ Control); (4) *E. coli* O157:H7 (Non-virulent); (5) *S.* Typhimurium ATCC 14028; (6) *P. aeruginosa* ATCC 27853; (7) *K. pneumoniae* ATCC 13882; (8) *C. jejuni* NCTC 11168; (9) *S. flexneri* ATCC 12022; (10) *E. aerogenes* ATCC 13048; (11) *E. coli* ATCC 23739; (12) *E. coli* ATCC 23723; (13) *E. faecalis* ATCC 19433; (14) 100bp M.M.

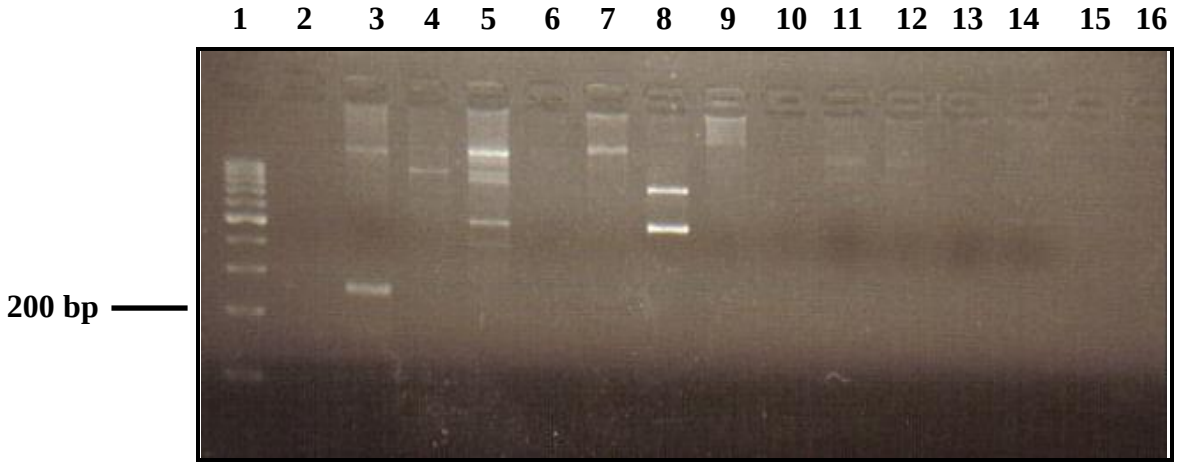

FIG. 4.6. Specificity assay for primers targeting the *invA* gene (*S.* Typhimurium). L to R: (1) 100bp M.M.; (2) NTC; (3) *S.* Typhimurium ATCC 14028 (+ Control); (4) *E. coli* O157:H7 ATCC 700927 (5) *E. coli* ATCC 11229; (6) *C. jejuni* NCTC 11168; (7) *E. aerogenes* ATCC 13048; (8) *S. flexneri* ATCC 12022; (9) *P. aeruginosa* ATCC 27853; (10) *E. faecalis* ATCC 19433; (11) *K. pneumoniae* ATCC 13882; (12) *K. pneumoniae* ATCC 13887 (13) *E. coli* ATCC 23739; (14) *E. coli* ATCC 23723; (15) *E. coli* ATCC 25922; (16) *S. faecalis* ATCC 19432.

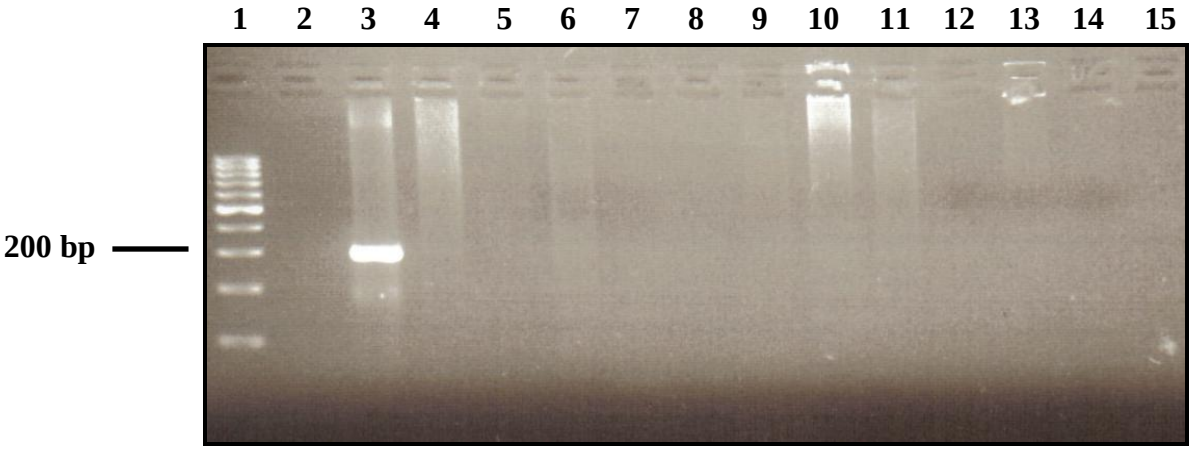

FIG. 4.7. Specificity assay for primers targeting the exoT gene (*P. aeruginosa*). L to R: (1) 100bp M.M.; (2) NTC; (3) *P. aeruginosa* ATCC 27853; (+ Control); (4) *S.* Typhimurium ATCC 14028; (5) *K. pneumoniae* ATCC 13882 (6) *E. coli* O157:H7 ATCC 700927; (7) *E. faecalis* ATCC 19433; (8) *E. coli* ATCC 23723; (9) *C. jejuni* NCTC 11168; (10) *E. coli* ATCC 11229; (11) *E. coli* ATCC 23739; (12) *E. coli* ATCC 25922; (13) *K. pneumoniae* ATCC 13887; (14) *S. flexneri* ATCC 12022; (15) *S. faecalis* ATCC 19432.

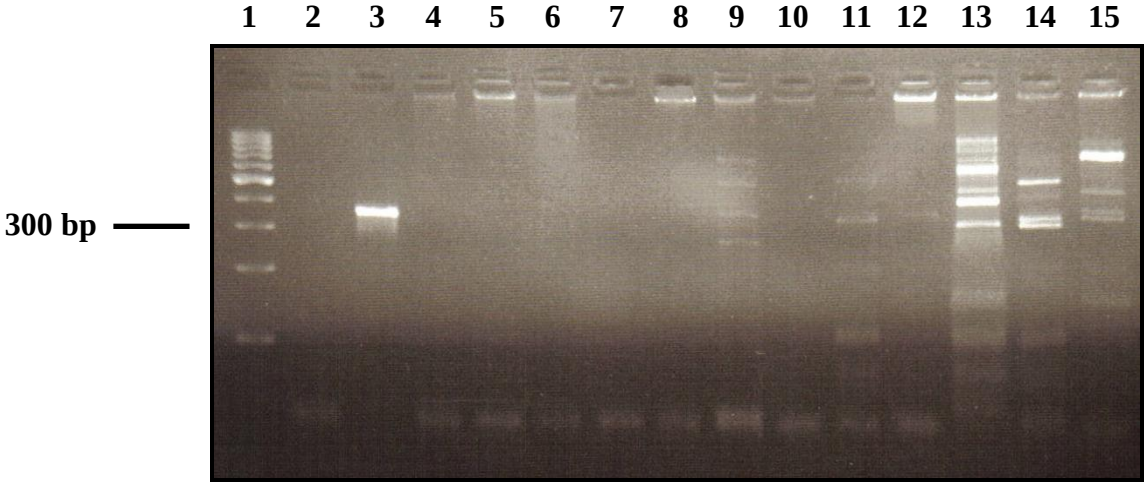

FIG. 4.8. Specificity assay for primers targeting the VS1 gene (*C. jejuni*). L to R: (1) 100bp M.M.; (2) NTC; (3) *C. jejuni* NCTC 11168 (+ Control); (4) *E. coli* O157:H7 ATCC 700927 (5) *K. pneumoniae* ATCC 13887 (6) *E. coli* ATCC 25922; (7) *E. faecalis* ATCC 19433; (8) *E. coli* ATCC 11229 (9) *S.* Typhimurium ATCC 14028; (10) *K. pneumoniae* ATCC 13882; (11) *S. flexneri* ATCC 12022; (12) *P. aeruginosa* ATCC 27853; (13) *E. coli* ATCC 23723; (14) *E. coli* ATCC 23739; (15) *E. aerogenes* ATCC 13048.

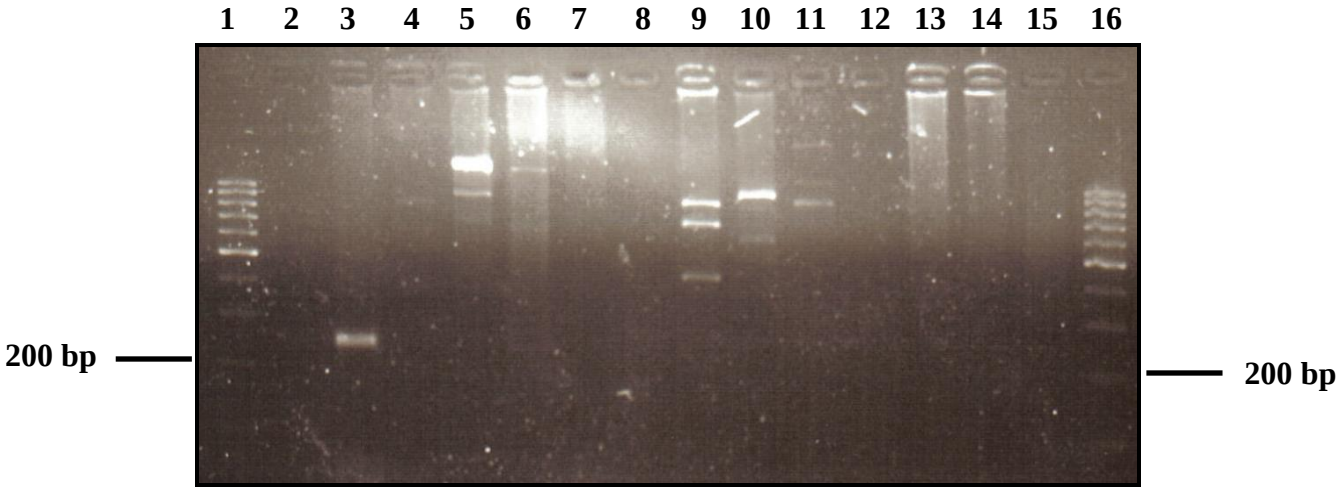

FIG. 4.9. Specificity assay for primers targeting the *ipaH* gene (*S. flexneri*). L to R: (1) 100bp M.M.; (2) NTC; (3) *S. flexneri* ATCC 12022 (+ Control); (4) *E. coli* O157:H7 ATCC 700927; (5) *S.* Typhimurium ATCC 14028; (6) *P. aeruginosa* ATCC 27853; (7) *C. jejuni* NCTC 11168 (8) *E. faecalis* ATCC 19433; (9) *E. aerogenes* ATCC 13048; (10) *S. enterica* ATCC 13314; (11) *K. pneumoniae* ATCC 13882 (12) *E. coli* ATCC 23723; (13) *E. coli* ATCC 11229 (14) *E. coli* ATCC 23739; (15) *S. faecalis* ATCC 19432; (16) 100 bp M.M.

It is important with qPCR studies to differentiate the detection limit from the linear range of quantification, the lowest point at which accurate, quantifiable data can be obtained. Amplicons were successfully detected at a lower limit of 10 cells/mL for all primer sets, with the exception of VS1 where a detection limit of 200 cells/mL was obtained. The developed protocols were therefore able to detect the number of cells corresponding to the literature minimum infectious dose of each individual pathogen (Table 4.3) via qPCR. At lower cell concentrations, melting curve analysis was needed to confirm successful generation of the expected product, since quantification of less than 100 cells/mL was unreliable.

In general, the C_p values for these cell densities were within the 30 to 35 cycle range in the amplification process, while higher cell concentrations appeared within 15 to 25 cycles (Appendix D). As seen in Fig 4.11 a to e, relative to linear range of each standard curve, melting peaks can be seen for many of the lower cell concentrations, however the concentrations could not be reliably quantified. The actual quantitative, linear portion of the calibration curves therefore did not extend as low as the limit of detection (Figs 4.10 a to e). For the ETIR primer set, the linear range was determined to extend as low as 10^2 cells/mL, SINV was 10^2 cells/mL, exoT was 10^2 cells/mL, ipaH2 was 10^3 cells/mL and VS1 was 200 cells/mL as indicated by the lowest cell concentration value on each of the standard curves. Points which caused the curves to deviate from linearity (mostly those with lower concentrations) were excluded. The detection of single cells was also tested, however they were unquantifiable and C_p values were greater than 40 cycles.

Reaction efficiencies were found to be within the range of 1.8 to 2.0 when calculated from the standard curves using the LightCycler® analysis software (Version 4.0) (Roche Diagnostics, Canada) with a formula of $E = 10^{-1/slope}$ (Table 4.3). For the exoT primer set, the reaction efficiency reached a value slightly greater than 2, at 2.053, which would suggest an efficiency of 103%. Efficiencies greater than 100% can be obtained, however they suggest that non-ideal conditions are present.

To confirm amplification of the correct products following qPCR cycling, melting curve analysis was performed. For each primer set, single peaks at a specific melting temperature (Table 4.2) were observed for reactions containing diluted template, including those outside of

TABLE 4.2. Summary of the experimental results of QPCR analyses for each primer set under reference conditions

Primer Set	Optimized qPCR Protocols			Correlation Coefficient (R^2)	qPCR Reaction Efficiency	I.D. of Target Pathogen[a] (# cells)	Melting Peak (°C)	qPCR Detection Limit (cells/mL)
	Denaturation	Annealing	Elongation					
ETIR	95°C for 15s	58°C for 10s	72°C for 10s	0.995	1.933 (93.3%)	10	85.4 ± 0.18	10
ipaH2	95°C for 20s	64°C for 10s	72°C for 10s	0.996	1.928 (92.8%)	$10 - 10^4$	86.4 ± 0.08	10
SINV	95°C for 20s	60°C for 20s	72°C for 20s	0.962	1.958 (95.8%)	$10^4 - 10^7$	85.1 ± 0.29	10
exoT	95°C for 15s	60°C for 15s	72°C for 15s	0.940	2.053 (105.3%)	10^8	91.7 ± 0.19	10
VS1	95°C for 0s	56°C for 10s	72°C for 20s	0.994	1.800 (80%)	500	76.3 ± 0.18	200

[a] I.D. is abbreviated for the infectious dose of each target pathogen which is defined as the minimum number of cells necessary to initiate infection in a suitable host environment. Infectious dose values were obtained from Liu *et al*., 2008; Theron *et al*., 2001; Maki and Hicks, 2002; Fok, 2005; Yang *et al*., 2004 for each pathogen respectively.

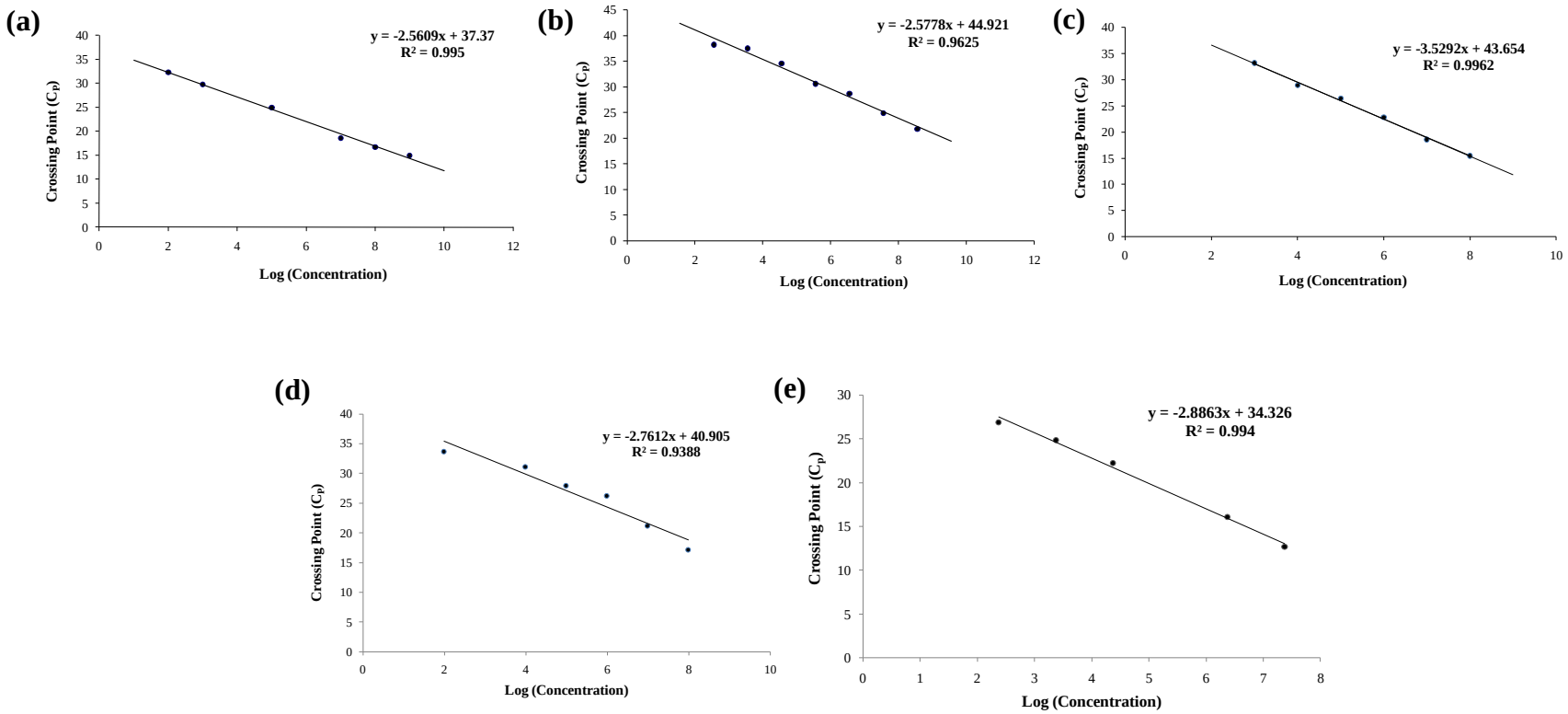

FIG. 4.10. Standard curves generated via qPCR for each of the tested primer sets under reference conditions. (a) ETIR; (b) SINV; (c) ipaH2; (d); exoT; (e) VS1. Each was constructed by plotting the C_p relative to the concentration of cells at each interval.

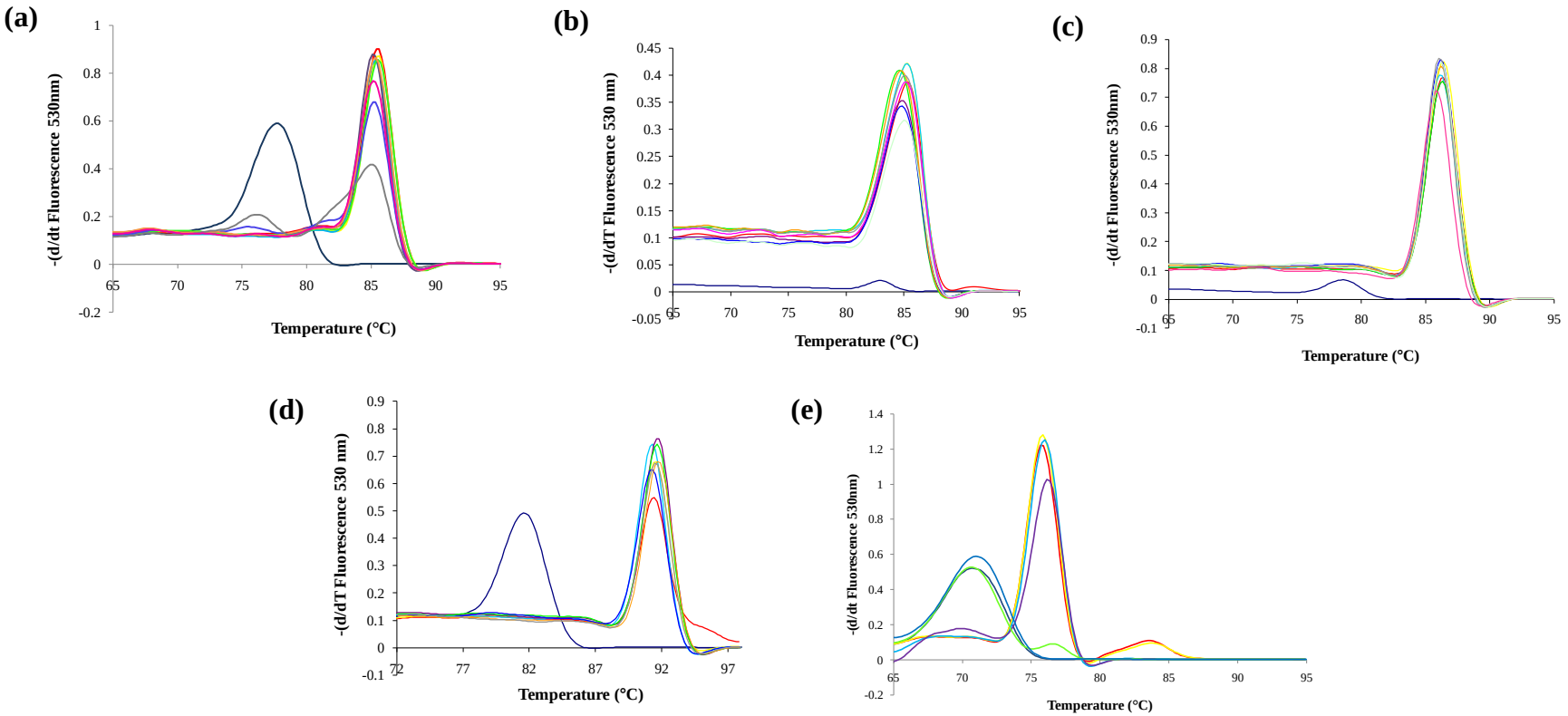

FIG. 4.11. Melting curves generated for each of the tested primer sets following qPCR cycling under reference conditions. (a) ETIR; (b) SINV; (c) ipaH2; (d); exoT; (e) VS1. Each was constructed from plotting -(d/dT Fluorescence) at 530 nm against temperature (ºC). Colours (with the exception of (e) where 10^8 cells/mL is represented by red, with concentrations decreasing in sequential order) are indicative of:

NTC 10^9 cells/mL 10^8 cells/mL 10^7 cells/mL 10^6 cells/mL 10^5 cells/mL
10^4 cells/mL 10^3 cells/mL 10^2 cells/mL 10^1 cells/mL 10^0 cells/mL

the linear range of quantification (Fig 4.11 a to e). These peaks correspond to the point at which the desired product denatured while the reactions were heated from 65 to 95°C. The amplicons of the ipaH2, ETIR and SINV primers denatured at 84 to 86°C as seen in Fig 4.11 a to c. An additional secondary peak easily distinguishable from the product of interest was observed in the NTC reactions (negative controls), corresponding to a small amount of dimerization of the complementary primers in the absence of template.

The exoT primer set showed a melting peak at a significantly higher melting temperature (91°C) than the others. This suggests that the fragment contains a large percentage (55%) of G and C, which would thus require a much higher melting temperature to disrupt the hydrogen bonds holding the double-stranded amplicon together. With the VS1 primer set, a secondary peak was observed at approximately 70°C, which is consistent with the observations by Yang *et al*. (2003, 2004) following qPCR cycling. Confirmation that each of the desired products was successfully amplified via qPCR was achieved by 2% agarose gel electrophoresis (Appendix E).

4.5. Optimization of DNA recovery

The efficiency of recovering DNA from *S.* Typhimurium cells retained by the 0.22 µm and 0.45 µm filters was tested with each of the methods previously described (Fig. 4.12). Cells (10^7 cells) were spiked into 100 mL samples of autoclaved environmental water to examine recovery. Autoclaving and UV-sterilization was necessary to remove any biological background, such as *S.* Typhimurium cells or DNA which were not part of the quantified artificial contamination, since the presence of these cells could potentially contribute to overestimates of DNA recoveries.

The average percentage of DNA recovered from $1x10^7$ membrane-bound cells of the model pathogen with each of the 10 methods ranged from 10.4 ± 1.34% to 73.3 ± 5.2%. This indicates a relatively large variability in cell and subsequent DNA recovery across each of the tested filter-purification variants (Table 4.3, Figure 4.12). The trend appeared dependent on both the filter type and extraction method. The 0.22 µm GSWP filters were found to allow recovery of the largest fraction of DNA (29.45 ng) from the membrane-bound cells isolated by extraction Method 8. All recoveries were determined relative to the amount of DNA isolated from a 1 mL aliquot of $1x10^7$cells/mL without using filtration (40.2 ng). The 40.2 ng yield and $1x10^7$ cell concentrations were then used as recovery references to normalize the experimental data, since initial cell concentrations were variable. This also accounted for the original DNA loss during isolation/purification. It is known that 100% recovery cannot be achieved by any DNA purification technique therefore the present study used 40.2 ng as an estimated 80.4% ideal recovery. With this assumption, approximately 19.6% of the theoretical DNA (5.0 fg per cell) contained within $1x10^7$ *S.* Typhimurium cells (50 ng) is lost during the regular purification process, without membrane filtration. The results suggest that a loss of target cells occurred when membrane filtration was incorporated prior to DNA extraction, since an even smaller proportion of the theoretical 80.4% was then recovered.

Methods 2, 3 and 6 produced the lowest DNA yields, with less than 15% of the DNA recovered from the initial cells. This indicates a decrease in cell recovery by several orders of magnitude (from 10^7 to approximately 10^5 cells), eliminating them as potential extraction aids (Table 4.3). The commercially available UltraClean™ Water DNA Isolation kit (Method 3) was also found to have the least efficient recovery, with only 10% of the recoverable DNA isolated.

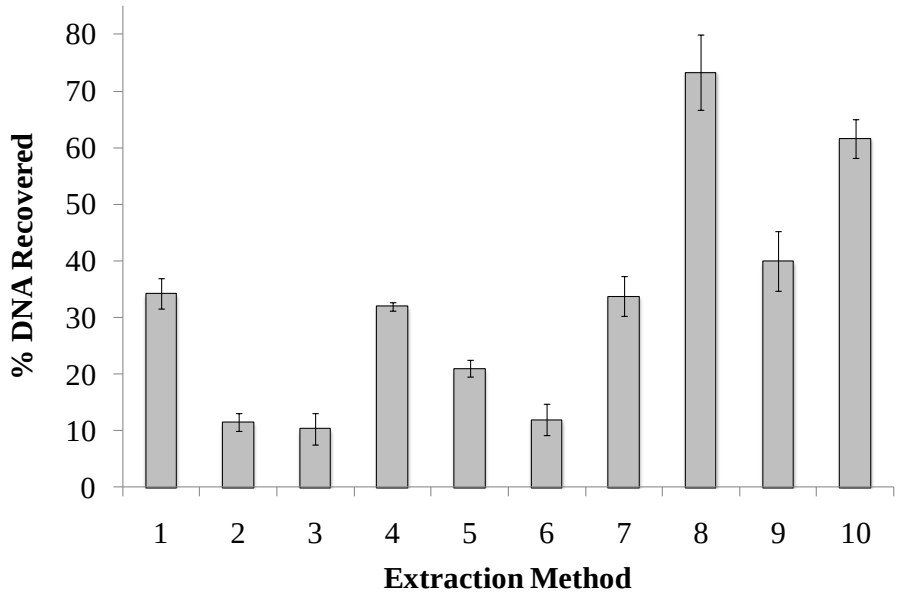

FIG. 4.12. Differences observed in DNA recovery amongst each of the 10 methods tested with autoclaved source water samples artificially contaminated with $1x10^7$ *S. Typhimurium* cells. Method 8 was selected for further use with field samples.

Method 8 was adopted for environmental sample analysis. This method had the highest yields, a low standard deviation between replicate measurements and the resulting DNA appeared with significant intensity following PCR amplification (Fig. 4.13). DNA extracted/purified following each of the 10 methods was amplifiable via PCR with the SINV primers, irrespective of final yields. Methods where lytic enzymes and proteases were incorporated also increased purity of the final products, as seen in Table 4.3. Interference in the form of non-specific binding was observed with methods 7 and 9, consistent with the low purity suggested by the $A_{260/280}$ (Table 4.3). From the gel image, the differences in the recovered DNA concentrations are also apparent from the varying band intensities. Several tests were also performed to determine whether low cell densities (<1000 cells) were recoverable and

consequently amplifiable by this method. Generally, DNA concentrations were unquantifiable by spectrophotometry and cell recovery could not be accurately estimated by endpoint PCR.

4.6. Nested PCR for qualitative examination of cell recovery

As a qualitative means of ensuring that DNA isolated using Method 8 was amplifiable using PCR, specifically at low DNA concentrations, a nested PCR was performed. This also functioned as an alternative to pre-enrichment during validation of the concentration-extraction protocol and a simple, inexpensive way to examine changes in detection sensitivity with the introduction of environmental samples. The newly designed invA-nested primer set was first optimized (Appendix F), and determined to function with a 66°C annealing temperature and 20 cycles in the first stage of PCR to produce the 640 bp product. Serially diluted DNA recovered from the 0.22 µm GSWP filter was determined to have an estimated minimum detection limit of 16.02 fg of DNA after two rounds of PCR (Fig 4.14 and 4.15). This corresponds to approximately 4 cells/mL of the reference *S.* Typhimurium strain, indicating that the DNA was suitable for use in qPCR.

4.7. QPCR sensitivity assay in source water

As a means of ensuring the sensitivity and validity of the newly designed methods with environmental samples, using *S.* Typhimurium as a model, a standard curve was replicated via qPCR with cell dilutions recovered using Method 8. From the experimental determination it can be seen that the linear range ($R^2 = 0.995$) extends to between approximately 10^8 cells/100mL to 10 cells/100 mL (Fig. 4.16 a). This suggests that there was a 1-log difference in sensitivity

TABLE 4.3. Comparison of cell and DNA recovery methods from membrane filters using artificially contaminated source water with known concentrations of *S.* Typhimurium cells. Initial values were normalized to a density of $1x10^7$ cells/mL and 40.2 ng DNA for consistency.

Method	Components	Pore Size & Type (µm)	Recovered DNA[a] (ng/µL)	% DNA Recovered[b]	$A_{260/280}$[c]
1	STE buffer	0.45	13.5 ± 2.7	34.2% ± 2.03%	+
2	STE buffer	0.22 GSWP	4.63 ± 1.6	11.5% ± 1.37%	-
3	UltraClean™ kit	0.22 GSWP	4.18 ± 2.8	10.4% ± 1.84%	-
4	STE buffer	0.22 GSWP	12.85 ± 6.1	32.0% ± 1.61%	+
5	SDS, Tris-HCl (x2)	0.22 GSWP	8.45 ± 1.4	21.0% ± 0.45%	+
6	SDS, Tris-HCl	0.22 GSWP	4.81 ± 2.8	12.0% ± 0.91%	++
7	SDS, Tris-HCl	0.45	13.6 ± 3.5	33.7 % ± 1.15%	-
8	Tris-HCl, SDS, Lys., Prot. K	0.22 GSWP	29.45 ± 6.6	73.3% ± 5.2%	++
9	Tris-HCl, SDS, Lys., Prot. K	0.22 GSWP	16.1 ± 5.2	40.0% ± 3.0 %	-
10	Tris-HCl, SDS, Lys., Prot. K	0.22 Dura.	24.8 ± 3.4	61.6% ± 1.50%	++

[a] Recovered DNA is the amount of DNA which was retrieved from the membrane filters as determined spectrophotometrically; [b] The % DNA recovered refers to the percentage of DNA successfully recovered from cells following bead beating and DNA purification, relative to the 40.2ng control; [c] $A_{260/280}$ is a measure of DNA purity. DNA with low purity (< 1.8) (Maniatis *et al*., 1982) is denoted -, while borderline purity +, and pure DNA ++.

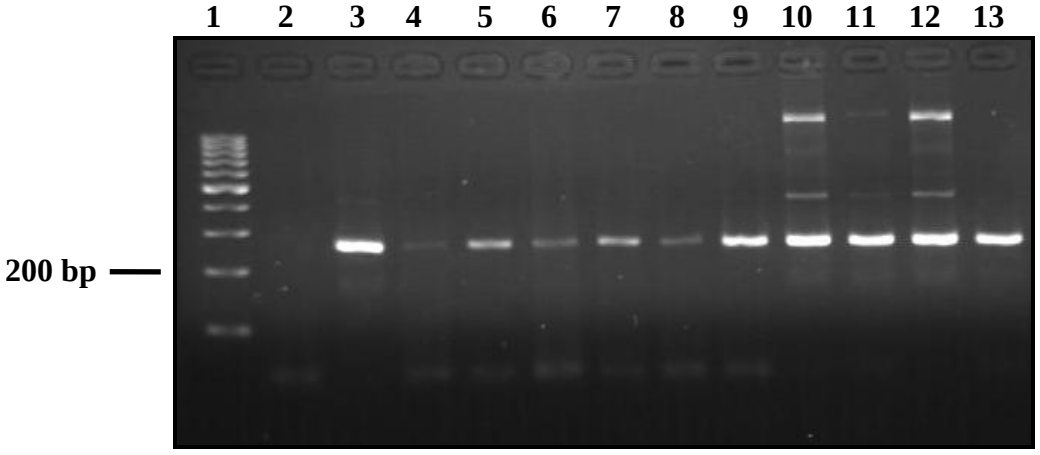

FIG. 4.13. DNA (~10^7 cells) recovered using each extraction method, amplified with the SINV primers. (1) 100bp M.M.; (2) NTC; (3) 10^9 cell positive control; (4) Method 1; (5) Method 2; (6) Method 3; (7) Method 4; (8) Method 5; (9) Method 6; (10) Method 7; (11) Method 8; (12) Method 9; (13) Method 10.

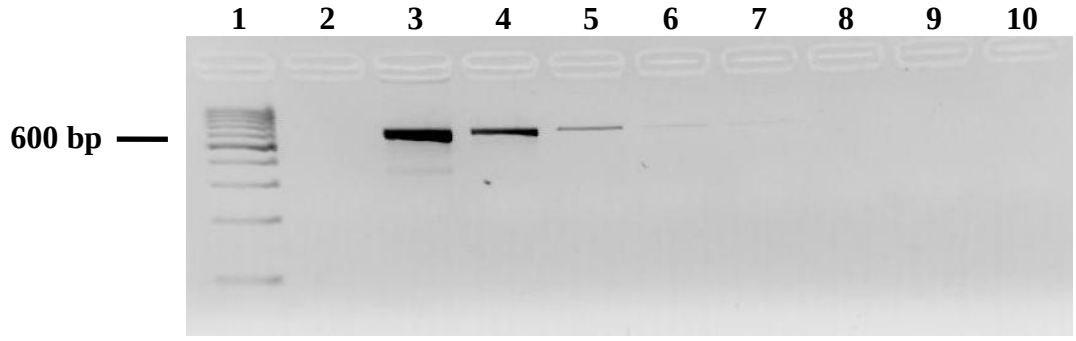

FIG. 4.14. Agarose gel of nested PCR products generated in the first round of amplification using DNA isolated by extraction method 8, with the invAnested primer set. (1) 100bp M.M.; (2) NTC; (3) 10^9 cell positive control; (4) 160.2 ng; (5) 16.02 ng; (6) 1.60 ng ; (7) $1.6x10^{-1}$ ng; (8) $1.6x10^{-2}$ ng; (9) $1.6x10^{-3}$ ng; (10) $1.6x10^{-4}$ ng.

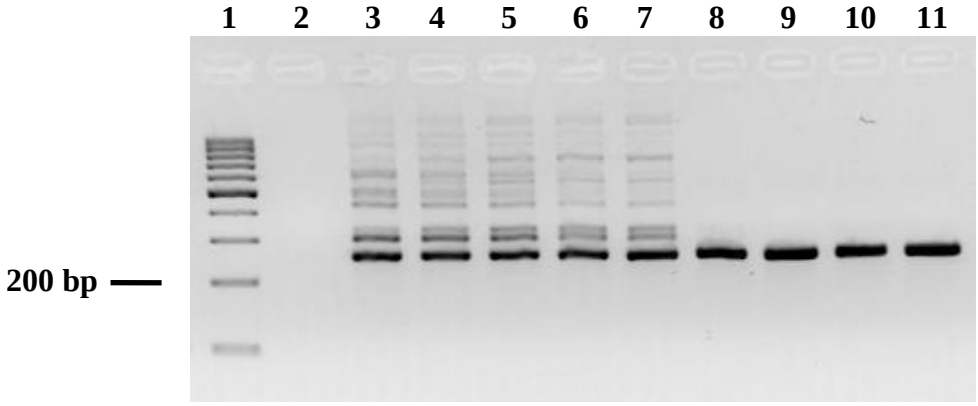

FIG. 4.15. Agarose gel of nested PCR products generated in the second round of amplification using DNA isolated by extraction method 8, with the SINV primer set. (1) 100bp M.M.; (2) NTC; (3) 10^9 cell positive control; (4) 160.2 ng; (5) 16.02 ng; (6) 1.60 ng; (7) $1.6x10^{-1}$ ng; (8)$1.6x10^{-2}$ ng; (9) $1.6x10^{-3}$ ng; (10) $1.6x10^{-4}$ ng; (11) $1.6x10^{-5}$ ng (16.02 fg).

between the reference and environmental conditions. Similarly, the melting curve (Fig. 4.16 b) of 85.05°C ± 0.19°C was identical to that under reference conditions (85.10°C ± 0.29°C), confirming that only the product of interest was amplified. The C_P values for the environmental

sample (Fig. 4.16 a) are visibly shifted relative to the reference with the lowest cell concentrations (≤100 cells) appearing > 40 cycles. As confirmed by melting curve analysis (Fig 4.16 b), the detection limit was identical to that obtained under reference conditions, with a quantitative limit of 10 cells/100 mL being detected following 50 cycles of amplification.

4.8. Microscopic examination of membrane filters

In the original overnight culture used for the microscopy portion of the experiment ($2.18x10^7$ cells/mL), it was determined that a relative 99:1 visual ratio of live to dead cells was seen, indicating that there were few dead cells present in the initial inoculum. After examining the filters under 400x and 630x magnification following the initial filtration, it could be seen that cells were attached to all filter types (Fig 4.17). These consisted of both live and dead cells, as indicated by the emission of green and red fluorescence respectively. At this point the ratio of live:dead cells decreased, to approximately 75% live, suggesting that some living cells lost viability. When the second filters were treated by Method 8, no cells were visible (neither live or dead) at either magnification (not depicted). This would suggest that all cells were removed from the filter; however it does not account for any cell losses.

To determine if any cells passed through the filters, filtrates were plated parallel to the microscopy. Physical observation of the triplicate samples indicated that no *S.* Typhimurium colonies were present following incubation at 37°C for 24 h. Therefore, it is suggested that no whole, viable cells passed through the filter or the filtration unit itself. Filtrates were also examined for the presence of extracellular DNA following filtration by amplification with the newly constructed nested primers. DNA present following filtration was not detected by nested PCR, as indicated by the absence of the 252bp product of the SINV primers in Fig. 4.18.

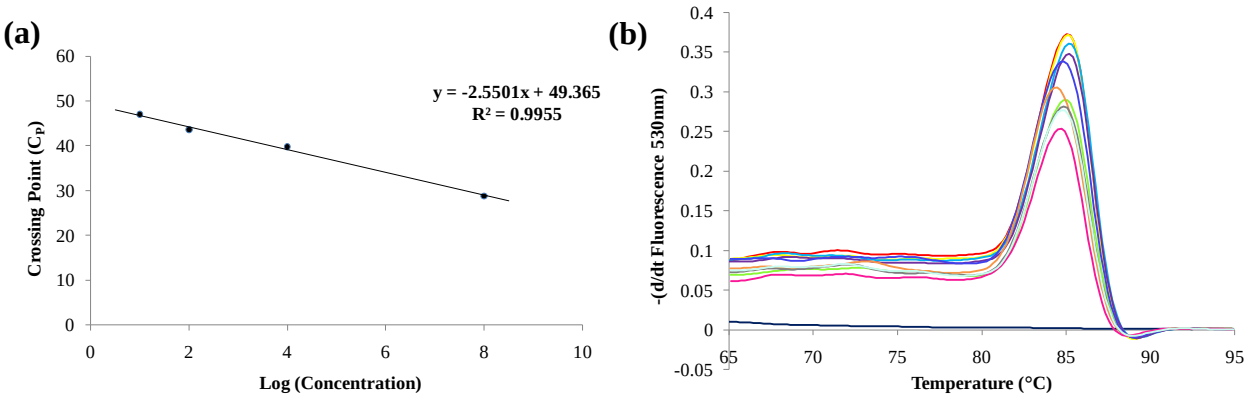

FIG. 4.16. QPCR sensitivity assay in source water using SINV primers measured by: (a) Standard curve generated using serial cell dilutions of 10^8 to 10 cells in 100 mL of source water; (b) Melting curve generated from the serial cell dilutions of 10^8 to 10 cells in 100 mL of source water. The melting peak was determined to be 85.05 ±0.19°C. The detection limit was determined to be 10 cells/100mL using Method 8. Colours in (b) are indicative of:

NTC — 10^8 cells/mL — 10^7 cells/mL — 10^6 cells/mL — 10^5 cells/mL — 10^4 cells/mL — 10^3 cells/mL — 10^2 cells/mL — 10^1 cells/mL — 10^0 cells/mL

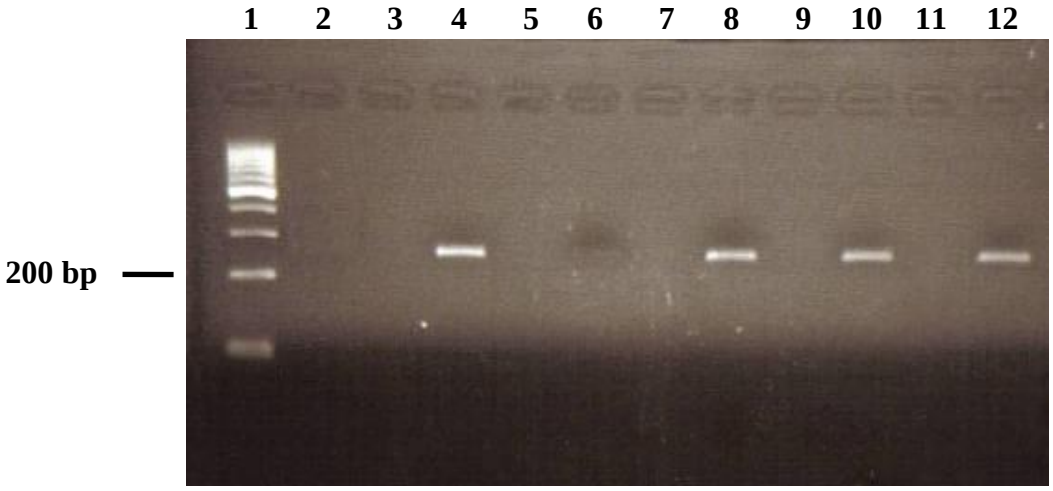

FIG. 4.18. Nested PCR amplification of DNA recovered from each type of membrane filter relative to each of the filtrates to check for the passage of DNA. (1) 100 bp M.M.; (2) NTC; (4) 10^7 cells/mL positive control; (6) Lakewater negative control; (7) Filtrate from 0.45 µm filter; (8) 0.45 µm filter; (9) Filtrate from 0.22 µm GSWP filter; (10) 0.22 µm GSWP filter; (11) Filtrate from 0.22 µm Durapore filter; (12) 0.22 µm Durapore filter.

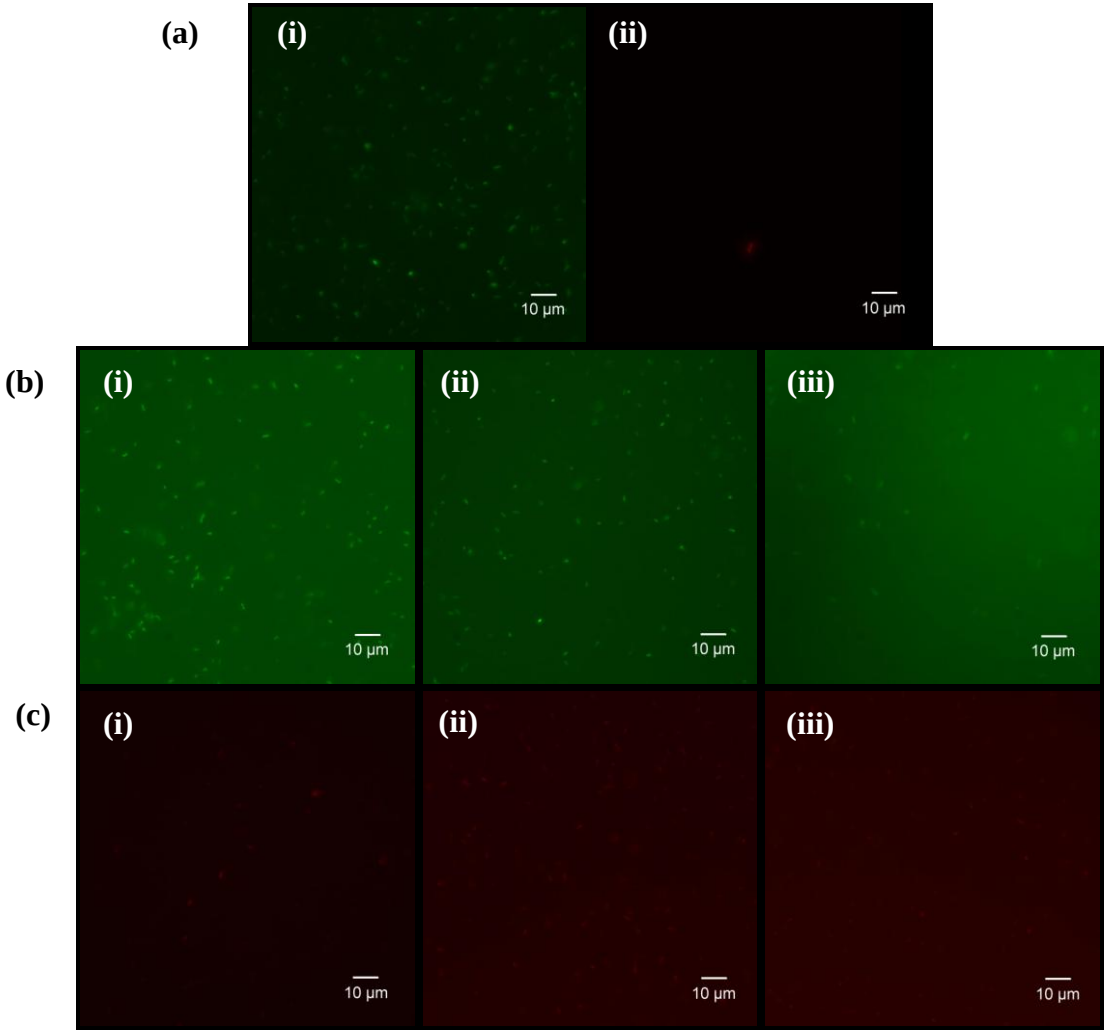

FIG. 4.17. Microscopic examination of membrane filters using epifluorescence at 400x (GFP) and 630x (dsRed) (A) Overnight culture (10^7 cells) (i) portion of live cells (ii) portion of dead cells. (B) Live cells trapped on (i) 0.45 µm filter; (ii) 0.22 µm GSWP filter; (iii) 0.22 µm Durapore filter. (C) Dead cells trapped on (i) 0.45 µm filter; (ii) 0.22 µm GSWP filter; (iii) 0.22 µm Durapore filter. No cells were observed following cell removal treatment. Scales indicate 10 µm.

CHAPTER 5: DISCUSSION

5.1. Primer design & selection process

In the present study, the objective was to examine the feasibility of using qPCR as the integral component to a detection system for monitoring the pathogenic content of influent and effluent waters at drinking water treatment facilities. For this study, oligonucleotide primers were selected to target six waterborne pathogens, which have been previously linked to water-related diseases of varying degree in Canada, specifically; *E. coli* O157:H7, *S.* Typhimurium, *S. flexneri*, *C. jejuni*, *K. pneumoniae* and *P. aeruginosa*. To date, no single study has examined the simultaneous detection of the above microorganisms using qPCR in water.

Reference cultures were selected based on each pathogen's degree of infectivity and status as a waterborne health risk, as outlined by Health Canada (Health Canada 2006a). *Escherichia coli* O157:H7, *S.* Typhimurium, *S. flexneri* and *C. jejuni* are representatives of the common waterborne pathogen group. *Pseudomonas aeruginosa* was chosen because of its dual function as an environmental heterotroph and an opportunistic pathogen, while *K. pneumoniae* was chosen as a representative of the fecal indicator group. This selection allowed for testing the ability to detect and differentiate pathogens from both heterotrophs and indicators via PCR. Low-copy, virulence-associated genes were chosen as detection targets to increase primer specificity and facilitate quantification of the pathogens. Each gene target was chosen because of their reported low copy numbers (accounting for both chromosomally-integrated and plasmid-encoded forms) (Stonnet and Guesden, 1993; Sánchez-San Martín *et al*., 2001; Fey *et al*., 2004; Yu *et al*., 2006; Ashida *et al*., 2007), as well as being specific to the desired reference strains and

associated in some way with their pathogenicity.

When PCR has been previously examined as an analytical technique to detect waterborne pathogens, researchers often attempted to "re-invent the wheel" with respect to primer design. Primers are being constantly modified and re-designed to target many of the same genes as existing primer sets, whose adequate functionality has already been demonstrated. There are generally no universally accepted primer sets for many functional genes across scientific literature. The most likely reasoning is the result of varied preferences in design criteria among researchers. As previously stated, the adoption of a series of primers acceptable for "standardized" use was what the present study aimed to achieve. Careful primer design was critical for progression, since the selection of non-optimal design parameters could incorrectly amplify unwanted DNA and cause problems further downstream. The characteristics of the ideal primer set for the purpose of this research were that they would not only have high specificity for their intended targets, but also carry a suitable robustness to reduce the occurrence of mispriming, cross-reactivity with non-target bacteria and be adequate for development into a standardized test that could be applied by water treatment personnel. To ensure that the most suitable primers were produced, stringent design criteria were followed.

As detection targets, low copy virulence-associated genes were specifically selected for this study. Many virulence-related genes are known to be present in only the pathogenic variants of a species, giving the benefit of allowing for strain-specific differentiation. In addition, for qPCR studies, the construction of calibration curves are generally performed using genes which have been cloned in single copy into commercially available vectors, as seen in qPCR work by

Sun *et al*. (2010), Ahmed *et al*. (2008) and Yang *et al*. (2004). This is advantageous since it facilitates quantification via copy number. In this study, the genes selected for analysis were already present in single or low copy, allowing for the same assumption to be valid for single cells isolated from the environment. The *tir*, *invA* and VS1 genes from *E. coli* O157:H7, *S.* Typhimurium and *C. jejuni* respectively were those present only in single copies on the respective host chromosomes (Goosney *et al*., 2000; Fey *et al*., 2004; Stonnet and Guesdon, 1993). The *ipaH* gene in contrast was present at the highest copy number, with 7 copies both chromosomally and plasmidborne (Ashida *et al*., 2007) while *exoT* is present in 2-5 copies (Feltman *et al*., 2001). As a result, detection limit calculations were altered to reflect this knowledge.

The scientific community also has conflicting views on the considerations for the most ideal primer design conditions. For instance, Apte and Daniel (2003) and Dieffenbach *et al*. (1993) suggest that the T_m between forward and reverse primers should be similar, however SantaLucia Jr. (2007) suggests the opposite. Design software also plays a factor in these conflicting opinions. No two design programs will generate identical series of primers for the same DNA sequence, since matches are calculated by different mathematical algorithms. In the literature, when primers are newly designed, researchers rarely dwell on the characteristics used to select a particular primer set and in turn only make reference to the primer design software used. As a result, for this study, a hybrid of the design criteria of both Dieffenbach *et al*. (1993) and Apte and Daniel (2003) was used to select the best primers generated by each program and evaluate those taken from external sources. Using these guidelines (See Section 3.2), suitable primers were produced, as confirmed in the initial optimization studies.

Computerized primer design programs Primer3 and the LightCycler® Probe Design 2.0 platform were used to select two novel primer sets, the exoT and ipaH2 primers, targeting the *exoT* gene of *P. aeruginosa* and *ipaH* gene of *S. flexneri*. Primer3 in particular has been previously demonstrated to be exceptional for designing primers for sequences with both AT and GC-rich regions and has a high success rate (Chavali *et al*., 2005), while the LightCycler® Probe Design 2.0 software has had limited use for this type of pathogen detection. A third primer set was designed, the KmagA primers for the *magA* gene of *K. pneumoniae*, however because of intense non-specific binding and the absence of the product of interest following PCR amplification, the primers could not be used further. Neither of the *K. pneumoniae* strains used in specificity studies was found to harbour the target gene, so it was eliminated from the analysis. The apparent rarity of the *magA* gene amongst the currently available *K. pneumoniae* type strains (ATCC, NCTC, etc...) also meant that it was not a desirable target.

While computer software was used to enhance the selection of the most optimal target regions on the gene of interest, certain parameters were user-specified. This included the selection of desired annealing temperatures (58-65°C) and both the oligo (18-22bp) and final amplicon sizes (150-350bp). These were then used by the software to generate primers for the defined criteria. User input was further required to select the most ideal primer set from the top 5 matches generated algorithmically and as a result, a high degree of skill and design knowledge was required by the operator. These primers were deemed suitable because of the fulfillment of three critical design parameters, the closeness in T_m of their forward and reverse primers, the % GC and minimal secondary structure formation. Mismatch in T_m between both forward and reverse primers of each set was determined to be within a 1-2°C range. This falls within the 2-

3°C temperature difference which has been preferred by Apte and Daniel (2003). Also, the GC percentage was above 50% for both primer sets and within the ≥ 50% suggested range. The importance of satisfying these two parameters lies within their role in primer specificity. Generally, specificity is lost when primer pairs are poorly matched in both T_m and % GC (Dieffenbach *et al*., 1993). The situation of both parameters within these estimated ranges equipped them with a theoretically high efficiency for annealing and greatly reduced chances for mispriming (Dieffenbach *et al*., 1993). In addition, it was determined experimentally that minimal dimerization occurred between the tested primers. The optimized conditions therefore greatly reduced the occurrence of the > 4 duplex formations predicted by both the OligoAnalyzer and GeneRunner software.

Also included in the study were the previously assembled ETIR and SINV primers, which were designed in the laboratory by Haffar and Gilbride (2010) to target the *tir* gene from *E. coli* O157:H7 and *invA* gene from *S.* Typhimurium. These primers were previously untested for sensitivity with environmental water samples. The VS1 primers from Stonnet and Guesdon (1993) for the detection of the VS1 gene of *C. jejuni* were examined as a result of their pre-determined high specificity and sensitivity. Thus the need to redesign these primers was unnecessary. Haffar and Gilbride followed the Dieffenbach *et al*. (1993) criteria for primer design, as well as using the LightCycler® Probe Design 2.0 software. These primers were generated by the same algorithms as the exoT primers, and therefore suited the design criteria used in this study. Stonnet and Guesdon (1993) in comparison designed the primers by hand and were only concerned with placing the 3′ end of the primers in a GC rich region. The entire VS1 gene sequence contains only 25.32% GC (Stonnet and Guesdon, 1993). To obtain primers with

3′ regions rich in G and C, only a limited number of primers fit this criterion. These primers therefore contain a low % GC of < 35%, under the ideal limits outlined in the present study. This did not have any effect on their functionality, as determined experimentally and in previous work by Yang *et al*. (2003, 2004).

5.2. Specificity and Sensitivity Assays

For the evaluation of each primer set, both specificity and sensitivity tests were performed. Initial BLAST homology searches revealed that the newly designed primers had increased affinities for sequences corresponding to their intended targets. This was also confirmed experimentally, when the desired amplicons were produced only by the respective reference strains with their optimized temperature profiles. Products of interest were also unamplified in the presence of template DNA from non-target bacteria, indicating their potential suitability for pathogen detection amongst a mixture of background DNA. In addition, no cross-reactivity was observed amongst the primers and the 5 pathogens of interest, giving the primer set a degree of robustness. The occurrence of false positives generated in a standard method as a result of specificity issues would therefore be limited or entirely non-existent. These results also confirmed the earlier exclusivity found by Haffar and Gilbride (2010) for the ETIR and SINV primer sets. In addition, the VS1 primer set had not been previously examined against such an array of non-target microorganisms in any of 3 separate studies by Stonnet and Guesdon (1993) or Yang *et al*. (2003, 2004). Non-specific binding was observed with the *E. coli* ATCC 23723 and 23739 and *E. aerogenes* ATCC 13048 type strains tested. This observation was however dismissed, since these strains do not carry the target gene, bands were easily differentiable by electrophoresis and the corresponding product of interest was unamplified.

For each primer set, qPCR assays with the SYBR Green I dye were developed. The selection of SYBR Green was favoured because of its suitability for optimization studies, high-throughput quantitative analyses and low cost relative to its counterpart the hydrolysis/FRET (TaqMan®) probes (Inglis and Kalischuk, 2004; Yang *et al*., 2004). It has also been shown to successfully amplify pathogenic DNA from a variety of complex samples, including milk (Yang *et al*., 2003), feces (Inglis and Kalischuk, 2004) and environmental water (Yang *et al*., 2003; Nam *et al*., 2005). In addition, it is better suited for the analysis of environmental samples since it is said to be more tolerant of targets having slight changes in their genetic makeup (Ahmed *et al*., 2008). The switch from endpoint to qPCR was not direct. Profile adjustments were necessary in order to reflect the rapid cycling conditions associated with these quantitative assays and account for variation in the catalytic efficiencies of the differing enzymes. The levels of primer dimer and non-specific products were reduced in some cases via optimization of temperature profiles, and increasing the concentration of available $MgCl_2$ within the reactions. In addition, the total cycling time for each primer set was under an hour and a half in length, with the ETIR primers taking only 50 minutes to cycle. This is a significant finding, since it indicates that in a single afternoon, samples could be prepared and analyzed by the novel qPCR protocols.

With respect to sensitivity, the primers adopted for this study were determined to have low detection limits when tested initially with reference strains. The expected linear relationship between C_p and the logarithm of cell concentrations was observed for each primer set, with amplification occurring between cycles 12 and 35 of the reactions. The quantitative range extended from DNA at the femtogram level at 100 cells/mL up to 10^7 cells/mL in all cases, as determined by the standard plots. Within this dynamic range, there was therefore a high degree

of reliability and confidence in accurately detecting the target organisms. These values are also in agreement with the pure culture work in many qPCR studies where SYBR Green I has been applied for the detection of the pathogens of interest. A 2006 study by Wolffs *et al*. defined a linear detection range of $5x10^2$ to $5x10^8$ CFU/mL of *S*. Typhimurium, when standard plots were created from whole cells. Comparatively, a 2005 study by Nam *et al*. proposed a reproducible SYBR Green assay for detecting *S*. Typhimurium from 10^2 to 10^8 CFU/mL. In addition, Heijnen and Medema (2006) developed an assay for the *stx* genes of *E. coli* O157:H7 which could comfortably detect as few as 10 cell equivalents of the reference strains. These results indicate that the linear quantifiable ranges determined in the present study are therefore highly comparable to those previously reported.

Detection below the quantifiable range was also observed to a minimum of 10 cells/mL. However, because of the high C_p values at which the fluorescence signal rose above the background, cell concentrations were not accurately quantified by the LightCycler® instrument. Confirmation of the desired amplicons for each primer set was then achieved using melting curve analysis and agarose gel electrophoresis. A similar observation was reported by Khan *et al*., 2007 where 1 cell/mL of an *E. coli* ATCC 35128 reference strain was weakly amplified in distilled water, however the C_T could not be calculated. Ahmed *et al*. (2008) also noted the occurrence of irreproducibility in qPCR data at lower (<5) copies of the *invA* (*S*. Typhimurium) and *mapA* genes (*C. jejuni*), which is consistent with results seen in this study. In the original VS1 research by Stonnet and Guesdon (1993), it was shown that a minimum of approximately 16 genome equivalents could be detected from the reference *Campylobacter* strains. Yang *et al*., (2004) also reported a lower limit of 1 cell/mL via qPCR. In comparison, only 200 cells/mL could be

detected when using the same reference strain (*C. jejuni* NCTC 11168) in this study. The reasons for the differences observed here are most likely attributed to the inherent limitations in accurately estimating *C. jejuni* cell concentrations, because of their small size and fastidious growth requirements. This may have resulted in overestimation of the assumed starting cell concentration, resulting in the higher cell counts required for detection. It was also determined that the ETIR and SINV primers had better detection limits than the original 150 (ETIR) and $2x10^3$ (SINV) cells/reaction previously reported (Haffar and Gilbride, 2010). In both cases, the inaccurate estimation of cell and template DNA concentrations in the original study are the most likely causes of these discrepancies, since the same reference strains were also used here.

The binding efficiency between the primers and template was also determined from the standard plots. Efficiencies are dependent on both primer design and optimal amplification conditions (Mygind *et al*., 2002). The ideal reaction efficiency is given a value of 2, which would suggest 100% amplification of the target, however values between 1.8 and 2 are considered acceptable. In this situation, reactions obey the 2^n rule of PCR, whereby theoretical doubling of the reaction products occurs following each successive cycle. The efficiencies determined in this study ranged from 80% (a value of 1.8) for the VS1 primers to 103% for the exoT primers (a value of 2.03). In theory, the exoT primer set would appear to be over-efficient, however when regressions are re-formatted for use in data analysis software, the programs do not recognize that the slope of qPCR standard curves can only reach a maximum value of -3.32 (a 100% efficiency rate) (Mygind *et al*., 2002). Mygind *et al*. (2002) observed a similar scenario with a qPCR study of *Chlamydia pneumoniae*, where reaction efficiencies reached as high as 2.94. The combination of linearity, high reaction efficiencies and low detection limits further suggests that the adopted

primer sets are well suited for quantitative assays for each of the target pathogens.

The detection limits obtained in this study, albeit not entirely reproducible at the single cell level, did correspond to at least the minimum infectious doses of each pathogen. In the cases of the SINV and exoT primers, detection occurred well below the suggested infectious doses of *S.* Typhimurium (10^4 cells) and *P. aeruginosa* (10^8 cells) under reference conditions. As well, the detection limits of 10 cells for *E. coli* O157:H7 and *S. flexneri* are in agreement with their predicted infectious doses. This is significant since it could give treatment facility operators an estimated assessment of the potential health risk associated with the 5 tested pathogens in an unknown water sample prior to ingestion/exposure. It would also allow time to react and adjust treatment measures accordingly to prevent the reoccurrence of outbreaks similar to Walkerton. The data collected reveals that the primers selected were both highly specific for their intended targets and sensitive for low pathogen loads.

5.3. Membrane Filtration and DNA Recovery

For the analysis of environmental samples, a membrane filtration step was added prior to the DNA extraction procedure. This served two purposes; (i) to enhance the probability of encountering pathogenic microorganisms in field samples, assuming a concentration effect would occur on dilute pathogen numbers, and (ii) to potentially address the issue of detecting extracellular DNA associated with lysed bacterial cells by the PCR process. The fractionation of samples in this fashion was assumed to prevent the detection of total DNA which is generally associated with traditional "spin down" extractions of freshwater DNA with samples (Mull and Hill, 2009; Liu *et al*., 2009; Khan *et al*., 2007). Filtration increases the likelihood of detecting

nucleic acids originating only from whole cells.

To date, very few studies have quantitatively compared the use of different variants for isolating DNA from freshwater by physical means. The most recent, a 2008 analysis by Horáková *et al*. examined differences in several isolation methods, using both 0.45 µm and 0.22 µm membrane filters. They did not perform an in-depth examination of cell recovery in their analysis so quantitative data could not be assessed. In the present study, a total of 10 different variations of the extraction methods were tested in an attempt to develop a rapid concentration-extraction procedure to minimize recovery of free DNA and maximize the recovery of whole cells and their DNA from the membrane filters. In all cases, the extractions included a combination of mechanical, chemical and enzymatic lysis. This form of treatment has been shown to work effectively with respect to final yields and DNA purity, when previously applied to soil (Yeates *et al*., 1998; Krsek and Wellington, 1999) and water samples (Horáková *et al.*, 2008). In the current study, a combination of Tris-HCl, 20% SDS and mechanical lysis by 0.5 mm Zirconia/Silica beads as well as both lysozyme and proteinase K treatments was found to be the most effective at removal, and lysis and purification of cells and DNA respectively (Method 8), when coupled with the original column-based DNA purification method. The combination of a surfactant (SDS) along with the Tris-HCl was ideal for permeabilizing the gram negative cell membranes, the effect of which was most likely enhanced when filters were heated to 70°C during the proteinase K treatment. Lytic enzymes were also thought to contribute significantly towards this enhancement, since increased yields were consistently obtained when both lysozyme and proteinase K treatments were performed (Methods 8, 9, 10). Differing efficiencies can also be attributed in part to the components of each tube, vessel size, and filter types.

The average final yield as determined from the 0.22 µm GSWP filters was 73.3% (29.5 ng), using the assumption that each *S.* Typhimurium cell in the normalized $1x10^7$ concentration contained approximately 5.0 fg of DNA (Malorny *et al*., 2003) and that a representative 40.2 ng of DNA could be isolated from $1x10^7$ cells experimentally, relative to the 50 ng calculated theoretically which was confirmed by Maynard *et al*. (2005). This method was selected because of its high % recovery and increased purity of the final DNA product relative to the other 9 tested methods, as confirmed by spectrophotometric quantification and both endpoint and nested PCRs. It is also necessary to note that in all cases, DNA concentrations were compared to a control. There was a 19.7% difference calculated between the recovery obtained using the HighPure PCR Template Preparation kit on its own relative to the theoretical 50 ng of DNA that would be expected from $1x10^7$ cells, indicating a loss of cells throughout the entire purification process. This also indicates that it is impossible to achieve 100% efficiency with respect to DNA recoveries, and as a result, for the present study it was assumed that the maximal possible recovery was therefore 80.4%. Ahmed *et al*. (2009) performed a similar analysis; however their recoveries were reported based on estimated cell numbers extrapolated using C_T values from qPCR calibration curves, and cell losses were unaddressed. Bernhardt *et al*. (1991) also documented that approximately 57 to 90% of seeded bacterial cells were released by 0.22 µm filters when applied to blood samples, supporting the current findings.

The components of each extraction solution may have potentially played key roles in the final recoveries among the differing extraction protocols. With respect to methods 1, 2 and 4, STE buffer consisting of NaCl, Tris-HCl and EDTA was applied as a filtration solution in a similar fashion to Ahmed *et al*. (2008, 2009). From the experimental results it can be suggested

that this buffer solution may have affected recoveries in the present study. Decreased yields were obtained in all methods that used STE buffer in comparison to methods without STE buffer, including those methods where a shear force was applied. An estimated 33% difference in recovery was also observed with method 1 (34%), relative to the 67% recovery found by Ahmed *et al*. (2009), where identical procedures were followed. In comparison, with the newly formulated methods 8, 9 and 10, where STE was removed and replaced by both Tris-HCl and SDS, significantly higher yields were obtained. The EDTA which is meant to have a chelating effect on free metal ions is thought to have elicited a shielding effect on the DNA in the current study. In addition, it is thought that any EDTA impurities remaining in the final DNA solution may have lowered polymerase activity by sequestering a portion of the available Mg^{2+} ions when this DNA was used for PCR (van Pelt-Verkuil *et al*., 2008).

It is believed that the efficiency of extraction relied on the size of the extraction vessels. The tube size was decreased from the original 15 mL seen in Methods 1 and 3, to 2 mL in the remaining novel methods. Experimental results suggest that higher recovery was achieved when smaller volumes of filtration buffer were used. The smaller size brought the beads in closer proximity to the filters, reducing the surface area and allowing for more efficient cell lysis. This decreased size was also much more convenient to use, since the 25 mm diameter filters fit easily into the 2 mL tubes. In addition, cells from the 15 mL tubes could not be pelleted as easily at the lower speeds required for the larger standalone centrifuges.

With respect to method 3 yielding the lowest final DNA concentration, Horáková *et al*. (2008) determined that the commercial UltraClean™ Water DNA Isolation kit resulted in a

significant loss of DNA within the final concentration steps. This may account for the extremely low yields observed in the present study. In addition, the filtration volumes suggested by the manufacturer range from 100 mL to 10 L, depending on the clarity of the water. Our results may indicate that the kit has limitations at the 100 mL lower sample size, and may be better suited for large volume sample concentrations. The remaining methods either did not produce DNA with suitable purity for qPCR or gave yields much lower than those observed with method 8, making them inefficient for our purpose.

An initial concern with the membrane filtration component was that some environmental pathogens may pass directly through the pores of the standard 0.45 µm filters. As a result, the 0.22 µm pore-size filters were initially preferred for this analysis. A study by Shirey and Bissonette (1991) found that several opportunistic pathogens such as *P. aeruginosa*, were detected with 0.22 µm filters while undetectable by standard 0.45µm filters, strengthening the decision to use the smaller-pore filters. Environmental cell sizes are much smaller than laboratory strains because of necessary cellular adaptations, including a reduction of metabolism in response to oligotrophic environments. With the smaller pore size, it was assumed that the majority of the bacteria in the environmental samples would be retained. Carter (1996) also suggested that there were no differences in recovery from either 0.22 µm or 0.45 µm filters, which was an additional incentive for their application. The present study has shown however that there are clear differences with respect to final DNA yield and cell recovery from filters of both pore sizes.

The hydrophilic 0.22 µm GVWP-Durapore and mixed cellulose-ester GSWP variants were tested. The GSWP has been previously tested by both Bej *et al*. (1991) and Oyofo and Rollins (1993), while the Durapore has been examined by Horáková *et al*. (2008) and Wolffs *et al*. (2006). Both studies on GSWP determined that no detectable signals were obtained following PCR amplification of DNA isolated from these filters. The results of the present study disagree with these findings, since nearly all samples were amplifiable by PCR and contained a suitable amount of DNA following extraction from the GSWP filters. This suggests that the DNA extraction method presently adopted is efficient. In comparison, Wolffs *et al*., (2006) found a significantly high recovery (93 ± 36%) from the Durapore filters following direct lysis. In the current study, 61.6% of the initial DNA was recovered from the same filters. It is necessary to note in this instance that while the % recovery from Wolffs *et al*. (2006) appears high, the standard deviation was much larger than those determined in the present work. This suggests that Wolffs *et al*. may have obtained values similar to those presently observed in their quadruplicate replicate samples. Regardless of the filter type, the recovery obtained by method 8 is on a comparable scale to those recoveries obtained by Wolffs *et al*., and Ahmed *et al*., (2008, 2009) for direct extraction from the membrane filter. The benefit of the concentration-purification procedure in the present work is that there was less variation in cell recovery than documented in previous studies.

It was also noted that in all of the tested methods, cell losses were obtained. This suggests that DNA from all of the original spiked cells was not recovered from the filters. With membrane filtration, a degree of cell loss is expected since the elution of bacterial cells and DNA from these filters is often incomplete (Stevens and Jaykus, 2004). Research by Bej *et al*. (1991) and Oyofo

and Rollins (1993) believed that the solution to this problem was to amplify the DNA in the presence of the filters. Their studies showed however that PCR was inhibited by the presence of many filter types. In the present study, we looked to determine the answer to this problem microscopically by examining filters before and after treatment by isolation method 8. It was determined that for each filter type, cells (both live and dead) were present on the filters following vacuum filtration. Relative to the original aliquot of overnight culture, a proportion of the cells (an estimated 1%) were not viable, as indicated by the binding of propidium iodide to their DNA, emitting red fluorescence under epifluorescence. This may be explained by the incidence of stress, in the form of slight vacuum pressure from the filtration process, and their addition to a hypotonic solution (distilled water) prior to filtration may have influenced the immediate death of some cells. From a microbiological perspective, cell death in an overnight culture is theoretically a valid observation. It would be expected that a portion of the bacterial population in the initial inoculum would have reached the death phase within the overnight incubation period since newly doubling cells will sequester the majority of the minimal nutrients provided from the TSB medium. The physical state of these cells is also unknown, however they may be present in whole, or partially lysed forms, which would be easily ruptured by the application of a vacuum pressure.

When filters were re-examined following treatment with method 8, there were no visible cells (green or red) at either 400x or 630x magnification, which would suggest that all cells were removed from the filters. There are several possible explanations for the disappearance of cells which may support these observations; however in most cases these losses are unavoidable. Some may be attributed to cells that became embedded within the filter pores or attached to the

upper surfaces of the filters as documented in earlier work on the recovery of bacteria and yeasts from beverages by Thomas (1988). These cells would not be visible upon microscopic examination, and could account for the filters appearing "clean" following bead beating, despite differences in the final recovery. A certain percentage of DNA may have also been lost as a result of degradation or shearing during the bead beating process. It is also possible that a proportion of the dead cells which were seen on each filter type may have lysed, and the extracellular DNA may have passed through the filter, as it was intended. This may account for the 21.6% difference in DNA obtained from $1x10^7$ cells using both the "spin down" (80.4%) and filter-purification procedures (73.3%) relative to the theoretical concentration of 50 ng. In this case, the theory that filtration removes the majority of extracellular DNA would have validity. Some cell loss may also be attributed to the DNA purification procedure itself, during the precipitation and elution stages, should the DNA have not fully eluted from the column.

The filter concentration process implemented in this study also serves an additional purpose, to function as an alternative to the culture enrichment process used to enhance low cell numbers to detectable thresholds. In these situations, although it is beneficial to detect such few cells, it is almost impossible to obtain a reliable estimate of the associated health risks. Cell concentrations can increase uncontrollably when cells are enriched, leading to inaccurate quantification (Wolffs *et al.*, 2006). By filter concentrating the samples, a much larger cell number could be concentrated from the sample to enhance PCR amplification. Previous work by Liu *et al*. (2008) has demonstrated that enrichment is unnecessary, contrary to popular belief, when approximately 7 CFU/mL of *E.coli* O157:H7 was detected from 1 L of source water. The present study has also demonstrated that with the newly developed filter-extraction procedure, as

few as 4 cells/100mL (16.02ng) could be detected, as confirmed by nested PCR and 10 cells/100mL by qPCR, without the need for enrichment. In comparison, Ibekwe and Grieve (2003) were able to detect only $3.5x10^3$ CFU/mL of *E. coli* O157:H7 in artificially contaminated soil samples prior to enrichment. Following the enrichment, they determined that a 2-log increase in the lowest cell concentration was observed, however the initial cell concentration prior to the experimentation was defined. With the filtration-concentration, a more accurate and reliable estimate of the original cells/mL value could be calculated, based on the amount of water which has passed through the filtration apparatus.

A sample size of 100 mL was used with seeded samples to be in accordance with *Standard Methods* protocols for coliform detection via traditional plate counts. This was performed to demonstrate that the number of pathogenic cells in the 100 mL aliquot that were undetected by the standard coliform membrane filtration methods could be detected via qPCR, highlighting the discrepancies in water quality practices. The *Standard Methods* protocols also suggest that for the detection of some microorganisms including *S.* Typhimurium and *C. jejuni,* large volume samples (1 L and 5 L respectively) must be filtered. For the purpose of this study, this was not an initial concern, due to the fact that the concentration of the *S.* Typhimurium reference culture used (10^7 cells on average) could reflect environmentally relevant concentrations. If for example a 5 L sample contained $2x10^3$ cells/mL or a 10 L sample contained $1x10^3$ cells/mL initially, the final concentration would therefore be at the 10^7 cell level applied here. This could also be further addressed when procedures are adapted for industrial use.

5.4. Extracellular DNA

In order to address the issue of detecting free-floating, extracellular DNA associated with lysed bacterial cells, source water samples were filtered prior to DNA extraction. It is necessary to eliminate the extracellular DNA sources, since overestimates are imparted on PCR techniques. Double-stranded DNA from the environment was assumed to pass through the pores of a membrane filter, having a pore diameter of either 0.22 or 0.45 µm. This assumption is supported by studies on the retention of DNA by membrane filters from the late 1960s by Phillips (1969) and Krasna (1970). Phillips (1969) determined that native DNA from reference *E. coli* cells was able to pass through membrane filters at a rate of 85%. Similarly, Krasna (1970) found that double-stranded DNA passed through membrane filters with ease, while approximately 20-30% of denatured DNA was retained on 0.22 µm filters. A small percentage may also be dissolved in aquatic environments, evidence of which has been previously documented (DeFlaun and Paul, 1989). In addition, the integrity of some virulence genes in aquatic environments has been examined. DNA can persist for up to 3 months as seen with the virulence plasmid of *S.* Typhimurium in 10°C seawater (Dupray *et al*., 1997), stressing the importance of addressing this issue when working towards developing a standardized method with molecular techniques.

In the present study, following filtration of fresh cultures with each of the 3 tested filter types, no quantifiable data was obtained when PCR was performed on each of the resulting filtrates. This suggests that no extracellular DNA from the *S.* Typhimurium reference strain had passed through any of the 3 filter types. Wolffs *et al*. (2006) had previously reported that as little as 0.3% of the original concentrations of pathogenic cells were recovered in the filtrate following filtration with 0.22 µm Durapore filters. It is known that the majority of cells placed into the

samples in this study were viable; therefore the likelihood that extracellular DNA may have been present was very low. This observation may be attributed to the notion that the membranes of dead cells, which were seen microscopically, may not have been permeabilized, preventing the release of extracellular DNA from these cells. In addition, PCR inhibitors may have passed through the filter and could have contributed significantly to enzymatic inhibition, preventing the amplification of the low DNA concentrations ($< 10^4$ cells). Finally, after filtration the physical state of the DNA was unknown. The DNA may have dissociated from its double stranded form and been retained on the filter, as seen by Krasna (1970), resulting in detection of the single strands by PCR. Finally, a percentage of the DNA may have become sheared as a result of the vacuum filtration, or dissolved entirely upon contact with the distilled water. The experimental results were therefore inconclusive as to whether the nucleic acids were retained or eluted and further investigation is necessary. Recent studies have examined the chemical treatment of environmental water samples with ethidium monoazide bromide (Nocker and Camper) or propidium monoazide bromide (Bae and Wuertz, 2007; Luo *et al.*, 2010) to prevent amplification of dead cells and extracellular DNA from cells with compromised membranes. This could also be used in conjunction with the present study to produce a more definitive answer. These methods do however tend to rely on the integrity of the outer membrane of bacterial cells. Therefore they do not take into account the differences in membrane integrity between viable cells that are injured or have weakened membranes and true non-viable cells.

5.5. Sensitivity assays in source water

When testing the newly developed filter-extraction procedure with serial cell dilutions of the *S.* Typhimurium model, several key observations were made. Melting curve analysis confirmed that the detection limit was identical to that obtained under reference conditions, with a semi-quantitative limit of 10 cells/100mL being detected following 50 rounds of amplification. This indicates that there was very minimal sensitivity loss observed between reference and field conditions, a trend rarely encountered in environmental PCR.

Many water-related studies have documented at least a 10-fold decrease in sensitivity when their methods are tested with field samples (Khan *et al*., 2007; Liu *et al*., 2008; Ram *et al*., 2008b). This occurs specifically with those having high turbidity. A small number of viable cells cannot generally be detected because of strong interference from dead cells and inhibitors (Gilbride *et al*., 2006; Liu *et al*., 2008). The initial results suggest that both the primers and the concentration-purification procedure are of suitable robustness to detect a small number of pathogenic cells in an environmental matrix, without prior enrichment. While probe-based detection is more specific, the SYBR Green assays developed in the present study have shown comparable results to the molecular beacon assays created by Ram *et al*., (2008b) and Sandhya *et al*. (2008). With the Sandhya *et al*. (2008) study in particular; a 1 CFU/mL detection limit was again only achieved after filters were enriched for 18 hours in peptone broth, which was entirely avoided in the present study. In addition, the average C_T value obtained for 1.2 CFU/mL in their study (18.5 cycles) would equate to a final post-enrichment concentration of approximately 10^6 cells based on their standard plot, differing significantly from the original cell number.

The experimental C_P values for DNA isolated from lake water were shifted to later cycles relative to those obtained with the same DNA concentrations in reference conditions. For example, the 10 cell dilution appeared at a C_P of 47, beyond the quantifiable range. This may suggest that some slight sample inhibition was observed following purification, preventing a percentage of the *S.* Typhimurium template from being amplified by the SINV primers. It would therefore be necessary to test the method in more highly turbid water samples, to ensure that it would be useable under conditions with high turbidity and suspended solids, for example a flowing river after intense precipitation. The present study has however developed assays suitable for direct, culture-independent detection of pathogenic cells in an environmental matrix with the aid of a membrane filtration-purification procedure which can be completed within 24 hours of the initial sampling. This strengthens the potential utility of the qPCR method as a tool for water quality monitoring.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

The present study highlighted many areas which are rarely addressed with respect to standardization of PCR for water quality monitoring. An array of highly specific and sensitive primers to detect virulence-associated genes of five waterborne pathogens was successfully assembled. This included two novel designs, and two previously unoptimized primer sets determined to be well suited for quantitative assays. The development of a rapid filter-extraction procedure to isolate pathogenic DNA from environmental samples was also accomplished and produced DNA of suitable quantity and purity for qPCR purposes, with 73.3% of the DNA from membrane-bound cells being recovered. This method was also successfully validated with artificially contaminated source water samples. The 10 cells/100mL detection limit determined following filtration-extraction with the model *S.* Typhimurium strain in source water showed comparability to the 10 cells/mL limit obtained from four of the five (*E. coli* O157:H7, *S.* Typhimurium, *P. aeruginosa*, and *S. flexneri*) tested pathogens under reference conditions, as predicted. Detection was therefore at or below the suggested infectious doses of each pathogen, as would was expected. The suggested analysis method (qPCR) is therefore adequate and feasible for future use in conjunction with indicator technology. It is necessary to note that the aim of the present study was not to eliminate the indicator-reliant methods entirely, but rather to highlight the differences with respect to analysis times, quantitative data collection and specificity.

6.2. Recommendations

Further research is necessary to complete validation of the methods developed herein. A field study is required in order to better define the capabilities of the newly developed methods for the analysis of naturally contaminated environments. It is preferred that samples of varying turbidity be collected from different geographical areas on a regular basis. For analysis of these samples, it is highly advised that the presently designed qPCR detection protocols be used in conjunction with traditional coliform counts for comparative purposes. In addition, the primer array could be further extended to include both protozoan and viral pathogens.

With regards to laboratory trials, part of the validation and standardization process involves testing the developed methods in different laboratories, and with different operators within the same laboratory. Should the robustness obtained within the present study be universal, it would be expected that the proposed methods could be put forward to water quality officials following intense field testing. Variation in experimental outcomes could be analyzed to determine whether the detection limits are adequate and of sufficient reproducibility to accept or reject the protocols as possible standard practices for monitoring the microbiological quality of water.

Automation of the entire process would also enhance the rate at which these methods would function, since the frequency of sampling intervals could be increased. This would also reduce the potential for sampling bias and allow continuous, real-time monitoring at treatment facilities. There are currently both automated DNA extraction and qPCR thermal cyclers either presently available or in development, suggesting that this technology has a bright future.

To examine areas where PCR-based pathogen detection could be further strengthened, a separate study could be designed using the present primers and DNA isolation protocols to incorporate the novel chemical compounds ethidium monoazide bromide and propidium monoazide bromide for viability and extracellular DNA analysis. An additional study could be performed to examine the effect of carrier DNA on the detection of low target concentrations. This could function as an *in situ* biological alternative to culture enrichment processes since in some instances, carrier DNA molecules have been known to naturally amplify and ease precipitation of rare DNA sequences. This may increase the likelihood of detecting nucleic acids associated with low levels of pathogen contamination, however some sensitivity loss may be observed.

CHAPTER 7: REFERENCES

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CHAPTER 8: APPENDIX

APPENDIX A: PCR PRIMER ALIGNMENTS WITH GENE SEQUENCES

Location of ETIR primers within the *tir* gene of *E. coli* O157:H7

Forward primer sequence (5′ → 3′): GTCAGCTCATTAACTCTACGGG
Reverse primer sequence (5′ → 3′): GCCTGTTAAGAGTATCGAGCG
Amplicon size: 207 bp

***Escherichia coli* translocated intimin receptor Tir *(tir)* gene, complete cds**
GenBank Accession No. AF125993

5′
ATGCCTATTGGTAACCTTGGTCATAATCCCAATGTGAATAATTCAATTCCTCCTGCACCTCCATTACCTT
CACAAACCGACGGTGCAGGGGGGCGTGGTCAGCTCATTAACTCTACGGGGCCGTTGGGATCTCGTGCGCT
ATTTACGCCTGTAAGGAATTCTATGGCTGATTCTGGCGACAATCGTGCCAGTGATGTTCCTGGACTTCCT
GTAAATCCGATGCGCCTGGCGGCGTCTGAGATAACACTGAATGATGGATTTGAAGTTCTTCATGATCATG
GTCCGCTCGATACTCTTAACAGGCAGATTGGCTCTTCGGTATTTCGAGTTGAAACTCAGGAAGATGGTAA
ACATATTGCTGTCGGTCAGAGGAATGGTGTTGAGACCTCTGTTGTTTTAAGTGATCAAGAGTACGCTCGC
TTGCAGTCCATTGATCCTGAAGGTAAAGACAAATTTGTATTTACTGGAGGCCGTGGTGGTGCTGGGCATG
CTATGGTCACCGTTGCTTCAGATATCACGGAAGCCCGCCAAAGGATACTGGAGCTGTTAGAGCCCAAAGG
GACCGGGGAGTCCAAAGGTGCTGGGGAGTCAAAAGGCGTTGGGGAGTTGAGGGAGTCAAATAGCGGTGCG
GAAAACACCACAGAAACTCAGACCTCAACCTCAACTTCCAGCCTTCGTTCAGATCCTAAACTTTGGTTGG
CGTTGGGGACTGTTGCTACAGGTCTGATAGGGTTGGCGGCGACGGGTATTGTACAGGCGCTTGCATTGAC
GCCGGAGCCGGATAGCCCAACCACGACCGACCCTGATGCAGCTGCAAGTGCAACTGAAACTGCGACAAGA
GATCAGTTAACGAAAGAAGCGTTCCAGAACCCAGATAATCAAAAAGTTAATATCGATGAGCTCGGAAATG
CGATTCCGTCAGGGGTATTGAAAGATGATGTTGTTGCGAATATAGAAGAGCAGGCTAAAGCAGCAGGCGA
AGAGGCCAAACAGCAAGCCATTGAAAATAATGCTCAGGCGCAAAAAAAATATGATGAACAACAAGCTAAA
CGCCAGGAGGAGCTGAAAGTTTCATCGGGGGCTGGCTACGGTCTTAGTGGCGCATTGATTCTTGGTGGGG
GAATTGGTGTTGCCGTCACCGCTGCGCTTCATCGAAAAAATCAGCCGGTAGAACAAACAACAACAACTAC
TACTACAACTACAACTACAAGCGCACGTACGGTAGAGAATAAGCCTGCAAATAATACACCTGCACAGGGC
AATGTAGATACCCCTGGGTCAGAAGATACCATGGAGAGCAGACGTAGCTCGATGGCTAGCACCTCGTCGA
CTTTCTTTGACACTTCCAGCATAGGGACCGTGCAGAATCCGTATGCTGATGTTAAAACATCGCTGCATGA
TTCGCAGGTGCCGACTTCTAATTCTAATACGTCTGTTCAGAATATGGGGAATACAGATTCTGTTGTATAT
AGCACCATTCAACATCCTCCCCGGGATACTACTGATAACGGCGCACGGTTATTAGGAAATCCAAGTGCGG
GGATTCAAAGCACTTATGCGCGTCTGGCGCTAAGTGGTGGATTACGCCATGACATGGGAGGATTAACGGG
GGGGAGTAATAGCGCTGTGAATACTTCGAATAACCCACCAGCGCCGGGATCCCATCGTTTCGTCTAA
3′

Location of SINV primers within the *invA* gene of *S.*Typhimurium

Forward primer sequence (5′ → 3′): TATGCCCGGTAAACAGATGAG
Reverse primer sequence (5′ → 3′): GTATAAGTAGACAGAGCGGAGG
Amplicon size: 252 bp

***Salmonella* Typhimurium InvA (*invA*) gene, complete cds**
GenBank Accession No. AF125993

5′
GATATTGCCTACAAGCATGAAATGGCAGAACAGCGTCGTACTATTGAAAAGCTGTCTTAATTTAATATTA
ACAGGATACCTATAGTGCTGCTTTCTCTACTTAACAGTGCTCGTTTACGACCTGAATTACTGATTCTGGT
ACTAATGGTGATGATCATTTCTATGTTCGTCATTCCATTACCTACCTATCTGGTTGATTTCCTGATCGCA
CTGAATATCGTACTGGCGATATTGGTGTTTATGGGGTCGTTCTACATTGACAGAATCCTCAGTTTTTCAA
CGTTTCCTGCGGTACTGTTAATTACCACGCTCTTTCGTCTGGCATTATCGATCAGTACCAGTCGTCTTAT
CTTGATTGAAGCCGATGCCGGTGAAATTATCGCCACGTTCGGGCAATTCGTTATTGGCGATAGCCTGGCG
GTGGGTTTTGTTGTCTTCTCTATTGTCACCGTGGTCCAGTTTATCGTTATTACCAAAGGTTCAGAACGTG
TCGCGGAAGTCGCGGCCCGATTTTCTCTGGATGGTATGCCCGGTAAACAGATGAGTATTGATGCCGATTT
GAAGGCCGGTATTATTGATGCGGATGCCGCGCGCGAACGGCGAAGCGTACTGGAAAGGGAAAGCCAGCTT
TACGGTTCCTTTGACGGTGCGATGAAGTTTATCAAAGGTGACGCTATTGCCGGCATCATTATTATCTTTG
TGAACTTTATTGGCGGTATTTCGGTGGGGATGACTCGCCATGGTATGGATTTGTCCTCCGCCCTGTCTAC
TTATACCATGCTGACCATTGGTGATGGTCTTGTCGCCCAGATCCCCGCATTGTTGATTGCGATTAGTGCC
GGTTTTATCGTGACCCGCGTAAATGGCGATACGGATAATATGGGGCGGAATATCATGACGCAGCTGTTGA
ACAACCCATTTGTATTGGTTGTTACGGCTATTTTGACCATTTCAATGGGAACTCTGCCGGGATTCCCACT
GCCGGTTTTTGTTATTTTATCGGTGGTTTTAAGCGTACTCTTCTATTTTAAATTCCGTGAAGCAAAACGT
AGCGCCGCCAAACCTAAAACCAGCAAAGGCGAGCAGCCGCTCAGTATTGAGGAAAAAGAAGGGTCGTCGT
TAGGACTGATTGGCGATCTCGATAAAGTCTCTACAGAGACCGTACCGTTGATATTACTTGTGCCGAAGAG
CCGGCGTGAAGATCTGGAAAAAGCTCAACTTGCGGAGCGTCTACGTAGTCAGTTCTTTATTGATTATGGC
GTGCGCCTGCCGGAAGTATTGTTACGAGATGGCGAGGGCCTGGACGATAACAGCATCGTATTGTTGATTA
ATGAGATCCGTGTTGAACAATTTACGGTCTATTTTGATTTGATGCGAGTGGTAAATTATTCCGATGAAGT
CGTGTCCTTTGGTATTAATCCAACAATCCATCAGCAAGGTAGCAGTCAGTATTTCTGGGTAACGCATGAA
GAGGGGGAGAAACTCCGGGAGCTTGGCTATGTGTTGCGGAACGCGCTTGATGAGCTTTACCACTGTCTGG
CGGTGACCGTGGCGCGCAACGTCAATGAATATTTCGGTATTCAGGAAACAAAACATATGCTGGACCAACT
GGAAGCGAAATTTCCTGATTTACTTAAAGAAGTGCTCAGACATGCCACGGTACAACGTATATCTGAAGTT
TTGCAGCGTTTGTTAAGCGAACGTGTTTCCGTGCGTAATATGAAGTTAATTATGGAAGCGCTCGCATTGT
GGGCGCCAAGAGAAAAAGATGTCATTAACCTTGTGGAGCATATTCGTGGAGCAATGGCGCGTTATATTTG
TCATAAATTCGCCAATGGCGGCGAATTACGAGCAGTAATGGTATCTGCTGAAGTTGAGGATGTTATTCGC
AAAGGGATCCGTCAGACCTCTGGCAGTACCTTCCTCAGCCTTGACCCGGAAGCCTCCGCTAATTTGATGG
ATCTCATTACACTTAAGTTGGATGATTTATTGATTGCACATAAAGATCTTGTCCTCCTTACGTCTGTCGA
TGTCCGTCGATTTATTAAGAAAATGATTGAAGGTCGTTTTCCGGATCTGGAGGTTTTATCTTTCGGTGAG
ATAGCAGATAGCAAGTCAGTGAATGTTATAAAAACAATATAAGGGCTTAATTAAGGAAAAGATCTATGCA
ACATTT
3′

Location of VS1 primers within the *VS1* gene of *C. jejuni*

Forward primer sequence (5′ → 3′): GAATGAAATTTTAGAATGGGG
Reverse primer sequence (5′ → 3′): GATATGTATGATTTTATCCTGC
Amplicon size: 358 bp

***C. jejuni* VS1 DNA**
GenBank Accession No. X71603

5′
AAGCTTGTGATACTTTTAAGTGCTATAGAAAGTGAAAATGAAATTTCTTTAGCAGGCATATATAGAGCGT
ATTGTTCCAAATTTGATTTAAAGAATGAAATTTTAGAATGGGGTCTTAAAATATTTAAAAACAATAATGC
CTTAAAAGATCTTGTAGAAAAAGAAGATATATACAATCCTATTGTTGTAAGTAGTTTGGTTTCTAAGCTA
GAAAATTTAGAAAATTTAGAGCTTTTATATACTTTAACTTGGCTAAAGGCTAAGGCTTTAAATTATAATG
CTTTTTATTTTAGAGTTCTTGATAAACTTTTAGAAAATGCAAAACAAGGTTTTGAAGATGAAAATCTACT
TGAAGAAAGTGCAAGAAGGGTAAAAAAAGAATTAACACTTAAAAGAAGTAAGATTTTTTTAGAGCAAGAT
GAAATTTTGCAGGATAAAATCATACATATCAAATCAAATCTTTTTATTATAAAAAATACTTTTGAAGATA
TTGTTATGATTTCTAAATTAGCCAAAGAAAATGATTTTAAATTTTGGTTTAGTAATGAAACAAATCTTAG
TTTGCAAATTGTTGCACCACTTCATTTTAATATTGCCATTATTTTAAGTTCTTTAACAAATTTAAATCTT
ATTTTTATGAATTTTTTTGAACTTTTTGATGATAAAATTTATTTAAGGTTTGAATATGATAATATTATCA
GTGATGAGCAAAAACTAAAACTTTGTGAGCTTTTAAATTCAAATCTTTCTGGTTTTAATTTGAAAAAAAT
TAAAAAGCCAATCATTAAAAAAGAGGAGTTAAAATTAGACTTAAACTATTCTAAAATGTATGCCAAATTA
GGTCTTAATACTAAAGATCAGCAAGGTTTAATGGCGTATTTGATGAATGTTTTTAATGAACTTGAACTTG
TTTTATGTGCAGCAAAAATTCAAACCATAAGACAAAGGACGCGTAATATTTTTATTTTTCAAAAGAATGA
AAAATTAGAACATAGCGAGCAAAAGTTAGTTAATTTATTAATAAGTGAGTAAAAAAATGTGTGGAATCGT
AGGCTATATAGGAAATAATGAAAAAAAACAAATTATACTAAATGGACTTAAAGAATTAGAATATCGTGGC
TATGATAGTGCGGGTATGGCAGTGATGCAAGAAGGCGAACTTAGTTTTTTTAAAGCTGTAGGAAAGCTT

3′

Location of ipaH2 primers within the *ipaH* gene of *S.flexneri*

Forward primer sequence (5′ → 3′): ATAATGATACCGGCGCTCTG
Reverse primer sequence (5′ → 3′): CGGCTTCTGACCATAGCTTC
Amplicon size: 247 bp

***S. flexneri* invasion plasmid antigen H (*ipaH*) gene, complete cds**
GenBank Accession No. M32063

5′
TGACCTAGCATTATGTTCTCTGTAAATAATACACACTCATCAGTTTCTTGCTCCCCCTCTATTAACTCAA
ACTCAACCAGTAATGAACATTATCTGAGAATCCTGACTGAATGGGAAAAGAACTCTTCTCCCGGGAAGAG
CGAGGCATTGCTTTTAACAGACTCTCCCAGTGCTTTCAGAATCAAGAAGCAGTATTAAATTTATCAGACC
TAAATTTGACGTCTCTTCCCGAATTACCAAAGCATATTTCTGCTTTGATTGTAGAAAATAATAAATTAAC
ATCATTGCCAAAGCTGCCTGCATTTCTTAAAGAACTTAATGCTGATAATAACAGGCTTTCTGTGATACCA
GAACTTCCTGAGTCATTAACAACTTTAAGTGTTCGTTCTAATCAACTGGAAAACCTTCCTGTTTTGCCAA
ACCATTTAACATCATTATTTGTTGAAAATAACAGGCTATATAACTTACCGGCTCTTCCCGAAAAATTGAA
ATTTTTACATGTTTATTATAACAGGCTGACAACATTACCCGACTTACCGGATAAACTGGAAATTCTCTGT
GCTCAGCGCAATAATCTGGTTACTTTTCCTCAATTTTCTGATAGAAACAATATCAGACAAAAGGAATATT
ATTTTCATTTTAATCAGATAACCACTCTTCCGGAGAGTTTTTCACAATTAGATTCAAGTTACAGGATTAA
TATTTCAGGGAATCCATTGTCGACTCGCGTTCTGCAATCCCTGCAAAGATTAACCTCTTCGCCGGACTAC
CACGGCCCGCAGATTTACTTCTCCATGAGTGACGGACAACAGAATACACTCCATCGCCCCCTGGCTGATG
CCGTGACAGCATGGTTCCCGGAAAACAAACAATCTGATGTATCACAGATATGGCATGCTTTTGAACATGA
AGAGCATGCCAACACCTTTTCCGCGTTCCTTGACCGCCTTTCCGATACCGTCTCTGCACGCAATACCTCC
GGATTCCGTGAACAGGTCGCTGCATGGCTGGAAAAACTCAGTGCCTCTGCGGAGCTTCGACAGCAGTCTT
TCGCTGTTGCTGCTGATGCCACTGAGAGCTGTGAGGACCGTGTCGCGCTCACATGGAACAATCTCCGGAA
AACCCTCCTGGTCCATCAGGCATCAGAAGGCCTTTTCGATAATGATACCGGCGCTCTGCTCTCCCTGGGC
AGGGAAATGTTCCGCCTCGAAATTCTGGAGGACATTGCCCGGGATAAAGTCAGAACTCTCCATTTTGTGG
ATGAGATAGAAGTCTACCTGGCCTTCCAGACCATGCTCGCAGAGAAACTTCAGCTCTCCACTGCCGTGAA
GGAAATGCGTTTCTATGGCGTGTCGGGAGTGACAGCAAATGACCTCCGCACTGCCGAAGCTATGGTCAGA
AGCCGTGAAGAGAATGAATTTACGGACTGGTTCTCCCTCTGGGGACCATGGCATGCTGTACTGAAGCGTA
CGGAAGCTGACCGCTGGGCGCAGGCAGAAGAGCAGAAGTATGAGATGCTGGAGAATGAGTACTCTCAGAG
GGTGGCTGACCGGCTGAAAGCATCAGGTCTGAGCGGTGATGCGGATGCGCAGAGGGAAGCCGGTGCACAG
GTGATGCGTGAGACTGAACAGCAGATTTACCGTCAGCTGACTGACGAGGTACTGGCCCTGCGATTGTCTG
AAAACGGCTCACGACTGCACCATTCATAATCACGTCGCATAAGCATAAACCGCAGACCGGATTGACTCCG
GAAAAACTGTGACCCGATTACGGACCTTAACAACAACCCGTAAATCCTCGCTCAATACCGGCAGGGATTT
ACGGCGTGCAACTGACTTTTTTGAGGGGATAACCAACCAGATCGTTTGCTATGGGAATATCGAGACAGTA
ATGAGTTAAATGATAAAAATTGTTTGAAAATATAGGGGATAAAGATCAATCCAAACTGGATGAAAGTAGA
ACTGGTCACATTAACATGGGTAGACTGATATAACAATCGACGGTTACTGGAAAGACAGGAACATATTCCT
CCAGCCGGAATGAAAACGCCGATAAAGCTCTAGGATTGTTTTTTTAAAGACTTTCTCGTTTTATTTGCAT
TAATAGACCAAGATATGAATAGTGAGGGGTTAATAAATGAAACCGATCAACAATCATTCTTTTTTTCGTT
CCCTTTGTGGCTTATCATGTATATCTCGTTTATCGGTAGAAGAACAGTGTACCAGAGATTACCACCGCAT
CTGGGATGACTGGGCTAGGGAAGGAACAACAACAGAAAATCGCATCCAGGCGGTTCGATTATTGAAAATA
TGTCTGGATACCCGGGAGCCTGTTCTCAATTTAAGCTTACTGAAACTACGTTCTTTACCACCACTCCCTT
TGCATATACGTGAACTTAATATTTCCAACAATGAGTTAATCTCCCTACCTGAAAATTCTCCGCTTTTGAC
AGAACTTCATGTAAATGGTAACAACTTGAATATACTCCCGACACTTCCATCTCAACTGATTAAGCTTAAT
3′

Location of exoT primers within the *exoT* gene of *P. aeruginosa*

Forward primer sequence (5′ → 3′): GGTCTCTATACCAACGGCGA
Reverse primer sequence (5′ → 3′): GAACAGGGTGGTTATCGTGC
Amplicon size: 285 bp

***Pseudomonas aeruginosa* exoenzyme 53 (*exoT*) gene, complete cds**
GenBank Accession No. L46800

5′
GATATCCATCGGGTTCTCCGCCCCGGTGTAGGCGCACGGGAGCTGGCGTAGGGAAAGTCCGCTGTTTTTC
GGCCGCTGACGGTTCTCTTTCCGCGTGCTCCGACGGCCGCCAACAGTAAAAAAACCACGGCCAATCCTGA
TAGGCGGAGGGGCGCCTCGTTCCTAGACTGGCGGGGAAACATCAGGAGACGTCAATCATCATGCATATTC
AATCATCTCAGCAGAACCCGTCTTTCGTGGCTGAGTTGAGCCAGGCCGTGGCCGGGCGCCTGGGACAGGT
CGAGGCCCGCCAGGTGGCCACTCCCCGGGAGGCGCAACAACTGGCCCAGCGCCAGGAAGCACCGAAGGGC
GAGGGCCTGCTCTCCCGCCTGGGGGCCGCCCTCGCGCGTCCCTTCGTGGCGATCATCGAGTGGCTGGGCA
AACTGCTGGGGAGCCGTGCCCACGCCTCCACCCAGGCGCCGCTCTCCCGTCAGGACGCGCCGCCTGCCGC
CAGTCTCTCGGCCGCCGAGATCAAGCAGATGATGCTGCAAAAGGCACTGCCCCTGACCTTGGGCGGACTT
GGCAAGGCGAGCGAGCTGGCGACTTTGACAGCGGAGAGACTGGCGAAGGATCACACGCGCCTGGCCAGCG
GCGACGGCGCCCTGCGCTCGCTGGCCACCGCCCTGGTCGGGATTCGCGATGGCAGCCGGATCGAGGCTTC
CCGTACCCAGGCTGCCCGCCTGCTCGAACAGAGCGTTGGGGGGATCGCGCTGCAACAGTGGGGGACCGCG
GGCGGTGCCGCCAGCCAGCATGTACTCAGCGCAAGCCCGGAGCAACTGCGCGAAATCGCCGTCCAACTGC
ATGCGGTAATGGACAAGGTCGCCCTGTTGCGCCACGCGGTAGAGAGCGAGGTAAAGGGCGAGCCTGTCGA
CAAGGCGCTGGCGGATGGCCTGGTGGAGCACTTCGGGCTGGAGGCGGAGCAGTACCTAGGCGAACACCCG
GACGGGCCGTACAGCGATGCCGAGGTGATGGCGCTCGGTCTCTATACCAACGGCGAGTACCAGCACCTGA
ATCGTTCCCTGCGTCAGGGGCGAGAGCTGGATGCTGGCCAGGCGTTGATCGACCAGGGCATGTCTGCCGC
GTTCGAAAAGAGCGGACCGGCTGAACAGGTCGTGAAGACCTTCCGCGGCACCCAGGGCAGGGATGCCTTC
GAGGCGGTGAAAGAGGGCCAGGTCGGCCACGACGCCGGCTATCTCTCCACCTCCCGGGACCCCAGCGTTG
CCAGGAGCTTCGCGGGCCAGGGCACGATAACCACCCTGTTCGGCAGATCCGGGATCGATGTCAGCGAGAT
ATCGATCGAGGGCGATGAGCAGGAGATCCTCTACGACAAGGGGACCGACATGCGCGTACTGCTCAGCGCC
AAGGATGGGCAGGGTGTGACCCGTCGGGTGCTCGAAGAGGCCACGCTGGGGGAACGGAGCGGCCACGGCG
AGGGACTGCTCGATGCCCTGGACCTGGCAACCGGGACGGATCGTTCAGGCAAGCCCCAGGAACAGGACCT
GCGCCTGAGAATGCGCGGCCTCGACCTGGCCTGACCGGTCGACGGCAGAGACGGACACTCCCAAGGGGTG
TCCGTTTTCATTTGCGCCGTACAGCGTCGGGCGCAATGGGCGGCAAGGAGGCCT
3′

TABLE B.1. Primers designed using the selection criteria but rejected from further analysis.

Primer Set	Target Organism	Sequence (5' → 3')	Gene Target	Product Size (bp)	Status
Camp	*Campylobacter* spp.	F: CACGTGCTACAATGGCATAT R: GGCTTCATGCTCTCGAGTT	*16S rRNA*	108	Not Working
EcoOCP1	*Escherichia coli*	F: CTGATATGTAGGTGAAGTCCC	*23s rRNA*	230	Not working
EUID2	*Escherichia coli* spp.	F: TCAGCGTTGGTGGGAAAG R: CGTTTCGATGCGGTCACT	*uidA*	184	Not working
KmagA2	*Klebsiella* spp.	F: GGTGATTCAAGCACTATACCTC R: ACTGCCATTCCACTTATAGC	*magA*	266	Not working
KmagA3	*Klebsiella* spp.	F: GGTGCTCTTTACATCATTGC R: CCATCTGCGAATTTAAACCT		224	Not working
Paer23S	*Pseudomonas* spp.	F: AGAAGTGCCGAGCATGGGAG R: CAAACCACACACCGAAGCTGC	*23s rRNA*	240	Not working
ipaH1	*Shigella* spp.	F: CACAGGTGATGCGTGAGACT R: CCGTAATCGGGTCACAGTTT	*ipaH*	168	Not working

TABLE C.1. Data compiled from Primer-BLAST search for sequence homologies with each of the primers tested.

Primers	Primer-BLAST Sequence Homologies (Most Common)	Associated Feature
ETIR	CP001846.1 *Escherichia coli* O55:H7 str. CB9615, complete genome	Translocated intimin receptor
	GQ338312.1 *Escherichia coli* strain 71074 enterocyte effacement pathogenicity island gene locus, partial cds	N/A
	CP001368.1 *Escherichia coli* O157:H7 str. TW14359, complete genome	Translocated intimin receptor
	CP001164.1 *Escherichia coli* O157:H7 str. EC4115, complete genome	Tir
	EU871628.1 *Escherichia coli* strain 33264 enterocyte effacement gene locus, partial sequence	N/A
	EU871627.1 *Escherichia coli* strain EDS-58 enterocyte effacement gene locus, partial sequence	N/A
	EU871626.1 *Escherichia coli* O157:H7 strain ECI-1717 enterocyte effacement gene locus, partial sequence	N/A
	BA000007.2 *Escherichia coli* O157:H7 str. Sakai DNA, complete genome	Translocated intimin receptor
SINV	CP001363.1 *Salmonella enterica* subsp. enterica serovar Typhimurium str. 14028S, complete genome	Export protein
	FN424405.1 *Salmonella enterica* subsp. enterica serovar Typhimurium str. D23580 complete genome	Secretory protein
	CP000857.1 *Salmonella enterica* subsp. enterica serovar Paratyphi C strain RKS4594, complete genome	Secretory protein
	AE006468.1 *Salmonella enterica* subsp. enterica serovar Typhimurium str. LT2, complete genome	Invasion protein
	CP001144.1 *Salmonella enterica* subsp. enterica serovar Dublin str. CT_02021853, complete genome	Invasion protein InvA
	FM200053.1 *Salmonella enterica* subsp. enterica serovar Paratyphi A str. AKU_12601 complete genome	Secretory protein
	EU348369.1 *Salmonella enterica* subsp. enterica serovar Senftenberg strain JXS-04#01 invasion protein	N/A
	EU348368.1 *Salmonella enterica* subsp. enterica serovar Pullorum strain 1794 invasion protein (invA) gene	N/A
VS1	X71603.1 *C.jejuni* VS1 DNA	N/A
	CP001876.1 *Campylobacter jejuni* subsp. jejuni IA3902, complete genome	Putative nucleotidyltransferase
	CP000814.1 *Campylobacter jejuni* subsp. jejuni 81116, complete genome	GlnD family protein
	CP000768.1 *Campylobacter jejuni* subsp. doylei 269.97, complete genome	GlnD family protein
	CP000538.1 *Campylobacter jejuni* subsp. jejuni 81-176, complete genome	GlnD family protein
	AL111168.1 *Campylobacter jejuni* subsp. jejuni NCTC 11168 complete genome	putative nucleotidyltransferase
	DQ493922.1 *Campylobacter jejuni* subsp. jejuni 81-176 putative subtilase family serine protease	N/A
	CP000025.1 *Campylobacter jejuni* RM1221, complete genome	GlnD family protein
ipaH2	CP001384.1 *Shigella flexneri* 2002017 plasmid pSFxv_1, complete sequence	Invasion plasmid antigen
	CP001383.1 *Shigella flexneri* 2002017, complete genome	Invasion plasmid antigen
	FJ227542.1 *Shigella flexneri* invasion plasmid antigen H (ipaH) gene, partial cds	N/A
	EU743831.1 *Shigella boydii* invasion plasmid antigen (ipaH2) gene, complete cds	N/A
	CP001063.1 *Shigella boydii* CDC 3083-94, complete genome	Invasion plasmid antigen
	CP001062.1 *Shigella boydii* CDC 3083-94 plasmid pBS512_211, complete sequence	Invasion plasmid antigen
	EU340151.1 *Shigella boydii* strain hn03 IpaH-1-like gene, partial sequence	Invasion plasmid antigen
	CP000266.1 *Shigella flexneri* 5 str. 8401, complete genome	Invasion plasmid antigen
exoT	FM209186.1 *Pseudomonas aeruginosa* LESB58 complete genome sequence	Exoenzyme T
	CP000438.1 *Pseudomonas aeruginosa* UCBPP-PA14, complete genome	Exoenzyme T
	AE004091.2 *Pseudomonas aeruginosa* PAO1, complete genome	Exoenzyme T
	L46800.1 *Pseudomonas aeruginosa* exoenzyme 53 (exoT) gene, complete cds	Exoenzyme T

APPENDIX D: QPCR AMPLIFICATION CURVES

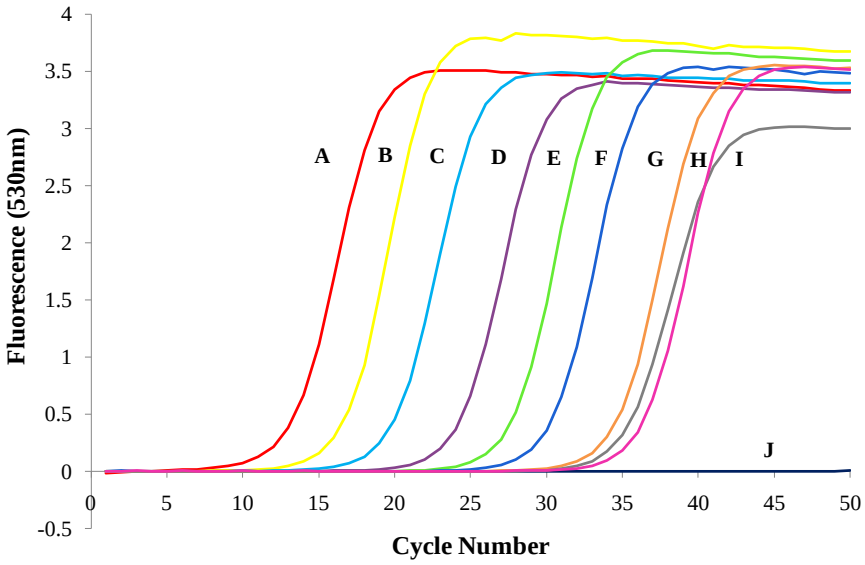

FIG. D.1. Amplification curve generated from serial dilutions of *S. flexneri* ATCC 12022 culture with ipaH2 primer set. (A) 10^8 cells; (B) 10^7 cells; (C) 10^6 cells; (D); 10^5 cells; (E)10^4 cells; (F) 10^3 cells; (G)10^2 cells; (H)10^1 cells; (I) 10^0 cells; (J) NTC.

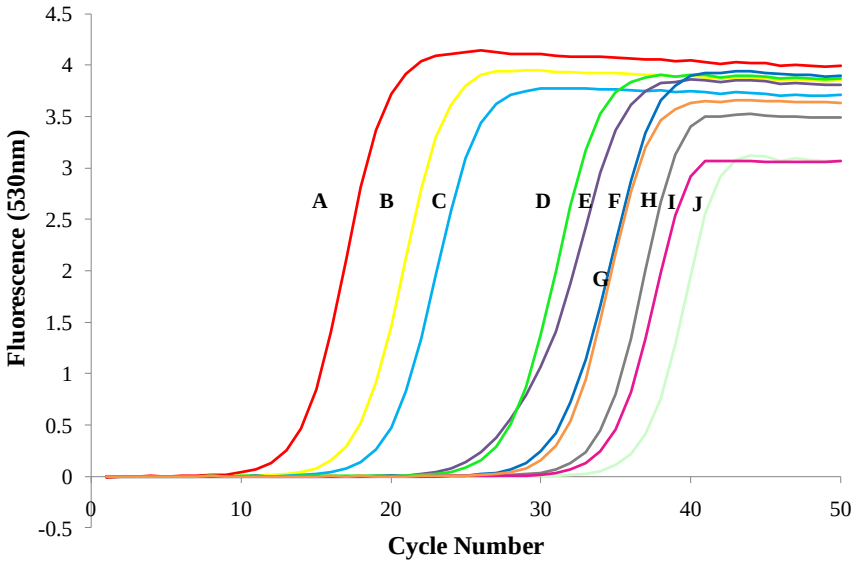

FIG. D.2. Amplification curve generated from serial dilutions of *E. coli* O157:H7 ATCC 700927 culture with ETIR primer set. (A) 10^8 cells; (B) 10^7 cells; (C) 10^6 cells; (D); 10^5 cells; (E)10^4 cells; (F) 10^3 cells; (G)10^2 cells; (H)10^1 cells; (I) 10^0 cells; (J)

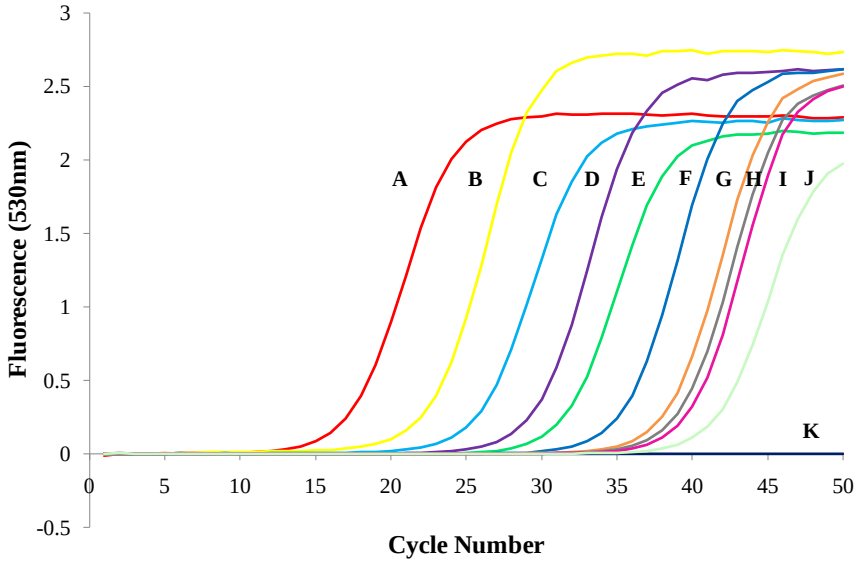

FIG. D.3. Amplification curve generated from serial dilutions of *S.* Typhimurium ATCC 14028 culture with SINV primer set. (A) 10^9 cells; (B) 10^8 cells; (C) 10^7 cells; (D); 10^6 cells; (E) 10^5 cells; (F) 10^4 cells; (G) 10^3 cells; (H) 10^2 cells; (I) 10^1 cells; (J) 10^0 cells; (K) NTC.

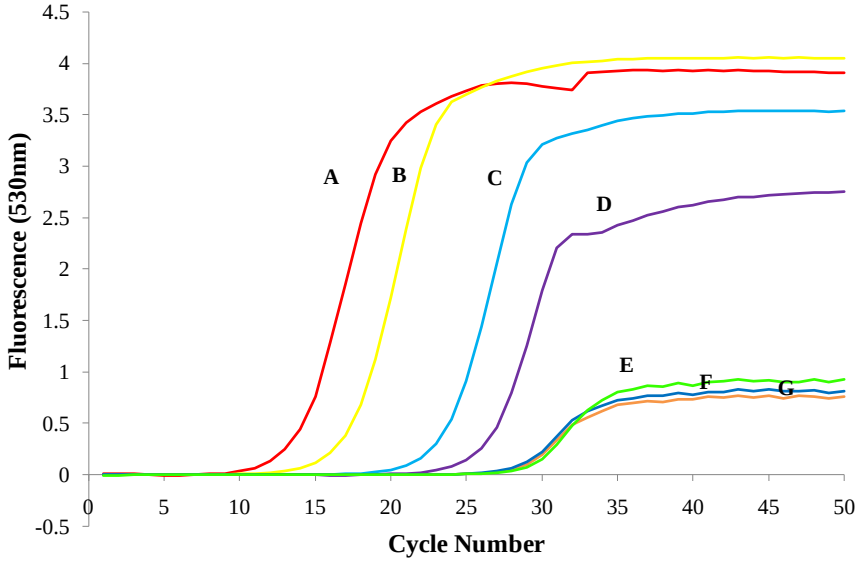

FIG. D.4. Amplification curve generated from serial dilutions of *C. jejuni* NCTC 11168 culture with VS1 primer set. (A) 10^6 cells; (B) 10^5 cells; (C) 10^4 cells; (D); 10^3 cells; (E)10^2 cells; (F) 10^1 cells; (G) NTC.

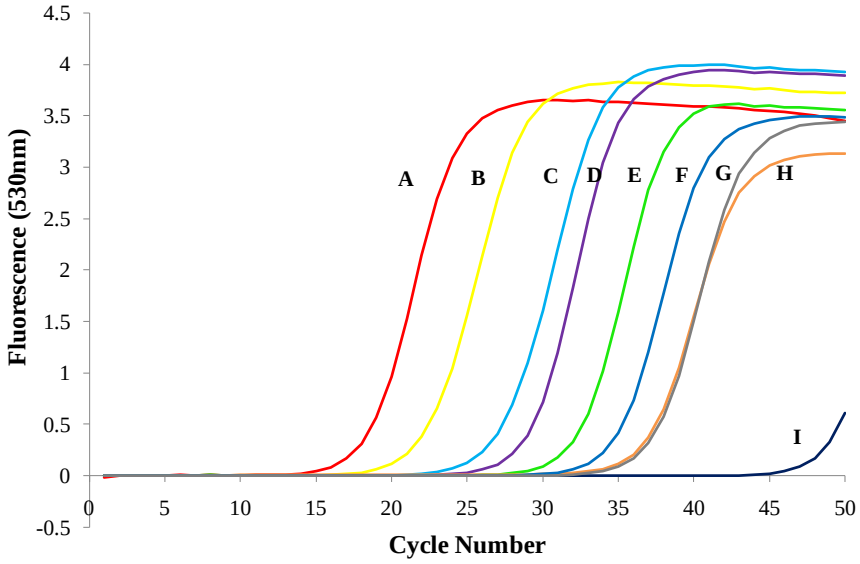

FIG. D.5. Amplification curve generated from serial dilutions of *P. aeruginosa* ATCC 27853 culture with exoT primer set. (A) 10^7 cells; (B) 10^6 cells; (C) 10^5 cells; (D); 10^4 cells; (E)10^3 cells; (F) 10^2 cells; (G) 10^1 cells; (H) 10^0 cells; (I) NTC.

APPENDIX E: CONFIRMATION OF QPCR PRODUCTS BY ELECTROPHORESIS

For each primer set with the exclusion of the VS1 primers, amplicons generated from the qPCR standard curves were confirmed by agarose gel electrophoresis (2%, 120V for 30 min). Slight dimerization can also be seen in the NTC reactions.

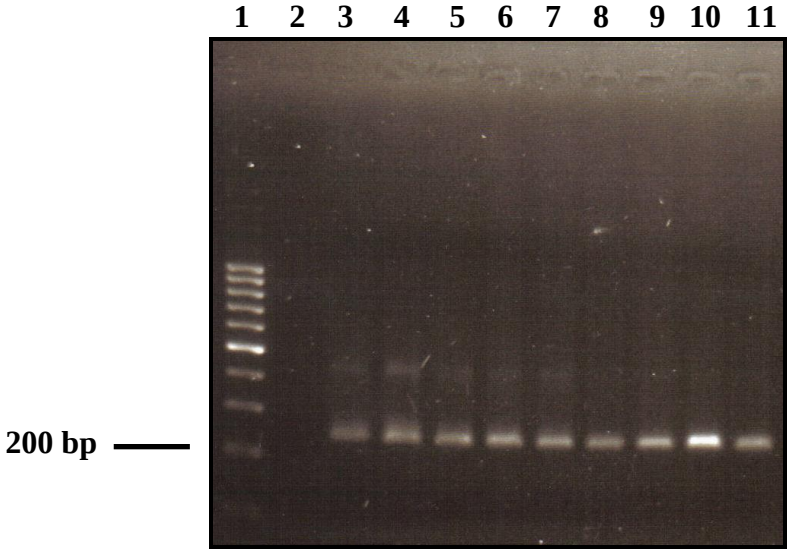

FIG. E.1. Amplicons generated with ipaH2 primer set via qPCR. (1) 100 bp MM; (2) NTC; (3) 10^8 cells; (4) 10^7 cells; (5) 10^6 cells; (6); 10^5 cells; (7)10^4 cells; (8) 10^3 cells; (9)10^2 cells; (10)10^1 cells; (11) 10^0 cells.

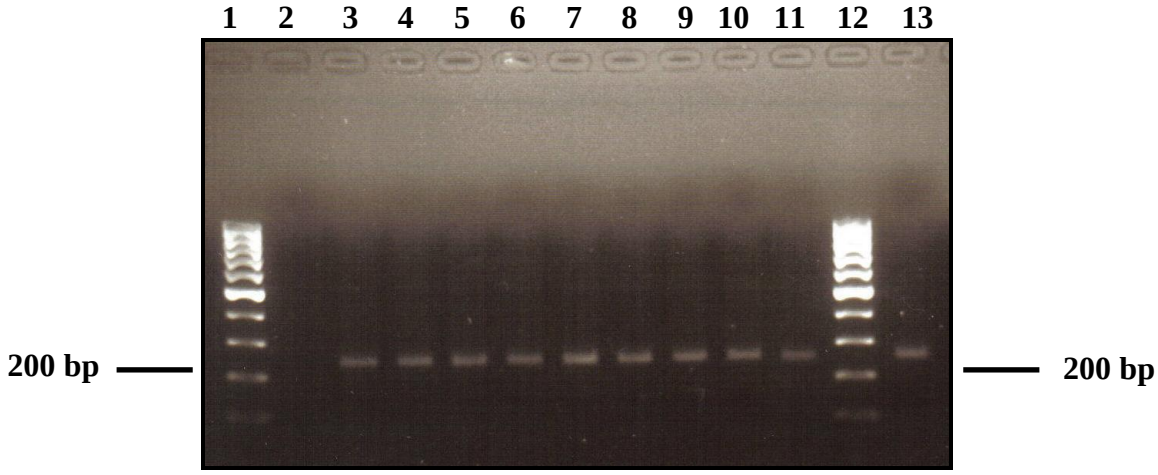

FIG. E.2. Amplicons generated with SINV primer set via qPCR. (1) 100 bp MM; (2) NTC; (3) 10^9 cells; (4) 10^8 cells; (5) 10^7 cells; (6); 10^6 cells; (7) 10^5 cells; (8) 10^4 cells; (9) 10^3 cells; (10) 10^2 cells; (11) 10^1 cells; (12) 100 bp MM; (13) 10^0 cells.

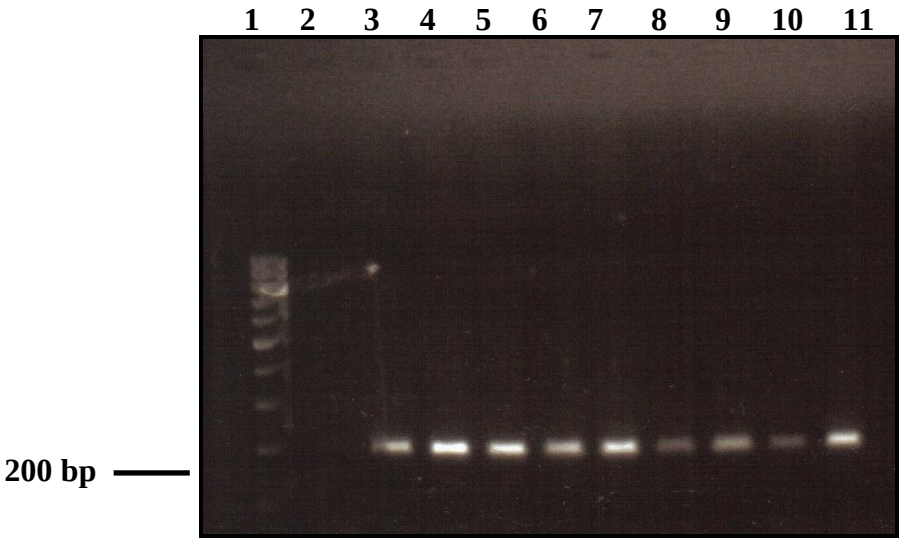

FIG. E.3. Amplicons generated via qPCR with ETIR primer set. (1) 100 bp MM; (2) NTC; (3) 10^8 cells; (4) 10^7 cells; (5) 10^6 cells; (6); 10^5 cells; (7)10^4 cells; (8) 10^3 cells; (9) 10^2 cells; (10) 10^1 cells; (11) 10^0 cells.

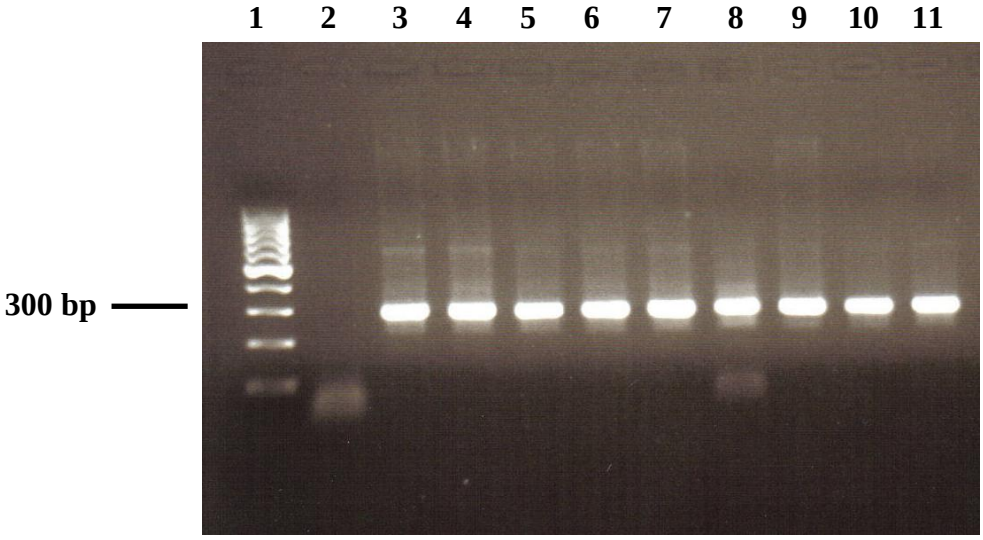

FIG. E.4. Amplicons generated via qPCR with exoT primer set. (1) 100 bp MM; (2) NTC; (3) 10^8 cells; (4) 10^7 cells; (5) 10^6 cells; (6); 10^5 cells; (7) 10^4 cells; (8) 10^3 cells; (9) 10^2 cells; (10) 10^1 cells; (11) 10^0 cells.

APPENDIX F: OPTIMIZATION OF invA PRIMERS FOR NESTED PCR ASSAY

Forward primer sequence: invA-nested F: (5′ → 3′): TGTCACCGTGGTCCAGTTTA
Reverse primer sequence: invA-nested R: (5′ → 3′): CTCGCCTTTGCTGGTTTTAG

A temperature gradient was used to determine the optimal temperature profiles for the novel *invA* nested primer set. Annealing temperatures ranging from 54 to 66°C were tested and 66°C was determined as the optimal temperature, since a single band was observed (640bp), and no cross-reactivity was seen upon secondary amplification with the SINV primer set.

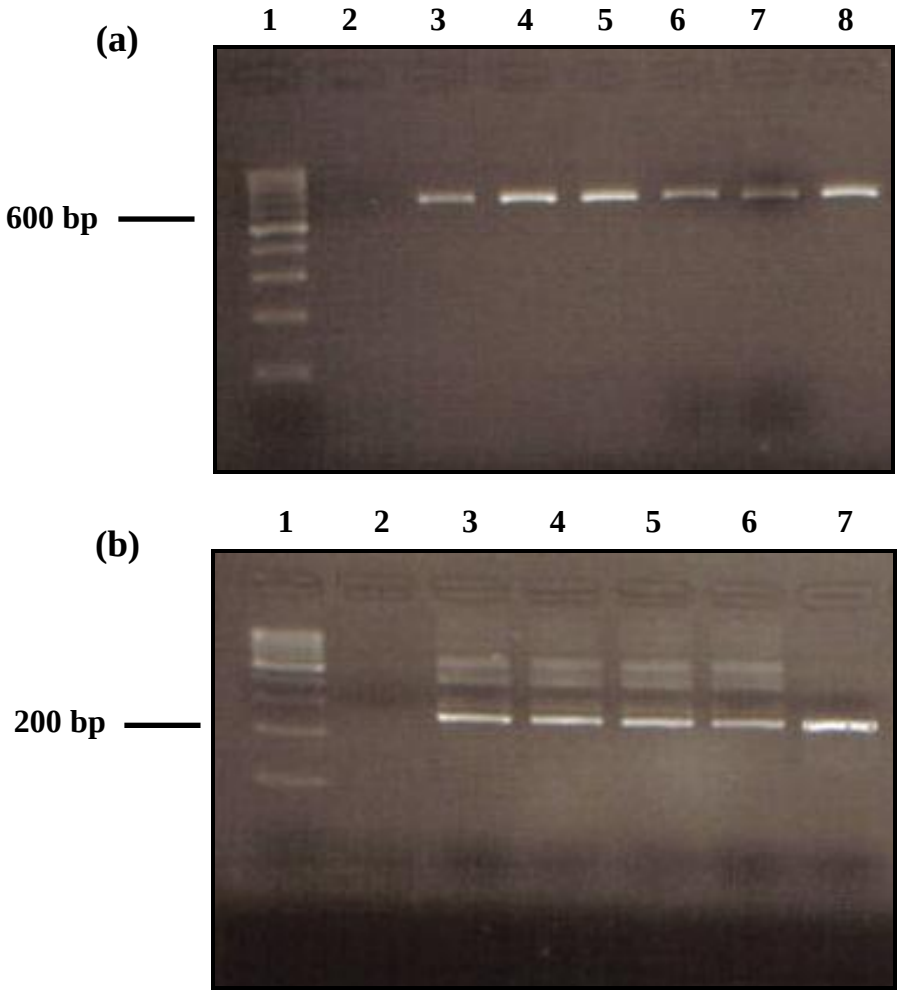

FIG. F.1. Electrophoresis (2% agarose, 120V) of amplicons generated by nested PCR with the invA-nested and SINV primer sets following annealing temperature optimization. **(a)** invA-nested amplification: (1) 100 bp M.M.; (2) NTC; (3) 61°C; (4) 62°C; (5) 63°C; (6) 64°C; (7) 65°C. **(b)** SINV amplification with 2 µL of primary product: (1) 100 bp M.M.; (2) NTC; (3) 62°C; (4) 63°C; (5) 64°C; (6) 65°C; (7) 66°C.