THE UNIVERSITY OF CALGARY

A Molecular Tool for the Detection of Waterborne Pathogens

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF CIVIL ENGINEERING

CALGARY, ALBERTA

DECEMBER, 1998

Abstract

Recent outbreaks of infectious diseases underline the need for improvements in the detection of waterborne pathogens. The development of a species-specific molecular tool offers great potential for the protection of public health. This study involved laboratory investigations and computer analyses of microbial chaperonin 60 (cpn60) genes. Species-specific 600 bp target fragments were amplified and DNA probes developed from several pathogenic eukaryotic and prokaryotic microorganisms. Detection limits were found to be greater than 105 000 cfu/100µL for *E. coli O157:H7*. The identification of *S. typhimurium LT2* in contaminated water samples was successful, as was the identification of *E. coli O157:H7* in wastewater samples. Additionally, the InstaGene DNA isolation method rendered consistent and reliable results. In sum, the detection of waterborne pathogens by DNA probing using the cpn60 ID method provided a species-specific method of microbiological water testing.

Acknowledgments

I would like to express my gratitude to the research team in Donald E. Woods laboratory, Department of Microbiology and Infectious Diseases, University of Calgary, for their unfaltering support and helpful discussions.

For his contributions and insightful ideas, I would like to thank my supervisor, Angus Chu, Department of Civil Engineering, University of Calgary.

I express my sincere gratitude to Donald E. Woods, Scientific Director of the Canadian Bacterial Diseases Network (CBDN), who generously allowed me the opportunity to work as part of his research team. His foresight and support were exemplary.

This project was supported by the Canadian Bacterial Diseases Network, Centres of Excellence Program and a grant from the National Science and Engineering Research Council (NSERC).

Finally, warm thanks to Kelly J. Duregon for her ongoing friendship and support.

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Abbreviations

A	Adenine
A260	Absorbance at 260 nm
A280	Absorbance at 280 nm
bp	Base pair
C	Cytosine
cfu	Colony forming unit
cpn60 gene	Chaperonin 60 gene
DNA	Deoxyribonucleic acid
dNTP	Deoxy-N-5-triphosphate, N={A, G, C, T}
G	Guanine
H279A	Upstream primer
H280A	Downstream primer
I	Inosine
ID	Identification
kb	Kilobase
NA	Nucleic acid
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
16S rRNA	Specific RNA molecule involved in ribosome structure and function
T	Thymine

1. Introduction

Recent outbreaks of infectious diseases in both the developing world and industrialized countries underline the need for improvements to current microbiological detection methods and changes in existing water quality guidelines.

According to the 1997 World Health Report, approximately 17 million people died in 1996 due to infectious diseases and parasitic illnesses (WHO, 1997). Understandably, the strategic importance of water reservoirs and their protection is increasing globally, requiring new technologies and analysis techniques for the provision of access to safe drinking water for communities. In many parts of the world water shortage is a reality, and contamination of both surface waters and groundwater is becoming an urgent international concern (Meakin, 1993). The gravity of this issue has made water contamination and disagreements about water usage and water access rights, the source of increasing tensions both within and between regions and countries (OCED, 1997). In fact, Meakin (1993) estimated that many countries share approximately 200 main rivers and lakes around the globe, and therefore, international pollution control and monitoring is inevitable. In addition, the world's population topped 5.8 billion in mid-1996, and at a growth rate of approximately 80 million people per year, the overall world population will surpass 6.12 billion by the year 2000. Accordingly, the need for water resource protection and water quality evaluation will become even more urgent for communities worldwide (WHO, 1997). Recent research has shown that the majority of water quality violations result from microbial related

contamination, a phenomenon that is both common and global in nature (Zhou, Smith and Stanley, 1997). In fact, the World Health Organization (WHO, 1997) also concluded that microbial quality continues to be the most important factor in the protection of public health, a challenge that is met with safe, potable water. In addition, as demand on the water supply increases, the deterioration and/or lack of infrastructure to support the appropriate treatment and carriage of potable water and sewage will become an unavoidable health concern. Fortunately, the Canadian Federal Infrastructure Works Program implemented in 1995, was aimed at improving and extending existing water and sewer facilities nationwide (Thompson, Gow and Associates, 1995). Furthermore, the introduction of water purification systems, monitoring programs, and educational programs have already resulted in dramatic decreases in water-related disease outbreaks (Environment Canada, 1998). At present, the US EPA recognizes that similar large-scale investment funds are needed in order to modify and the upgrade of existing water purification devices and potable water supplies in the United States. In fact, it is estimated that $ 12.1 billion are needed immediately for both upgrading existing facilities and for the establishment of new treatment plants to protect water supplies in the near future (Weisberg, 1997). Accordingly, vigilance in the development of cost-conscious, effective water monitoring techniques is a critical part of the process to ensure access to safe drinking water and recreational waters around the world. The fact remains that even when traditional detection and surveillance methods are in place, outbreaks of infectious diseases caused by microbiological contamination of water supplies and recreational water occur, confirming the fact that more sophisticated techniques are needed.

For example, the largest disease outbreak correlated to water contamination in the US was reported in 1993/94 where over 400 000 people became ill with cryptosporidiosis. Although indicator counts did not reflect the actual contamination (i.e. fecal and total coliform counts were not above existing water guidelines), an outbreak this extent had never been reported before (Kramer *et al.*, 1996). Not surprisingly, researchers found that there is no significant relationship between indicator organisms and pathogenic species in water (Falcao *et al.*, 1993, Ferguson *et al.*, 1996, Hurst *et al.*, 1997, Lemming and Nichols, 1996, Mroz and Pillai, 1994).

A water-related outbreak of shigellosis reported by Bolstein (1991), in which approximately 65 people were infected with *Shigella sonnei* from swimming, further underlines the need for adequate monitoring systems. In this case, water analyses indicated that fecal coliform counts were in compliance with present water guidelines, and any evidence that sewage contamination had occurred from nearby facilities or from improperly working facilities, could not be found.

The microorganism *Shigella sonnei* cannot be detected by traditional fecal coliform counts, however, recent discoveries and developments in molecular microbiology may provide a new approach for both advancements in water monitoring techniques and the precise detection of microbial contamination.

Traditionally, water quality monitoring techniques have employed indicator organisms (total and fecal coliform bacteria) to estimate water contamination levels. However, the absence of such indicator organisms does not guarantee an acceptable level of water quality (Emde, Smith and Stanley, 1995). The inability of traditional indicator methods to both detect

protozoan cysts and enteric viruses, and prevent waterborne disease outbreaks through the water supply, has prompted study directed at pathogen-specific detection methods.

Recent developments in molecular and medical microbiology have resulted in the application of biotechnology in the field of medicine, the pharmaceutical industry, and in agricultural and food sectors. The medical applications of this technology are primarily twofold; the establishment of new diagnostic tools, and the subsequent prevention of disease. However, the extension of the knowledge gained in biotechnology also offers great potential to other disciplines, and could potentially help solve environmental problems.

In fact, experts at the 1996 Organization for Economic Co-operation and Development (OCED) conference in Mexico concluded that biotechnological detection methods would provide an excellent tool for many microbiological water contamination problems. In addition, the very nature of epidemic outbreak overrides the concept of national boundaries, and requires solutions that are constructed on a global scale. As a result, it is imperative that international organizations be essential players in addressing the detection of waterborne pathogens in the environment (OCED, 1997).

The explosive increase in global economic networking has resulted in increased communication and cross-border trade. This has also substantially increased the circulation of both microbes and potential hosts. The mixing of previously isolated microorganisms and individuals has increased the potential for diseases to spread, and ultimately, this has put public health at significant risk (Kaferstein, Motarjemi and Bettcher, 1997). In fact, recent cases of influenza from Asia have demonstrated that no country will be isolated and protected from such hazards (Logan *et al.*, 1998). Therefore, the development of strategies

and technologies that are applicable on a global scale and consistent with international guidelines, will provide a more encompassing approach to protecting global public health.

Logan *et al.* (1998) stated that existing detection methods for pathogens are inadequate for the detection of specific microorganisms in water. In addition, the problem of pathogen detection has emerged partly because many microbes were newly identified in the last decade, and have not yet been integrated into existing detection protocols. For example, *G. lamblia*, *C. parvum*, and infectious hepatitis have been responsible for many waterborne disease outbreaks, however, detection methods have yet to be established in order to prevent future outbreaks of these infectious diseases. Furthermore, changing water technologies such as water reuse and wastewater recycling, increase the potential health hazards associated with contaminated drinking water and emphasize the need for improvement and development of new detection techniques.

In the same way that a fingerprint is a unique, person-specific detector, small bits of genetic code conserved in microorganisms act as pathogen-specific markers, identifying each microbe as unique. Accordingly, the development of a detection method that can identify species-specific organisms using molecular tools, offers significant potential for quantifying waterborne pathogens in water and wastewater samples. The primary objective of this thesis involved the development of a species-specific detection method and its application to pathogen recognition in water and wastewater.

2. Principles of Molecular Techniques

This section deals with selected molecular techniques utilized in the identification of pathogenic microorganisms.

2.1 Primers

Basically, primers are oligonucleotides that anneal to complementary segments of the template DNA. In general, there are primer designs, in which the sequence of primer bases matches the sequence of the target DNA, and cpn60 primers are those that anneal to conserved sites (Maloy *et al.*, 1996). For the amplification of target fragments, at least one set of primers consisting of an up-stream primer and a down-stream primer has to be present. The up-stream primer binds at the beginning of the variable region on the gene, and the down-stream primer anneals to the end of the target fragment.

2.2 Polymerase Chain Reaction

Polymerase chain reaction is a powerful tool in molecular microbiology and is found in a wide variety of applications in diagnostic and clinical microbiology. This brief overview

outlines some of the basic principles surrounding the generation of DNA probes and polymerase chain reaction (PCR).

The polymerase chain reaction is a technique that enables the multiplication of target fragments, which increases the concentration of these sequences. A thermocycler is utilized in order to provide the appropriate temperature and to facilitate amplification.

PCR amplification involves the use of reagents at defined concentrations and characteristic temperatures. DNA *Taq* polymerase is a highly processive enzyme that functions optimally at temperatures of approximately 75°C to 80°C. It is able to extend characteristically short primers up to 10 kb. DNA *Taq* polymerase is isolated from the thermophilic bacteria *Thermus aquaticus*, and is widely used for PCR amplification, however, many cycles at elevated temperatures decrease its efficiency. As a result, optimization procedures are required in order to find optimal reaction conditions for amplification.

In order to extend the primer sequences with DNA polymerase, a sufficient amount of nucleotides or dNTPs must be present. Typically, the concentrations of each dNTP are in the order of 200 μM, and are added to the reaction mixture. An increase of dNTP concentration increases the error rate of the DNA polymerase, and a decrease of dNTPs increases the amounts of misincorporation of base pairs. Accordingly, the addition of dNTPs requires a balancing of error rate changes to produce an optimal reaction.

An additional reagent that plays a crucial role in PCR amplification is the concentration of magnesium ions (Mg^{++}). The presence of magnesium ions facilitates the action of *Taq* polymerase and influences the denaturing temperature and primer annealing. In addition, the uptake of magnesium ions by the template DNA and the dNTPs must be taken into account

and therefore, the Mg^{++} concentration must be adjusted accordingly. As was the case with the addition of dNTPs, the addition of Mg^{++} results in a decrease in target yield and so these factors must be balanced in order to optimize reaction conditions.

The numbers of cycles influence the efficiency of the PCR amplification. In general, the cycle number is 30, which results in an amplification rate of 10^4 to 10^5 DNA molecules. Of significant importance is the correlation between cycle number and *Taq* polymerase life span, that is, as cycle number increases, *Taq* life span decreases. Ultimately, an increase in cycle number may not necessarily increased product yield.

Before denaturing, annealing and elongation are carried out, and the DNA is denatured in an initial cycle. Then, the three critical steps for amplification of target DNA are carried out (Figure 2.1). First, the double stranded DNA molecules are denatured at temperatures ranging from 94°C to 97°C. Secondly, primers anneal to the target DNA at lower temperatures that are primer specific. Finally, the fragments are elongated in a third step that generally takes place at 72°C. As explained above, the number of cycles influences the fragment yield. After amplification is complete, an additional cycle at 72°C for approximately 10 minutes completes the elongation stage. Finally, samples are held at 4°C in the last cycle.

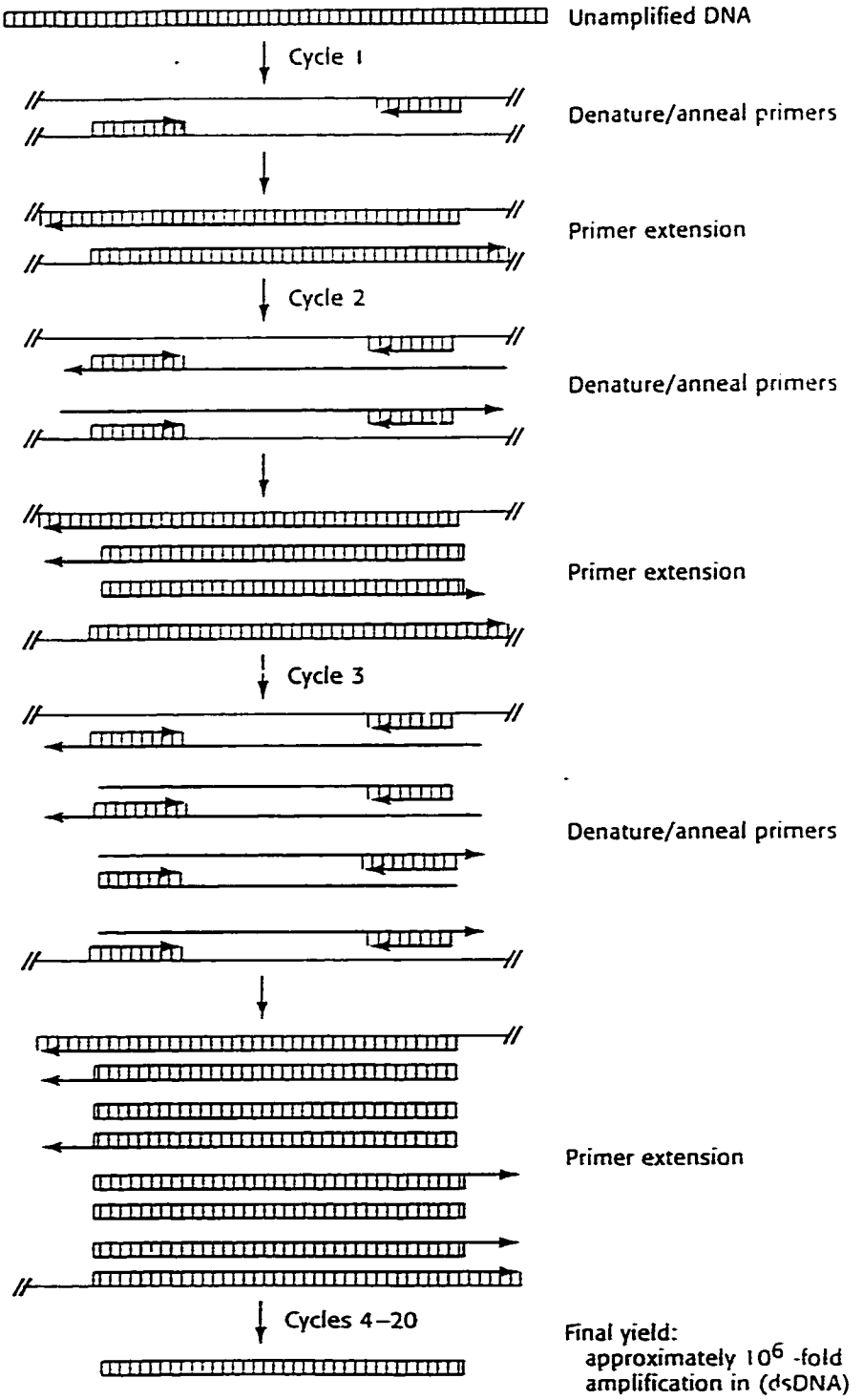

Figure 2. 1 PCR amplification (taken from Maloy *et al.*, 1996).

2.2 Gel Electrophoresis

The separation of negatively charged DNA fragments is done with gel electrophoresis analysis. Applied current and the size of the DNA fragments are the main parameters that influence the electrophoretic separation. Basically, the various sizes of DNA fragments migrate through the gel and separate according to their lenghts at various positions. The longer the segments are, the shorter the travelling distance will be in the gel. The applied current determines the velocity of fragment migration. Visualization of the gel is carried out by ethidium-bromide staining and photography under UV light. To assist in determining lengths of fragments, a molecular size standard is employed to compare the migrated PCR products to fragments of known size. In general, a 1kb DNA ladder is used to determine the product sizes.

2.3 DNA Probing

The utilization of DNA probes allows researchers and laboratory staff to identify species-specific microorganisms in water samples. Basically, these DNA probes are applicable for the detection of bacteria, protozoa, and viruses. The key element of DNA probing is the use of specific genes and their variable or diagnostic regions. These diagnostic regions, as a result of their species-specificity, facilitate the detection of individual pathogenic strains.

In essence, the generation of DNA probes is based on diagnostic sites within a gene. DNA probes are actually copies of a species-specific region in a gene, and consequently, the DNA probes are able to recognize species-specific regions in any prepared sample under appropriate reaction conditions. Many of these DNA probes are already available commercially, and have been applied mainly in the medical community for the identification of pathogenic microorganisms (Olsen, 1991).

The development of a DNA probe from the chaperonin 60 gene, an essential gene present in all living organisms, and the gene segment investigated in this thesis, is outlined in Figure 2.2.

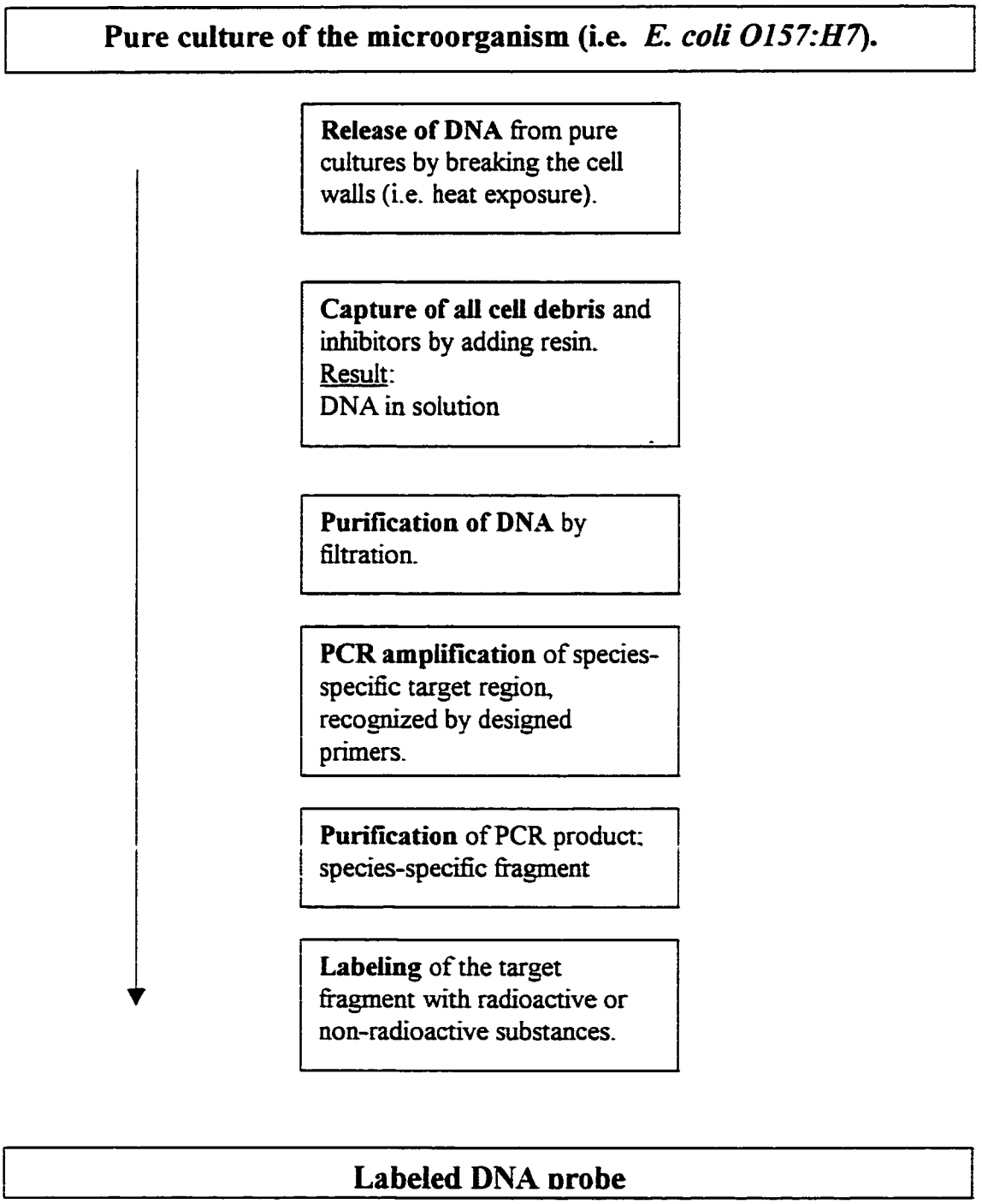

Figure 2. 2 Development of DNA probes.

2.4 Restriction Pattern

The application of restriction enzymes that 'digest' or break long DNA segments into short fragments at defined sites, results in both the identification of specific pathogens and the determination of DNA sequences. Restriction enzymes (i.e. *Hin*dIII and *Eco*RI) produce DNA fragments of specific lengths, that are analyzed on agarose gels. With the application of electrical current, the DNA fragments migrate according to their molecular size through the gel and are separated. The larger molecules travel shorter distances than the smaller fragments. Finally, after staining with a DNA binding compound such as fluorescent ethidium-bromide, bands appear as distinct patterns that can be visualized well under UV light. As such, these restriction patterns are used to differentiate microorganisms (Brock and Madigan, 1991).

3. Waterborne Disease-Causing Microorganisms

In the past, massive outbreaks of cholera, typhoid fever and plagues in Europe and North America have clearly shown the direct relationship between water quality, sanitary facilities and infectious diseases. The occurrence of pathogenic microbes in water supply systems and recreational waters around the world, indicates that water quality monitoring is a crucial element of public health protection. The following summary presents a list of several important waterborne pathogens and their characteristics. The pathogens listed below were investigated in experimental trials, in the development of species-specific DNA probes and polymerase chain reaction (PCR) amplification.

3.1 Bacteria

Bacteria cause many gastrointestinal illnesses and infectious diseases. Some waterborne pathogenic bacteria such as *E. coli O157:H7*, are classified as emerging pathogens, that is, microorganisms that were previously unknown or previously thought to be non-pathogenic in human populations (Morse, 1995). *E. coli O157:H7* gained widespread attention after a severe outbreak in 1993 that was related to undercooked hamburger meat served at a local fast-food restaurant (Feng, 1995). Even though the first appearance of *E. coli O157:H7* was seen in 1982, the transmission route to cattle has yet to be established and is still under

investigation (Potera, 1998). Of great consequence to this investigation, was the identification of contaminated water as an excellent route of transmission for *E. coli O157:H7* (Swerdlow *et al.*, 1992). Luckily, as is the case for most bacterial pathogens, *E. coli O157:H7* is susceptible to chlorination, and can be eliminated in regular water treatment facilities. However, post-treatment contamination could result in its transmission via the drinking water distribution system, as was seen in the Missouri outbreak of 1989 (Swerdlow *et al.*, 1992).

Not surprisingly, *E. coli O157:H7* can also be transmitted by recreational waters. The outbreak of 1991 in Portland, Oregon included 59 people, all of whom had direct contact with the water. It is clear that *E. coli O157:H7* poses a significant health risk, and the fact that *E. coli O157:H7* may persist in water for prolonged periods is of consequence, because of the potential for vector transmission via contaminated water (Keene *et al.*, 1994).

Recently, *E. coli O157:H7* has been labeled one of three major enteric pathogens in Canada (Ng *et al.*, 1997).

Salmonella typhimurium LT2 is another representative pathogenic bacteria that causes infectious disease in humans. Most *Salmonella* spp. are characterized by their low infectious dose of 100 to 1000 organisms when ingested, and their prolonged survival rate in the environment. *Salmonella* spp. are grouped among the top three enteric pathogens in Canada, and it is not surprising that approximately 2 to 3 million infections are caused by *Salmonella* spp. in the US per year alone (LCDC Canada, 1997). In Canada, *Salmonella* spp. infections are thought to affect approximately 2.4% of the population, an equivalent of 627 200 cases per year (Ng *et al.*, 1997). *Salmonella* spp. is mainly associated with

foodborne outbreaks, but many outbreaks in which contaminated water was the primary route of transmission, have also been reported (Angulo *et al.*, 1997).

The group D classification of *Shigella* spp. includes the strain *Shigella sonnei*, the causative agent for many waterborne disease outbreaks. The fatality rate for *S. dysenteriae* is as high as 20% of hospitalized patients, while *S. sonnei* does not cause significant mortality (LCDC Canada, 1997). Approximately 2/3 of all outbreaks around the world are associated with *Shigella* spp., which causes the highest incidence of mortality in children under the age of 10 years (LCDC Canada, 1997). A low infectious dose of approximately 180 organisms (ingested dose) stresses its importance as an identifiable pathogen in water pollution monitoring and detection. In addition, its ability to survive in water for up to 3 days presents a relatively unique public health risk. Many outbreaks of *Shigella* spp. have been reported worldwide. Recently, an outbreak caused by contaminated drinking water in Island Park, Idaho in 1995, was associated with *Shigella sonnei*, and resulted in the infection of approximately 82 people (CDC, 1996).

Outbreaks caused by *V. parahaemolyticus* occur mainly in Japan, Southeast Asia, and North America during the summer months. Its main route of transmission is orally, through ingestion of undercooked seafood that has been contaminated by seawater. *V. parahaemolyticus* has a survival rate of approximately 120 days in shrimp and has been shown to survive for up to 72 days in laboratory swab cultures. A high infectious dose of 10^6 organisms, when taken in orally, causes a variety of intestinal disorders (LCDC Canada, 1997).

Burkholderia pseudomallei is associated with infections occurring in Southeast Asia, mainly in Thailand. Its transmission routes include contaminated soil and water in combination with open wounds, in addition to, inhalation and ingestion of contaminated dust. At present, the infectious dose is unknown, the mortality rate is approximately 50%, and *B. pseudomallei* has been shown to survive in soil for several years. (LCDC Canada, 1997).

In general, bacterial infections cause mild to severe intestinal disturbances, sometimes causing death, and treatment for most species is available in the form of antibiotics. Unfortunately, the antibiotic arsenal is rapidly disappearing with the emergence of resistant strains and therefore, other methods of prevention and treatment are highly sought at present. Appropriately, pathogen-specific recognition and identification of bacterial contamination, would act as a front line prophylaxis, to prevent many of the microbiological infections caused by water contamination.

3.2 Protozoa

The most frequently occurring causative agent in this group is identified as *Giardia lamblia*. *Giardia* spp. cause outbreaks where sanitary facilities are inadequate or absent, and unfiltered drinking water is contaminated by human and animal feces (LCDC Canada, 1997). The low infectious dose of less than 10 cysts, coupled with its long survival rate in cold waters and its resistance to chlorination, makes this pathogen a prime target for many

investigations. Clearly, the occurrence of numerous outbreaks in areas with advanced water treatment, stresses its capacity to pass through water treatment systems undetected, and as a result, *G. lamblia* is a commonly investigated waterborne pathogen.

In fact, a recent study investigated Canada geese as suspect carriers of *G. lamblia*. It focused on the spread of this pathogen to surface water reservoirs across Canada and the US, implicating the birds as mere carriers, rather than infected transmitters, of the pathogen *G. lamblia* in their feces (Graczyk *et al.*, 1998). Given the wide seasonal migration patterns of these birds, these findings have a significant impact on water quality monitoring issues across the continent. This study represents a significant shift away from investigations of infected populations to carrier populations in the search for transmission routes and subsequent disease outbreaks. Accordingly, it comes as no surprise that *G. lamblia* has been identified as the most causative agent in waterborne disease outbreaks in the US (Marshall *et al.*, 1997). Outbreaks of giardiasis commonly occur in travelers and backpackers, who have ingested unfiltered stream waters (Marshall *et al.*, 1997). The specific detection of *G. lamblia* is clearly necessary in order to prevent future outbreaks and to ensure the safety of the water supply.

The first *Cryptosporidium* spp. infection was recorded in 1976. Many frequent waterborne outbreaks have been related to contamination by *Cryptosporidium parvum*, an organism that causes an infection rate ranging between 0.6% and 2% in industrialized countries worldwide (LCDC Canada, 1997). An outbreak of cryptosporidiosis in 1994 was reported in Washington State, where well water contamination was identified as the cause (Dworkin *et al.*, 1996). The frequency of cryptosporidiosis was not reported, and generally not

registered by local or national health departments. In addition, it was apparent that most physicians were not familiar with the identification of *Cryptosporidium* oocysts, and therefore, recognition by health departments did not occur (Marshall *et al.*, 1997). At present, the identification methods available range from microscopic evaluation of stool and water samples to the identification of the organism by PCR amplification of DNA probes, however, to date, a sensitive and accurate detection method for most parasites (including *Cryptosporidium parvum*) does not exist (Jaykus, 1997).

In summary, *Giardia lamblia* and *Cryptosporidium parvum* cause parasitic illnesses, and the detection of such pathogens by water-testing laboratories may not be sufficient (Marshall *et al.*, 1997). In addition, the ability of these microorganisms to survive chlorination of drinking water and their proven survival rates in the environment, are factors that present significant public health risks. New detection methods currently under investigation in both the medical and scientific communities may eventually provide better tools for the evaluation of surface, ground, and drinking water.

3.3 Viruses

There are several hundred known viruses of potential health risk that are associated with contaminated water environments. Recent studies have indicated, however, that little information is available about the correlation between viral contamination and potential

health threats to the public (Rose, 1997). At least one study found that approximately 20 million cases of waterborne virus infections causing 2010 deaths were observed worldwide in one year (Bennett *et al.*, 1987).

Between 1974 and 1987, approximately five virus related outbreaks occurred in Canada, affecting approximately 229 people. Norwalk virus and hepatitis A virus (HAV) were the main causes of these outbreaks. However, hepatitis E viruses (HEV) and rotaviruses have also been associated with waterborne disease outbreaks, and in fact, many of these viruses are found in contaminated groundwater (Health Canada, 1995). Despite these findings, in its summary of the Candidate Contaminant List (CCL) for microorganisms, the US EPA (1998) determined that rotavirus, HAV and HEV should not be listed on the CCL, because new water guidelines and treatment requirements will effectively solve the risk of infectious diseases caused by these viruses. However, coxsackie viruses, echoviruses, adenoviruses, and caliciviruses will be listed on the CCL, because recent research has shown that viral resistance to disinfection and subsequent detection in treated waters has been documented. The US EPA recommended that intensive research be carried out in order to determine the behaviour of such pathogens, and until such information is available these viruses should be listed on the CCL (US EPA, 1998).

Finally, the risks associated with viral infections exceed those associated with gastrointestinal diseases, due to the potential for further, more serious chronic conditions to arise (Rose, 1997). A recent study indicated that coxsackie B viruses were associated with myocarditis (inflammation of the heart muscle), a finding that represents a relatively new perspective on mortality due to heart disease. Given that approximately 41% of elderly

people die as a result of various heart diseases, this discovery may link viral infections to serious chronic conditions (Klingel *et al.*, 1992). In summary, viral resistance to disinfection and month long survival rates in groundwater are characteristics that demonstrate potential health risks, and future research should be directed at determining both the extent and the effects of viral contamination in the environment (Rose, 1997). Unfortunately, the cpn60 ID method is based upon the presence of conserved genomic sequences that exist in pro- and eukaryotic organisms only, thereby excluding viral detection from the set of potentially detectable pathogens in environmental waters.

4. The Bacteriological Evaluation of Water

Indicator organisms, such as coliform bacteria, are widely used for the evaluation of water and the determination of possible fecal contamination. Coliform tests are also employed to determine water quality in recreational, shellfish harvesting, and drinking waters (Toranzos and McFeters, 1997).

Basically, the coliform tests are categorized into two main classifications. The first group, named the total coliform group, consists of aerobic and facultatively anaerobic, gram-negative, non-sporeforming, rod-shaped bacteria that ferment lactose with gas and acid production in 24 to 48 hours at an incubation temperature of 35°C (Toranzos and McFeters, 1997). This group includes microbes such as *Escherichia coli*, *Klebsiella* spp., and *Citrobacter* spp. Total coliform tests are utilized to determine fecal contamination or water impurities from wastewater effluents, however, the presence of coliform bacteria from other sources such as plant materials and the wood industry could result in false-positive enumerations. Therefore, an important aspect of the total coliform test is its interpretation, which represents a significant contribution to the final evaluation of a water environment. The second group, labeled the fecal coliform or thermotolerant coliform group, are defined by exactly the same criteria as the first group, with the exception of lactose fermentation with gas and acid production at elevated temperatures. Accordingly, the determination of fecal coliforms is carried out at an incubation temperature of 44.5°C. Again, the interpretation of fecal coliform counts must take into consideration the diverse array of

living environments, both fecal and non-fecal in origin, that *E. coli* thrives in (Toranzos and McFeters, 1997).

4.1 History of the Coliform Test

The Drinking Water Standards set out by the US Federal Security Agency in 1946, included the determination of coliform bacteria according to the Standard Methods for Water Analysis, the American Public Health Association, and the Public Health Services Drinkling Water Standards (Simmons and Gentzkow, 1955). As early as 1955, the coliform test and the membrane filter technique (MF) provided the foundation for the bacteriological evaluation of water. In fact, early analyses described membrane filtering as a reliable bacteriological analysis of water (Simmons and Gentzkow, 1955).

In epidemiological studies done on the Ohio River, Lake Michigan, and Long Island Sound, Stevenson (1953) found that 1000cfu/100mL of total coliform bacteria could be used as a standard for recreational water.

In the US, the Federal Water Pollution Control Act and the Water Quality Act of 1965 both made water use categorizations the basis of upon which water quality standards were set (Gallagher and Spino, 1967). For example, these standards determined that non-contact recreational waters used for boating could have coliform counts of up to 5000cfu/100mL. Unfortunately, such numbers had no epidemiological basis nor did any bacteriological study

support these standards, and as such, these coliform values were random evaluations of water quality.

Additionally, Gallagher and Spino (1967) concluded that coliform determination was the best technique for the evaluation of water quality, although it was found that *Salmonella* spp. were the only species clearly correlated with coliform concentrations. Further investigations demonstrated that the most accurate determination of fecal pollution from warm-blooded animals at that time could be carried out by the coliform test. (Geldreich, 1970). In 1979, the US EPA introduced a new program called 'Health Effects - Recreational Water Quality Standards' and they determined that an appropriate microbial indicator should be used to determine water quality (Campbell *et al.*, 1979). Carter *et al.* (1987) concluded that the fecal coliform test and total coliform enumeration from water samples are routinely used to estimate potential health risks. At that time it was not clear, however, if a relationship between pathogenic microorganisms and indicator counts actually existed. This trend has persisted, as demonstrated by Keefe and Green's (1989) confirmation that the enumeration of fecal coliforms was, in fact, the standard method for microbiological evaluation of water environments. Even at this point, no relationship between indicators and pathogens had been established.

Discussions by Falcao *et al.* (1993) evaluated the significance of fecal coliform bacteria in pollution determination and subsequently concluded that this test was strongly correlated with recent fecal contamination. However, the investigators could not correlate the presence of indicators with the occurrence of pathogens. More recently, Leeming and Nichols (1996) stated that the reluctance to both adopt new standards for pathogen

determination and establish more appropriate techniques, due to the lack of epidemiological evaluation of present microbial indicators. At present, the US EPA, the World Health Organization, and the European Economic Community recommend that drinking water and recreational water be evaluated by the coliform indicator method (Toranzos and McFeters, 1997). However, a significant correlation between such indicator organisms and the presence of pathogenic microorganisms has not been established (Falcao *et al.*, 1993, Hurst *et al.*, 1997, Leeming and Nichols, 1996, Mroz and Pillai, 1994).

In summary, the standard protocols for evaluating drinking and recreational water by total and fecal coliforms do not effectively address the potential public health risks associated with microbiological water contamination.

5. Environmental Regulations and Guidelines

In general, regulations and guidelines for water pollution protection are based on scientific knowledge about the individual properties of specific substances. However, regulation of maximum concentration levels (MCL) and the lack of data reflecting correlations between potential health hazards and substance concentrations, make a practical application to actual regulations and guidelines difficult at best (Environment Canada, 1995).

Valiante (1993) discussed the limitations of standards-based pollution control and stressed one major issue. He pointed out that standards-based evaluations focus on environmental damages that have already occurred, rather than on the source of pollution. Future policy must involve prophylactic solutions that incorporate the latest technology available, in order to achieve effective environmental and occupational health protection. Clearly, standards have to be dynamic and involve the incorporation of recent technological advances.

Finally, pollution control is and will continue to be a dynamic learning process, and consequently, flexibility in regulatory bodies will be essential in order to adopt new findings from other disciplines with ease and optimally regulate state-of-the-art policies. Furthermore, the integration of environmental law in conjunction with economic growth and development will ensure that it becomes a founding element in future societies.

5.1 Global Perspectives

Due to the global nature of environmental issues, international collaboration in the area of environmental protection and disease prevention is absolutely essential to the establishment of a surveillance network worldwide. The ultimate goal of such connections is, effectively, the global regulation of pollution and contamination. In accordance with such directives, the United Nations Environmental Programme Report of 1990 (United Nations, 1990), illustrated the effectiveness of international co-operation associated with environmentally related issues. In this report, the specialized role Non Governmental Organizations (NGOs) play in local, national, but mainly international environmental issues was shown. In addition, the ongoing co-operation between other international organizations such as the Organization for Economic Co-operation and Development (OECD), the Council for Mutual Economic Assistant (CMEA), the European Economic Community (EEC), the Association of South East Asian Nations (ASEAN), the Organization of African Unity (OAU), and the Gulf Co-operation Council, demonstrate the increasing desire for global solutions.

Historically, the first NGO that dealt with environmental issues was established in the nineteenth century, as public concerns arose over damage to the environment and the global disappearance of wildlife species. In the 1960s and 1970s the growth of NGO activities developed in concert with increasing public awareness of and interest, in environmental issues.

Recently, the Earth Summit of 1992 in Rio de Janeiro, Brazil represented the first of a series of international conferences addressing international awareness of and concern for, environmental pollution. The Earth Summit II of 1997 in New York, USA , was a major international platform for experts to discuss possible solutions for water pollution and drinking water issues. According to recent estimates by the UN, approximately one billion people around the world lack access to safe drinking water, and appropriately, the timing of this summit as a forum to address this urgent concern was no coincidence (United Nations, 1997). It is clear that improvements in water and wastewater technology are needed on a global scale in order to increase the population served by water and wastewater facilities and decrease the rate of infectious diseases cause by contaminated water. Given the historically dynamic nature of regional and national politics, the accomplishment of such directives may eventually require the participation, co-operation, and leadership capabilities of NGO's.

5.2 The Status of Canadian Water

Water consumption in Canada ranks second largest in the world with respect to water volume per capita, a consumption that reaches approximately 326 liters per capita, per day (Environment Canada, 1998). Industry, agriculture, transportation, energy and recreational users, in addition to municipalities comprise the main sources of water consumption in Canada. Approximately 9% of the world's renewable fresh water can be found in Canada,

however, most water flows occur in northern directions, which is opposite in direction to the location of urban centres. As a result, water scarcity occurs primarily in southern regions and water protection and management will become very important in order to ensure stable and safe water supplies for growing communities. Public health hinges on clean, potable water, and therefore, water needs to be protected. Appropriate quantification systems could improve the optimization of water monitoring and assist in water reservoir protection.

It is estimated that in some Canadian regions, drinking water quality and sanitation facilities are below average, falling short of World Health Organization guidelines. A direct effect of this shortfall is an increased risk of water related diseases, and subsequently, an increased risk to public health (Meakin, 1993).

Recently, global and local interests in public health and environmental concerns have caused a positive change to legislated protective laws (e.g. environmental issues in international trade agreements). Unfortunately, the lack of international collaboration with respect to water reservoir and groundwater protection, complicates cross-border enforcement of environmental laws (i.e. Canada-US border). In addition, the investigation of environmental damage in other countries is difficult to prosecute when agreements are not in place.

A recent inventory of global pollution indicated that the quality of fresh water and marine water has decreased, and the need for environmental protection programs has become essential to provide access to safe drinking water. A growing global population, coupled with increased socio-economic growth and global connectedness, are realities that must be addressed with more stringent regulations via environmental legislation. Lundqist (1992)

captured these ideas simply, by defining two main issues surrounding water management: firstly, the protection of water reservoirs, and secondly, the optimization of water utilization. Furthermore, water management should focus on demand strategies rather than on supply-based strategies. Environmental concerns about increased utilization of water resources have intensified over the past decades to the point where exploration of new water supplies is politically risky. In addition, the restrictive costs incurred through the funding of new water facility projects make supporting such endeavors by governments, virtually impossible (Dzieglewski and Baumann, 1992).

5.3 Federal Water Legislation

In Canada, water protection is a very complex but shared issue. As such, responsibilities are divided between Federal and Provincial/Territorial Legislation. Under the Canadian Constitutional Act of 1982, the provinces were made responsible for both surface and groundwater, in addition to flow regulations, water use development, pollution control, thermal and hydro-electrical power development, and finally, they were given the authority to legislate in the areas of water and supply. At present, Canadian Water Quality Guidelines are developed by the Federal Government, however, these guidelines are not enforceable. Federal Drinking Water Standards were adopted into the legislation of only two provinces in Canada, namely, those of Alberta and Quebec (Droste, 1997). Primarily, it is the Canadian

Environmental Protection Act (CEPA) and the Fisheries Act that act as the main framework, regulating and standardizing environmental protection in Canada. The relatively new Canadian Environmental Protection Act was first proclaimed in 1988, and has since been reviewed in 1995. As a result of this review, the Standard Committee recommended that more federal government involvement in pollution prevention was required, in order to protect both Canadian health and the environment (Environment Canada, 1997).

In contrast, The Fisheries Act is the oldest environmental protection act in Canada. It regulates any physical alteration of fish habitats, ultimately providing for the protection and conservation of those habitats. Both the CEPA and the Fisheries Act are federal legislation, and therefore, apply nationwide to water related issues. Federal jurisdiction also applies to boundary and trans-boundary waters. Additional federal laws protect the National Parks, territories and reserves found in Canada.

Finally, there are regulations in place to support these sometimes complicated interactions between federal, provincial, and interprovincial jurisdictions with respect to water issues, agriculture, significant national water issues, and health (Environment Canada, 1997).

Historically, the collaboration between federal and provincial governments in the sector of drinking water quality was established over 20 years ago. The Federal-Provincial Committee on Environmental and Occupational Health consists of representatives from both regional/federal health departments and environment ministries, and involves drinking water quality issues. The national guidelines include microbiological, chemical, physical and radiological parameters and their maximum acceptable concentration levels for drinking

waters. Territorial and provincial governments (Environment Canada, 1998) carry out the implementation of individual regulations and guidelines.

5.4 Water Contamination in Alberta

As water demand rises, water quality becomes more of concern in establishing water management strategies (APE, 1997).

Planning, developing and managing water resources are essential factors for water utilization in irrigation, hydro-electric devices and/or flood protection (APE, 1997). In addition, inter-provincial and international agreements for water sharing, water quality control, pollution evaluation and prevention should be based on a network of measurement controls and enforcement of established standards and guidelines by federal legislation.

In the Province of Alberta, the Environmental Protection and Enhancement Act (EPEA) outlines standards and guidelines for environmental quality control. Industrial and municipal effluent guidelines and standards were originally set out by the Alberta Environmental Protection Agency and continuously incorporate provincial, federal and international surface water quality guideline changes.

According to the Alberta Ambient Surface Water Quality Interim Guidelines, raw water that is to be used for drinking purposes after treatment, or for non-contact outdoor recreational uses, should not exceed 5000 total coliform bacteria per 100 mL and not more than 1000

fecal coliform bacteria per 100 mL in 90% of all samples. In this case, a definition of the treatment facility required to appropriately deal with such raw water limits is essential, because chlorination alone is ineffective for most parasites which are also not predicted by fecal or total indicators (Marshall *et al.*, 1997). In addition, conventional state-of-the-art methods of wastewater treatment never produce totally safe final effluents (Mascher *et al.*, 1996). That is, wastewater treatment effluents contaminate receiving waters to some extent with pathogens. Consequently, improvements to existing regulations and guidelines based on the most recent research findings will be unavoidable in the future, in order to sufficiently protect public health.

Bacteriological contamination guidelines for direct-contact recreational waters and crop irrigation require not more than 1000 total coliforms per 100 mL and not more than 200 fecal coliforms per 100 mL in more than 5 samples taken over a 30 day period. Overall, 2400 total coliforms per 100 mL should not be exceeded any time (AEP, 1997).

Unfortunately, effective guidelines for viruses and protozoa do not exist at this time. Regardless, the Summary of Guidelines for Microbiological Parameters published by the Environmental Health Directorate, Health Canada (1996) concluded that both viral and protozoal contamination should not be present in drinking water environments, however, this directive will fail to be upheld until a set of effective guidelines based on solid scientific study is developed.

6. Indicators and Disease Outbreaks

Coliform bacteria indicators were introduced several decades ago. Many authors have observed that a correlation between pathogenic microorganisms and the presence of indicator bacteria could not be established, and that positive coliform test results, and their interpretation required careful evaluation in order to estimate public health risks. (Falcao *et al.*, 1993, Hurst *et al.*, 1997, Leeming and Nichols, 1996, Mroz and Pillai, 1994).

6.1 1997 World Health Organization Report

The recent publication of the World Health Organization's 1997 World Health Report, noted that 2.5 million people died in 1996 as a cause of water related diarrhoeal diseases. Waterborne pathogens are a major cause of diarrhoeal diseases worldwide (Toranzos and McFeters, 1997). Furthermore, over 17 million deaths were due to infectious or parasitic illnesses in 1996. In addition, infectious and parasitic diseases caused 43% of the 40 million deaths that occurred in developing nations. In developed countries, the increasing number of infectious diseases and parasitic illnesses are due, at least in part, to international travelling, tourism and trade. According to WHO (WHO, 1997), infectious diseases are correlated to non-communicable diseases, specifically cervical, gastric and hepatic cancer. Such findings have prompted a call for global efforts to prevent, treat, and cure infectious diseases. In addition, the 1997 World Health

Report listed international concerns by priority, and noted that disease control could be carried out through proper disease detection and management, in addition to improvements in detection methods for pathogen identification (WHO, 1997).

6.2 Disease Outbreaks from Recreational Waters

Swimming related illnesses due to bacterial infections are a major cause of gastroenteritis and shigellosis (Bolstein, 1991, Toranzos and McFeters, 1997). A 1989 shigellosis outbreak in Oakland County, MI was correlated to the presence of *Shigella sonnei* in a recreational park pond in which the transmission route was identified as contaminated water and associated with swimming in the pond (Bolstein, 1991). Furthermore, a study of the available indicator test results from the pond water presented no evidence of fecal coliform or total coliform counts above the US EPA standards for recreational waters. It was concluded that the negative test results of fecal coliforms did not determine that the swimming water was pathogen free. Simply put, the standards and guidelines for recreational waters at that time were not based on epidemiological studies and the correlation between indicator bacteria and gastrointestinal diseases could not be established (Bolstein, 1991). More sensitive indicators and appropriate guidelines are much needed to prevent such incidents in the future.

6.3 Drinking Water Contamination

In related studies, Mroz and Pillai (1994) examined bacterial populations found in groundwater in the El Paso County region of Texas, USA. It should be noted that groundwater is the main source of potable water for individuals living on the US Mexico border in El Paso County. A total of 73 wells were included in this study, and according to the results, no correlation between indicator bacteria and pathogens was established. Due to the fact that water is a very scarce resource in El Paso County, access to safe drinking water is a critical issue that requires effective protection. The researchers found that both false positive results and false negative results occurred in tests performed on well water samples. In fact, indigenous microorganisms grew in test media, indicating fecal pollution, even though no waste discharges occurred. The coliform test did not detect the presence of pathogenic organisms such as *Aeromonads* spp. in groundwater and resulted in false negative samples.

Is this study, 13 wells were found to be polluted with fecal contaminants as indicated by fecal coliform counts, and therefore, a potential health risk existed. Furthermore, using a selective isolation method, they found pathogenic bacteria in 34 wells in El Paso County. These differential results indicated that 34 wells were potentially polluted, although fecal coliform counts were not above US EPA guidelines.

To date, the determination of indicator, bacterial, and pathogenic movement through the groundwater domain has not been undertaken. Different growth and die off rates, in addition to different organism shapes and sizes could result in significant microorganismic concentration gradients in shallow wells and could cause infectious disease outbreaks (Morz and Pillai, 1994).

In another example, Arvanitidou (1991) found no correlation between *Campylobacter jejuni* and indicator microorganisms. An analysis of 364 drinking water samples found 220 samples to be clear of coliform bacteria, however, *Campylobacter* spp. were isolated. As a result, drinking water contamination was found, but again, the indicator did not show significant counts. As a result, Arvanitidou (1991) concluded that a test for *Campylobacter* spp. must be incorporated into drinking water evaluations to ensure the prevention of waterborne outbreaks and secure access to safe drinking water, especially where chlorination is not standard practice in water treatment facilities.

In addition, Brock and Madigan (1991) pointed out that *Cryptosporidium* spp. and *Giardia* spp. are significantly resistant to chlorination and that many giardiasis outbreaks are related to drinking water supplies that are only equipped with a chlorination stage of treatment. Protozoa survive beyond free chlorine concentrations that are effective in killing off free bacteria cells in water (King *et al.*, 1988). It is estimated that 4 % of the US population is asymptomatically carrying *Giardia lamblia*. However, rapid growth within the body most commonly causes diarrhea, dehydration, mucous secretion, and flatulence (Atlas, 1995).

In a similar investigation, Goldstein *et al.* (1996) observed an outbreak of cryptosporidiosis in Nevada, although the state-of-the-art water treatment facility there produced drinking water that met the current standards for potable water. According to the authors, this outbreak clearly demonstrates the need for regulations that include organisms that cannot recovered by existing test (Goldstein *et al.*, 1996).

Congruent studies in the Yukon, found that giardiasis and cryptosporidiosis outbreaks occur on a regular basis (Roach *et al.*, 1996). In fact, the Whitehorse water treatment facility had

occasionally contained water contaminated with *Giardia* spp. and *Cryptosporidium* spp. In the Yukon, chlorination continues to be the only stage used in water treatment devices and therefore, *Giardia* spp. and *Cryptosporidium* spp. were not eliminated by treatment intervention. It is not surprising that an endemic infection rate of 0.1% was found, although this rate was deemed insignificant by the author in terms of public health concern (Roach *et al.*, 1996). More accurate detection techniques could improve existing treatment facilities and produce valuable data for the introduction of new guidelines.

A 1994 outbreak of cryptosporiosis occurred in a Washington community that received drinking water from deep wells. A significant relationship between increasing water consumption and illnesses was found. Furthermore, the cause of drinking water contamination was found to be occurring through a defect pipeline containing wastewater. In light of this finding, Dworkin *et al.* (1996) concluded that standard water testing should be performed on a regular basis for deep drinking water wells, because these findings actually showed that pathogenic contamination may travel far distances and eventually, into groundwater. In addition, Dworkin *et al.* (1996) proposed that drinking water wells be inspected for structural defects and surface contamination as part of a scheduled maintenance routine program.

In 1991, 44 residences in London, United Kingdom were infected by *Cryptosporidium* spp. In keeping with epidemiological findings, a significant relationship between quantity of water consumption and illnesses was established. No irregularities in the drinking water system had occurred at the time, and in addition, the standard indicator failed to detect the presence of contamination by *Cryptosporidium* spp. (Maguire *et al.*, 1995). Overwhelmingly, the evidence

suggested that present standards for bacteriological contamination detection should be replaced by more accurate technologies, in order to prevent such public health incidents.

By far the largest documented outbreak in the US occurred in 1993, in Milwaukee, Wisconsin where approximately 403 000 people were infected by *Cryptosporidium* spp. through contaminated drinking water (Osewe *et al.*, 1996). Individuals who consumed municipal drinking water developed cryptosporidiosis, even though routine testing in the form of coliform tests were preformed in the water treatment plant on a regular basis (i.e. several times a day). In the Milwaukee outbreak, the water screening technique failed to indicate hazardous contamination of drinking water by an undetectable pathogen, and widespread cryptosporidiosis was the end result. Clearly, specific pathogen testing and additional drinking water regulations can limit such incidences. Advanced molecular microbiological detection methods are an example of better detection tools that are capable of rapidly identifying microorganisms.

7. 16S rRNA vs Cpn60 ID Method

In the past, the main focus of the detection of waterborne pathogens by polymerase chain reaction (PCR) analysis and DNA probe development, involved the 16S rRNA gene targets. The discovery of the cpn60 ID method as an alternative to the 16S rRNA method has been a valuable contribution to detection methodology. The following literature review deals with the pros and cons of investigating with the 16S rRNA detection method versus the cpn60 ID method.

Detection methods are available for many microbes, however, 16S rRNA and cpn60 ID techniques seem to be the most applicable for the detection of waterborne pathogens in both water and wastewater environments. These species-specific detection methods are based on specific genes of microorganisms in addition to gene sequences that contain species-specific diagnostic fragments. The specific genes that are utilized in the 16S rRNA ID method occur in high numbers in prokaryotic cells (Göbel *et al.*, 1987). However, the identification of pathogenic microorganisms by 16S rRNA genes is labour intensive, and this specific region has been shown to undergo evolutionary changes that inevitably led to false-positive and false-negative test results (Goh *et al.*, 1996). In addition, 16S rRNA sequences have only been found in prokaryotic cell structures, and therefore, the identification of only prokaryotic microorganisms, specifically bacteria, can occur with this detection method (Atlas, 1995).

Alternatively, the well-conserved DNA regions on the cpn60 gene allow for species-specific pathogen identification through stable diagnostic sites. As a result, the detection of cpn60 genes can be carried out on both prokaryotic and eukaryotic microbes, because the cpn60 gene is found

in all cell structures (Watson *et al.*, 1987). This is important because this one method alone is able to identify bacteria, algae, fungi, and protozoa.

In addition, it was observed that evolutionary changes did not significantly affect the combination of conserved and variable regions on the cpn60 gene, and therefore, the detection of waterborne pathogens based on their cpn60 gene sequences will provide consistent and accurate results (Goh *et al.*, 1996). Still, it should be noted that the dynamic processes of evolution play an important long-term role in the development of any genetic DNA probe. These probes, while perhaps temporally specific, are conserved genetic units. In sum, evolutionary changes fit well with the dynamic investigative nature of the PCR amplification technique.

Additionally, the identification of enteric microorganisms based on 16S rRNA genes requires the application of several restriction enzymes in order to produce species-specific patterns. The investigations involving the differentiation of various *Campylobacter* spp. carried out by Cardarelli-Leite (1996) were done prior to PCR amplification with specific primers, followed by six different restriction enzyme digestions, in order to produce species-specific DNA patterns. The species identification carried out by Goh *et al.* (1996) was based on the amplification of the cpn60 gene and one set of primers that produced species-specific probes for the detection of bacteria.

In related analyses, Wilson *et al.* (1990) investigated the amplification of bacterial 16S rRNA genes by PCR analysis. The specific primers utilized in this experiment showed that self-amplification of the primer 3′-OH ends occurred, leading to absence of target DNA diagnostic region amplifications. In addition, the secondary structure of the rRNA and its very complex components led Watson *et al.* (1991) to conclude that future research must recognize and deal

with this problem. Furthermore, complex gene structures require an increased denaturing temperature during the PCR cycling process in order to facilitate primer annealing processes (Innis *et al.*, 1995). Such elevated temperature exposure, however, reduces the period of enzyme activity and ultimately results in less yield of target fragments (Maloy *et al.*, 1996).

A recent study involved the use of the restriction enzyme *Hin*dIII to produce species-specific patterns for *Salmonella* spp. and discriminated *Salmonella typhimurium* but did not differentiate among other *Salmonella* spp. (Patton *et al.*, 1991). This investigation included the digestion of several bacterial strains with three restriction endonucleases (*Hin*dIII, *Bgl*II, and *Hho*I). Ultimately, the use of additional investigative procedures prolongs laboratory analyses and requires additional reagents that might be costly.

Similar analyses carried out by Thompson-Carter (1989), indicated that the digestion of 16S rRNA genes with *Hin*dIII and *Eco*RI produced sufficient differentiation. However, the digestion of target fragments from different strains with *Hin*dIII and *Eco*RI produce different pattern characteristics, and therefore, cannot be compared.

Goh *et al.* (1996) demonstrated that seven of 20 clinical isolates were misidentified by conventional screening methods, which are based on phenotypic characterization for the detection of coagulase-negative *Staphylococci* spp. More importantly, the analysis of the cpn60 genes revealed phylotypic information that could not be ascertained with the 16S rRNA-based method. Furthermore, Goh *et al.* (1996) designed a set of cpn60 primers that could amplify a species-specific fragment from 29 *Staphylococcus* spp. and 30 other bacterial isolates. It is these two primers and this amplification process that were found to be the most appropriate route of investigation for the detection of pathogenic microorganisms in water and wastewater

environments. It should be noted that further investigations are necessary to confirm and elaborate on the findings from Goh *et al.* (1998, 1997, 1996) and the results outlined in this investigation, and in effect, extend the list of species-specific probes.

Both the digestion, amplification of 16S rRNA genes and the amplification of species-specific DNA probes by the Cpn60 ID method represent powerful molecular tools in the medical community. The identification of pathogens in water and wastewater with these techniques could potentially open new avenues in setting more appropriate water guidelines that are based on the detection of specific human pathogenic microorganisms rather than traditional indicator techniques. The preservation, protection, and optimal use of existing water resources worldwide are highly dependent on accurate water quality evaluations, in order to limit public health risks associated with waterborne pathogens.

8. Methods and Materials

The rapid and ongoing developments in molecular techniques offer a variety of avenues to optimize and ultimately solve research tasks. Individual steps in protocols or even established protocols may be substituted in many ways and new findings may be incorporated into examination protocols. The protocols employed in this research were modified in some areas from the original protocols, but mainly follow previously outlined protocols.

8.1 Isolation of DNA

The isolation of deoxyribonucleic acid (DNA) was carried out according to two protocols. First, the sodium dodecyl sulfate (SDS)-proteinase K and phenol/chloroform isolation was applied to bacterial and protozoa samples. The extraction procedure was carried out after the samples were centrifuged. The pellet was re-suspended in 567 µL TE buffer. After addition of 30 µL of 10% SDS and 3 µL of 20mg/mL proteinase K the samples were vortexed and incubated for 60 minutes at 37°C. The detergent lysed the bacterial cell walls and the mixture became viscous, however, the eukaryotic cells of protozoa required predigestion by lysozyme.

The addition of 100 µL of 5M sodium chloride (NaCl) solution with agitation, provided an essential salt concentration that prohibited the forming of unsolulable hexadecyltrimethyl ammonium bromide (CTAB)-nucleic acid complexes. Adding 50 µL of CTAB/NaCl solution facilitated the removal of cell wall debris and denatured proteins, and as a result the DNA remained in solution.

The purification was carried out by the phenol/chloroform method that removed CTAB-debris complexes and the DNA was transferred to a new microcentrifuge tube and precipitated by adding isopropanol. After washing the isolated DNA fragments with 70% ethanol the samples were dried and re-suspended in double distilled sterile water and stored at -20°C (Sambrook *et al.*, 1989).

A second DNA isolation method was carried out by the InstaGene protocol from BioRad, USA (BioRad, 1998). The InstaGene matrix is a chelex resin that binds polyvalent metal ions. The styrene divinylbenzene copolymers, that are the main structural components of the matrix, include the chelating groups. The presence of these groups inhibits the breakdown of DNA by cationic catalysis when exposed to elevated temperatures (i.e. during boiling). The sample was pelleted by centrifugation. The pellet was re-suspended in 200 µL InstaGene matrix, vortexed and incubated for 20 - 30 minutes at 56°C in a heating block. After the incubation, the sample was placed in a boiling water bath for 10 minutes. Cell lysis occurred by boiling the sample. Prolonged incubation times were required for eukaryotic cells. The mixture was centrifuged and the supernatant containing the DNA was used for PCR amplification. The samples were stored at -20°C. The samples were centrifuged after thawing, in order to pellet the matrix and inhibitors.

8.2 Cpn60 Primers

The primer set used in this examination was developed by Goh *et al.* (1996). These primers anneal to conserved regions of the chaperonin 60 gene in both eukaryotic and prokaryotic cells and produce a species-specific diagnostic fragment. The cpn60 primers H279A and H280A were synthesized and obtained from University Core DNA Services, Bio/Can Scientific, and Gibco Life Technologies.

The degenerate primers H279A and H280A were 26 nucleotides in length, with the following sequences:

H279A 5′GAIIIIGCIGGIGA(TC)GGIACIACIAC 3′

and

H280A 5′(TC)(TG)I(TC)(TG)ITCICC(AG)AAICCIGGIGC(TC)TT 3′

The incorporation of inosine (I) resulted in a reduction in degeneration of the primer set (Goh *et al.*, 1996).

8.3 Polymerase Chain Reaction

The use of PCR enabled the multiplication of target fragments to elevated levels. In addition, probe development was carried out by PCR.

The experiments were carried out under the following thermal cycling conditions:

3 minutes at 95°C or 97°C	1 cycle
1 minute at 94°C, 2 minutes at 37°C, 5 minutes at 72°C	40 cycles
10 minutes at 72°C	1 cycle
stored at 4°C	

The most critical temperature during the PCR amplification is the annealing temperature, at which primers bind to the template DNA. In the experiments, the annealing temperature was low in order to optimally facilitate the binding process with cpn60 primers. As outlined by Maloy *et al.* (1996), the annealing temperature is calculated by adding 2°C for any A or T base and 4°C for any G or C base. However, in the following study, the universal nature of these primers required lower annealing temperatures (Goh *et al.*, 1996).

8.4 Gel Electrophoresis

The amplified samples were analyzed on a ethidium-bromide stained agarose gel. The agarose gel consisted of 2 g agarose added to 100 mL of double distilled sterile water. After boiling the mixture, it was poured in a prepared mold. The application of a comb incorporated wells into the set agarose. The wells had a volume of approximately 0.05 cm^3. Approximately 20 μL of PCR product was mixed with 5 μL dye and added to the prepared gel. The samples were separated over a period of approximately 3 – 4 hours which is equivalent to approximately 300 Vh (Volt hours). After the separation took place, the gel was stained with ethidium-bromide in a waterbath containing the stain. The staining process was completed after 1 – 2 hours. The evaluation of the fragments was carried out under UV light and the agarose gel was photographed.

8.5 DNA Labeling

After the polymerase chain reaction amplification of the target fragments, the products were labeled and utilized as DNA probes for the identification of complementary species. The labeling procedure was carried out with the Boehringer Mannheim DIG High Prime Labeling and Detection Kit. This labeling procedure is referred to as a random primed

labeling technique. This type of labeling involves a process in which the Digoxigenin (DIG) is coupled to dUTP via an alkali-labile ester bond.

The PCR products were denatured at 95°C, incubated at 37°C overnight with DIG High Prime and quantified by comparison with standard DNA concentrations.

The labeled 600 bp target fragments were stored at -20°C at various dilutions. Approximately 40 - 50 ng/mL of probe were used for the dot blot hybridization between probes and amplified water samples.

8.6 Dot Blot Hybridization

Water and wastewater samples were PCR amplified and analyzed by gel electrophoresis. Approximately 5μL of each PCR product was blotted onto a hybond-N nylon membrane (Amersham Life Science). The membrane was incubated for approximately 30 minutes at 120°C. After neutralization with 0.5 M Tris, (pH 7.5) the membrane was dried and pre-hybridized at 42°C for 60 minutes in hybridization solution. This solution contained 50% formamide, 5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS and a 1/5 volume of blocking solution (10X conc.). After pre-hybridization, the DIG labeled probe was added at a concentration of approximately 40 - 50 ng/mL and hybridized overnight at 42°C in a water bath.

The post hybridization washes were 2 X 15 minute washes in 2X SSC. 0.1% SDS at room temperature, followed by 2 X 15 minute washes in 0.1X SSC, 0.1% SDS at 68°C. The detection procedure was carried out by the CSPD chemiluminescence method, according to Boehringer Mannheim protocols and visualized on high performance autoradiography film (Amersham Life Science).

The signal evaluation (intensity of dot blots) involved the comparison of individual sample dot blots with the negative control dot blot. This examination procedure recognized that any sample application onto the membrane resulted in visual dot blots, however, only significant signals were recorded as positive intersections. The dot blot hybridization analysis is a qualitative method rather than a quantitive method.

9. Experimental Investigation

Previous studies carried out by Goh *et al.* (1998, 1997, 1996) have shown the successful species-specific identification of clinical isolates with the cpn60 method. In addition, the design and use of the cpn60 primers H279A and H280A confirmed that a single set of primers was necessary in order to amplify a variety of species and ensure their successful identification. This investigation involved the application of the cpn60 identification method in water and wastewater environments, and the identification of waterborne pathogens in such environments. Additionally, modifications and optimizations were carried out in order to facilitate amplification and identification of water and wastewater samples.

Overall, five main experiments were carried out to investigate the application of the cpn60 identification method, and are listed as follows:

1. The amplification of several pathogenic isolates was carried out utilizing the cpn60 primers H279A and H280A.
2. Further studies determined detection limits of *E. coli O157:H7* when amplified and analyzed by dot blot hybridization using existing protocols.
3. The analysis of laboratory water samples involved the amplification and dot blot analysis of several mixtures of pathogens (*E. coli O157:H7, S. typhimurium LT2,* and *S. sonnei*).
4. Wastewater samples were analyzed by the cpn60 ID method detecting *E. coli O157:H7* in spiked and unspiked samples.

5. The InstaGene DNA isolation method was evaluated in order to determine its consistency and applicability.

9.1 Amplification of Waterborne Pathogens

First, the isolation of DNA from all supplied bacterial and protozoa strains was investigated and analyzed. After successful DNA isolation and DNA concentration determinations, the polymerase chain reaction was carried out on all isolates. The PCR conditions and thermal cycling conditions employed in these investigations are outlined in the Methods and Materials section.

9.1.1 Results

After isolation of DNA by the InstaGene method, the samples were amplified by PCR. The amounts of DNA were approximately 60 ng for each PCR reaction vial, as evaluated by UV spectrophotometry for nucleic acid analysis. The 600 bp fragments were amplified, as seen in Figure 9.1 at the 600 bp position, by comparison with the 1 kb DNA ladder bands at standardized positions (Figure 9.1). The bands at the 600 bp level suggested that the primers H279A and H280A amplified the target region of *E. coli O157:H7, S. typhimurium*

LT2, *S. sonnei*, *V. parahaemolyticus*, *B. thailandensis*, *B. pseudomallei*, and *S. enteritidis* (Figure 9.1, Lanes 1 through 8). The amplification of *G. lamblia*, however, did not occur as indicated by the absence of the 600 bp target bands (Figure 9.1, Lane 8). The negative control indicated no contamination of the PCR master mix by the lack of bands in the agarose gel (Figure 9.1, Lane C).

The enumeration of the investigated microorganisms showed concentrations in the magnitude of 10^7 to 10^8 cfu/mL (Table 9.1). The nucleic acid analysis produced values indicating amounts ranging from approximately 31.6 µg/mL to 335.1 µg/mL for *S. typhimurium LT2* and *S. sonnei*, respectively (Table 9.1).

The PCR amplification of the strains *B. thailandensis* and *B. pseudomallei* were carried out at elevated denaturing temperatures of 97°C. These strains contain DNA with a high GC content. Further, it was observed that the target fragments of *B. thailandensis* and *B. pseudomallei* were visualized at the 650 bp level on the agarose gel.

Table 9. 1 Pathogen Concentrations for PCR Amplification

Organism	PCR Product	Microorganism Concentration[+] [cfu/mL]	Nucleic Acid Concentration [µg/mL]
E. coli O157:H7	1	10.60 X 10^8	292.5
S. typhimurium LT2	2	1.72 X 10^7	31.6
S. sonnei	3	8.70 X 10^8	335.1
V. parahaemolyticus	4	N/A	N/A
B. thailandensis	5	N/A	100.0
B. pseudomallei	6	N/A	100.0
S. enteritidis	7	8.93 X 10^8	176.5
G. lamblia	8	Approx. 10^{7*}	155.0

* The concentration for *G. lamblia* was measured as trophozoites/mL.
N/A Not available
[+]Duplicate enumeration

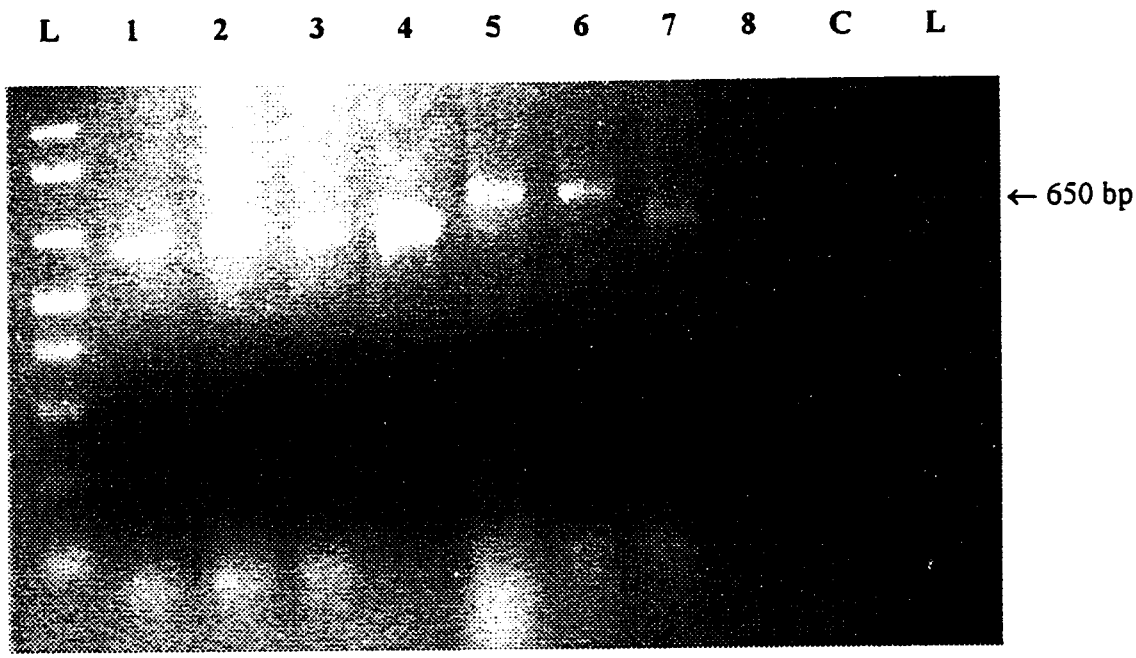

Figure 9. 1 Ethidium-bromide stained 2% agarose gel of PCR amplified target microorganisms 1, 2, 3, 4, 5, 6, 7, 8, and C.

1	*E. coli O157:H7*
2	*S. typhimurium LT2*
3	*S. sonnei*
4	*V. parahaemolyticus*
5	*B. thailandensis*
6	*B. pseudomallei*
7	*S. enteritidis*
8	*G. lamblia*
C	Negative control
L	DNA ladder

9.1.2 Discussion

The successful amplification of all target microorganisms, except *G. lamblia*, indicated that the cpn60 gene, when amplified with the primers H279A and H280A, resulted in 600 bp fragments from all examined bacterial isolates. Therefore, it is possible to project that the cpn60 gene consists of a conserved region that is present in all of the above investigated pathogenic bacteria. Goh *et al.* (1996) observed that the cpn60 gene sequences offer highly stable segments and diagnostic fragments that are conserved during evolutionary changes. In fact, the computer-supported evaluation of the cpn60 gene sequences (outlined under 11. The Examination of Cpn60 Primers) of the organisms *C. perfringens*, *E. histolytica*, *G. lamblia*, *H. pylori*, *S. cerevisiae*, *S. epidermidis* and *Y. enterocolitica* indicated that the set of cpn60 primers H279A and H280A also anneal to these cpn60 genes and amplify a 600 bp fragment. Therefore, experimental analysis and theoretical evaluation of the target cpn60 gene sequences demonstrated the amplification of all investigated pathogenic microorganisms.

However, the amplification of *G. lamblia* could not be demonstrated, and the absence of the 600 bp target fragment clearly indicated that the primers were not able to amplify the target. The lack of amplification for *G. lamblia* was most likely due to enzymatic inhibition. The more complex structure of eukaryotic cells requires extensive DNA purification techniques in order to eliminate PCR inhibitors. In fact, Persing *et al.* (1993) suggested inhibitor removal from extracted DNA on an Elutip minicolumn or an Extractor-15 column to counteract this problem. Unfortunately, such purification techniques ultimately reduce the

amount of template DNA significantly (Persing *et al.*, 1993). Furthermore, purification by the InstaGene method removes inhibitors from bacterial cells and while standard protocols are established for these targets, protocols for parasitic microorganisms are still under investigation (BioRad, 1998). In sum, the absence of 600 bp target fragments of *G. lamblia* was likely caused by the incomplete removal of PCR inhibitors.

Recently, Roger *et al.*, (1998) analyzed the cpn60 gene in *G. lamblia* and concluded that cpn60 gene sequences are highly conserved throughout the entire life cycle of *G. lamblia*. In addition, the sequence of the cpn60 gene for *G. lamblia* has been published in the GenBank (AF029695). A homology analysis (found in Section 10) of the cpn60 primers H279A, H280A and the cpn60 gene sequence resulted in the theoretical amplification of a 600 bp fragment. Therefore, the PCR amplification of *G. lamblia* under the outlined conditions should occur unless inhibitors restrain the DNA polymerase or the primer annealing process, as was likely the case in the above investigation.

9.2 Detection Limits of E. coli O157:H7

In a second experiment, the pathogenic strain *E. coli O157:H7* was investigated in more detail, in order to examine the detection limits for the PCR amplification of the cpn60 gene with the primer set H279A and H280A. At present, the pathogenic strain *E. coli O157:H7* is considered an emerging pathogen, and has been the causative agent responsible for many recent outbreaks of severe gastrointestinal diseases (Feng, 1995). In addition, it has been shown that the vehicles of transmission were both drinking water and recreational waters. The largest outbreak associated with *E. coli O157:H7* occurred in Missouri, in 1989 which involved more than 240 people, including 32 hospitalizations and four deaths (Feng, 1995). It is clear that a detection method able to differentiate between species, and more specifically, between pathogenic and non-pathogenic species, would be of great value. The investigations involving the detection limits of the cpn60 ID method were carried out in order to gain more knowledge about this technique's application potential for the water and wastewater industry.

9.2.1 Results

The results of the determination of various bacterial concentrations and nucleic acid (NA) concentrations from a dilution series are shown in Table 9.2. The bacterial concentrations range from approximately 92 500 cfu/100μL to 737 00 cfu/100μL. The NA concentrations decreased with decreasing bacterial concentrations. It was found that a positive PCR amplification occurred for the dilutions 1/200, 1/300, 1/400, 1/500, 1/600, and 1/700, however, amplification did not occur for the dilutions 1/100 and 1/800 (Figure 9.2). The detection limit for the strain *E. coli O157:H7* was found to be greater than approximately 105 000 cfu/100μL.

Figure 9.2 indicates amplification of the dilutions 1/200, 1/300, 1/400, 1/500, 1/600, and 1/700 which were observed as visualized bands on an ethidium-bromide stained 2% agarose gel at the 600 bp mark (Figure 9.2, Lanes 1/200, 1/300, 1/400, 1/500, 1/600, and 1/700). The dilutions 1/100 and 1/800 did not amplify, as indicated by the absence of visual bands on the agarose gel (Figure 9.2, Lanes 1/100 and 1/800). The negative control also failed to amplify (Figure 9.2, Lane C). A standard 1 kb DNA ladder (Gibco BRL) was used as a molecular weight reference (Figure 9.2, Lane L).

Table 9. 2 Dilution Series of *E. coli O157:H7*

Dilution	1/100	1/200	1/300	1/400	1/500	1/600	1/700	1/800
Amplification	-	+	+	+	+	+	+	-
NA Concentration [ng/100μL]	2.95	1.48	0.98	.74	0.59	0.49	0.42	0.37
Bacteria Concentration [10^3 cfu/100μL]	737	370	245	185	147	122.5	105	92.5

NA Nucleic acid

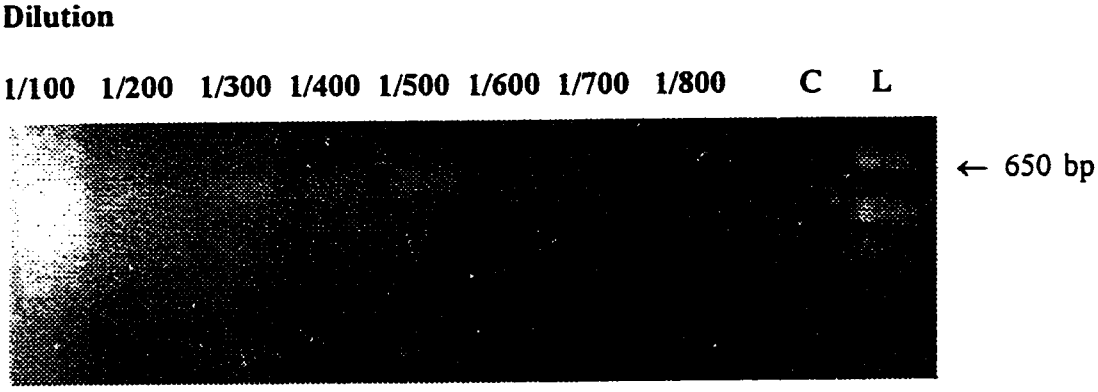

Figure 9. 2 Sensitivity of detection of *E. coli O157:H7*. PCR products of a serial dilution of *E. coli O157:H7* cells amplified by the universal primers H279A/H280A and visualized on ethidium-bromide stained 2% agarose gel.

C	Negative control
L	DNA ladder

9.2.2 Discussion

PCR amplification utilizing the cpn60 primers H279A and H280A was investigated to determine detection limits of bacterial isolates, as shown in Table 9.2. Based on the data provided in Table 9.2, there is a defined relationship between the nucleic acid concentration and the bacterial concentration. In fact, a distinct detection limit of approximately 105 000 cfu/100μL (*E. coli O157:H7*) was observed on agarose gel (Figure 9.2, Lane 1/700). This observation was supported by the presence of a 600 bp fragment amplified by the cpn60 primers. The lack of amplification (Figure 9.2, Lanes 1/800) demonstrated the acuity of the detection limits. The random amplification of sample 1/100 (Figure 9.2, Lane 1/800) was attributed to contamination. Interesting to note is the observation that the absence of reagent contamination as indicated by negative controls does not provide information regarding sporadic contamination problems (Kwok and Higuchi, 1989). Therefore, the random amplification of sample 1/100 was thought to be caused by sporadic contamination of the sample.

Previous experiments carried out by Goh *et al.* (1996) did not indicate any determination of detection limits using this technique. However, the investigations did suggest a 24 hour incubation time of clinical samples in brain heart infusion (BHI) broth prior to PCR amplification, in order to significantly increase the amount of target organisms (Goh *et al.*, 1996).

It is known that a single bacterial cell can reach a concentration of approximately 1 X 10^6 cells after 10 hours of incubation under optimal growth conditions with a doubling time of

30 minutes (Brock and Madigan, 1991). Therefore, a prior incubation time of 24 hours improves the detection of low microorganism concentrations, however, this would not apply to non-culturable strains.

Many detection methods for non-culturable pathogens in very low environmental concentrations were improved by increasing the sample volumes. The studies carried out by Mahbubani *et al.* (1992) investigated water samples of 4 - 400 liters of river, filtered and analyzed for *G. lamblia*. It has been well documented that standard procedures for the detection of *G. lamblia* and *Cryptosporidium* oocysts require large amounts of sample volumes, that are usually in the order of 100 - 1 000 liters (Jaykus, 1997). Additionally, several pathogens require prolonged incubation time, such as *G. lamblia*. Keister (1983) suggested an incubation time of 72 - 96 hours for *G. lamblia* on TYI-S-33 medium. These modifications could improve existing detection limits and increase the sensitivity of species-specific detection by the cpn60 ID method.

9.3 Laboratory Water Sample Analyses

The specificity of the cpn60 ID method was investigated using laboratory water samples contaminated with several pathogenic isolates. The experiments involved the isolate *S. typhimurium LT2* from which a DNA probe was amplified and labeled. *Salmonella* spp. are the causative agents for approximately 2 - 3 million infections per year in the United States (LCDC Canada, 1997). The outbreaks are associated with both food contamination and transmission via water. The low infectious dose of approximately 100 - 1000 organisms when ingested (LCDC Canada, 1997) underlines the need to be able to detect this pathogen in drinking water and recreational water environments and protect public health.

9.3.1 Results

The investigations involving the applicability of the cpn60 ID method were carried out on laboratory water samples consisting of combinations of several pathogens at known concentrations and included PCR amplification and dot blot hybridization. The serial diluted water samples were contaminated with *E. coli O157:H7*, *S. typhimurium LT2*, and *S. sonnei* (Table 9.3). The bacterial concentrations were in the order of approximately 10^7 cfu/mL in the mixture. An overall mixture volume of 750 µL was utilized for the examination (Table 9.3).

In addition, the nucleic acid concentrations of the individual isolates and the various mixtures were recorded. The nucleic acid concentrations of the isolates were lower than the nucleic acid readings of the mixtures (Table 9.4).

The PCR products were visualized on an ethidium-bromide stained 2% agarose gel (Figure 9.3). The bands at the 600 bp mark indicated that the primers amplified, as well as a 1/10 dilution of these samples, labeled M11, M21, M31, M41, respectively. The pathogens *E. coli O157:H7*, *S. typhimurium LT2* and *S. sonnei* were successfully amplified, as indicated by the presence of target fragments. A negative control indicated the absence of PCR reagent contamination.

A hybridization was carried out between the PCR products M1, M11, M2, M21, M3, M31, M4, M41, 1, 2, 3, and the DIG-labeled *S. typhimurium LT2* probe (probe #2) to determine the specificity of this method. The identification of the target species was indicated by strong chemiluminescent signals on the nylon membrane (Figure 9.4, Samples 2, M1, M11, M2, M21, M3, and M31). The probe did not hybridize with the samples that did not contain *S. typhimurium LT2* (Figure 9.4, Samples 1, 3, C, M4, M41).

Table 9. 3 Contamination Concentrations of Laboratory Water Samples

Sample [750µL]	1 [cfu/mL]	2 [cfu/mL]	3 [cfu/mL]	dH_2O^* [µL]
M1	3.68 X 10^7	0.43 X 10^7	0.81 X 10^7	0
M2	3.68 X 10^7	0.43 X 10^7	0	250
M3	0	0.43 X 10^7	0.81 X 10^7	250
M4	3.68 X 10^7	0	0.81 X 10^7	250
C	0	0	0	750

*deionized sterile water

M1	Mixed water sample containing *E. coli O157:H7*, *S. typhimurium LT2*, and *S. sonnei.*
M2	Mixed water sample containing *E. coli O157:H7*and *S. typhimurium LT2.*
M3	Mixed water sample containing *S. typhimurium LT2* and *S. sonnei.*
M4	Mixed water sample containing *E. coli O157:H7* and *S. sonnei.*
1	*E. coli O157:H7*
2	*S. typhimurium LT2*
3	*S. sonnei*
C	Negative control

Table 9. 4 Hybridization Results of *S. typhimurium LT2* Probe and Water Samples.

	1	2	3	M1	M2	M3	M4
Hybridization	-	+	-	+	+	+	-
***S. typhimurium LT2* present**	-	+	-	+	+	+	-
Nucleic Acid [µg/mL]	46.2	31.6	38.5	83.3	61.0	35.8	63.3

M1	Mixed water sample containing *E. coli O157:H7, S. typhimurium LT2,* and *S. sonnei.*
M2	Mixed water sample containing *E. coli O157:H7*and *S. typhimurium LT2.*
M3	Mixed water sample containing *S. typhimurium LT2* and *S. sonnei.*
M4	Mixed water sample containing *E. coli O157:H7* and *S. sonnei.*
1	*E. coli O157:H7*
2	*S. typhimurium LT2*
3	*S. sonnei*
C	Negative control

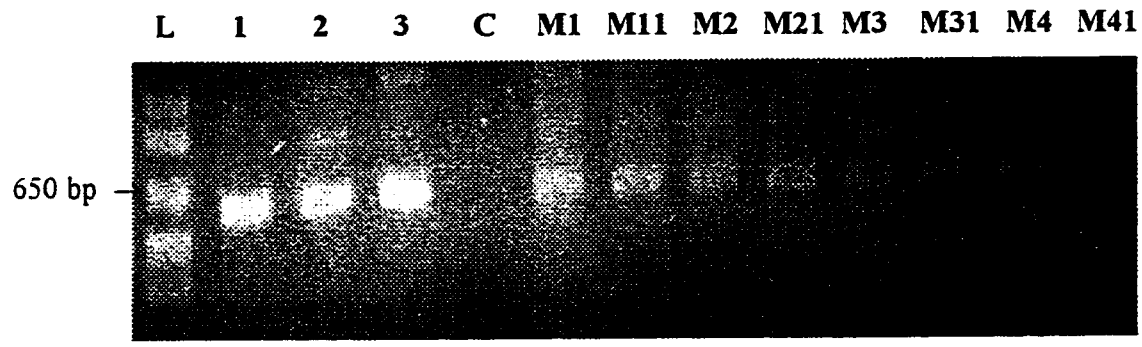

Figure 9. 3 Ethidium-bromide stained 2% agarose gel of PCR amplified samples 1, 2, 3, C and M1 through M4.

M1	Mixed water sample containing *E. coli O157:H7, S. typhimurium LT2,* and *S. sonnei.*
M11	A 1:10 dilution of M1.
M2	Mixed water sample containing *E. coli O157:H7* and *S. typhimurium LT2.*
M21	A 1:10 dilution of M2.
M3	Mixed water sample containing *S. typhimurium LT2* and *S. sonnei.*
M31	A 1:10 dilution of M3.
M4	Mixed water sample containing *E. coli O157:H7* and *S. sonnei.*
M41	A 1:10 dilution of M4.
1	*E. coli O157:H7*
2	*S. typhimurium LT2*
3	*S. sonnei*
C	Negative control

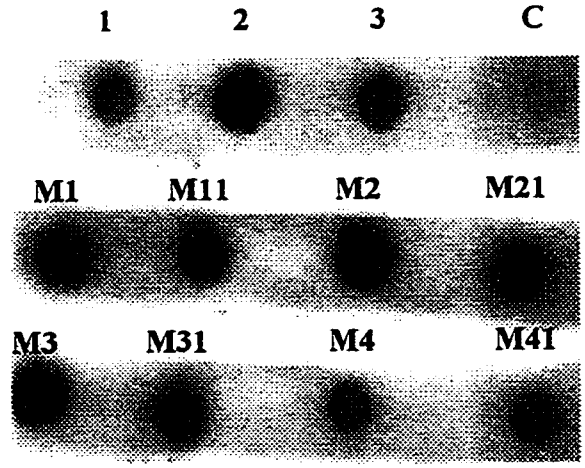

Figure 9. 4 Determination of species-specificity in mixed water samples by dot blot hybridization with PCR amplified samples 1, 2, 3, C, M1 through M4 and *S. typhimurium LT2* probe.

M1	Mixed water sample containing *E. coli O157:H7, S. typhimurium LT2,* and *S. sonnei.*
M11	A 1:10 dilution of M1.
M2	Mixed water sample containing *E. coli O157:H7* and *S. typhimurium LT2.*
M21	A 1:10 dilution of M2.
M3	Mixed water sample containing *S. typhimurium LT2* and *S. sonnei.*
M31	A 1:10 dilution of M3.
M4	Mixed water sample containing *E. coli O157:H7* and *S. sonnei.*
M41	A 1:10 dilution of M4.
1	*E. coli O157:H7*
2	*S. typhimurium LT2*
3	*S. sonnei*
C	Negative control

9.3.2 Discussion

This experiment involved determining the specificity and sensitivity of the *S. typhimurium LT2* probe through the analysis of mixed laboratory water samples containing several pathogenic isolates at known concentrations (Table 9.3). The identification of *S. typhimurium LT2* in mixed water samples was successful, as demonstrated by the intersection of *S. typhimurium LT2* probe and PCR amplified *S. typhimurium LT2* contaminated water samples. To reiterate significant chemiluminescent signals were judged quantitatively, that is, based on their intensity rather than on presence/absence. This indicated that the cpn60 ID method can be utilized for pathogen identification. These results are consistent with the findings outlined by Goh *et al.* (1997), in which the identification of 30 *Staphylococcus* spp. was carried out successfully. In addition, it was demonstrated that the identification of *Streptococcus iniae* by the cpn60 ID method was successful and resulted in positive cross hybridization between the probe and the target DNA (Goh *et al.*, 1998). According to the initial studies carried out by Goh *et al.* (1998, 1997, 1996), the cpn60 identification method clearly demonstrated the specificity and accuracy of this detection technique. Further investigations will undoubtedly increase the pool of pathogenic isolates identifiable by the cpn60 gene sequence, using the cpn60 primers outlined here.

9.4 Wastewater Analyses

Further studies were conducted on wastewater samples from the Bonnybrook wastewater treatment facility in Calgary, in order to test the performance of the cpn60 ID method. Before the analysis was done, the wastewater samples were concentrated by centrifugation from 200 mL to 1 mL. After concentration, the samples WW2 and WW21 were spiked with *E. coli O157:H7*, amplified, and tested for the presence of *E. coli O157:H7*. Again, the presence of *E. coli O157:H7* in wastewater effluents presents a potential health hazard for populations accessing the water body down stream from the point of contamination. Feng (1995) observed that *E. coli O157:H7* has a survival rate of up to 14 days in cold waters and therefore, this organism has the potential to contaminate rivers and lakes even if they are only remotely connected to the main contaminated stream. Therefore, identification of *E. coli O157:H7* is an essential tool for monitoring and protecting drinking water and recreational water bodies.

9.4.1 Results

The PCR products of the concentrated wastewater samples were visualized on an ethidium-bromide stained 2% agarose gel (Figure 9.5). The amplification of all original samples was positive, as indicated by the visual 600 bp fragments. The nucleic acid concentrations are

provided in Table 9.5. Additionally, the amounts of bacteria added to the wastewater samples WW2 and WW21 were recorded (Table 9.5). The dot blot hybridization resulted in positive chemiluminescent signals in samples WW1, WW2, WW21 and 1.2 when hybridized with the DNA probe #1 (*E. coli O157:H7* probe) (Figure 9.6). Sample M was contaminated with approximately 100 μg/mL *B. pseudomallei* DNA, amplified, and hybridized, which resulted in a chemiluminescent signal (Figure 9.6, Sample M).

During the initial investigation of the wastewater samples, the polymerase chain reaction conditions were set at a denaturing temperature of 95°C. The amplification did not occur, as indicated by the absence of 600 bp target fragments on the ethidium-bromide stained agarose gel (Figure 9.7). In a second trial, the PCR amplification was carried out at an elevated denaturing temperature of 97°C. The PCR products were visualized on ethidium-bromide stained agarose gel (Figure 9.5). The presence of the target 600 bp fragments indicated a positive amplification of all samples (Figure 9.5, Lanes WW1, WW11, WW2, WW21, and M).

Additionally, samples of *B. pseudomallei* DNA (6, 6.1, 6.2) at various concentrations were amplified and hybridized with probe #1. The PCR products were visualized on agarose gel (Figure 9.5, Lanes 6, 6.1, 6.2). The dot blot analysis indicated that probe #1 linked to the samples containing *E. coli O157:H7*, and did not intersect with samples that lack this microorganism (Figure 9.6). The resulting significant chemiluminescent signals demonstrated the species specificity of probe #1 (Figure 9.6).

A second experiment dealing with wastewater samples was done according to the same protocols. The dilutions and sample preparations were carried out before DNA isolation.

The samples and their respective specifications, in addition to hybridization results are outlined in Table 9.6. The nucleic acid concentrations of spiked wastewater samples W2 and W21 were significantly lower than those concentrations of samples W1 and W11 (Table 9.6).

After PCR amplification of all concentrated and spiked wastewater samples, in addition to the individual pathogenic isolates *E. coli O157:H7, S. typhimurium LT2* and *S. sonnei*, the PCR products were visualized on ethidium-bromide stained 2% agarose gel (Figure 9.8). The presence of the 600 bp target fragments indicated successful amplification (Figure 9.8, Lanes W1, W11, W12, W13, W2, W21, 1, 2 and 3). However, the amplification of wastewater samples W22 and W23 could not be visualized on agarose gel (Figure 9.8, Lanes W22 and W23). The significant intersections between the amplified PCR products containing *E. coli O157:H7* DNA and the *E. coli O157:H7* probe suggested the identification of *E. coli O157:H7* (Figure 9.9).

Table 9. 5 Wastewater Sample Concentrations and Hybridization

Sample	WW1	WW11	WW2	WW21	M	1.2
Nucleic Acid [µg/mL]	71.8	7.2	55.0	5.5	N/A	3.5
***E. coli O157:H7* added [cfu/mL]**	0	0	13.7×10^6	13.7×10^5	0	13.7×10^6
Hybridization	+	-	+	+	+	+

N/A Not Available

WW1 Concentrated Wastewater sample without additional contamination

WW11 A 1:10 dilution of WW1

WW2 Concentrated Wastewater sample spiked with *E. coli O157:H7* cells

WW21 A 1:10 dilution of WW2

M Concentrated Wastewater sample spiked with *B. pseudomallei* DNA

1.2 A 1:100 dilution of *E. coli O157:H7* sample

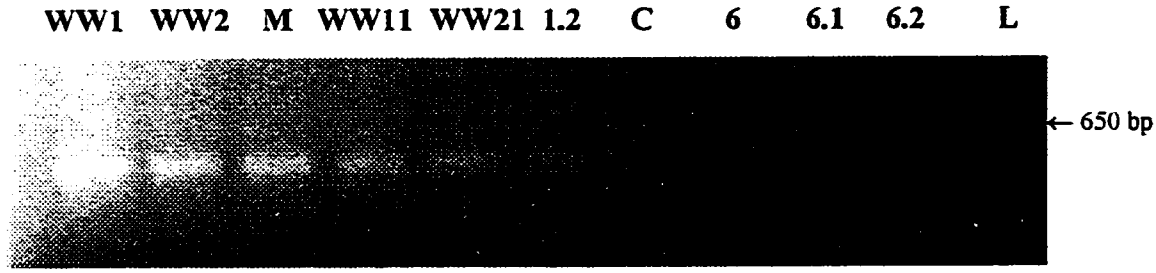

Figure 9. 5 PCR products WW1, WW11, WW2, WW21, M, 1.2, 6, 6.1, 6.2, and C on ethidium-bromide stained 2% agarose gel.

WW1	Concentrated Wastewater sample without additional contamination
WW11	A 1:10 dilution of WW1
WW2	Concentrated Wastewater sample spiked with *E. coli O157:H7* cells
WW21	A 1:10 dilution of WW2
M	Concentrated Wastewater sample spiked with *B. pseudomallei* DNA
1.2	A 1:100 dilution of *E. coli O157:H7* sample
6/6.1/6.2	A serial dilution: pure/1:10/1:100, respectively of *B. pseudomallei* DNA
C	Negative control

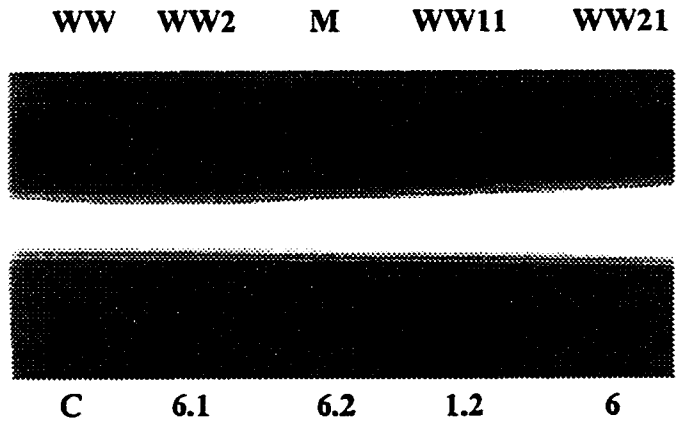

Figure 9. 6 Dot blot hybridization between samples WW1, WW11, WW2, WW21, M, 1.2, 6, 6.1, 6.2, C, and *E. coli O157:H7* probe.

WW1	Concentrated Wastewater sample without additional contamination
WW11	A 1:10 dilution of WW1
WW2	Concentrated Wastewater sample spiked with *E. coli O157:H7* cells
WW21	A 1:10 dilution of WW2
M	Concentrated Wastewater sample spiked with *B. pseudomallei* DNA
1.2	A 1:100 dilution of *E. coli O157:H7* sample
6/6.1/6.2	A serial dilution: pure/1:10/1:100, respectively of *B. pseudomallei* DNA
C	Negative control

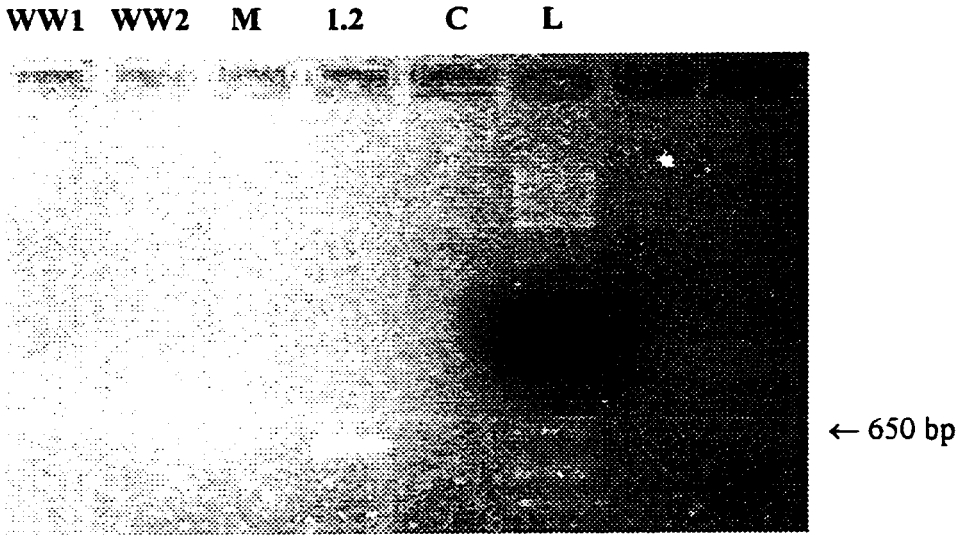

Figure 9. 7 PCR products WW1, WW2, M, 1.2, and C on ethidium-bromide stained 2% agarose gel.

WW1	Concentrated Wastewater sample without additional contamination
WW2	Concentrated Wastewater sample spiked with *E. coli O157:H7* cells
M	Concentrated Wastewater sample spiked with *B. pseudomallei* DNA
1.2	A 1:100 dilution of *E. coli O157:H7* sample
C	Negative control

Table 9. 6 Analysis of Wastewater Samples

Sample	Nucleic Acid [µg/mL]	*E. coli O157:H7* added [cfu/mL]	Hybridization with probe #1
W1	45.3	0	+
W11	4.5	0	-
W12	ND	0	-
W13	ND	0	+
W2	21.5	17.1×10^6	+
W21	ND	17.1×10^5	+
W22	ND	17.1×10^4	+
W23	ND	17.1×10^3	+
1	603.8	0	+
2	776.8	0	-
3	728.0	0	-
C	0	0	-

ND Not Detectable

W1/W11/W12/W13 A serial dilution of concentrated wastewater samples without additional contamination (pure/1:10/1:100/1:1000).

W2/W21/W22/W23 A serial dilution of concentrated wastewater samples with additional *E. coli O157:H7* contamination (pure/ 1:10/ 1:100/ 1:1000).

1 *E. coli O157:H7* sample

2 *S. typhimurium LT2* sample

3 *S. sonnei* sample

C Negative control

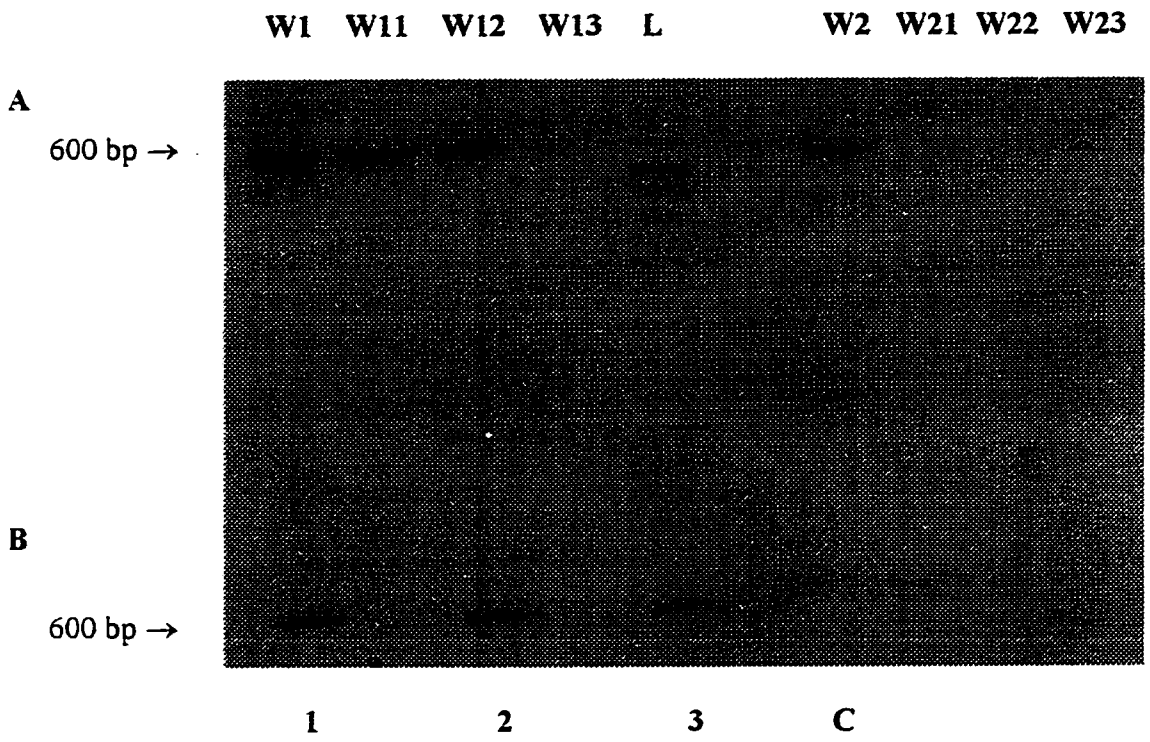

Figure 9. 8 Ethidium-bromide stained 2% agarose gel with two sets of wells (A and B) of PCR amplified samples W1 throughW13, W2 through W23, 1, 2, 3, and C.

W1/W11/W12/W13	A serial dilution of concentrated wastewater samples without additional contamination (pure/1:10/1:100/1:1000).
W2/W21/W22/W23	A serial dilution of concentrated wastewater samples with additional *E. coli O157:H7* contamination (pure/ 1:10/ 1:100/ 1:1000).
1	*E. coli O157:H7* sample
2	*S. typhimurium LT2* sample
3	*S. sonnei* sample
C	Negative control

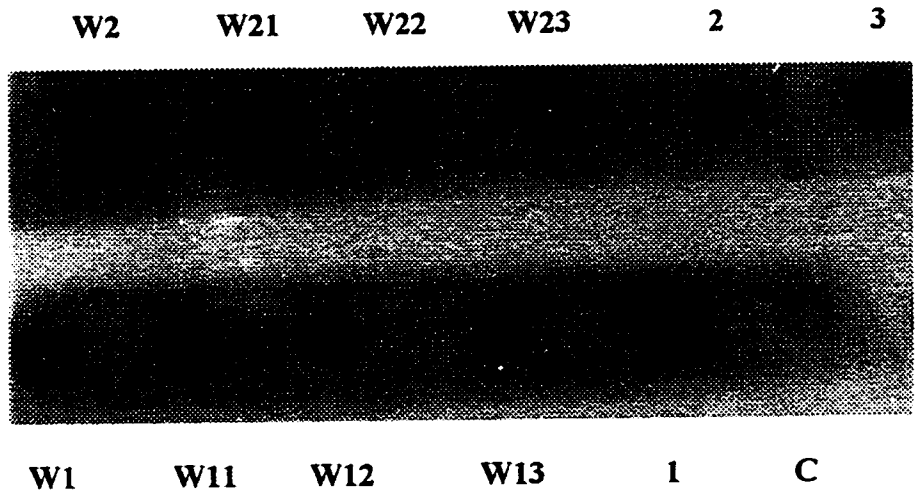

Figure 9. 9 Determination of the species-specificity of the *E. coli O157:H7* probe by dot blot hybridization with PCR amplified samples W1 through W13, W2 through W23, 1, 2, 3 and C.

W1/W11/W12/W13	A serial dilution of concentrated wastewater samples without additional contamination (pure/1:10/1:100/1:1000).
W2/W21/W22/W23	A serial dilution of concentrated wastewater samples with additional *E. coli O157:H7* contamination (pure/ 1:10/ 1:100/ 1:1000).
1	*E. coli O157:H7* sample
2	*S. typhimurium LT2* sample
3	*S. sonnei* sample
C	Negative control

9.4.2 Discussion

Further investigations involved analyses of environmental wastewater samples by the cpn60 ID method using the *E. coli O157:H7* probe. It was demonstrated that the probe intersected with the target DNA from the same isolate (Figures 9.6 and 9.9). During evaluations of both wastewater samples, PCR denaturing temperatures were increased to 97°C, in order to amplify high GC content DNA and complex DNA structures (Greisen *et al.*, 1994). The lack of amplification in samples using 95°C denaturing temperatures, indicated that complex DNA structures and high GC content DNA might be present (Figure 9.7). In addition, the amplification of wastewater samples containing *B. pseudomallei* DNA (that also consists of high GC fragments) (Brett, 1998), required increased denaturing temperatures (Innis *et al.*, 1995). Furthermore, the amplification of *E. coli O157:H7*, *S. typhimurium LT2* and *S. sonnei* at elevated denaturing temperatures was carried out successfully, indicating that most bacterial isolates can be amplified using a denaturing temperature of 97°C (Figure 9.8). However, increased denaturing temperatures decrease the period for *Taq* polymerase activity during the amplification process and therefore, decrease the efficiency of the polymerase chain reaction (Maloy *et al.*, 1996). Several additives were tested to overcome this phenomena, and it was observed that through the addition of glycerol, or with concentration adjustments of K^+ or Mg^{++} the product yield was increased during amplification using elevated denaturing temperatures (Innis *et al.*, 1995). The utilization of such additives could be applied to this investigation, which, in turn, could facilitate amplification using the cpn60 primers H279A and H280A at higher

denaturing temperatures. In fact, the intersection of *E. coli O157:H7* DNA and probe (Figure 9.9, Sample 1) suggested that the *E. coli O157:H7* sample was successfully amplified and therefore, the elevated denaturing temperature had minimal affect on the polymerase chain reaction for *E. coli O157:H7*, even without the use of PCR additives. Therefore, the amplification of wastewater samples at elevated denaturing temperature is recommended in order to amplify a wide spectrum of microorganisms occurring in water and wastewater environments.

The hybridization results of the wastewater samples demonstrated that positive identification of *E. coli O157:H7* occurred with use of the *E. coli O157:H7* cpn60 DNA probe (Figures 9.6 and 9.9). The results suggested that the wastewater samples (without additional *E. coli O157:H7* contamination) may have been initially contaminated with *E. coli O157:H7*, as seen by the intersection of DNA probe and sample DNA (Figure 9.6, Sample WW1, Figure 9.9, Sample W1). However, a serotype specific identification by this investigation could not be confirmed and further examination are needed in order to detect *E. coli O157:H7* contamination in these samples. The lack of cross hybridization between the *E. coli O157:H7* probe and the 1/10 dilution of the sample WW1 (labeled WW11), indicated that the contamination was less than that of the *E. coli O157:H7* spiked samples WW2 and WW21 (Figure 9.6). These findings were also observed when the wastewater samples W1, W11, W12 and W13 were analyzed (Figure 9.9). The *E. coli O157:H7* probe intersected with W1 (unspiked sample), which suggested initial water contamination with *E. coli O157:H7*. The cross hybridization between the *E. coli O157:H7* probe and the samples W2, W21, W22, and W23 indicated the successful identification of this pathogen in spiked

wastewater samples (Figure 9.8). The negative controls indicated no contamination of the PCR mixtures, as seen in the absence of chemiluminescence (Figures 9.6 and Figure 9.9, Samples C). The positive intersection between the *E. coli O157:H7* probe and the sample 1.2 and 1 (consisting of *E. coli O157:H7* amplified DNA), indicated the specificity of the probe (Figures 9.6 and 9.9). However, the positive signal of W13 (Figure 9.9) suggested cross contamination. In fact, in similar studies, sporadic contamination problems have occurred, despite the fact that negative controls have not indicated any contamination of the PCR reagents (Kwok and Higuchi, 1989). Therefore, the positive intersection demonstrated in the above experiment can be explained by sporadic cross contamination.

In addition, the lack of intersection between the *E. coli O157:H7* probe and the *B. pseudomallei* DNA (Figure 9.6, Samples 6, 6.1, 6.2) further demonstrated the specificity of the cpn60 ID method. This experiment showed that the identification of *E. coli O157:H7* did occur in environmental wastewater samples with the cpn60 identification method.

Given that even state-of-the-art wastewater treatment facilities discharge effluent waters containing significant amounts of pathogenic microorganisms, these findings provide an optimistic solution to present detection limitations (Lòpez-Pila *et al.*, 1996, Mascher *et al.*, 1996). Similarly, Mascher *et al.* (1996) confirmed that wastewater treatment processes in general, do not eliminate pathogens, but suggested that wetland treatment or sand filtration employed following the biological treatment level, reduced pathogen levels. Therefore, the identification of pathogenic microorganisms in water environments that receive wastewater treatment plant effluents would be beneficial for public health protection and produce valuable data for disease outbreak prevention. This approach is in alignment with the

primary goals of water and wastewater treatment, and further studies involving drinking water, surface water and groundwater contamination should be investigated in order to determine the extent of the cpn60 gene ID method's application in the field.

9.5 InstaGene Analysis

The DNA release utilizing the InstaGene method from BioRad was carried out according to existing standard protocols (BioRad, 1998). The matrix consisted of styrene divinylbenzene co-polymers that removed PCR inhibitors such as polyvalent metals and restraining enzymes and proteins (Walsh *et al.*, 1991). Past studies using the matrix have found that the presence of this resin during the boiling process for cell lysing, possibly prevents the degradation of DNA (Walsh *et al.*, 1991). The utilization of the InstaGene DNA purification method was chosen, due to its rapid, and replicable DNA purification capabilities.

9.5.1 Results

In a sub-experiment, the pathogenic strain *E. coli O157:H7* was utilized for the investigation of the relationship between nucleic acid concentrations that were released by the InstaGene method, and the amount of colony forming units (cfu).

In Figure 9.10 the spectrophotometric readings for nucleic acid, A260 (260 nm) and for proteins, A280 (280 nm) were plotted versus the bacterial concentration of *E. coli O157:H7*. Upon analysis, two linear functions were found ($P_{A260}<0.0001$, $r^2_{A260}=0.88$, $P_{A280}<0.0001$, $r^2_{A280}=0.94$). As indicated in Figure 9.10 nucleic acid concentration

increased with increasing bacterial concentration. The increase in protein concentrations characterized by the A280 values was more gradual in comparison to the nucleic acid concentrations determined by the A260 values. The background calibration was carried out at a wavelength of 320 nm and incorporated into the results.

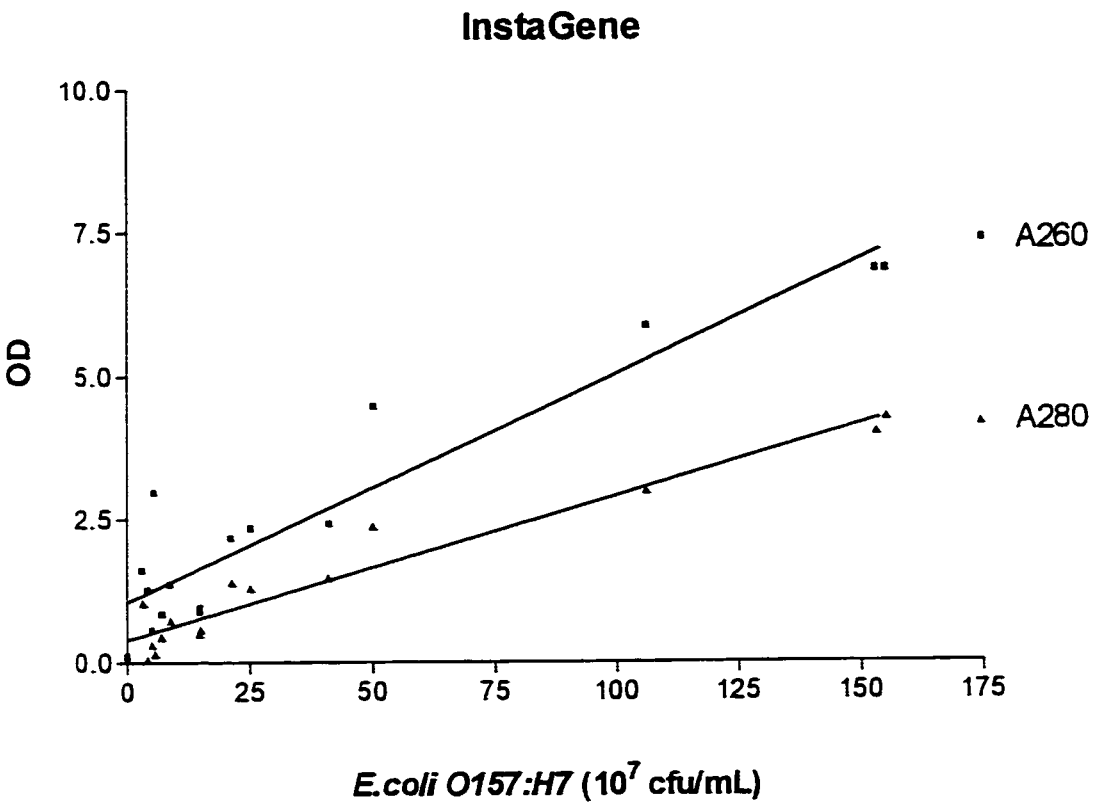

Figure 9. 10 ***E.coli O157:H7*** **(10^7 cfu/mL) versus nucleic acid and protein concentrations (OD).**

A260 Absorbance at a wavelength of 260 nm for nucleic acid detection

A280 Absorbance at a wavelength of 280 nm for protein detection

9.5.2 Discussion

The investigation of the InstaGene DNA purification technique revealed that this method is fast, inexpensive, and consistent with respect to isolating DNA from bacteria. The correlation between bacterial concentrations and NA concentrations outlined in this study, clearly supports the reliability of this technique. The linear regression performed on the obtained data resulted in a linear relationship between bacterial concentration and nucleic acid concentration (Figure 9.10). This relationship indicated that after carrying out a serial dilution of water or wastewater samples and nucleic acid release, the amounts of nucleic acid were directly related to the bacterial concentrations in the diluted water or wastewater samples. The consistency of the InstaGene method allows DNA release after bacterial dilution and delivers accurate results, as indicated in Figure 9.10. A study carried out by Woo *et al.* (1997) involved the analysis of several DNA isolation methods and demonstrated that the inhibition of PCR amplification was reduced by approximately 15% using the InstaGene matrix. Therefore, the capturing and removal of PCR inhibitors by the InstaGene matrix provides reliable PCR amplification of bacterial template DNA.

Finally, further investigations of the application of this DNA release and purification method should involve eukaryotic cell lysing, for example, from *G. lamblia* contaminated samples. The fast and accurate nature of the InstaGene method opens new avenues for optimizing molecular identification methods. Overall, it is hoped that the identification of waterborne pathogens by molecular methods will be an ongoing pursuit, as new protocol developments for DNA isolation offer potentially faster detection times.

10. Computer Analyses of Cpn60 Genes

The computer supported gene analysis was carried out in order to demonstrate the application potential of the cpn60 ID method for other important waterborne pathogens. The availability of sequenced cpn60 genes in the GenBank for several of these pathogens theoretically extended the scope of the investigations outlined in this thesis. The cpn60 genes were only available for the pathogenic microorganisms outlined here. The analyses of these known sequences were carried out by DNASIS, Version 3.7, Hitachi Software (1997). The results reflect the analysis of the set of primers H279A and H280A and cpn60 genes from the following isolates:

Pathogen	**Infectious Dose**
- *Clostridium perfringens* (X62914)	10/g food
- *Entamoeba histolytica* (AF029366)	unknown
- *Giardia lamblia* (AF029695)	<10 cysts
- *Helicobacter pylori* (X73840)	-
- *Saccharomyces cerevisiae* (M33301)	-
- *Staphylococcus epidermidis* (U13618)	-
- *Yersinia enterocolitica* (X82212)	unknown

This theoretical investigation resulted in amplification of cpn60 genes, however, conclusions regarding the respective PCR conditions cannot be obtained from this study. Further examinations involving the cpn60 genes should be carried out using a laboratory trail, in order to precisely determine realistic, optimal PCR conditions and protocols.

The US EPA suggested in its summary on the Candidate Contaminant List (CCL) for microorganisms, that the organism *H. pylori* will be added to the list because of lack of scientific knowledge surrounding primary routes of transmission and negative health effects (US EPA 1998). *Yersinia enterocolitica* was recently recognized as the causative agent for many waterborne outbreaks. In addition, Carter *et al.* (1987) concluded that coliform indicator organisms did not correlate with the presence of *Y. enterocolitica*. Furthermore, the increasing rate of *Y. enterocolitica* infections during the winter period suggested that this pathogen is able to survive and grow at low temperatures found in the winter (Bergen and Argaman 1983). Therefore, the detection of these pathogens would result in valuable information that could be utilized before infectious disease outbreaks, during, and also after such incidents for evaluation and data collection purposes.

The theoretical evaluation of the cpn60 genes in pathogens demonstrated successful amplification and may lead to the development of additional cpn60 probes for the pathogen identification method outlined here.

The published cpn60 gene sequences from several pathogens were analyzed by DNASIS Version 3.7, Hitachi Software (1997). The redundancies were as follows: R=A+C, Y=C+T, K=G+T, M=A+C, N=Idosine.

A	Adenine	**R**	binds to either to Adenine or Cytosine
C	Cytosine	**Y**	binds either to Cytosine or Thymine
G	Guanine	**K**	binds either to Guanine or Thymine
T	Thymine	**M**	binds either to Adenine or Cytosine

10.1 Clostridium perfringens

The primer set annealed between positions 1336 and 1940. The primer intersected fully with the target gene and there were no mismatches recorded.

```
1330 actaatgatgtggcaggagatggaactactacagctaccttattagctca 1379
            |||||||||||||||||||||||||  →
        5'-gannnngcnggngayggnacnacnac-3'

                    (H279A)
...

1901 cttgtgttggagttaaagcacctggatttggtgatagaagaaaagaaatg 1950
               ←  |||||||||||||||||||||||||
               3'-aargcnccnggnttyggnganmrnmr-5'

                          (H280A)
```

The target fragment for the development of the species-specific probe had a length of 605 bp and the following sequence:

```
  5'-gatgt   ggcaggagat   ggaactacta   cagctacctt   attagctcaa
gcaattataa   gagaaggatt   aaaaaatgta   acagcagggg   caaatcctat
attaataaga   aatggaatta   aaactgcagt   tgaaaaagct   gtagaggaaa
tacaaaaaat   ttctaagcct   gtaaatggaa   aagaagacat   agctagagtt
gctgcaattt   cagcagctga   tgaaaaaatt   ggtaagttaa   ttgcagatgc
tatggaaaag   gtaggaaatg   aaggagttat   aactgtagaa   gaatctaaat
caatgggaac   tgagttagat   gttgttgaag   gtatgcaatt   tgatagggga
tatgtatcag   cttatatggt   tactgatact   gaaaaaatgg   aagctgtttt
agataatcca   ttagtattaa   taacagataa   gaaaataagc   aatatacaag
atttattacc   attacttgag   caaatagttc   aagcaggtaa   aaaactttta
ataatagctg   atgatataga   aggcgaagct   atgacaacat   tagttgttaa
taaattaaga   ggaacattta   cttgtgttgg   agttaaagca   cctggatttg
gtgatagaag   a- 3'
```

10.2 Entamoeba histolytica

The annealing positions for primer H279A and H280A were 268/293 and 844/869, respectively.

```
251 ttgcaagtaaagttaatgatcgttctggagatgggacaacaacagcaaca 300
                      |||||| |||||||||||||||||||  →
                   5'-gannnngcnggngayggnacnacnac-3'

                              (H279A)
...

830 caattgctgcagtaagagctccaggttttggagaaactagaaaaggaatt 880
              ←  | |||||||||||||||||||| |||
                  3'-aargcnccnggnttyggnganmrnmr-5'

                              (H280A)
```

The alignment of primer H279A and the cpn60 sequence involved one mismatch (between g and t, position 274) and resulted in 96% annealing. In addition, there were two mismatches recorded between primer H280A and the cpn60 gene sequence, which resulted in an alignment window of 92% (positions 845 and 866).

The amplified target fragment had a length of 601 bp and the following sequence:

```
    5'-gat   cgttctggag   atgggacaac   aacagcaaca   tgtttattaa
gaaaagtagc   atgtgaagga   gttcaagcta   ttaatacagg   gttatctggt
actgatttat   taaaaggaat   atcaattgca   aaagatatag   tattaaaaga
aattacaaaa   caatcaaagc   caacactaaa   agaagacatt   atttcagttg
caagagtatc   agcaaacaat   gatgaaaaaa   ttggagaaat   ggtaggtgat
atttttggta   aaattggaag   agatggtgct   gttgatattg   aaacagggaa
aggaacaaaa   gacattgtta   atattgttga   aggaatggtt   cttgaccaag
gatttttatc   tcgttatttt   actactgatg   aaaaaaatac   taaagtagat
ataagaaata   cagatgttat   tgtatgtgat   tataaactaa   gctcatcaca
aagtgtagtt   cctttattag   aattatgttt   aaaaagaaaa   agaccattag
ttgttatttc   agacactata   gatggtgatg   cattaacaac   attagtatta
aataaattaa   gaggattacc   aattgctgca   gtaagagctc   caggttttgg
agaaacta-3'
```

10.3 Helicobacter pylori

The intersection between the primers and the target gene was successful at the positions 301/326 for the H279A primer and 883/908 for the H280A primer, respectively.

```
290 gcaaaaccgctgatgctgccggcgatggcacgaccacagcgaccgtgctag 340
                |||||||||||||||||||||||||  →
           5'-gannnngcnggngayggnacnacnac-3'
```

(H279A)

...

```
870 tatcgcagcggttaaagctccaggctttggggacagaagaaaagaaatgct 920
              ←  |||||||||||||||||||||||||
              3'-aargcnccnggnttyggnganmrnmr-5'
```

(H280A)

Both primers intersected with 100% matching to the cpn60 gene sequence. The following sequence represents the amplified target product with a length of 608 bp and the sequence:

5'-
gatgctgccg gcgatggcac gaccacagcg accgtgctag cttatagcat
ttttaaagaa ggtttgagga atatcacggc tggggctaac cctattgaag
tgaaacgagg catggataaa gctgctgaag cgatcattaa tgagcttaaa
aaagcgagca aaaaagtagg cggtaaagaa gaaatcaccc aagtggcgac
catttctgca aactccgatc acaatatcgg gaaactcatc gctgacgcta
tggaaaaagt gggtaaagac ggcgtgatca ccgttgagga agctaagggc
attgaagatg aattggatgt cgtagaaggc atgcaatttg atagaggcta
cctctccccc tattttgtaa cgaacgctga gaaaatgacc gctcaattgg
ataatgctta catcctttta acggataaaa aaatctctag catgaaagac
attctcccgc tactagaaaa aaccatgaaa gagggcaaac cgcttttaat
catcgctgaa gacattgagg gcgaagcttt aacgactcta gtggtgaata
aattaagagg cgtgttgaat atcgcagcgg ttaaagctcc aggctttggg
gacagaag-3'

10.4 Giardia lamblia

The primer set annealed to the target gene at the positions 647/672 for the H279A primer and 1226/1251 for the H280A primer.

```
630 cgtctcttcgtacaaatacCATGGCAGGGGATGGAACCACAACttctctta 680
                         ||||||||||||||||||||||||  →
                  5'- gannnngcnggngayggnacnacnac-3'

                                (H279A)
...

1220 gccgtccgcGCTCCAGGCTACGGAGACGTTAAgaagggcgtactggagga 1269
       ←    ||||||||||| |||||||  |||
       3'-aargcnccnggnttyggnganmrnmr-5'

                      (H280A)
```

The primer intersection involved two mismatches for primer H279A (positions 677and 678) and six mismatches for primer H280A (positions 1226,1227,1228, 1239,1247,and 1248). The overall annealing efficiencies were 92% and 77% for H2797A and H280A, respectively.

The PCR product had the following sequence and a length of 605 bp:

```
5'-  acca   tggcagggga   tggaaccaca   acttctctta   ttctctccgg
caaactagtc  aatgagatga   acaagtatgc   attaagtggg   ttagggaatc
tccagttact  ccaggctctc   aattcagctg   gtgtagattg   cctccaatct
ctaagaaagc  aatccagagc   cattgagagc   aataagatgc   tttatagcgt
ggccacaatt  gcagccaaca   acgaccccaa   aattggtaag   gttgtttctg
acgcatttgc  ggctgttggc   cgagaaggaa   caataactgt   cgaggatgga
tatacagaca  ttgacagctt   aacgttaccg   acggatgctc   cattccaagt
gggtttttgt  ctccttattt   cgctctggga   ggctcccgtt   accttgagct
caccaacccg  ttggtggtca   ttaccgacac   agttctgttc   ctcagcagct
cctcttgttt  ctattctgga   acgttgcgtg   aaagagaaaa   gaccacttct
tattatagca  tcggatgtga   caggggacgc   attaagtaca   ctcgcaatca
atacactcaa  aggtactgtg   cgttgctgtg   ccgtccgcgc   tccaggctac
ggagacgtta  a-3'
```

10.5 Saccharomyces cerevisiae

The binding between the primers and the target gene was successful at the positions 683/708 (H279A) and 1265/1290 (H280A).

```
670 ctccaaaaccaatgaggctgctggtgacggtactacttctgctactgtctt 720
                  |||||||||||||||||||||||| |  →
              5'-gannnngcnggngayggnacnacnac-3'

                          (H279A)
...

1250 aaggtttgtgctgtgaaggcgcctggtttcggtgataatagaaagaatac 1299
                  ←  ||||||||||||||||||||||||
                  3'-aargcnccnggnttyggnganmrnmr-5'

                          (H280A)
```

The annealing resulted in a mismatch at position 707 for primer H279A and 100% intersection of primer H280A. The target fragment had a length of 608 bp and the sequence:

```
5'-
  gaggctgc   tggtgacggt   actacttctg   ctactgtctt   aggtagagcc
atcttcacag   aatccgtcaa   aaatgtcgcc   gctggttgta   accctatgga
tttgagaagg   ggttctaagt   tgcagttgaa   aaagtgattg   aatttttgag
cgccaacaag   aaagaaatta   ccacatctga   ggaaattgct   caagtagcaa
ccatttctgc   caatggggac   tctcatgttg   gtaagttact   agcttcagct
atggaaaagg   ttggaaaaga   aggtgtcatc   actatcagag   aaggtagaac
attggaagat   gaacttgagg   ttactgaagg   tatgaggttt   gatcgtggtt
ttatttctcc   atacttcatc   actgatccaa   agtcgagcca   aggtggaatt
tgaaaagcca   ttgctattgt   tgagtgaaaa   gaaaatttct   tccattcaag
atatcttgcc   agctttggaa   atttccaatc   aaagcagaag   acctttgttg
atcattgctg   aagatgttga   cggtgaagct   cttgcggctt   gtattttgaa
caagttgagg   ggtcaagtta   aggtttgtgc   tgtgaaggcg   cctggtttcg
gtgataatag   -3'
```

10.6 Staphylococcus epidermidis

The cpn60 gene sequence allowed for the annealing of the primer set at the positions 688/713 for H279A and 1267/1292 for H280A.

```
670 gttgcgaataaaacaaatgaaatcgctggggacggtacaactacagcaaca 720
                      |||||||||||||||||||||||||  →
                  5'-gannnngcnggngayggnacnacnac-3'

                              (H279A)
...

1260 agcagttaaagccccaggatttggtgatgcacgtaaagcaatgttagaag 1309
       ← |||||||||||||||||||||||||
       3'-aargcnccnggnttyggnganmrnmr-5'

                    (H280A)
```

The intersection between the primer set and the cpn60 gene resulted in 100% matching and the amplified 605 bp target fragment involved the following sequence:

```
    5'-gaa   atcgctgggg   acggtacaac   tacagcaaca   gttttagcac
aatcaatgat   tcaggaaggt   cttaagaatg   ttacaagtgg   tgcaaatcct
gtaggcttaa   gacaaggtat   tgacaaagca   gtgcaagtgg   ctatagaagc
gcttcatgag   atttctcaaa   aggttgaaaa   taagaacgag   atagcgcaag
ttggagctat   ttcagcagca   gatgaagaaa   tcggtcgcta   catttctgaa
gcaatggata   aagtaggtaa   cgatggcgtt   atcactattg   aagaatcaaa
tgggtttaat   acagaattag   aagtagctga   aggaatgcaa   tttgatcgcg
gttatcaatc   accatatatg   gtaactgact   cagataaaat   gatagctgaa
ttagaacgtc   catatatatt   agtaacggat   aagaaaattt   catcattcca
agatattctt   ccattattag   aacaagttgt   gcaggctagt   cgaccaattt
taattgttgc   ggatgaagta   gaaggcgatg   cacttactaa   tattgtttta
aaccgcatgc   gtggaacatt   tactgctgta   gcagttaaag   ccccaggatt
tggtgatcga    cg-3'
```

10.7 Yersinia enterocolitica

The primer set H279A/H280A annealed to the target cpn60 gene at the positions 247/272 and 829/854, respectively.

```
230 ttgcctctaaagcgaacgacgctgcgggtgacggtaccactactgcaactg 280
                     |||||||||||||||||||||||||  →
                  5'-gannnngcnggngayggnacnacnac-3'

                                (H279A)
...

820 gccgctgttaaagcacctggtttcggcgaccgtcctaaagccatgctgcaa 870
          ←  |||||||||||||||||||||||||
          3'-aargcnccnggnttyggnganmrnmr-5'

                        (H280A)
```

The intersection between both primers and the target cpn60 gene involved 100% annealing.

The product was a 608 bp fragment with the following sequence:

```
   5'-gacg ctgcgggtga cggtaccact actgcaactg tattagctca
atccattatc actgaaggcc tgaaagcagt tgcggccggc atgaacccaa
tggatctgaa acgcggtatc gacaaagccg ttatcgctgc ggttgaagag
ctgaaaaaac tgtctgtacc atgctctgat tctaaagcaa ttgctcaggt
agggaccatc tctgcaaact cagactcaac tgtaggtgag ctgattgcac
aagcgatgga aaaagtcggt aaagaaggyg ttatcaccgt tgaagaaggt
tcaggcctgc aagacgagct ggacgttgtt gaaggtatgc agttcgaccg
cggctacctg tctccttact tcatcaataa accagaaacc ggttctattg
aacttgaaag cccattcatc ctgctggctg acaagaaaat ctctaacatc
cgcgaaatgc tgccagttct ggaagccgta gcgaaagcgg gtaaaccact
gctgatcatt gcagaagatg ttgaaggcga agctctggca actctggtgg
ttaacaccat gcgcggtatt gttaaagttg ccgctgttaa agcacctggt
ttcggcgacc gtcg-3'
```

10.8 Discussion

The DNASIS analyses of the target pathogen cpn60 gene sequences suggested that the PCR products of all analyzed microbes were approximately 600 bp in length when amplified by the primer set H279A/H280A. These findings are in agreement with the laboratory results and suggest that the identification of waterborne pathogens by the cpn60 gene ID method can be applied to both prokaryotic and eukaryotic microorganisms. The significant differences between the PCR products of the DNASIS examination demonstrated that a species-specific identification of the analyzed pathogens could occur. In fact, the DNASIS analysis indicated that there were significant differences between all amplified 600 bp target fragments (Table 10.1) The homologies ranged from 67.7% to 45.3% according to the DNASIS analysis. It was observed that *E. coli* and *S. typhimurium* differ for 58% of their protein coding genes (Ochman and Wilson, 1987). The laboratory experiments involving the identification of *E. coli O157:H7* and *S. typhimurium LT2* demonstrated clear identification of these species by the cpn60 ID method. Therefore, an average homology between species of approximately 58% was sufficient to differentiate these pathogens. In conclusion, the homology values resulting from the DNASIS analysis demonstrated that an identification of those pathogens according to the cpn60 ID protocol would be successful. There were several pathogens where the primer set did not anneal completely. This might suggest that the PCR conditions and reagent concentrations need to be optimized in order to yield the desired target fragments. However, there was no specific information obtained

about PCR conditions and/or reagent concentrations in the DNASIS analysis. Therefore, a laboratory analysis is strongly recommended in order to support and confirm these findings. Finally, the DNASIS analyses demonstrated that an approximately 600 bp fragment from cpn60 genes from several different microorganisms could theoretically be amplified and therefore, a species-specific identification would be possible, if DNA probes were developed.

Table 10. 1 Homology of Analyzed 600 bp Target Fragments (%)

Isolates	*C. perfringens*	*E. histolytica*	*G. lamblia*	*H. pylori*	*S. cerevisiae*	*S. epidermidis*	*Y. enterocolitica*
C. perfringens	100	58.5	50.3	64.7	58.5	67.6	59.1
E. histolytica	58.5	100	53.1	52.8	51.5	53 9	49.4
G. lamblia	50.3	53.1	100	45.3	47.4	47 4	50.4
H. pylori	64.7	52.8	46.8	100	58.4	61 6	63.3
S. cerevisiae	58.5	51.5	47.4	58.4	100	56.4	64.4
S. epidermidis	67.6	53.9	47.4	61.6	56.4	100	59.1
Y. enterocolitica	59.1	49.4	50.4	63.3	64.4	59.1	100

11. The Examination of Cpn60 Primers

The primer set H279A/H280A consists of oligonucleotides, each of which are 26 bp in length and anneal at a temperature of 37°C to the target cpn60 gene of several pathogenic isolates. The primers were designed by Goh *et al.* (1996) and their universal nature enables them to anneal to a variety of microorganisms. The low stringency of the PCR protocol supports both the amplification of microbes, and the development of species-specific DNA probes for the detection of these microorganisms in both laboratory settings and environmental samples.

Initial difficulties in dealing with the degenerated primers subsequently led to an examination and optimization of the PCR protocol in the early stages of this research.

11.1 Source of Primers

Three different sources of primers were examined. The three sources were the University Core DNA Services Laboratory, Bio/Can Scientific Laboratory and Gibco Life Technologies, respectively.

The primer sequences were:

H279A 5'- GAIIIIGCIGGIGAYGGIACIACIAC - 3'

H280A 5'- YKIYKITCICCRAAICCIGGIGCYTT -3'

Redundancies:

R=A+G I=Idosine

Y=C+T K=G+T

The primer concentrations in the PCR protocol were 0.5μg/μL of each primer. The evaluation of the primers was carried out by the standard PCR protocol.

11.2 Primer Analyses

The investigation of the primers included an examination of performance after several storage periods, involving PCR runs on the same day of synthesis, after four days, six days and seven days when stored at -20°C. The primers were thawed and frozen for each protocol, and therefore, the amplification after four days involved one additional thawing/freezing cycle. The remaining primer samples went through two and three thawing/freezing cycles, respectively.

The performance of the primers synthesized by the University Core DNA Services was visualized on ethidium-bromide stained 2% agarose gel (Figure 11.1). The amplification of *E. coli O157:H7* DNA by the cpn60 primers after a number of thawing/freezing cycles resulted in different target yields. However, all primers amplified the 600 bp target sequences (Figure 11.1). The different signal strengths correlated with the age of the primer. Accordingly, the 7d primer set produced the weakest band on the stained agarose gel (Figure 11.1, Lane 7d) and the 0d primer set amplified the most yield, as indicated by the strongest band on the agarose gel (Figure 11.1, Lane 0d).

All negative controls where the template DNA was substituted by double distilled sterile water suggested no contamination of the PCR reagents as indicated by the absence of fragments on the stained agarose gel (Figure 11.1, Lanes C7, C6, C4, C0).

A second investigation involved the evaluation of the primer set from different sources. PCR amplification was carried out according to the standard protocol. The organisms *E. coli O157:H7*, *S. enteritidis* and *S. sonnei* were investigated. The amplification of serial dilutions of each target microorganism was done and resulted in bands visualized on ethidium-bromide stained 2% agarose gel (Figure 11.2). The amplification by the primers from the University Core DNA Services Laboratory produced more multiple banding than the primers from Bio/Can Scientific Laboratory (Figure 11.2), however, both primer sets amplified the target fragments indicated by the presence of the 600 bp fragments (Figure 11.2). In addition, the amplification of the 10^{-2} dilution (approximately 10^5 cfu/mL) of *S. sonnei* by the primers from Bio/Can produced a stronger band on the agarose gel (Figure 11.2, Lane 3'.2). However, none of the primers were able to amplify at the 10^{-4} level, reflecting a level of approximately 10^3 cfu/mL.

The dot blot hybridization between DNA probe #7 (*S. enteritidis*), probe #1 (*E. coli O157:H7*), and amplified samples of *E. coli O157:H7, S. enteritidis*, and *S. sonnei* are shown in Figure 11.3 and 11.4. Probe #7 intersected with samples 7, 7' and 7" which represent PCR products amplified by primers from University Core DNA Services, Bio/Can Scientific, and Gibco Life Technologies, respectively (Figure 11.3). In addition, probe #1 intersected with samples 1, 1', and 1" as indicated by the presence of strong chemiluminescent signals (Figure 11.4). There was no intersection between probes and negative controls as demonstrated by the absence of strong chemiluminescent signals at positions C, C' and C" (Figure 11.3 and 11.4).

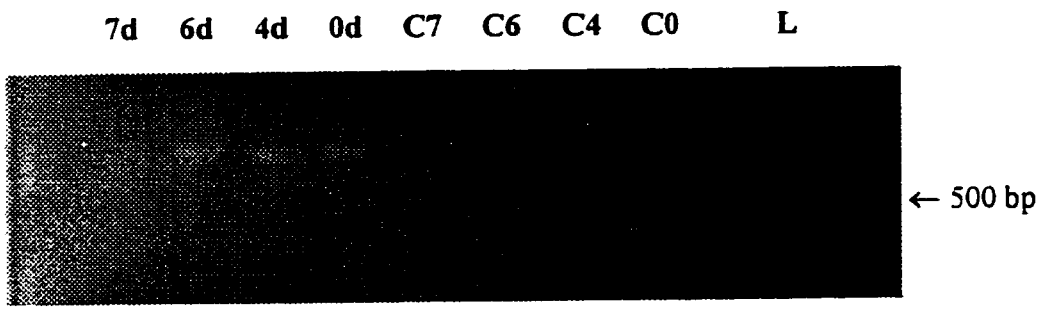

Figure 11. 1 Ethidium-bromide stained 2% agarose gel of PCR products 7d, 6d, 4d, 0d, and negative controls C7, C6, C4, C0 amplified by the universal primers H279A and H280A.

7d PCR product amplified by the primer set H279A/H280A after 7 thawing/freezing cycles.

6d PCR product amplified by the primer set H279A/H280A after 6 thawing/freezing cycles.

4d PCR product amplified by the primer set H279A/H280A after 4 thawing/freezing cycles.

0d PCR product amplified by the primer set H279A/H280A after no thawing/freezing cycle.

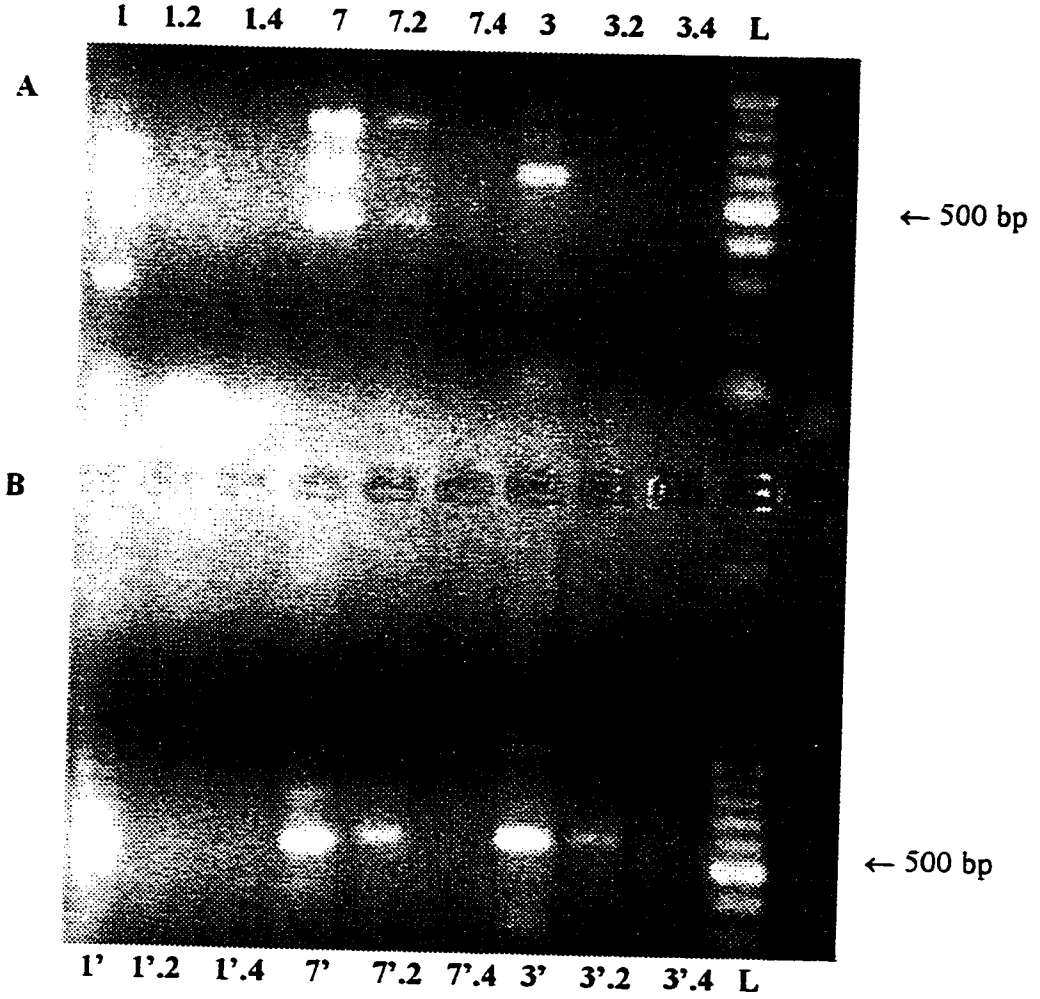

Figure 11. 2 Ethidium-bromide stained 2% agarose gel of PCR products amplified by the primer set H279A/H280A.

A Primer set synthesized by the University Core DNA Services.

B Primer set synthesized by Bio/Can Scientific.

University Core DNA	1/1.2/1.4	*E. coli O157:H7* samples; pure/1:100/1:10000.
Services primer set	7/7.2/7.4	*S. enteritidis* samples; pure/1:100/1:10000.
	3/3.2/3.4	*S. sonnei* samples; pure/1:100/1:10000.
Bio/Can Scientific	1'/1'.2/1'.4	*E. coli O157:H7* samples; pure/1:100/1:10000.
primer set	7'/7'.2/7'.4	*S. enteritidis* samples; pure/1:100/1:10000.
	3'/3'.2/3'.4	*S. sonnei* samples; pure/1:100/1:10000.

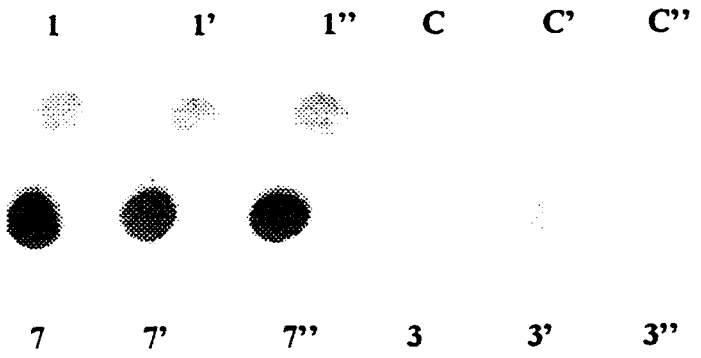

Figure 11. 3 Determination of the specificity of the cpn60 ID method by dot blot hybridization with PCR amplified samples 1, 3, 7, C and *S. enteritidis* probe. (The amplification was carried out with the primer set H279A/H280A from three different sources).

1/1'/1''	*E. coli O157:H7* sample amplified with primers H279A/H280A from University Core DNA Services / Bio/Can Scientific / Gibco BRL
3/3'/3''	*S. sonnei* sample amplified with primers H279A/H280A from University Core DNA Services / Bio/Can Scientific / Gibco BRL
7/7'/7''	*S. enteritidis* sample amplified with primers H279A/H280A from University Core DNA Services / Bio/Can Scientific / Gibco BRL
C/C'/C''	Negative control amplified with primers H279A/H280A from University Core DNA Services / Bio/Can Scientific / Gibco BRL

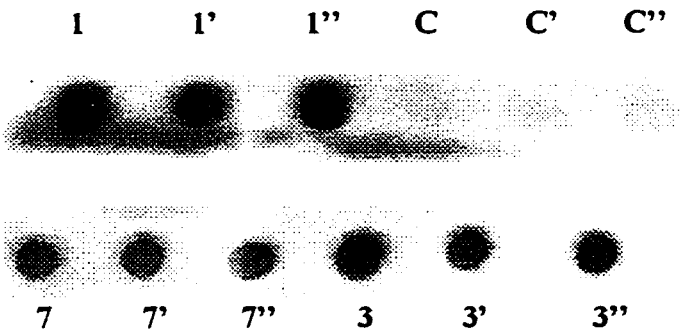

Figure 11. 4 Determination of the specificity of the cpn60 ID method by dot blot hybridization with PCR amplified samples 1, 3, 7, C and *E. coli O157:H7* probe. (The amplification was carried out with the primer set H279A/H280A from three different sources).

1/1'/1''	*E. coli O157:H7* sample amplified with primers H279A/H280A from University Core DNA Services / Bio/Can Scientific / Gibco BRL
3/3'/3''	*S. sonnei* sample amplified with primers H279A/H280A from University Core DNA Services / Bio/Can Scientific / Gibco BRL
7/7'/7''	*S. enteritidis* sample amplified with primers H279A/H280A from University Core DNA Services / Bio/Can Scientific / Gibco BRL
C/C'/C''	Negative control amplified with primers H279A/H280A from University Core DNA Services / Bio/Can Scientific / Gibco BRL

11.3 Discussion

The evaluation of the primers was carried out according to standard protocols and followed the same procedures as outlined in the Methods and Materials section. Differences between the respective primer performances were investigated, and according to the findings, the source and storage conditions were determined.

As demonstrated in Figure 11.1, the primer handling and storage conditions were critical to their performance. In fact, cpn60 primers should be stored separately and should be individually aliquoted in order to prohibit primer degeneration due to thawing/freezing cycles (Kwok and Higuchi, 1989). In addition, all other PCR reagents should be separated from the template DNA storage facilities in order to limit the extent of potential contamination problems that ultimately result in lack of amplification. Accordingly, the primers used in this investigation were aliquoted into 10μL portions and stored at -70°C. Furthermore, all additional PCR reagents were individually stored and used once for each PCR protocol. Clearly, limiting the thawing/freezing to no cycles resulted in optimal PCR yields (Figure 11.1, Lane 0d).

In the second experiment, the amplification differences between different primer sources indicated that all primers amplified the 600 bp target fragments. However, multiple banding was observed for the primers from the University Core DNA Services (Figure 11.2, Lanes 1, 7, and 7.2). The multiple bands suggested that the amplification of higher molecular weight fragments occurred, and that these PCR products could not be used for the development of DNA probes. In sum, investigations for this thesis primarily involved use the primer set H279A/H280A from Bio/Can Scientific for the development of DNA probes and the amplification of samples. In

addition, the higher yield of the Bio/Can primer amplification suggested optimal conditions for further investigations using the cpn60 ID method. The presence of bands using lower template DNA concentrations (Figure 11.2, Lanes 7'.2 and 3'.2) suggested that the Bio/Can primers anneal at a higher rate to the template, and therefore it could be expected that the Bio/Can primers produce optimal experimental conditions.

Finally, the dot blot hybridization of DNA probes and PCR products amplified by primers from different sources indicated successful identification of targets (Figure 11.3 and 11.4). The presence of equally strong chemiluminescent signals on the photographic film of the intersection between probes and samples suggested that the amplification using primers from three different sources had little effect on the performance of the hybridization. The *S. enteritidis* probe clearly intersected with *S. enteritidis* DNA, and therefore identified *S. enteritidis* when amplified by the outlined primer sets (Figure 11.3, Positions 1, 1', and 1"). In addition, the intersection between *E. coli O157:H7* probe and *E. coli O157:H7* DNA suggested that the source of primers did not influence the performance of the dot blot hybridization (Figure 11.4, Positions 7, 7', and 7"). Finally, the lack of hybridization between the probes and negative controls indicated that no contamination had occurred during the amplification process (Figure 11.3 and 11.4, Positions C, C', and C").

In conclusion, potentially damaging processes that can occur during the handling and storage of primers demand that care must be taken when using the cpn60 primers H279A and H280A. Individual storage at -70°C, coupled with limited thawing/freezing cycle numbers greatly improved the performance of amplification. In addition, the primer source investigations demonstrated that differences could occur in the performance of the PCR

amplification. However, proper handling and storage of both the primers and the PCR reagents may assist in limiting performance errors.

12. Conclusions

1. The cpn60 ID method required one set of primers (H279A and H280A) and one set of reaction conditions in order to amplify and identify a variety of species-specific pathogens in water and wastewater.
2. The amplification of several pathogenic microorganisms was successful and resulted in the development of species-specific DNA probes.
3. Both laboratory investigations and computer analyses of cpn60 genes confirmed that the primers H279A and H280A amplified diagnostic 600 bp fragments that ultimately resulted in species-specific DNA probes.
4. Specifically, the identification of *E. coli O157:H7* was successful in spiked environmental wastewater samples using the developed cpn60 *E. coli O157:H7* probe.
5. The detection limits of the cpn60 *E. coli O157:H7* probe were found to be greater than 105 000 cfu/100μL.
6. The cpn60 *S. typhimurium LT2* probe was able to detect *S. typhimurium LT2* in *E. coli O157:H7*-, *S. typhimurium LT2*-, and *S. sonnei*-contaminated water samples.
7. The cpn60 *E. coli O157:H7* probe identified *E. coli O157:H7* in *B. pseudomallei* contaminated wastewater samples.
8. The InstaGene DNA isolation method was capable of removing PCR inhibitors from water and wastewater samples.

9. The isolation of DNA from the investigated isolates by the InstaGene method demonstrated reliable and consistent results.

10. The amplification of high GC content DNA and wastewater samples was successful after increasing the denaturing temperature form 95°C to 97°C. This increase did not affect the amplification of other investigated pathogens.

11. The storage and handling conditions of both the cpn60 primers (H279A and H280A) and PCR reagents had a significant influence on PCR yield.

Finally, the aforementioned investigations involving the cpn60 ID method clearly demonstrated great potential for the future development of alternative tools for water conservation and public health protection.

It was estimated that approximately 1 billion people around the word do not have access to safe drinking water (United Nations, 1997). In addition, the growing global population and increasing water consumption worldwide suggest that water conservation and water reservoir protection will play a vital role in human survival in the near future (United Nations, 1997). The detection of waterborne pathogenic microorganisms in water and wastewater will provide valuable information about future water usage and water treatment technologies. Moreover, it was observed that state-of-the-art wastewater treatment facilities do not produce pathogen free effluents, thereby contaminating receiving waters that may be fresh water resources for downstream communities (Mascher *et al.*, 1996). In summary, the establishment of new detection methods for the determination of waterborne pathogens in both freshwater and wastewater streams is essential for public health protection, a concept that is in keeping with the basic directives of water and wastewater management strategies.

Secondly, the traditional approach for the determination of microbiological water contamination involves fecal and total coliform indicator tests. However, as discussed, Emde *et al.* (1995) determined that the potential risk associated with microbiological pollutants may be higher than the risk associated with chemical contaminants. Unfortunately, policy does not yet correlate with such information. An example of this is the treated water survey done by the Alberta Environmental Protection Agency, that

requires water treatment facilities to determine 250 chemical and physical parameters of drinking water, versus only two microbiological indicators (Emde *et al.*, 1995). Such decisions occur despite evidence that many researchers have yet to observe a correlation between human pathogens and indicator organisms (Falcao *et al.*, 1993, Ferguson *et al.*, 1996, Hurst *et al.*, 1997, Lemming and Nichols, 1996, Mroz and Pillai, 1994). There is an urgent need for the expansion of existing water guidelines to include the identification of specific pathogens in order to secure public health at present, and in the future.

Not surprisingly, international collaboration in water protection and conservation has traditionally been the focus of many international debates (United Nations, 1997). Despite the conflicts that exist, it has been suggested repeatedly that the establishment of both national and international regulations and guidelines and their implementation undoubtedly plays a key role in providing access to safe drinking water. Although local and regional communities are directly affected by water pollution, these interests are innately upheld, as the compatibility and transferability of collected water quality data will provide a powerful tool for water protection and conservation on a global scale. Therefore, nothing less than an all inclusive, co-operative alliance for the collection of data and ultimately, the protection of water resources worldwide, will adequately support humanity in the future (United Nations, 1997).

The investigations involving the cpn60 ID method resulted in the provision of valuable information about future developments in detection techniques for the examination of water and wastewater environments. The molecular approach has undoubtedly provided great potential for improvements to traditional water evaluation methods. Increasing awareness

of public health risks associated with water contamination further demonstrate the growing need for changes in water guidelines and the development of new technologies.

In fact, the most complete understanding of the correlation between human waterborne diseases and water treatment technologies is necessary, in order to protect public health from the various risks associated with water contamination (Hellard *et al.*, 1997).

Finally, the improvement of detection tools for the identification of human pathogens in water and wastewater environments demonstrates that alternative technologies can provide additional instruments for the protection and conservation of finite water resources. This, in addition to water saving strategies will become essential parts of water management as the world's population expands in concert with the global need for food supplies. Therefore, the monitoring of microbiological contamination in water supports the optimal use of existing water resources, as well as provides a much needed supplement for existing and future water conservation strategies in an attempt to secure widespread access to potable water for many generations.

13. Recommendations

1. Further examination involving the expansion of pathogens detectable by the cpn60 ID method should be carried out.
2. Finding optimal solutions for decreasing detection limits are necessary in order to determine low concentrations of pathogens in environmental samples. (i.e. concentration of large sample volumes)
3. The optimization of DNA isolation and purification methods for environmental water samples should be investigated.
4. PCR conditions should be revised in order to decrease valuable detection time and deliver results faster.

In general, this study demonstrated that an interdisciplinary approach to optimizing detection methods is a challenging, but inevitable endeavor.

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15. Glossary

(Adapted from Brock and Madigan, 1991)

Adenine	A purine base that pairs with thymine in the DNA structure.
Amplification	The production of many DNA copies from one master region of DNA.
Annealing	Spontaneous alignment of two single DNA strands to form a double helix.
Autoradiography	Detection of light emission in a sample, for example a gel or membrane, by placing it in contact with a photographic paper.
Biotechnology	The use of living organisms for the large-scale production of valuable products.
Catalyst	A substance that promotes a chemical reaction without itself being changed in the end.
Coccus	A spherical bacterium
Coliforms	Gram-negative, nonsporing, facultative rods that ferment lactose with gas formation within 48 hours at 35°C.
Colony	A population of cells growing on solid medium arising from a single cell.
Complementary	Nonidentical but related genetic structures that show precise base pairing.
Covalent	A nonionic chemical bond formed by a sharing of electrons between two atoms.
Culture	A particular strain or kind of organism growing in a laboratory medium.
Cyst	A resting stage formed by some bacteria and protozoa in which the whole cell is surrounded by protective layer: not the same as spore.
Cytosine	A base that pairs with guanine.

Denaturation	Irreversible destruction of a macromolecule, as for example the destruction of a protein of DNA fragments by heat.
Deoxyribonucleic acid (DNA)	A polymer of nucleotides connected via a phosphate-deoxyribose sugar backbone; the genetic material of the cell.
Dilution series	Serial dilution, i.e. 10^{-1}, 10^{-2}, 10^{-3}.
DNA ladder	Molecular size reference consisting of molecules with defined lengths.
Dot blot hybridization	Specific type of hybridization in form of dot blots.
Doubling time	The time needed for a population to double.
Downstream position	Refers to nucleic acid sequences on the *3' side* of a given site on the DNA of RNA molecule. Compare with upstream.
Elongation	Chemical synthesis of oligonucleotide.
Endemic	A disease that is constantly present in low numbers in a population.
Enteric	Intestinal.
Enterotoxin	A toxin affecting the intestine.
Enzyme	A protein functioning as the catalyst of living organisms, which promotes specific reactions or groups of reactions.
Epidemic	A disease occurring in an unusually high number of individuals in a community at the same time.
Eukaryote	A cell of organism having a true nucleus.
Facultative	A qualifying adjective indicating that an organism is able to grow either in the presence or absence of an environmental factor, for example oxygen.
Fluorescent	Having the ability to emit light of a certain wavelength when activated by light of another wavelength.

Gel	An inert polymer, usually made of agarose or polyacrylamide, used for separating macromolecules such as nucleic acids or proteins by electrophoresis.
Gene	A unit of heredity; a segment of DNA specifying a particular protein or polypeptide chain.
Genome	The complete set of genes present in an organism.
Genotype	The genetic complement of organisms.
Guanine	A base that pairs with cytosine.
Homology	Matching in structure.
Hybridization	The natural formation or artificial construction of stable hybrid nucleic acid complexes by complementary base pairing between two nucleic acid strands.
Inhibition	Prevention of growth or function.
Inoculum	Material used to initiate a microbial culture.
Intersection	Process of binding between two complementary DNA fragments.
Kilobase	A one-thousand base fragment of nucleic acid. A kilobase pair is a fragment containing one thousand base pairs.
Luminescence	Production of light.
Lysis	Rupture of a cell, resulting in loss of cell contents.
Lysozyme	Digestive enzyme
Macromolecule	A large molecule formed from the connection of a number of small molecules. A polymer.
Membrane	Any thin sheet or layer.
Molecule	The result of two or more atoms combining by chemical bonding.

Negative control	PCR reaction mixture without template DNA (Blank).
Nucleic acid	A polymer of nucleotides.
Nucleotide	A monomeric unit of nucleic acids, consisting of a sugar, phosphate, and nitrogenous base.
Nucleus	A membrane-enclosed structure containing the genetic material (DNA) organized in chromosomes.
Oligonucleotide	A short nucleic acid molecule, either obtained from an organism or synthesized chemically.
Parasite	An organism able to live on and cause damage to another organism.
Pathogen	An organism able to inflict damage on a host it infects.
Phenotype	The characteristics of an organism observable by experimental means.
Polymer	A large molecule formed by polymerization of monomeric units.
Polymerase chain reaction	A method for amplifying DNA in vitro involving the use of oligonucleotide primers complementary to nucleotide sequences in a target gene and copying the target sequences by the action of DNA polymerases.
Primer	A short stretch of RNA or DNA used as a starting point of nucleic acid synthesis.
Probe	A short stretch of nucleic acid complementary in base sequence to a nuclei acid from a given source, and used to selectively isolate the nucleic acid from a mixture of other nucleic acids.
Prokaryote	A cell or organism lacking a true nucleus, usually having its DNA in a single molecule.
Prophylactic	Treatment, usually immunologic, designed to protect an individual from a future attack by a pathogen.

Protein	A polymeric molecule consisting of one or more polypeptides.
Proteinase K	Digestive enzyme.
Pure culture	An organism growing in the absence of all other organisms.
Recombination	Process by which genetic elements in two separate genomes are brought together in one unit.
Redundancies	Ability of bases to bind to two or more types of bases.
Restriction endonucleases	Enzymes that recognize and cleave specific DNA sequences, generating either blunt or more commonly, single-stranded ends.
Ribonucleic acid	A polymer of nucleotides connected via a phosphate-ribose backbone, involved in protein synthesis.
Ribosome	A cytoplasmic particle, which is part of the protein-synthesizing machinery of the cell.
Species	A collection of closely related strains.
16S rRNA	Specific RNA molecule involved in ribosome structure and function.
Spore	A general term for resistant resting structures formed by many bacteria and fungi.
Sterile	Free of living organisms.
Strain	A population of cells all descended from a single cell.
Swab	Sterile sampling device.
Template	A molecule "mold" that shapes the structure or sequence of another molecule.
Thymine	A base that pairs with adenine.
Toxin	A microbial substance able to induce host damage.
Trophozoite	Protozoal cells

Upstream position	Refers to nucleic acid sequences on the *5' side* of a given site on a DNA or RNA molecule.