Ecology of pathogenic *Escherichia coli* in water: Survival and Molecular Detection

by

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An Abstract of the Thesis

Recently there has been a surge of interest in the ability of enterohemmorhagic *Escherichia coli* (EHEC) to survive in aquatic environments. It has been speculated a correlation may exist between the genotype of an *E. coli* strain and its ability to persist in an aquatic environment. One of the objectives of this study was to determine if any correlation could be made between the persistence of EHEC strains in natural well water and their ERIC-genotype. Sixteen strains of EHEC were monitored, individually, in untreated well water microcosms incubated at 10°C and 22°C for 56 days. The strains were selected from three serogroups (O26, O111 and O157) and represented six distinct genotypes determined by ERIC-PCR. The microcosms were prepared, in triplicate, each sample having a final cell concentration of approximately 10^7 cfu/ml well water. Levine Eosin Methylene Blue agar was used to determine the cell density of culturable *E. coli* cells. At 10°C, cell density declined depending on the strains by 1.78 to 6.07 log units in 8 weeks, with six strains falling below the detection limit of 0.8 log cfu/ml by day 56. Of the ten persisting strains, four showed superior survival with cell density decreasing to an average of approximately 5 log cfu/ml while the remaining six strains showed moderate levels of survival, decreasing to a average cell density of approximately 3.5 log cfu/ml. At 22°C all strains eventually dropped by 4.87 to 6.52 log in 56 days with 14 strains dropping below the detection limit. The 16 strains demonstrated highly variable levels of survival with no correlation between ERIC-genotype and the strain's ability to persist being evident.

The second objective of the study was to develop a molecular detection method for pathogenic *E. coli* employing multiplex-PCR and DIG-labeled DNA probes. The molecular detection of EHEC and other pathogenic *E. coli* of virotypes, entertoxigenic (ETEC), enteropathogenic (EPEC) and enteroinvasive (EIEC), has been the topic of numerous studies. A multiplex PCR-DNA probing assay to detect the four major *E. coli* virotypes was developed. Six highly specific PCR primer sets and DIG-labeled chemiluminescent probes were designed to target the shiga-like toxin I and II genes (*sltI* and *sltII*) of EHEC, heat stable and heat labile toxin genes (*hs* and *hl*) of ETEC, EPEC-adherence factor gene (*eaf*) of EPEC and the invasiveness plasmid (ial) of EIEC. The primer sets generate amplicons 350, 262, 170, 322, 293 and 390 bp in length, respectively. The multiplex primers were tested for specificity against 31 pathogenic *E. coli* strains, various non-pathogenic *E. coli* strains and non-*E.coli* enteric and environmental bacterial strains. The results showed a high degree of specificity for strains from their corresponding virotypes and minimal non-specific reaction with the non-target *E. coli* and control bacterial strains. To improve the specificity of the multiplex-PCR amplification, six DIG-labeled oligonucleotide probes were designed to hybridize with the six multiplex-PCR amplicons. Chemiluminescent detection of probe hybridization against multiplex-PCR dot blots revealed high degrees of specificity of probes for the target amplicons. The proposed multiplex PCR-DNA probing assay provides rapid and specific detection of four major *E. coli* virotypes.

Ecology of pathogenic *Escherichia coli* in water: Survival and Molecular Detection

Chapter 1

Introduction to the Thesis

Pathogenic *Escherichia coli* have become a major health concern as they have been associated with the contamination of many foods (Samadpour *et al.*, 1994; Janisiewicz *et al.*, 1999; Gyles *et al.,* 1998), drinking water (Swerdlow *et al.*, 1992; Rasmussen and Casey, 2001) and recreational water (Ackman *et al.*, 1997). Six virotypes of *E. coli* have been identified based on the virulence factors they express and the gastrointestinal diseases they cause. The four major virotypes are enterohemmorhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and enteroinvasive *E. coli* (EIEC) (Salyers and Whitt, 1994).

E. coli O157 is one of the most well known serotypes of pathogenic *E. coli* (Law, 2000). This serotype belongs to the virotype EHEC and has been the focus of numerous studies. Although this serotype is important to public health, other members of the EHEC virotype can be equally pathogenic as over 100 EHEC serotypes have been identified (Law, 2000). Recurrence of illness is often a result of their ability to persist in host animals and the environment (LeJeune *et al.*, 2001). The persistence of *E. coli* O157 has been monitored in a variety of studies focusing on water (Rice *et al.*, 1992; Warburton *et al.*, 1998; Wang and Doyle, 1998), soil and manure (Fukushima *et al.*, 1999; Topp *et al.*, 2003).

Investigation of characteristics allowing this pathogen to persist has been limited. Numerous influences have been suggested including particulate niche protection

(Brettar and Höfle, 1992), internalization by protozoa (Maule, 2000) and intrinsic differences between strains (Wang and Doyle, 1998). The genetic composition, or genotype, of the organism is one intrinsic difference which has been proposed to influence the survival of *E. coli* in manure (Topp *et al.*, 2003). Typing methods have been applied to *E. coli* illustrating that the genomes of *E. coli* strains are very diverse (Topp *et al.*, 2003). It is important to determine the influence this diversity has on the survival of *E. coli* strains. The first objective of this thesis was to investigate the potential influence of ERIC-genotype on the persistence of EHEC in natural well water. The second component involves the development of a molecular detection method to detect the four major virotypes of diarrheic *E. coli* simultaneously.

The large variety of serotypes and phenotypic characteristics associated with EHEC and the other *E. coli* virotypes, EPEC, ETEC, and EIEC, makes the identification of pathogens by standard methods a time consuming and labour intensive process. Problems with the current methods of detection are emerging as strains with aberrant biochemical results (Ware *et al.*, 2000) begin to appear and other strains lose their antigenicity (Hara-Kudo *et al.*, 2000). These issues with standard detection methods for pathogenic *E. coli* have spurred the development of numerous molecular based methods. The methods developed have focused detection on a number of genes associated with phenotypic and virulence traits. Multiplex-PCR is a common molecular detection method applied to pathogenic *E. coli*. The methods developed so far target, primarily, a single virotype, EHEC (Osek, 2002; Pollard *et al.* 1990; Paton and Paton, 1998) or a single serotype, namely *E. coli* O157:H7 (Cebula *et al.,* 1995; Fratamico *et al.*, 1995; Venkateswaran *et al.*, 1997; Campbell *et al.*, 2001; Wang *et al.*, 2002). Two

studies have developed assays which target multiple virotypes, but they divide the multiplex-PCR into three (Rappelli *et al.*, 2001) or four (Pass *et al.*, 2000) different reactions.

This introductory chapter will review the significance of *Escherichia coli* as a pathogen, its persistence in host animals and the environment and new means for its detection.

Escherichia coli

Escherichia coli are facultatively anaerobic, Gram-negative, rod shaped bacteria which belongs to the family Enterobacteriaceae. *E. coli* are generally harmless natural commensal organisms found in the intestine of warm-blooded animals (Holt *et al.*., 1994). Specific strains, such as *E. coli* K12, have long been used as tools for research, while others cause severe disease in both animals and humans (Smith, 1992).

E. coli can be subdivided based on the serology of the lipopolysaccharide (O), flagellar (H) and capsular (K) antigens or by the presence of specific virulence factors (Holt *et al.*, 1994) resulting in distinctive serotypes or virotypes, respectively. Six virotypes of *E. coli* have been identified: enterohemorrhagic (EHEC), enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAggEC) and diffusely adherent (DAEC). The division of these diarrhea inducing *E. coli* strains is based on the virulence factors they possess and differences in the signs of disease generated (Smith, 1992). The virulence factors of EAggEC and DAEC have not yet been fully characterized (Rappelli *et al.*, 2001) so only the four virotypes EHEC, EPEC, ETEC and EIEC will be studied here.

Enterohemorrhagic *Escherichia coli* (EHEC)

Enterohemorrhagic *E. coli* has become a major culprit in food and water borne illness. The resultant diseases include watery diarrhea, bloody diarrhea, neurologic complications, thrombotic thrombocytopenic purpura (TTP), hemolytic uremic syndrome (HUS) and even death. The production of toxins by the infectious agents has been identified as one of the primary causes of symptom development (Nakao and Takeda, 2000).

Shiga toxins (Stx) have been identified as the major virulence factors of EHEC. The nomenclature associated with these toxins has become increasingly complicated since first reported in *E. coli* in 1977. At this time the toxin was referred to as VT for verotoxin (Nakao and Takeda, 2000). Further investigation into toxins produced by *E. coli* found that some of the cytotoxic activity could be neutralized by an antitoxin prepared against the *Shigella dysenteriae* 1 toxin and this toxin was called SLT for Shiga-like toxin. These toxins were later found to be the same and to be the common virulence factor for hemorrhagic colitis (HC) and HUS. It has been established that the VT toxin is equivalent to SLT which is equivalent to Stx (Nakao and Takeda, 2000). Irrespective of name, the toxins generated are of major importance in the generation of disease. This has resulted in a great deal of research into the structure and activity of these toxins.

The Stx toxins are A-B toxins consisting of two major groups called Stx1 and Stx2 (Schmidt, 2001). A-B toxins are composed of two portions, A and B. Portion B is responsible for binding the toxin to the target cell while portion A mediates the enzymatic activity (Salyers and Whitt 1994). The genes encoding both Stx variants are

thought to be generally encoded in the genome of lambdoid prophages (Schmidt, 2001), which integrate into the bacterial chromosome. Both toxins have been found to be similar in biological activity and over all structure, although differing in immunological and physicochemical traits. Stx1 is conserved within *E. coli* with few variants being reported, while more than 20 potential variations have been reported for Stx2. The Stx toxins possess at least three activities: cytotoxicity, enterotoxicity and neurotoxicity (Nakao and Takeda, 2000; Mainil, 1999).

For these toxic effects to occur, however, the *E. coli* cells must adhere to their target of infection. Following ingestion by a host organism and successful passage through the stomach, pathogenic EHEC must adhere to the mucosal lining to avoid being passed out of the gastrointestinal tract. Conflicting results have arisen regarding the expression and activity of fimbrial structures involved in the adhesion of EHEC, specifically serotype O157. Sequencing of the 60 MDa pO157 plasmid did not recover a fimbrial gene cluster such as those encoded on the large plasmid of EPEC strains, which are characterized by their intimate attachment to intestinal cells. This suggests that it is unlikely that fimbrial adhesin is encoded on this plasmid and any adhesin(s) generated must be encoded chromosomally. A variety of outer membrane proteins associated with *E. coli* O157 adherence have been identified. These proteins are distinct from the intimin protein associated with EPEC (Law, 2000). It is the attachment of the cells that allows them to become intimately associated with their target host cells and brings the toxins into close enough proximity to the cell to initiate their biological activity.

Enteropathogenic *Escherichia coli* (EPEC)

EPEC are organisms which are capable of adhering to the surface of mucosal cells and causing changes to the microvilli and structural rearrangement of the host cells actin cytoskeleton (Rappelli *et al.*, 2001). This *E. coli* virotype causes diarrhea that predominates in children in developing nations. EPEC causes disease in very young children, usually younger than six months and rarely over the age of one year. This virotype is also a recognized cause of chronic diarrhea (Donnenberg and Kaper, 1992). The EPEC virotype is characterized by infection of the small intestine and the presence of bacteria clusters at the epithelial surface. The attachment of this organism is vital to disease development (Tobe *et al.*, 1999). EPEC adherence induces the formation of pedestal-like structures on epithelial cells beneath the adherent bacteria. Microvilli close to the site of attachment are also lost. Initially the loss of microvilli induced by EPEC infection alone was thought to be the cause of fluid secretion and diarrhea associated with EPEC as this could lead to malabsorption and osmotic diarrhea. It has been determined, however, that elevation of intracellular calcium ions is also a potential cause of fluid loss initiated by EPEC infection (Donnenberg and Kaper, 1992)

Initial attachment of the EPEC cells to the target host cell is caused by intimin, the 94 kDA outer membrane protein product of the *eaeA* gene (Donnenberg and Kaper, 1992). In vitro studies of EPEC infection have demonstrated that attached bacteria transduce signals into host cells via the secretion of effector molecules. The effect is termed attaching and effacing, the results of which are cytoskeletal rearrangement and a dramatic loss of microvilli. It was established early that EPEC strains produce distinct microcolonies, referred to as localized adherence (LA), rather than uniformly covering

the host cell (Donnenberg and Kaper, 1992). The localized adherence (LA) phenotype in EPEC has been correlated with the carriage of a large plasmid in the range of 50 to 70 MDa depending on the serotype and strain. This family of related plasmids have been denoted the EPEC adherence factor (EAF) plasmids. The importance of these plasmids was demonstrated in a volunteer study which found that a plasmid-cured EPEC strain was significantly less pathogenic than the parent strain. In addition to being less virulent the plasmid-cured strains were found to lack the LA phenotype (Tobe *et al.*, 1999). This localized adherence is dependent upon adherence to both the host cell and neighbouring EPEC cells. The EAF plasmid encodes the necessary bundle-forming pili (BFP) which are produced within adherent microcolonies. The BFP create a meshwork of interbacterial fibers that physically stabilize the attached colony (Tobe *et al.*, 1999). Without colony formation there will be limited disease development. The diarrhea and other symptoms associated with EPEC are thought to be the result of the bacterial invasion of the host cell and the resulting impact on signal transduction systems (Salyers and Whitt, 1994).

Enterotoxigenic *Escherichia coli* (ETEC)

ETEC are strains of *E. coli* that produce, either a heat labile enterotoxin (LT), a heat stable enterotoxin (ST) or both (Rappelli, *et al.*, 2001). They cause acute, watery diarrhea in humans and animals and are responsible for a significant amount of morbidity and mortality among young children and the elderly in developing countries (O'Brien and Holmes, 1996). ETEC is one of the most common etiological agents of traveler's diarrhea in developed and developing countries and may account for 30-50%

of cases reported. Infants in third world countries are also prone to developing diarrhea due to ETEC infection and these episodes can occur sporadically or in outbreaks. Young domestic animals are susceptible to diarrhea due to ETEC, but adults do not seem to be afflicted (Gyles, 1992). Virulence of ETEC also requires the production of a species-specific colonization factor antigen in addition to enterotoxins (O'Brien and Holmes, 1996).

LT are closely related to cholera toxin of *Vibrio cholerae* in both structure and function. LTs are classified as either LT-I or LT-II based upon polyclonal antibody neutralization tests. The LT mode of action involves activation of the enzyme adenylate cyclase that causes an increase in intracellular cyclic AMP (O'Brien and Holmes, 1996). Increased cAMP levels disrupts the activity of sodium and chloride transporters of the host cell which results in an ion imbalance. This imbalance causes excess water loss by the cell resulting in diarrhea (Salyers and Whitt, 1994).

ST are low-molecular weight, extracellular polypeptides which are capable of altering the movement of fluid and electrolytes across the intestinal epithelium. There exist two variants of ST, designated ST-I and ST-II. These variants are unrelated and differ in both structure and mechanism of action (Gyles, 1992). Both are encoded by plasmids which vary greatly in molecular size and which may also encode LT, fimbrial colonization factor antigens and antibiotic resistance determinants. *E. coli* and a variety of other gram-negative bacteria produce ST-I. ST-I proteins bind to the extracellular domain of guanylate cyclase C and directly stimulate its intracellular catalytic activity. In enterocytes of the small intestine, ST-I initiates a cascade involving the intracellular accumulation of cyclic GMP and eventually results in the secretion of Cl^- ions into the

intestinal lumen as seen in LT activity. ST-II production has been demonstrated primarily by ETEC isolates from pigs, however, the gene for this toxin displays homology with DNA from ETEC strains isolated from humans. The activity of ST-II does not equal that of LT or ST-I, as ST-II proteins have been shown to cause elevated concentrations of intracellular calcium ions resulting in water loss (O'Brien and Holmes, 1996).

Enteroinvasive *Escherichia coli* (EIEC)

EIEC resemble *Shigella* in terms of pathogenicity and most virulence genes are encoded on large virulence plasmids almost identical to those of *Shigella* spp. (Rappelli *et al.*, 2001). EIEC cause occasional outbreaks of food-associated diarrhea and dysentery in adults in developed nations and can cause similar disease in children in less developed nations. These *E. coli* strains share both antigenic and pathogenic traits with *Shigella* spp. EIEC are capable of penetrating and multiplying within colonic epithelial cells. It has been determined that only those harbouring a large plasmid, termed the Invasiveness plasmid, display the invasive phenotype and that this plasmid therefore encodes the necessary products for invasiveness. The activity of enterotoxins and cytotoxins in EIEC mediated diarrhea and dysentery has been investigated. An enterotoxin was identified which is 68 to 80kDA in size but its activity was not found to be dependent on the presence of the large plasmid. Cytotoxic activity was also detected, but it was at lower levels and distinct from the enterotoxic activity observed (O'Brien and Holmes, 1996). Data on EIEC appear limited. This may be due to EIEC similarity to *Shigella* spp and thus *Shigella* being the focus of invasiveness research.

Reservoirs of *Escherichia coli*

EHEC have become a major cause of water borne outbreaks. Shiga toxin producing *E. coli* are a major cause of human illness resulting in a spectrum of clinical manifestations including watery diarrhea, bloody diarrhea and hemolytic uremic syndrome. A correlation has been made between cattle density and the incidence of *E. coli* associated disease (Valcour *et al.,* 2002).

Cattle are considered the primary reservoir for EHEC (Valcour *et al.*, 2002). Shiga toxin producing *E. coli* interact with cattle in two ways, either as an intestinal disease of calves, new born to 4 months old, or as an asymptomatic carriage in healthy animals, young calves and adults (Mainil, 1999). The carriage of these organisms by healthy cattle has stimulated large scale surveys for the presence of EHEC, especially serogroup O157, in beef and dairy herds including healthy adult cattle, healthy young calves, young bulls at slaughter, culled cows and veal calves (Mainil, 1999). The international surveys involved countries in Europe, North and South America and Asia (Mainil, 1999). Although each survey detected Shiga toxin producing *E. coli* in most herds the proportion of animals testing positive varied between studies (Mainil, 1999). There does not appear to be a definitive prevalence of *E. coli* O157 in beef over dairy cattle or *vice versa*. The involvement of cattle in the spread of *E. coli* O157:H7 has been the focus of much research, resulting in conflicting information. The conflicting data spurred a longitudinal study in the United State (Shere *et al.*, 1998) which investigated the age of first infection, levels and length of shedding of the organism, maintenance and dissemination in cattle, vehicles and/or sources and the impact of farm management on *E. coli* O157:H7 incidence in a herd. The findings of this study

conclude that herds found to be negative initially are capable of maintaining zero to low prevalence of *E. coli* O157:H7. In positive herds, seasonal shedding of *E. coli* O157:H7 was not observed. The shedding observed was intermittent and may have been a result of re-inoculation from an environmental source rather than colonization. Genomic typing of O157:H7 isolates indicated that a point source of *E. coli* O157:H7 was the cause of dissemination of a strain through a herd, most likely being a contaminant of the animals' drinking water (Shere *et al.*, 1998). Further investigations into the prevalence of O157:H7 in cattle have generated alternate conclusions on the subject of shedding, with an increase of shedding observed during the summer and early fall (Elder *et al.*, 2000). Numerous epidemiological studies have been conducted to identify the risk factors and management practices that may affect fecal shedding. Little agreement has been reached on the statistical significance of the factors evaluated (Rasmussen and Casey, 2001). Whatever the shedding triggers may be, it has been well established that cattle are the primary reservoir of *E. coli* O157:H7. There are however numerous alternative sources of this pathogen including pigs, birds and other domestic mammals.

Shiga toxin producing *E. coli* are the cause of a well-documented disease of piglets termed edema disease (ED) (Mainil, 1999). A disease with a potential morbidity rate of 30-40% in some farms and a case fatality rate greater than 90%. Symptoms include subcutaneous edema and nervous clinical signs (i.e. convulsions) with diarrhea occurring rarely. EHEC are the causal virotype, generating Shiga toxins, specifically Stx2e, which has been determined to be, with very few exceptions, pig specific. Shiga toxins other than Stx2e have been detected in porcine *E. coli.* These toxins however are not associated with ED. Public heath concerns have been raised as Stx2e

generating EHEC have been isolated from humans and the existence of clinical similarities between hemolytic uremic syndrome (HUS) of humans and ED of pigs have been determined. As porcine EHEC serogroups have not been observed in humans with HUS and few porcine EHEC isolates have been of the O157 serogroup, it has been concluded the piglets, pork and pork products are not a source of contamination of EHEC for humans (Mainil, 1999).

Other animals have also been identified as potential sources of pathogenic *E. coli*. Birds, though their association with the pathogen is uncertain, are believed to be potential disseminators of infection, especially among domestic ruminants and humans. Although rare and documentation is often incomplete, isolation of EHEC from pets such as cats, dogs and horses has also been observed, the latter two being cited as potential vectors for human contamination with O157:H7 (Mainil, 1999). Other domestic farm animals have also been identified as asymptomatic carriers of EHEC although there is little data available concerning the prevalence of EHEC in small ruminants. A study designed to investigate the prevalence of EHEC in the feces of healthy dairy ruminants, found that in addition to cattle, both sheep and goats were asymptomatic carriers of Shiga toxin producing *E. coli* (Zschöck *et al.*, 2000). Although not detected in this study, previous isolation and characterization of O157:H7 from sheep has been achieved. The detection of *E. coli* O157 in culture positive animals only during the summer (Zschöck *et al.*, 2000) once again suggests shedding of this organism is seasonal.

Drinking water has repeatedly been identified as a source of *E. coli* O157:H7 infection, for example, the outbreak in Cabool, Missouri in 1990 (Swerdlow *et al.*, 1992) and the case of the Walkerton, Ontario outbreak in the spring of 2000 (Rasmussen and

Casey, 2001). Exposure to recreational water has also been associated with the development of hemorrhagic colitis (HC), due to *E. coli* O157:H7 (Ackman *et al.*, 1997). Infection of the human population is not completely dependent upon the process of water contamination. A variety of foods have also been identified as potential sources of infection.

Undercooked meat, primarily ground beef, has been identified as a major cause of *E. coli* O157:H7 infection resulting in severe disease symptoms (Elder *et al.*, 2000). The primary culprit being contamination of raw meat during slaughter or processing with feces carrying O157:H7 cells. Because the culture based isolation of the pathogen can be difficult various approaches have been taken to detect such contamination. One study employed DNA probes targeting the *slt*I and *slt*II gene sequences to detect Shiga toxin producing *E. coli*. Although beef was the primary target, bacterial Shiga toxin genes were also detected in samples of pork, poultry, lamb, fish and shellfish (Samadpour *et al.*, 1994).

The contamination of food products is not restricted to meats. Although less frequent, outbreaks associated with the consumption of contaminated fruits and vegetables have also occurred. External barriers such as peels and rinds typically prevent the entry and subsequent growth of microorganisms in the interior of fruits and vegetables. However, it has been shown that wounds in the flesh of fruits and vegetables will allow growth of *E. coli* (Janisiewicz *et al.*, 1999). Raw milk has also been identified as a potential source of O157:H7 infection, either from direct consumption or the consumption of raw milk products such as cheese (Zschöck *et al.*, 2000). The ability

of *E. coli* to persist within animal host populations and the environment is the reason why there are such a variety of sources of EHEC infection.

Persistence of Enterohemorrhagic *E. coli*

Water

Contamination of water by *E. coli* O157:H7 has resulted in numerous outbreaks related to recreational water and drinking water. Numerous studies have been performed investigating the survival of *E. coli* in water such as natural river and lake water, municipally treated drinking water and bottled drinking water. *E. coli* surviving in aquatic systems can show reduced culturability making detection difficult (Arana *et al.*, 1997). This can greatly reduce the detection of water contamination. As in soil, persistence and survival of these organisms in natural water may be attributed to protective niches generated by particulate materials (Brettar and Höfle, 1992) and inside protozoa (Maule, 2000). A comparison of the ability of *E. coli* O157:H7 to survive in municipal water, reservoir water and recreational lake water revealed that *E. coli* O157:H7 survived better in sterilized municipal well water than in untreated recreational lake and reservoir water. The higher level of survival was attributed to the poor ability of O157 to compete with other microorganisms (Wang and Doyle, 1998). Although the data agree with data seen in previous studies of *E. coli* O157:H7 survival in municipal water by Rice *et al.* (1992), the survival rates differed. Studies monitoring the survival of *E. coli* O157:H7 from bottled water (Warburton *et al.*, 1998) and natural mineral water (Kerr *et al.*, 1999) also saw similar trends in survival, for example, greater recovery from lower temperature water. The only difference seen between the two studies being the

length of time of cell recovery which were, 309 days (Warburton *et al.,* 1998) and 63 days (Kerr *et al.,* 1999). The differences in recovery time and number from bottled and municipal water have been attributed to a variety of influences: presence of culture media in cell inoculum (since water was contaminated artificially), presence of organic matter (Kerr *et al*, 1999), temperature, differences in water composition and intrinsic differences in the *E. coli* O157:H7 strains (Wang and Doyle, 1998). Intrinsic genotypic differences of EHEC are a suspected cause of survival variability between strains of *E. coli.* This hypothesis is to be addressed in this study.

Cattle

Cattle have been identified as the primary reservoir of *E. coli* O157:H7 (Valcour *et al.*, 2002), but it is believed that environmental persistence of *E. coli* O157 plays a key role in the epidemiology of this infectious agent. Although fecal excretion of *E. coli* O157 is only transient, typically lasting 3 to 4 weeks, *E. coli* O157 can be repeatedly isolated from environmental sources for periods lasting several years. This suggests the presence of non-bovine reservoirs for *E. coli* O157 (LeJeune *et al.*, 2001). Surveys have shown that *E. coli* O157:H7 can be isolated from water troughs of cattle and it has been suggested that these troughs housing contaminated sediments are a source of continuous re-infection (LeJeune *et al.*, 2001; Rasmussen and Casey, 2001). Such domestic environmental reservoirs are one cause of the persistence of *E. coli* O157 in cattle populations. Although persistence in a domestic environment and within cattle populations is a great concern, *E. coli* O157 has been found to persist in soil and water, independent of a cattle population.

Soil

The shedding of *E. coli* O157:H7 by animals has been identified as a source of isolates detected in the environment such as soil. One study has followed the decay of *E. coli* O157 in soil after an outbreak associated with sheep. Following an outbreak of *E. coli* O157 amongst attendants of a scout camp it was determined that the field location for the camp had previously been used for grazing by approximately 300 sheep. Heavy rain had caused the contaminated fecal material to become widespread throughout the entire field. The study observed a 105 day survival period in the soil by the *E. coli* O157 contaminants. This survival was only slightly below the 130 days of survival observed in experimentally generated laboratory data (Ogden *et al.*, 2002). Lack of tilling, presence of plant roots (Ogden *et al.*, 2002) and soil protozoans (Barker *et al.*, 1999) have been suggested as possible causes for the enhanced survival of *E. coli* O157 observed in soil.

Soil protozoans have been identified as a reservoir for numerous human pathogens including, *Legionella* spp, *Listeria*, opportunistic mycobacteria, coliforms and *E. coli* O157 (Barker *et al.*, 1999). Intra-protozoal growth of these organisms has been linked with enhanced environmental survival, increased virulence and elevated resistance to biocides and antibiotics (Barker *et al.*, 1999). The ability to survive within the vacuoles of a protoazoan not only allows for extended persistence in the environment as they are protected from predation and stress but also contributes to enhanced dissemination in both soil and water (Maule, 2000).

Typing methods applied to *E. coli*

Most strains of *E. coli* are not pathogenic and different strains of *E. coli* cause different types of disease. For these reasons methods of strain differentiation were developed. Initially strain identification involved serological classification based on antigenic differences in highly variable bacterial surface molecules. This method is referred to as serotyping. As molecular biological techniques became well established, numerous methods evolved which differentiate *E. coli* based on the strain's genetic composition. These methods are referred to as genotyping methods (Salyers and Whitt, 1994).

Serotyping

Serotyping of *E. coli* is based on two surface components, the O antigen of lipopolysaccharide, and the H antigen the flagella. The O antigen identifies the serogroup of a strain while the H antigen identifies its serotype. Thus two strains may belong to the same serogroup but belong to different serotypes (Salyers and Whitt, 1994). For the virotype EHEC alone greater than 160 serogroups have been identified in humans and greater than 200 have been identified in cattle (Gyles *et al.*, 1998). Even though *E. coli* strains belonging to particular serogroups, i.e. O157 and O111, have been found to have greater virulence for humans (Paton and Paton, 1999) there is not always a correlation between pathogenicity and the expressed antigens (Rappelli *et al.*, 2001).

Genotyping

Genotypic analysis has many epidemiological applications for recognizing outbreaks of infection, detecting the cross-transmission of nosocomial pathogens, determining the source of an infection and recognizing particularly virulent strains of organisms. Typing or strain classification has been accomplished using a variety of approaches. The methods applied must meet three important criteria in order to be useful. All organisms within a species must be typeable by the method employed. Any typing method must possess a high differentiation capability of unrelated strains and demonstrate the relationship of related strains. Thirdly, a major concern of typing methodology is reproducibility, the ability of a technique to yield the same result when a particular strain is repeatedly tested (Olive and Bean, 1999). There are numerous typing methods available, however, four common methods are Pulse-field gel electrophoresis (PFGE), Amplified fragment length polymorphisms (AFLP), Repetitive extragenic palindrome (Rep) -PCR and Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR.

(i) Pulse-field gel electrophoresis (PFGE)

PFGE is a highly regarded method of molecular typing. The procedure involves embedding whole bacterial cells in agarose gel plugs. The cells are subjected to *in situ* detergent lysis and digestion with a low-cutting frequency restriction enzyme to generate large fragments. To clearly separate the large DNA fragments generated (often ranging from 10 to 800 kb), the agarose plugs are inserted into an agarose gel. The gel is subjected to a current that changes polarity at predetermined intervals. The changing of the current's polarity is necessary to effectively separate the large

fragments within the gel. The electrophoretic patterns are then visualized using a fluorescent stain such as ethidium bromide. The primary drawback of the method is the time required to complete the procedure which can take 2 to 3 days (Olive and Bean, 1999). The length of the procedure, however, has not hindered its application in the verification of *E. coli* transmission from dairy farms (Lahti *et al.*, 2002). The procedure has also been applied in a study to monitor *E. coli* O157 contamination of carcasses during slaughter. The study successfully eliminated outside contaminants as the source of carcass contamination and identified fecal material associated with the carcasses as the likely source (Barkocy-Gallagher *et al.*, 2001). The procedure has also been applied in another study to detect heterogeneity amongst Shiga toxin producing *E. coli* strains isolated from a variety of sample sources including HUS patients, cattle and food (Pradel *et al.*, 2001). The Centre for Disease Control in the United States of America also uses PFGE for surveillence of *E. coli* O157:H7 in food borne disease (PulseNet).

(ii) Amplified Fragment Length Polymorphisms (AFLP)

AFLP is a genome fingerprinting technique based on the selective amplification of a subset of DNA fragments generated by restriction enzyme digestion of genomic DNA. In the most common application bacterial DNA is extracted, purified and subjected to digestion with two different restriction enzymes. The fragments generated by digestion are then ligated to linkers containing the corresponding restriction site and a DNA sequence homologous to a pre-designed PCR primer-binding site. By employing primer sequences homologous to the linkers and containing one to two selective bases at the 3' end only a subset of the genomic restriction fragments will be amplified. The patterns generated by gel electrophoresis can then be analyzed (Olive and Bean,

1999). The method has been found to have a high level of reproducibility and discriminating power (Savelkoul *et al.*, 1999). AFLP has been applied to type *E. coli* isolated from poultry in South Africa where it was found that 30 different genotypes existed within the 50 isolates tested. The study also compared the use of plasmid profiling as a typing method with AFLP. It illustrated that even though specific plasmid profiles can be associated with specific AFLP fingerprints, the method of plasmid profiling alone is a poor typing method. This is a result of plasmid instability and low reproducibility due to variable bands resulting from open and linear forms of the plasmids (Georgnaras *et al.*, 2001).

(iii) Repetitive extragenic palindromic (Rep) PCR and Enterobacterial repetitive intergenic concensus (ERIC) PCR

Both Rep-PCR and ERIC-PCR are genomic fingerprinting methods employing repetitive elements found within bacterial genomes. Rep-PCR generates strain specific electrophoretic patterns by PCR amplification of repetitive sequences termed repetitive extragenic palindromic (Rep) elements. Rep elements are 38 bp sequences consisting of six degenerate positions and a 5 bp variable loop between each side of a conserved palindromic stem. Stem loop structures can be generated due to the palindromic nature of the Rep elements (Olive and Bean, 1999). A second set of repetitive elements has been identified which can also be used to type bacterial strains, enterobacterial repetitive intergenic consensus (ERIC) sequences. These sequence are 126 bp elements which possess a highly conserved central inverted repeat and are located in intergenic regions of the genome. The Rep or ERIC amplification methods can be performed with a single primer, a single set of primers or multiple sets of primers targeting the repetitive element sequences. Although ERIC patterns are less complex

than Rep patterns, both methods provide good discrimination at the strain level (Olive, and Bean, 1999). ERIC-PCR has been applied numerous times in the genotyping of *E. coli*. The method is able to discriminate between genotypes found within a single serotype, for example O157 (Adwan *et al.*, 2002). The ERIC-PCR procedure has also been applied to determine the genetic relatedness of *E. coli* isolated from pigs (Osek, 2000) and poultry (Carvalho de Moura *et al.*, 2001). With the ability to differentiate between genotypes one can then compare characteristics of specific genotypes in relation to each other.

As discussed above, the ability of *E. coli* to persist in soil and water has been attributed to numerous potential factors. Wang and Doyle (1998) suggest that the differences in survival of *E. coli* observed between studies may be the product of intrinsic differences between strains. No published study could be found that evaluates genotypic differences and their influence on survival of *E. coli* in water. It is the objective of this study to investigate the potential influence of EHEC genotype on survival in untreated, well water.

Applications of Polymerase Chain Reaction in the detection of *E. coli*

Being both a major etiological agent of diarrheal disease and natural commensal organism of the gastrointestinal tract, *E. coli* can pose a challenge for isolation and identification. The identification of diarrheagenic *E. coli* cannot be made on the basis of culture and biochemical criteria since they are indistinguishable from the non-pathogenic *E. coli* found in human feces (Rappelli *et al.*, 2001). Serotyping of O and H antigens is also of limited use in identifying pathogens. There is no correlation between

pathogenicity and expressed antigens (Rappelli *et al.*, 1992) and there is a growing concern of antigen loss as a product of long term exposure to starvation conditions (Hara-Kudo, *et al.*, 2000). For these reasons the objective of the second part of this study is to develop a detection method for EHEC, EPEC, ETEC and EIEC based upon the virulence factor genes they express.

The polymerase chain reaction (PCR) is a molecular biological technique that, through temperature cycling, can ideally multiply a single stand of deoxyribonucleic acid (DNA) to billions of copies. This is achieved by combining the target DNA of interest with the necessary building blocks and tools. The necessary materials include deoxyribonucleoside triphosphates (dNTPs), heat stable DNA polymerase, and primer sequences designed to target a specific region of the template DNA. A PCR protocol cycles through three temperatures: denaturing, annealing and extension. The denaturing temperature is one that results in the separation of a piece of double stranded DNA into two single strands of DNA. This is followed by a gradual cooling to the annealing temperature at which the primer sequences can anneal to their complementary sequence within the source DNA, an essential step in DNA amplification. The third step is to raise the temperature to the optimum for DNA polymerase catalytic function. At this temperature the DNA polymerase will initiate synthesis at the 3'-hydroxyl end of the primers using the dNTPs in solution as the building blocks. The procedure is carried out in a thermocycler, a programmable block heater in which the reaction tubes are immersed (Glick and Pasternak, 1998). The specificity of the amplification generated is one of the primary advantages of the PCR.

The amplification of DNA from a single source with a single primer pair has proven to have numerous applications. This method however could become much more labour intensive should the number of sequences to be targeted in a sample of source DNA be increased. To increase the information gained from a single PCR reaction one can mix a number of primer pairs together in a single tube. This will result in the simultaneous amplification of a variety of target sequences. This is termed Multiplex PCR (MP-PCR). By generating multiple amplification products or amplicons from a single DNA source one decreases the time required for reaction mixture preparation, the materials required and the need for multiple DNA preparations. This is especially useful if there is only a minimal amount of sample available.

Both single primer PCR and Multiplex PCR have been applied in the detection and identification of microorganism from a variety of sources and conditions. PCR has been applied to detect pathogenic bacteria that cannot otherwise be detected due to the development of a viable but non-culturable state (Bej *et al.*, 1991a; Brauns *et al.*, 1991). This method has also been applied to screen blood for pathogenic retroviruses (Vet *et al.*, 1999). The rapid method is also highly desirable for the detection of slow growing microorganisms such as members of the genus *Mycobacterium* (Fang *et al.*, 2002). PCR and MP-PCR protocols have been designed to detect food borne microbes such as *Shigella* (Villalobo and Torres, 1998) and *Clostridium botulinum* (Lindström *et al.*, 2001) respectively. The methods have also been used to detect specific microbes in and population changes of complex environments such as soil (Campbell *et al.*, 2001). *Escherichia coli* being a major cause of food and water borne illness has also become a subject for PCR and MP-PCR detection methods.

PCR and MP-PCR protocols have been developed to detect and identify a variety of nonpathogenic and pathogenic *E. coli* based upon their trait and virulence genes. One study performed illustrated the detection of coliform bacteria and *E. coli* based on the presence of the *lac*Z and *uid*A genes. The *lac*Z gene encodes the enzyme β-galactosidase, which is responsible for lactose hydrolysis. The *uid*A gene encodes β-glucuronidase, an enzyme capable of hydrolyzing derivatives of glucuronic acid (Bej *et al.*, 1991b). Lactose fermentation is one trait used in the identification of coliforms making the *lac*Z gene a clear target for coliform detection by PCR. β-glucuronidase activity is considered an indicator of *E. coli*. The value of detecting the *uid*A gene is also of concern as up to 15% of a *E. coli* population in water appear to be β-glucuronidase negative (Bej *et al.*, 1991b). By detecting the presence of the *uid*A gene one can not avoid the reporting of false negatives.

PCR and MP-PCR protocols have also been developed for the detection of individual virotypes of *E. coli*. The identification of EPEC was addressed in one study, targeting the *bfp*A gene. The gene is found on the EAF plasmid and encodes the inducible fimbrial structures called bundle-forming pili. This is a suitable target for EPEC as it appears to be specific to EPEC and the gene shows a high degree of homology between EPEC strains (Gunzburg *et al.*, 1995). A method to detect EIEC and *Shigella*, a genetically related genus, from feces was developed based on detection with IS630-specific and *rfc*-specific primers. IS630, believed to be a plasmid sequence, is found in both *Shigella* and EIEC strains (Houng *et al.*, 1997). Although the study claims to differentiate between the two genera, only evidence of *Shigella* serotype differentiation could be established. This would make the identification of EIEC amongst *Shigella* sp.

difficult if only IS630 primers are employed. ETEC detection by PCR technology has resulted in a MP-PCR which can detect ETEC based on multiple alleles of the heat stable (ST) and heat labile (LT) enterotoxins. ST and LT are plasmid-encoded genes that can appear individually or together on the same multicopy plasmid (Stacy-Phipps *et al.*, 1995). Because the assay targets multiple alleles of the LT and ST genes simultaneously, the detection of ETEC is enhanced as the method has the ability to more accurately detect ETEC from stool. The ST and LT enterotoxins are the most significant targets for PCR detection as they are the primary cause of ETEC pathogenicity (Stacy-Phipps *et al.*, 1995). Many studies have been published addressing the detection of EHEC by PCR technology. The most prevalent targets for MP-PCR detection of EHEC are the *sltI* and *sltII* genes, which encode the Shiga-like toxins. This may be due to the fact that *sltI* and *sltII* are the primary virulence genes responsible for disease symptoms. The current standard detection methods for EHEC employ antisera which detects the O157:H7 serotypes though evidence has shown that *slt*I and *slt*II positive non-O157 *E. coli* are responsible for equivalent pathologies (Paton and Paton, 1999). By targeting the *slt*I and *slt*II genes one can identify the presence of both O157 and non-O157 EHEC strains rapidly and determine the distribution of even non-O157 EHEC (Pollard *et al.*, 1990). The *slt*I and *slt*II toxin genes are not the only genes used to identify EHEC by PCR methods. The *uid*A gene (discussed earlier) has also been applied in combination with the *slt* genes for the identification of EHEC by MP-PCR. The *uid*A gene of O157:H7 has specifically been targeted to identify the serotype as it is unique relative to those of other *E. coli* strains. *E. coli* O157:H7 has been determined to be β-glucuronidase negative although it still possesses a *uid*A gene.

The *uid*A allele found in O157:H7 has a base change at position 92 which has been exploited for the identification of O157:H7 by PCR (Cebula *et al.*, 1995; Feng and Monday, 2000; Venkateswaran *et al.*, 1997). The uniqueness of this allele makes it ideal for the identification of O157:H7 strains of *E. coli,* although with the increase in significance of pathogenic non-O157 serotypes, for example O111, O113 (Paton and Paton, 1999) and O26, its usefulness may begin to wane. The genes encoding surface antigens have been the target of other studies performed for the detection of EHEC. The *fli*C and *rfb*E genes are the primary targets selected for the detection of EHEC based on surface antigens. The $fliC_{H7}$ gene encodes the flagellin structural gene of the H7 flagellar type and the *rfb*E gene encodes the enzyme involved in O157 antigen biosynthesis. The *rfb*E gene is divergent from the *rfb* loci encoding the O antigens of *E. coli* other than O157, making it ideal for the detection of O157 strains. These are the two most prevalent surface antigen encoding genes employed in PCR detection of EHEC (Fratamico *et al.*, 2000; Hu *et al.*, 1999; Osek, 2002; Paton and Paton, 1998; Radu *et al.*, 2001). EHEC are a diverse group with many strains belonging to other serotypes that are responsible for the development of EHEC associated disease. This has lead to the development of a MP-PCR which directs its primers to target the *rfb* genetic loci for the detection of alternate serotypes of EHEC in addition to O157 (Paton and Paton, 1999). The surface antigens are clearly important indicators of serotype and can be applied in the epidemiological tracking of the EHEC strains but as individual traits they are inadequate for the detection of EHEC. To be more effective they should be applied in combination with other primers, such as virulence factors, to most effectively identify pathogenic strains (Nagano *et al.*, 1998; Radu *et al.*, 2001). The *eae*

gene encodes an outer membrane protein called intimin that is associated with lesion formation resulting in disease. Four different alleles of the *eae* gene have been identified, α, β, δ, and γ. The *eae* genes have been applied in a MP-PCR to identify the specific alleles and classify suspected pathogens into either the EPEC or EHEC clonal groups (Reid *et al.*, 1999). The *eae*A gene for γ-intimin, which is associated with the EHEC 1 clonal group has been applied in MP-PCR assays alone to assist in the detection of EHEC strains (Feng and Monday, 2000; Fratamico *et al.*, 1995; Osek, 2002). As attachment is important in the cause of disease it would be assumed that the *eae*A gene is a significant gene to include in a detection assay EHEC. A final target used in the detection of EHEC is the gene for enterohemolysin. Three studies have used enterohemolysin genes as part of a MP-PCR detection assay: *hly*A (Fratamico *et al.*, 2000), *hly*933 (Paton and Paton, 1998) and *ehx*A a related *E. coli* hemolysin (Feng and Monday, 2000). Although enterohemolysin is a virulence factor carried by many EHEC, the gene encoding the protein has a high level of heterogeneity, making it a poor candidate for the consistent detection of EHEC by MP-PCR. The protocols designed for *E. coli* detection by MP-PCR are not restricted to single virotypes. A number of MP-PCR assays have been created with larger target ranges which can identify members of different virotypes simultaneously, thus increasing the screening capacity of a single reaction (Frankel *et al.*, 1989; Lang *et al.*, 1994; Pass *et al.*, 2000; Rappelli *et al.*, 2001; Tornieporth *et al.*, 1995; Tsen and Jian, 1998).

The use of MP-PCR is not restricted to the identification of bacteria alone there are numerous other applications. One study combined the detection of EHEC by detection of *slt* genes with a molecular typing method, RAPD-PCR, by including a pair

of short 10-mer primers for the random amplification of source DNA (Hopkins and Hilton, 2000). Another MP-PCR was designed in order to make a comparison of attaching and effacing gene subtypes from human and bovine isolates (China *et. al.*, 1999). MP-PCR has also been applied in the detection and analysis of virulence factor alleles. For example the *pap*P gene, which encodes the major structural subunit and antigenic determinant of P fimbriae of extraintestinal pathogenic *E. coli* (Johnson *et al.*, 2000) and the ST and LT toxins of ETEC (Stacy-Phipps *et al.*, 1995). MP-PCR has thus proven to be a highly adaptable method for the detection and analysis of bacterial populations.

There are, however, drawbacks to the use of PCR based methods alone. The detection of the target genes is dependent upon the fidelity of primer binding during the annealing step of amplification. Should the primers misalign on the source DNA nonspecific amplification can result. Most often PCR products are verified by product size using gel electrophoresis. A worthwhile exercise but useless should a nonspecific product generated be similar in size to the expected product. One method to verify that the sequence generated was from a complementary primer annealing is the use of linear DNA probes.

DNA Probes

DNA-DNA hybridization is one of the most reliable techniques for the recognition of specific DNA sequences. It has been used widely for the analysis of DNA, often complementing PCR (Fujiwara and Oishi, 1998). The use of DNA probe hybridization techniques has been shown to be useful in the detection of pathogenic *E. coli.* A study

performed by Gomes *et al.* (1987) developed two different DNA probes that identify distinct regions of the pInv, a 120-140 megadalton plasmid encoding genes necessary for host cell invasion by EIEC. The probes were developed by labeling DNA fragments generated from pInv plasmid by restriction enzyme digestion. The probes were applied to 81 *E. coli* strains representing 10 known EIEC associated serotypes, which had been tested previously for invasiveness using the Sereny test. The Sereny test being an assay to determine the ability of an organism to cause inflammation by inoculating it into the eye of a guinea pig, virulent strains will result in the development of keratoconjunctivitis. The colonies were screened by colony hybridization for the presence of gene sequences homologous with the DNA probes developed. The probes confirmed the presence of pInv plasmid in Sereny positive strains and Sereny negative strains, which had maintained the plasmid, but not in Sereny negative that were plasmid negative. The study suggested that even though the existing methods for the identification of EIEC were effective, they did not effectively detect EIEC which fall outside known invasiveness serogroups or strains that do not fit previously identified phenotypic patterns (Gomes *et al.*, 1987).

DNA probes have also been developed which can detect Shiga-toxin producing *E. coli* in food. Weagent *et al.* (1999) used PCR to generate DIG-labeled probes to target the virulence genes stx1 and stx2 of EHEC by incorporating DIG into the primer sequences used and including DIG-labeled dUTP in the PCR reaction mixture. Twenty-five *E. coli* strains were tested with the stx1 and stx2 probes created and the probes were able to detect their target sequences from both PCR amplicons and colony blots positive strains. The results of the two assays were identical (Weagent *et al.*, 1999). The

use of DNA probes has also been combined with MP-PCR in a number of studies applied to pathogenic *E. coli* (Hopkins and Hilton, 2000; Stacy-Phipps *et al.*, 1995; Lang *et al.*, 1994). A study by Hopkins and Hilton (2000) developed a MP-PCR assay which combined the random amplification of polymorphic DNA (RAPD) – PCR genotyping method with 2 sets of primers targeting *stx*1 and *stx*2 genes. The MP-PCR was designed to simultaneously determine the genotype of the *E. coli* strain and detect the presence of the *stx*1 and *stx*2 genes. The presence of stx1 and stx2 amplicons amongst the RAPD-PCR amplicons was detected using DIG-labeled DNA probes homologous to the stx1 and stx2 amplicons (Hopkins and Hilton, 2000). A similar study was developed by Stacy-Phipps *et al.* (1995) to target the detection of ETEC in stool. The PCR amplicons were screened by dot blot analysis with DNA probes complementary to the ST and LT amplicons of the ETEC. The application of DNA probes can also help differentiate the products of PCR. A study performed by Lang *et al.* (1994) had developed a MP-PCR assay for the detection of LT, stx1 and stx2 genes of ETEC and EHEC. It was discovered that when applied to some known LT positive strains, 2 PCR products were generated instead of the expected single amplicon. Southern blotting analysis using a probe designed for the LT amplicon confirmed that the target LT gene generated only one amplicon and the other amplicon must have been a product of a non-specific amplification. Therefore, the application of DNA probes to .detect and confirm specific PCR products is an important method to improve the specificity of a PCR assay.

Chapter 2

Persistence of enterohemorrhagic *Escherichia coli* in well water and its relatedness to ERIC-genotype

Introduction

The contamination of water by EHEC has resulted in numerous outbreaks associated with both recreational and drinking water. Drinking water has repeatedly been identified as a vehicle of pathogenic *E. coli* resulting in large outbreaks around the world. Two well known outbreaks of *E. coli* O157:H7 in North America include one in Cabool, Missouri in 1990 (Swerdlow *et al.*, 1992) and another in Walkerton, Ontario in the spring of 2000 (Rasmussen and Casey, 2001).

Numerous studies have examined the ability of *E. coli* to survive in water. The studies have involved looking at the persistence of pathogenic *E. coli* in river and lake water, municipally treated drinking water and bottled water. The persistence and survival of *E. coli* O157:H7 in natural, untreated water may be attributed to the protective niches provided by particulate matter (Brettar and Höfle, 1992) or to internalization by protozoans (Maule, 2000). A comparative study was performed by Wang and Doyle (1998) investigating the ability of *E. coli* O157:H7 to survive in municipal water (autoclaved and filtered), reservoir water and recreational water at 8°C, 15°C and 25°C. The study revealed the ability of *E. coli* O157:H7 to survive better in the treated municipal water at 8°C relative to the untreated waters. The observation was attributed to the low temperature and minimal competition present. Rice *et al.* (1992) had performed a similar study looking at the survival of *E. coli* O157:H7 in well water at 5°C and 20°C. The general trend observed was similar to that observed in the municipal water study performed by Wang and Doyle (1998), with greater persistence at the lower temperature. Although temperature is one influence on the survival of *E. coli* O157:H7, others have been proposed: the presence of organic matter (Kerr *et al.*, 1999),

differences in water composition, and intrinsic differences in the *E. coli* O157:H7 strains (Wang and Doyle, 1998). The intrinsic genotypic differences amongst strains of EHEC are a suspected cause of survival variability (Topp *et al.*, 2003). It is the influence of these genotypic differences that will be investigated in this study.

A variety of methods have been used to determine the genotype of enterohemorrhagic *E. coli* for a variety of applications. The enterobacterial repetitive intergenic concensus polymerase chain reaction (ERIC-PCR) method has demonstrated its ability to discriminate between different genotypes of *E. coli* found within a single serotype, such as O157 (Adwan *et al.*, 2002). The ERIC-PCR procedure has also been applied to determine the genetic relatedness of *E. coli* isolated from pigs (Osek, 2000) and poultry (Carvalho de Moura *et al.*, 2001). With the ability to differentiate between genotypes one can move forward to apply genotyping methods to help monitor characteristics, such as prolonged survival of specific genotypes, and determine the impact genotype may have on that characteristic.

The objective of this study is to determine if a correlation exists between the genotype of an EHEC strain and its ability to persist in untreated well water. The study will employ 16 EHEC strains selected from a collection 91 *E. coli* strains that have been genotyped using the ERIC-PCR method. The strains selected will reflect a combination of different genotypes and serotypes of EHEC. Comparisons between persistence and serotype and persistence and temperature will also be examined.

Materials and Methods:

Strains and Culturing: Ninety-one *Escherichia coli* isolates were initially used in this study. The *E. coli* collection was composed of 1 control strain (ATCC 25922), 51 non-clinical pig isolates and 39 EHEC strains (23 human isolates and 16 bovine isolates) of various known and unknown serotypes (Table 2.1). The non-clinical pig isolates, isolated from various locations across Ontario, were provided by Dr. E. Topp at Agriculture and Agri-Food Canada, London, Ontario and Dr. C. Poppe at Health Canada, Guelph, Ontario. The EHEC strains were isolated from hosts from across North America, Australia and Europe and generously provided by Dr. C. Gyles, University of Guelph, Guelph, Ontario. The *E. coli* isolates were grown at 37°C in trypticase soy broth (TSB) (Becton, Dickinson and Company, Cockneysville, MD) and stock cultures maintained at -80°C in TSB supplemented with 25% (v/v) glycerol.

Sixteen strains of enterohemmorhagic *Escherichia coli* were selected for the well water microcosm study (Table 2.2). These strains were selected to represent a combination of genotypes and serotypes in order that the survival of specific strains in well water could be made taking into consideration their relationship with other genotypes and serotypes. The strains were cultured in either 10 ml or 30 ml volumes of TSB for the purposes of DNA extraction and microcosm inoculation, respectively. The cells were grown overnight at 37°C under aerobic conditions with gentle shaking.

ERIC-PCR fingerprinting and typing:

Fingerprinting: A collection of 91 *E. coli* strains, composed of 23 human (EHEC), 16 bovine (EHEC) and 51 porcine (non-clinical) isolates, was applied to an ERIC-PCR

DNA fingerprinting assay. All isolates were cultured at 37°C in TSB and later harvested during the late log phase at an optical density (OD_{600nm}) of 1. An InstaGene DNA extraction kit (Bio-Rad Biochemicals) was used to extract DNA from the *E. coli* samples based on the manufacturer's instructions. The procedure involved combining an aliquot of culture with a cell suspension buffer. The sample was pelleted, the supernatant discarded and the pellet washed twice in the suspension buffer. After washing, the pellet was combined with InstaGene matrix and incubated at 65°C for 15 minutes. The sample was then vortexed briefly, centrifuged at 14,000 rpms and then 10 μl of the supernatant used in the 25 μl ERIC-PCR assay. The composition of the PCR reaction mixture was as follows: 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 1x PCR buffer, 1.5U Taq DNA polymerase, and 2 μM of each ERIC primer (ERIC2 5'-*AAG TAA GTG ACT GGG GTG AGC G*-3'; ERIC1R 5'-*ATG TAA GCT CCT GGG GAT TCA C*-3'). The PCR protocol involved an initial incubation at 94°C for 3 minutes, followed by 30 cycles consisting of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 3 minutes. A final extension step at 72°C for 5 minutes completed the amplification. The ERIC-PCR products generated were visualized by electrophoresis in 1% agarose containing 40 mM Tris-acetate, 1 mM EDTA (pH 8.0) and 0.2 μg/ml ethidium bromide.

ERIC-PCR typing: The ERIC-PCR fingerprint generated by each isolate was converted to a binary code number based on the presence or absence of the amplified bands (Table 2.1). The data generated were analyzed using the TREECON software (Yves Van de Peer, University of Konstanz, Germany) which is designed for the construction and illustration of evolutionary trees based on percentage similarities calculated from DNA fingerprint patterns. The percentage similarities between fingerprints were

calculated using the *Dice* correlation coefficient (S_D). $S_D = 2n_{AB}/ n_A+n_B$ where n_{AB} is the number of bands common for A and B; n_A is the total number of bands in A; and n_B is the total number of bands in B. Cluster analysis was performed for the 91 band patterns using the unweighted pair group method with averages (UPGMA).

Microcosm preparation and incubation: Sixteen strains of EHEC selected were cultured in 30 ml of TSB overnight at 37°C. The optical density (OD) of the cultures was measured using a Novaspec II visible spectrophotometer (Pharmacia Biotech, Cambridge, England) at a wavelength of 600 nm. The OD obtained was then used to determine the dilution required to obtain an inoculum of approximately 10^8 cells/ml. An aliquot of the diluted culture was taken and spun using a Centra CL3R centrifuge (ThermoIEC, Needham Heights, MA) for 5 to 10 minutes at 3200 x g. The supernatant was removed and discarded and the cells resuspended and washed twice in phosphate-buffered saline (0.14 M NaCl, 0.002 M KCl, 0.01 M Na_2HPO_4, 0.02 M KH_2PO_4). The cells were then suspended to an approximate concentration of 10^8 CFU/ml in sterile distilled water. Two ml of the inoculum were added to 198 ml of untreated well water in a sterile 250 ml polypropylene screw cap bottle for a final cell concentration of approximately $1x10^6$ CFU/ml. Microcosms were prepared in triplicate for each EHEC strain. Triplicates of uninoculated control microcosms were included to monitor the presence of background heterotrophic organisms in the untreated well water. The water for this study was provided by Don and Barb Barnes (Shuniah Township, Thunder Bay, Ontario) from a dug well to which no disinfecting agents had been added. The water was tested for the presence of coliforms and *E. coli* by the

Public Health Laboratory (Thunder Bay, Ontario) and found to be negative. These results were confirmed by an *E. coli* test in our lab. A 200 ml water sample was filtered through a 0.22 μm Millipore filter (Millipore Corporation, Bedford, MA) using a sterile funnel. The filter was incubated overnight at 37°C on a Levine Eosin Methylene Blue agar plate and inspected for the presence of colonies typical of *E. coli*. None was found.

Monitoring survival:

The microcosms were incubated stationary at 10°C and 22°C for 56 days. Samples were taken on day 0,1,3, 7, 14, 21, 28, 42 and 56. Before sampling microcosm bottles were inverted 3-5 times to re-suspend any cells that may have settled. A 0.5 ml to 1 ml aliquot of water was removed to a sterile 1.5 ml microcentrifuge tube for dilution. Samples were initially diluted 1000x in sterile 0.85% saline and were plated using a Autoplate 4000® Automated Spiral Plater (Spiral Tech, Inc., Bethesda, MD), an automated device which deposits liquid sample on the surface of an agar plate to determine the cell densities of the samples. Levine Eosin Methylene Blue (L-EMB) agar (Becton, Dickinson and Company, Cockneysville, MD) was plated with 50 μl of sample to monitor the levels of *E. coli*. Positive *E. coli* colonies counted were either dark brown or black in colour with or without a green metallic sheen. Any other colony type was not counted. Trypticase soy agar (TSA) (Becton, Dickinson and Company, Cockneysville, MD) was plated with 50 μl of sample to monitor the levels of total bacteria. All colony types were counted for the monitoring of total bacteria.

Microsoft Excel software was used to determine the average and standard deviations of the cell densities of each sample.

Results

ERIC-PCR Typing of EHEC

Figure 2.1 illustrates the genotypic relationship of 91 *E. coli* strains, including 39 EHEC human and bovine isolates, and 51 non-clinical porcine *E. coli* isolates determined by ERIC-PCR. The 91 strains of *E.coli* were divided into 18 different ERIC-genotypes. The ERIC-genotype of the organisms is based on the similarity of the ERIC-PCR electrophoretic fingerprints generated. Organisms with similar fingerprints were grouped into the same genotypes. These genotypes were then grouped arbitrarily into clusters. Clusters I to V show at least 75% similarity with each other with genotypes within each cluster showing greater than 80% similarity. Members of cluster VI were grouped together due to their low level of similarity (60%) with the rest of the genotypes analyzed. The ERIC-PCR assay generated a total of 18 PCR fragments (amplicons) with each *E. coli* strain displaying a different pattern composed of some combination of these amplicons. These patterns were converted into binary codes for analysis with TREECON software (Yves Van de Peer, University of Konstanz, Germany). Each strain was analyzed for the presence or absence of each of the 18 observed bands thus resulting in an 18-digit binary code number (Table 2.1).

The ERIC-PCR assay showed that *E. coli* strains with the same serotype could be very diverse genotypically (Figure 2.1). For instance, serotype O157 strains largely fall into cluster VI, but a single strain of O157, H19, falls into cluster I, showing only a 60% similarity with the O157 strains found in cluster VI. A second independent O157 strain, H21, falls in cluster V, showing only 75% similarity with the first independent strain (H19) and 60% similarity with the large cluster of O157 genotypes in cluster VI

thus illustrating that serotype similarity is a poor measure of genetic relatedness. The strains of O157 can be seen within three different clusters but possess four different genotypes, A, R, N and P (Figure 2.1). A second example involves strains of serotype O26. Member of this serotype can be found in two distinct clusters of the dendrogram, cluster I, with genotype B, and cluster III, genotype H, which show an approximate 85% similarity with each other. An independent O26 strain is also found in cluster I with genotype A. The members of the serotype O26 were found to have three different genotypes, A, B and H further illustrating the genetic variability between strains of the species *Escherichia coli* and the members of the associated serotypes. Also, members of multiple serotypes can belong to the same genotype. For instance, members of four different O serotypes, O111, O153, O26 and O157 all possess the genotype A. Variability can also be seen in the host specificity of strains within a genotype. Genotype F is composed of 17 *E. coli* strains with three different host specificities: human, bovine and porcine.

The 91 *E. coli* strains generated 3-7 ERIC-PCR bands each. Some of the bands seen are shared between the many genotypes for example those bands found between the range of the 100 bp and 200 bp. The majority of genotypic variability is seen in the band patterns found in the range of 200 bp and 1000 bp. The relatedness of these strains is based upon the differences seen in the band patterns formed.

Survival of EHEC in Well Water

Sixteen EHEC human and bovine isolates, representative of the genotypic and serotypic variations in the collection, were selected for the survival study (Table 2.2).

Their survival was monitored at 10°C and 22°C in untreated well water for 56 days. The data were grouped for comparisons between members of the same genotype and serotype at two different temperatures. Figure 2.2 includes the data for *E. coli* strains of serotype O157 with genotypes A, N, R and P. Figure 2.3 includes the data for *E. coli* stains of serotype O26 with the genotypes A, B and H. Figure 2.4 includes the data for *E. coli* strains of genotype A with serotypes O26, O111 and O157.

O157 Genotypes

Figure 2.2 reports data for eight different EHEC strains belonging to the serogroup O157. Five of the strains belong to genotype N (H8, H25, H32, H28 and H24) and the remaining three belonging to genotypes A (H19), R (H21) and P (H22). The five strains of genotype N showed varying degrees of survival at 10°C. The log cfu/ml value at day 0 was approximately 7.0 for these five strains. After 56 days of incubation at 10°C the decline in log cfu/ml ranged between 1.8 (H32) and 6.0 (H24). The decline of the three additional O157 strains of genotype A, R and P also fell within this range. The eight strains fell into three distinct groupings by day 56, strains with superior survival, strains with moderate survival and strains with poor survival. Strains H32 and H8 demonstrated the greatest survival rates falling to only 5.3 and 4.8 log cfu/ml from 7 log cfu/ml at day 0. Strains H21, H25 and H28 demonstrated moderate rates of survival having dropped from 7 log cfu/ml at day 0 to 2.8, 2.4 and 3.1 log cfu/ml respectively by day 56. Strains H19, H22 and H24 demonstrated the poorest rates of survival falling from approximately 6.5 log cfu/ml at day 0 to below the detection limit (less than 0.8 log cfu/ml) by day 56.

O26 Genotypes

The data pertaining to the three EHEC strains of genotype B, each belonging to serotype O26, and two additional O26 strains, genotypes A and H, are reported in Figure 2.3. Strains H4, H5 and H15, all genotype B, behaved very differently from each other at 10°C, with H4 and H5 cfu/ml dropping from approximately 6.8 log at day 0 to about 1.4 log at day 56. However, H15 dropped only 1.8 log by day 56 from 7.1 log at day 0. The 4 log difference between H15 and strains H4 and H5 after 56 days incubation at 10°C indicates their ability to survive in untreated well water varies despite belonging to the same genotype. Strains H35 and H18 are two additional strains belonging to serotype O26. At 10°C strains H35 and H18 decrease from 6.6 and 7.0 log cfu/ml at day 0 to 5.8 and 2.2 log cfu/ml respectively, after 56 days of incubation.

O111 Genotypes

Figure 2.4 illustrates the data obtained for the five strains selected belonging to genotype A. Three of these strains belong to serotype O111 (H12, H38 and H11) while the additional two strains belong to serotypes O157 (H19) and O26 (H18). Once again the strains showed a high degree of variability in their ability to persist at 10°C. The change in log cfu/ml by day 56 ranged between 2.2 log for H18 and 5.9 log for H19. The final log cfu/ml values for strains H11, H12 and H38 were very similar, 3.6, 3.8 and 4.1 respectively. Their rate of decline, however, over the 56 day period varied, with H11 dropping to 3.5 log by day 21 from 7.1 log at day 0. Strains H12 and H38 declined more slowly from their starting values of 6.7 and 6.6 log respectively, dropping to only 6.2 log cfu/ml and 5.9 log cfu/ml by day 21.

Effect of temperature on *E. coli* survival

Temperature has a significant influence on the survival of EHEC strains as the rate of decline, in general, is greater at 22°C than at 10°C (Figures 2.2, 2.3, and 2.4). Four survival trends were demonstrated by the 16 EHEC strains selected. These trends include (1) strong survival at both 10° and 22°C, (2) moderate to strong survival at 10°C with reduced levels of survival at 22°C, (3) poor survival at 10°C with superior survival at 22°C and (4) poor survival rates at both 10°C and 22°C. Strong or superior survival refers to the relative survival of a strain within a group at day 56 for 10°C and day 21 for 22°C. Poor survival refers to a relatively rapid decline in cell density to near or below the detection limit, 0.8 log cfu/ml.

Those strains showing superior survival at both 10°C and 22°C include strains H32, H8 (Figure 2.2), H15 (Figure 2.3). H32 demonstrated the highest level of survival at 10°C and 22°C during 56 day incubation period, decreasing from approximately 7.2 log cfu/ml at day 0 to 5.3 and 1.1 log cfu/ml at 10°C and 22°C respectively by day 56. H8 showed a similar trend dropping from approximately 7.2 log cfu/ml at day 0 to 4.8 and 0.8 log cfu/ml at 10°C and 22°C respectively, falling below the detection limit at 22°C only in the last week of monitoring. Strain H15 also demonstrated a high rate of survival at both 10°C and 22°C having the highest rate of survival of all O26 strains at 10°C. This superior rate of survival is also seen at 22°C as H15 dropped the least of all O26 strains from 7.3 log to 2.2 log cfu/ml.

Nine of the sixteen strains were found to show a superior or moderate rate of survival at 10°C while demonstrating a poor rate of survival at 22°C. These strains

include H19, H21, H24, H25, H28 (Figure 2.2), H4, H35 (Figure 2.3) and H18, H12 and H38 (Figure 2.4). While demonstrating moderate rates of survival at 10°C, discussed above, strains H19, H21, H24, H25 and H28 demonstrate poor rates of survival at the alternate temperature, 22°C, with each strain falling from approximately 7 log cfu/ml to below the detection limit, 0.8 log cfu/ml, by day 56. While showing moderate rates of survival at 10°C, strains H4 and H35 (Figure 2.3) demonstrated poor survival at 22°C falling rapidly before day 21 and each dropping from 6.4 to less than 0.8 log cfu/ml by day 56. While showing high rates of survival at 10°C strains H18 and H12 (Figure 2.4) fell relatively rapidly at 22°C from 7.1 and 6.6 log cfu/ml to less than 0.8 log cfu/ml by day 56 respectively. Strain H38 (Figure 2.4) showed moderate survival success at 10°C dropping at a slow rate from 6.6 log cfu/ml at day 0 to 2.5 log cfu/ml by day 56. This slow rate of decline in H38 was also observed at 22°C making the slow drop from 6.4 log to less than 0.8 log at day 56 a moderate success relative to other serotypes present.

Strains H5 (Figure 2.3) and H11 (Figure 2.4) demonstrated the more peculiar trend which resulted in poor survival at 10°C with moderate to superior survival at 22°C. H5 falls below the detection limit, 0.8 log cfu/ml, by day 56 at both 10°C and 22°C, from 7 log at day 0, however, the rate of decline is greater at 10°C. H5 reaches 2.8 log cfu/ml at 10°C by day 21 but only drops to 4.4 log cfu/ml by day 21 at 22°C. The survival of strain H11 (Figure 2.4) differs in that at 10°C the log cfu/ml almost plateaus at approximately 3.5 log cfu/ml having dropped rapidly from 7 log to 3.5 log by day 21 while at 22°C, by day 21, H11 had dropped to only 5.2 log from 7 log at day 0, eventually dropping further to less than 0.8 log by day 56.

Only one of the EHEC strains demonstrated poor overall survival at both 10°C and 22°C, strain H22 (Figure 2.2). At 10°C the cell density of H22 was consistently less than the cell densities of other strains, dropping to below the detection limit by day 42 from approximately 6 log cfu/ml at day 0. At 22°C H22 again dropped rapidly from 5.7 log cfu/ml at day 0 to less than 0.8 by 21.

Heterotrophic Bacterial Counts

During the 56 day incubation period untreated well water without inoculum, included in the study as a negative control, was monitored for changes in cfu/ml of heterotrophic organisms at both 10°C and 22°C. Figure 2.5 illustrates the findings of the uninoculated control relative to strains with superior survival abilities (H32) and poor survival abilities (H22). At both 10°C and 22°C the heterotrophic bacterial cell densities for the uninoculated control showed only marginal changes. Heterotrophic organisms at 10°C increased from 4.1 log cfu/ml at day 0 to 5.2 log fu/ml at day 14 then returned to approximately 4 log cfu/ml at day 56. At 22°C heterotrophic organisms increased from 4.1 log cfu/ml at day 0 to 6.1 log cfu/ml by day 14 then fell to approximately 3.4 log cfu/ml by day 56.

On day 0 the total bacterial counts (*E. coli* plus background enumerated on TSA) and *E. coli* alone (enumerated on EMB) for both H32 and H22 were approximately 6.2 and 7 log cfu/ml at 10°C, respectively. These values are almost identical due to the dominance of the *E. coli* inoculum over the relatively low background heterotrophic bacterial cell density in the well water, which were approximately 4.1 log cfu/ml. H32 shows high level of persistence at day 56 at a log cfu/ml of 5.3 and the total bacterial

count at 5.9 log cfu/ml. H22, however, drops from 6.2 log cfu/ml to less than 0.8 log cfu/ml while the total bacterial count for the H22 treated water sample remains high, at approximately 5-6 log from day 0 to day 28, then falls to approximately 4 log cfu/ml which is approximately equivalent to the background number of organisms in untreated water.

The total bacterial counts and *E. coli* counts for strain H32 are very similar at 22°C on day 0, approximately 7.3 log cfu/ml, while for H22 the total and *E. coli* counts were slightly different at 6.4 and 5.7 log cfu/ml, respectively. Unlike at 10°C, strains H32 and H22 showed greatly reduced levels of survival by day 56 at 1.1 log and less than 0.8 log, respectively, though H32 did show a much slower rate of decline relative to H22. The total bacterial counts for both H32 and H22 microcosms were 3.2 and 3.5 log at day 56, respectively, almost equivalent to the background counts obtained from untreated well water at 3.4 log cfu/ml.

Table 2.1: Binary codes generated for 91 E. coli isolates from their ERIC-PCR electrophoretic band pattern for analysis using TREECON

E. coli Strain	Serotype	Host[a]	Band Number																	
			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
ATCC 25922	ND	P	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	0
1.2.1	ND	P	1	0	0	0	1	1	0	0	0	0	1	0	0	1	0	0	1	0
1.2.2	ND	P	1	0	0	0	1	1	0	0	0	0	1	0	0	1	0	0	1	0
5.1.4	ND	P	0	0	0	0	0	1	0	1	0	1	0	0	0	1	1	0	1	0
6.2.2	ND	P	0	0	1	0	0	0	0	1	0	0	1	0	0	0	1	0	1	0
9.1.1	ND	P	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0
13.1.3	ND	P	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0
14.1.5	ND	P	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0
14.2.2	ND	P	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1	0	1	0
21.1.2	ND	P	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1	0	1	0
21.1.3	ND	P	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0
12.2.1	ND	P	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0
1.2	ND	P	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1	0	1	0
1.3	ND	P	0	0	1	0	0	1	0	1	1	0	0	0	0	1	1	0	1	0
2.2	ND	P	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	1	0
2.3	ND	P	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	1	0
4.1	ND	P	0	0	1	0	0	0	0	1	0	0	0	1	0	0	1	0	1	0
5.1	ND	P	0	0	1	0	0	1	0	1	0	0	1	0	0	0	1	0	1	0
6.1	ND	P	0	0	1	0	0	1	0	1	0	0	1	0	0	0	1	0	1	0
8.5	ND	P	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0
10.5	ND	P	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0
12.2	ND	P	0	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0	1	0
12.5	ND	P	0	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0	1	0
16.5	ND	P	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0
20.3	ND	P	0	0	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0
30.1	ND	P	0	0	1	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0
H1	O26:H11	B	0	0	0	0	1	0	1	1	0	1	0	0	0	0	1	0	1	0
H2	O26:H-	B	0	0	0	0	1	1	0	1	0	1	0	0	0	0	1	0	1	0
H3	O113:H21	H	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0
H4	O26:H-	H	0	0	0	0	1	1	0	1	0	1	0	0	0	0	1	0	1	0
H5	O26:H11	H	0	0	0	0	1	1	0	1	0	1	0	0	0	0	1	0	1	0
H6	O111:H-	B	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	0	1	0

: B = Bovine, H = Human, P = Porcine

E. coli Strain	Serotype	Host[a]	Band Number																
			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
H7	O113:H21	B	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0
H8	O157:H7	B	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	1
H9	O111:H8	B	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	0	1	0
H10	O26:H11	H	0	0	0	0	1	1	0	1	0	1	0	0	0	0	1	0	1	0
H11	O111:H8	H	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	0	1	0
H12	O111:H-	H	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	0	1	0
H13	O26:H-	H	0	0	0	0	1	1	0	1	0	0	0	0	0	0	1	0	1	0
1.1.1R	ND	P	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0
2.1.3R	ND	P	0	0	0	0	0	0	1	1	0	1	0	0	0	0	1	0	1	0
3.1.1R	ND	P	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	0	1	0
4.1.1R	ND	P	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0
4.2.2R	ND	P	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	0	1	0
5.1.1R	ND	P	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0
6.1.1R	ND	P	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0
1.2.1d	ND	P	1	0	0	0	1	1	0	0	0	0	1	0	0	1	0	0	1	0
7.1.1R	ND	P	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0
8.1.1R	ND	P	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	1	0
8.2.1R	ND	P	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0
9.1.2R	ND	P	0	0	0	0	0	1	0	0	1	0	0	0	1	0	1	0	1	0
9.2.1R	ND	P	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0
10.1.1R	ND	P	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0
10.1.2R	ND	P	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0
11.1.1R	ND	P	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0
11.2.3R	ND	P	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0
12.1.5R	ND	P	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0
12.2.1R	ND	P	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	1	0
13.1.2R	ND	P	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0
13.2.3R	ND	P	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0
14.1.1R	ND	P	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	0
14.2.1R	ND	P	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	0
15.1.1R	ND	P	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	0
15.2.2R	ND	P	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0
16.1.1R	ND	P	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0

: B = Bovine, H = Human, P = Porcine

E. coli Strain	Serotype	Host[a]	Band Number																	
			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
H14	O111:H8	B	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	0	1	0
H15	O26:H11	H	0	0	0	0	1	1	0	1	0	1	0	0	0	0	1	0	1	0
H16	O153:H25	H	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0
H17	O111:H-	B	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	0	1	0
H18	O26:H-	H	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	0	1	0
H19	O157:H7	H	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	0	1	0
H20	O113:H21	B	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0
H21	O157:H7	H	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0
H22	O157:H7	H	0	1	0	0	1	1	0	0	0	0	0	0	0	1	0	0	1	0
H23	O153:H25	B	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0
H24	O157:H-	H	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	1
H25	O157:H-	H	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	1
H26	O113:H21	H	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0
H27	O111:H8	H	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	0	1	0
H28	O157:H-	B	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	1
H29	O157:H7	H	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0
H30	O26:H11	B	0	0	0	0	1	1	1	1	0	1	0	0	0	0	1	0	1	0
H31	O26:H-	B	0	0	0	0	1	1	0	1	0	1	0	0	0	0	1	0	1	0
H32	O157:H7	B	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	1
H33	O153:H25	H	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	0	1	0
H34	O111:H-	H	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	0	1	0
H35	O26:H11	H	0	0	0	0	1	1	1	1	0	1	0	0	0	0	1	0	1	0
H36	O153:H25	B	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	0	1	0
H37	O113:H21	B	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0
H38	O111:H-	H	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	0	1	0
H39	O113:H21	H	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0

: B = Bovine, H = Human, P = Porcine

Table 2.2: Genotype and serotype classifications for 16 EHEC selected for use in the survival study.

Strain	Serotypes	ERIC-PCR Genotype◆	Host Source
H8	O157:H7	N	Bovine
H19	O157:H7	A	Human
H21	O157:H7	R	Human
H22	O157:H7	P	Human
H24	O157:H-	N	Human
H25	O157:H-	N	Human
H28	O157:H-	N	Bovine
H32	O157:H7	N	Bovine
H4	O26:H-	B	Human
H5	O26:H11	B	Human
H15	O26:H11	B	Human
H18	O26:H-	A	Human
H35	O26:H11	H	Human
H11	O111:H8	A	Human
H12	O111:H-	A	Human
H38	O111:H-	A	Human

◆ See Figure 2.1

Figure 2.1: Dendrogram derived from ERIC-PCR data from multiple laboratory strains of *Escherichia coli*.

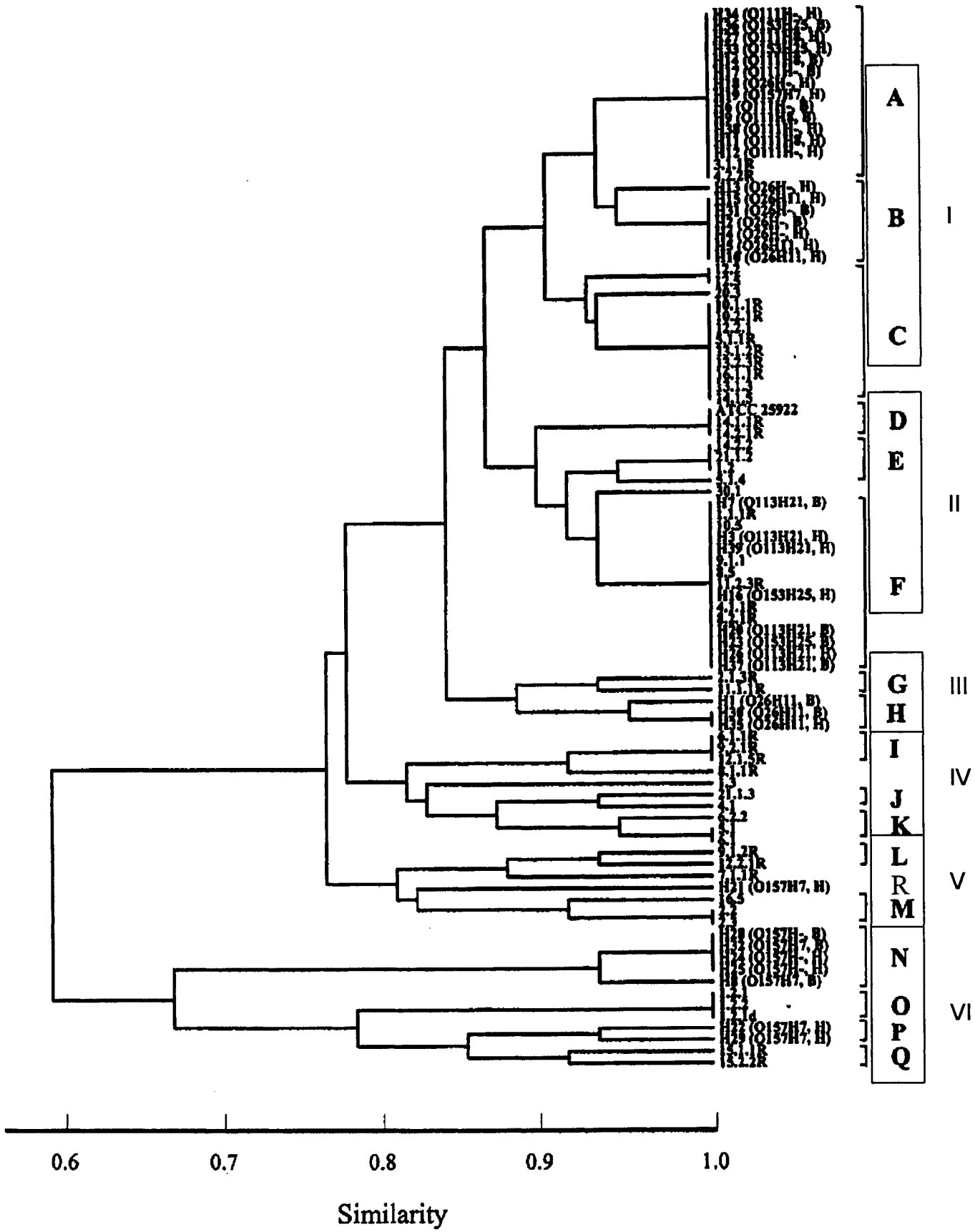
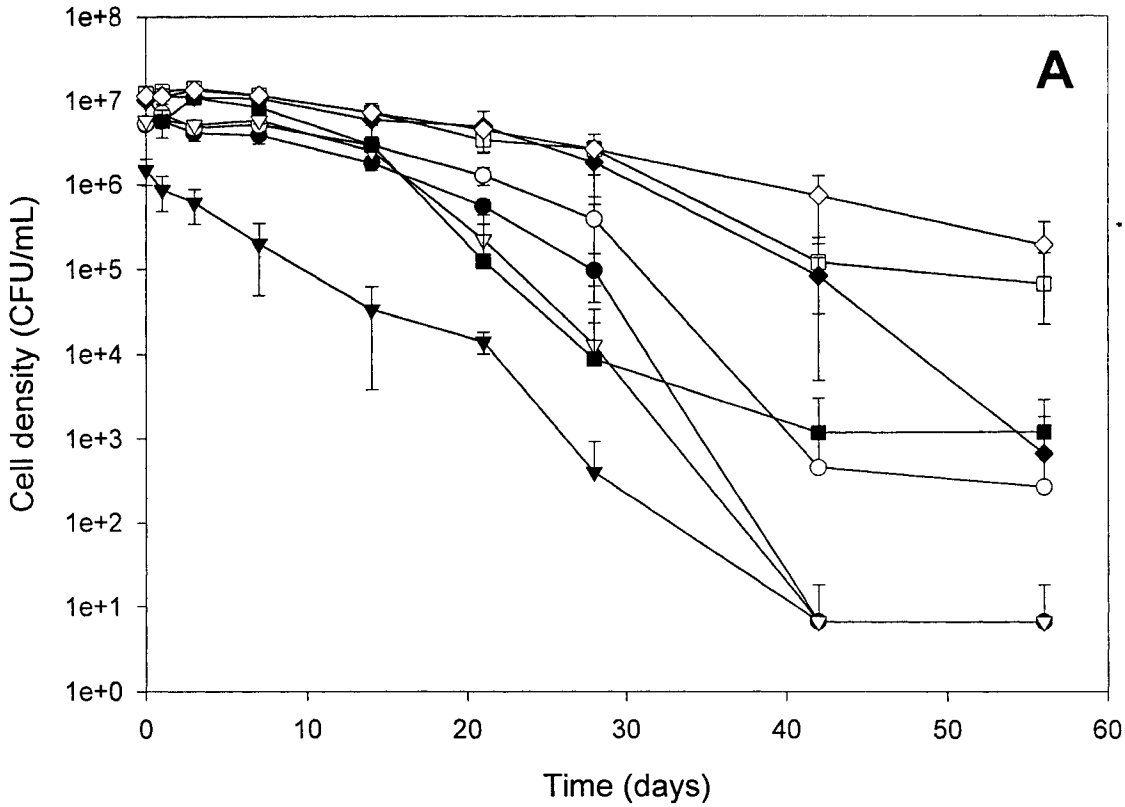


Figure 2.2. Survival of enterohemmorhagic *Escherichia coli* belonging to ERIC-PCR genotype N and serotype O157 at (A) 10°C and (B) 22°C. ○, strain H28; ■, strain H21; ●, strain H19; ▼, strain H22; ▽, strain H24; ◆, strain H25; ◊, strain H32; □, strain H8.



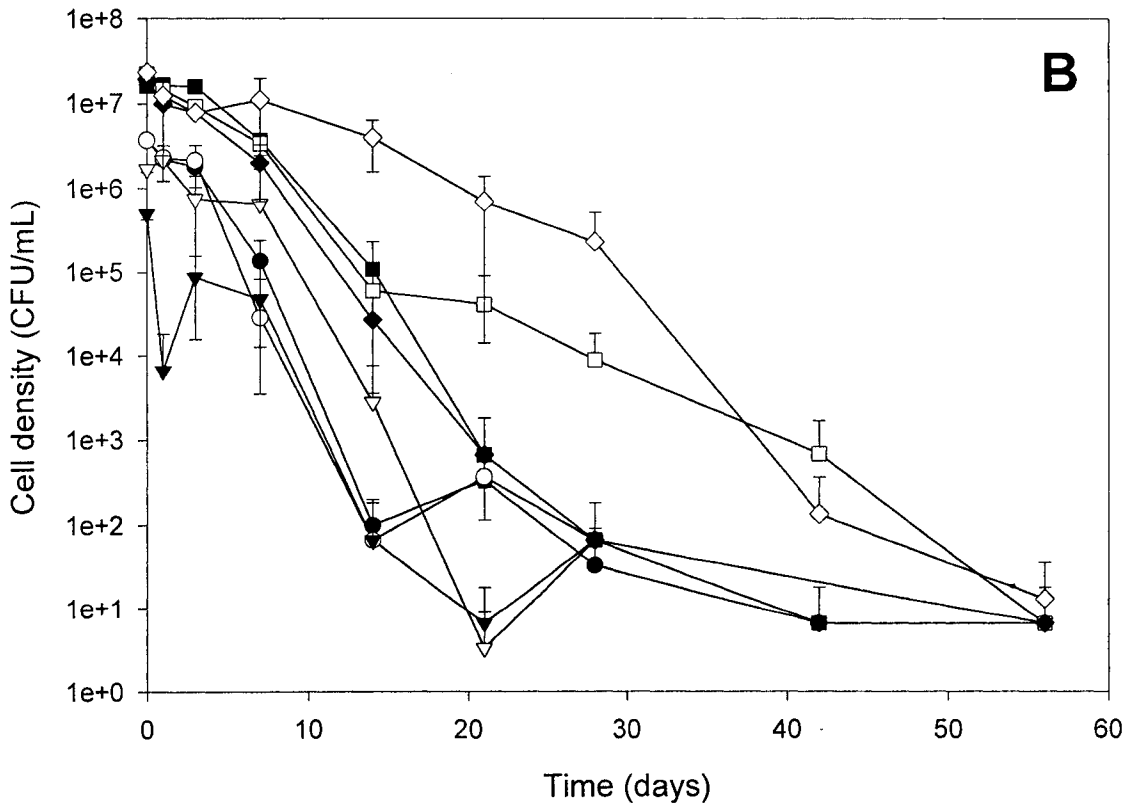
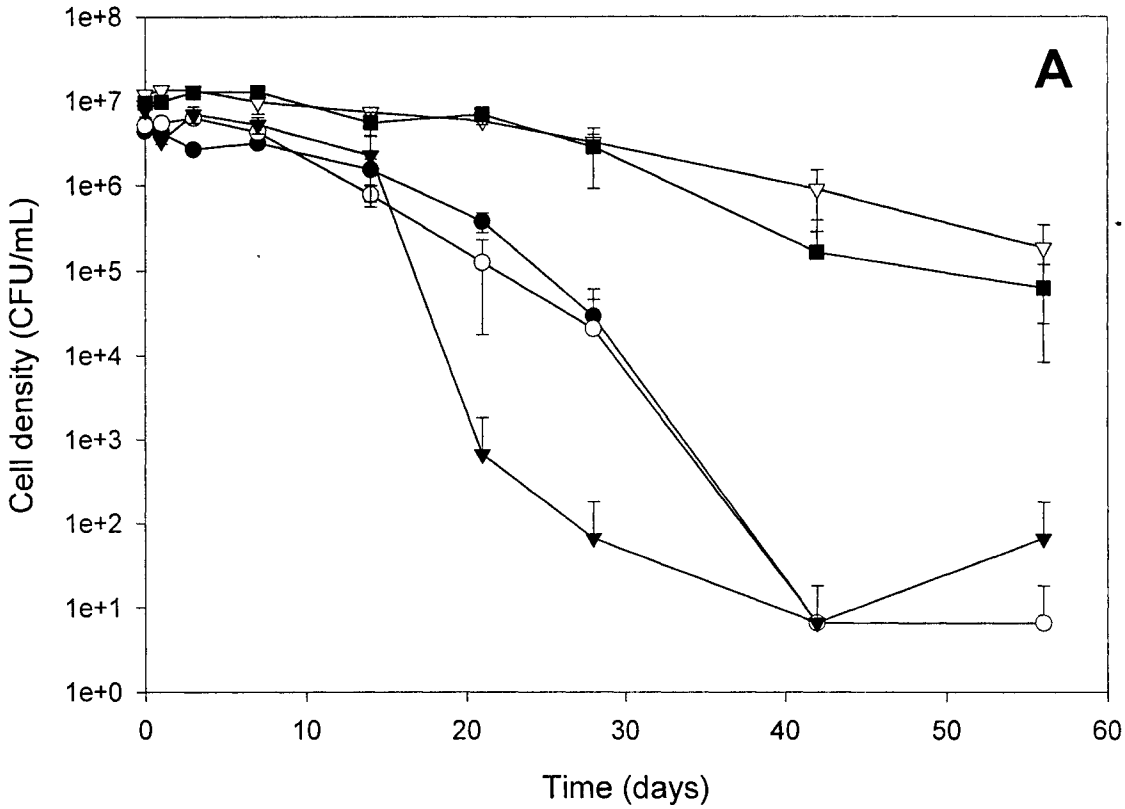


Figure 2.3. Survival of enterohemmorhagic *Escherichia coli* belonging to ERIC-PCR genotype B and serotype O26 at (A) 10°C and (B) 22°C. ▼, strain H5; ∇, strain H15; ○, strain H4; ■, strain H18; ●, strain H35.



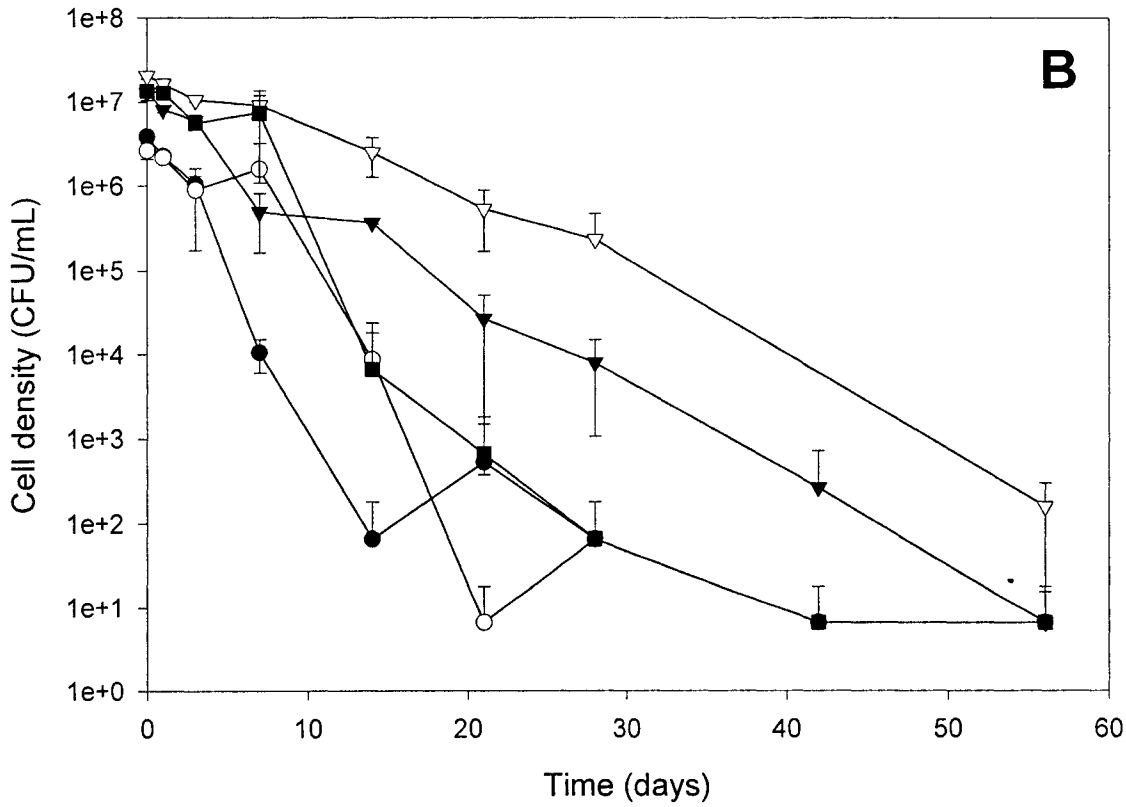
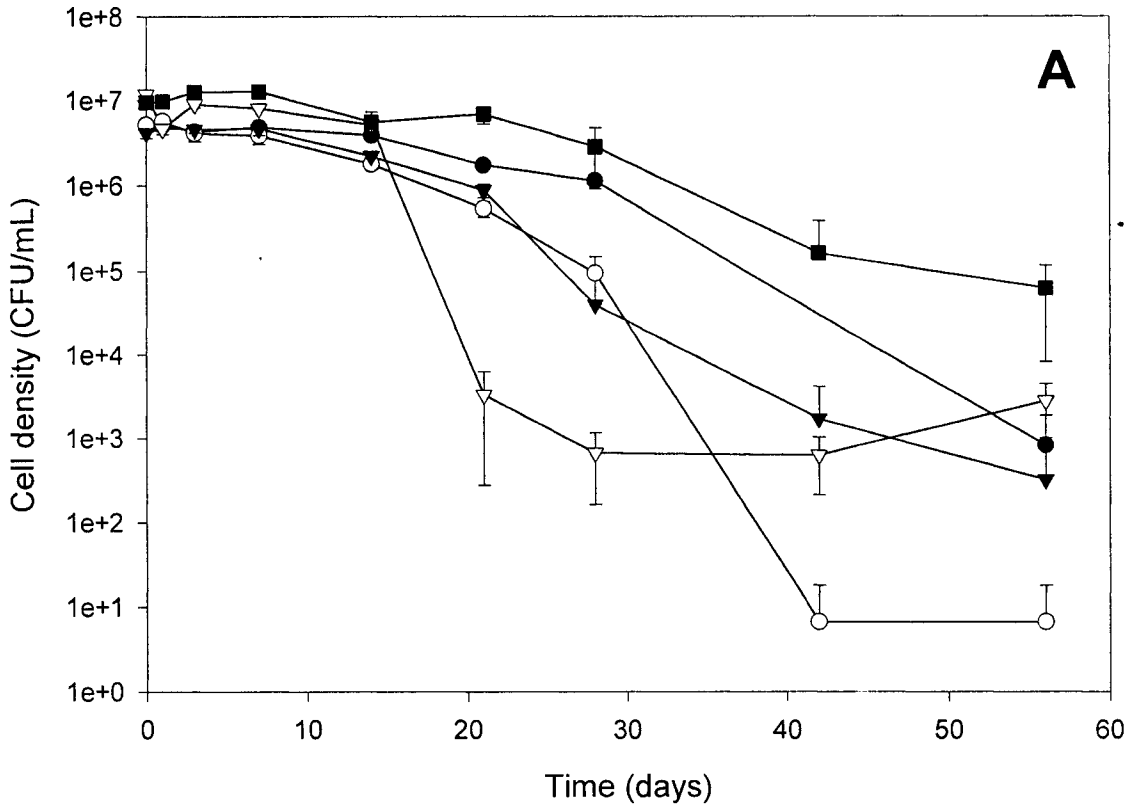


Figure 2.4. Survival of enterohemmorhagic *Escherichia coli* belonging to ERIC–PCR genotype A and serotype O111 at (A) 10°C and (B) 22°C. ▼, strain H38; ■, strain H18; ○, strain H19; ●, strain H12; ∇, strain H11.



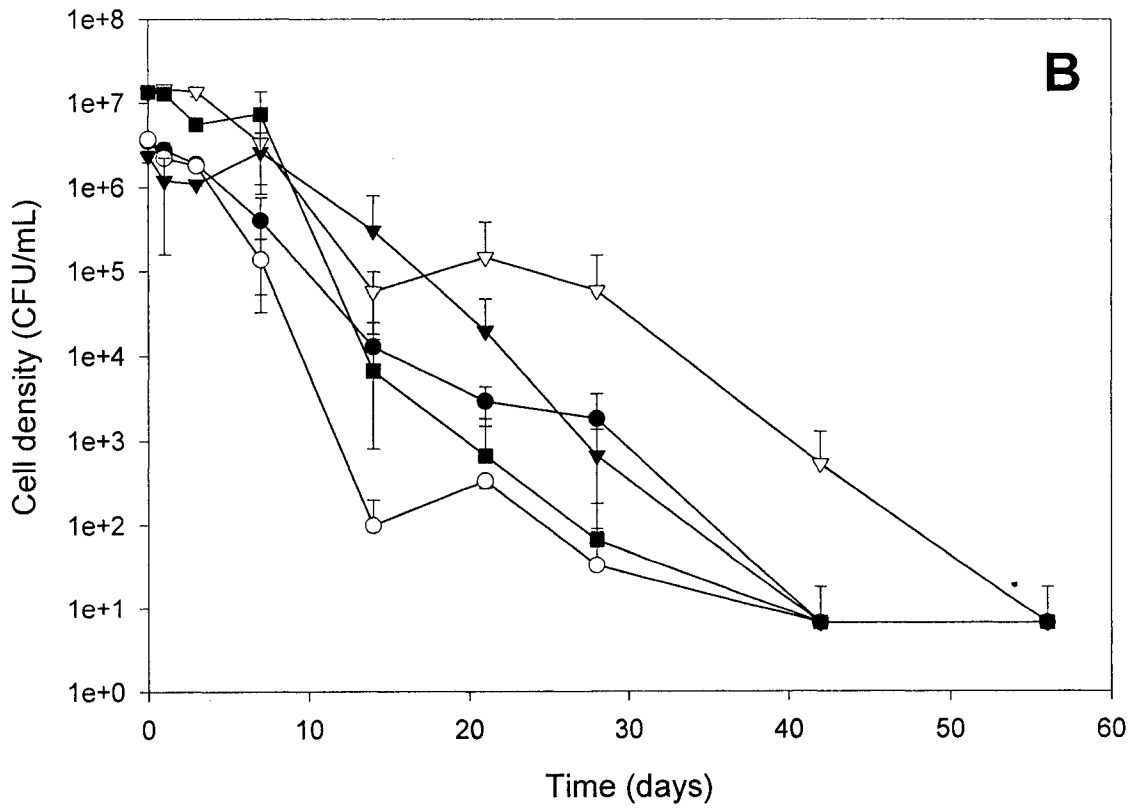
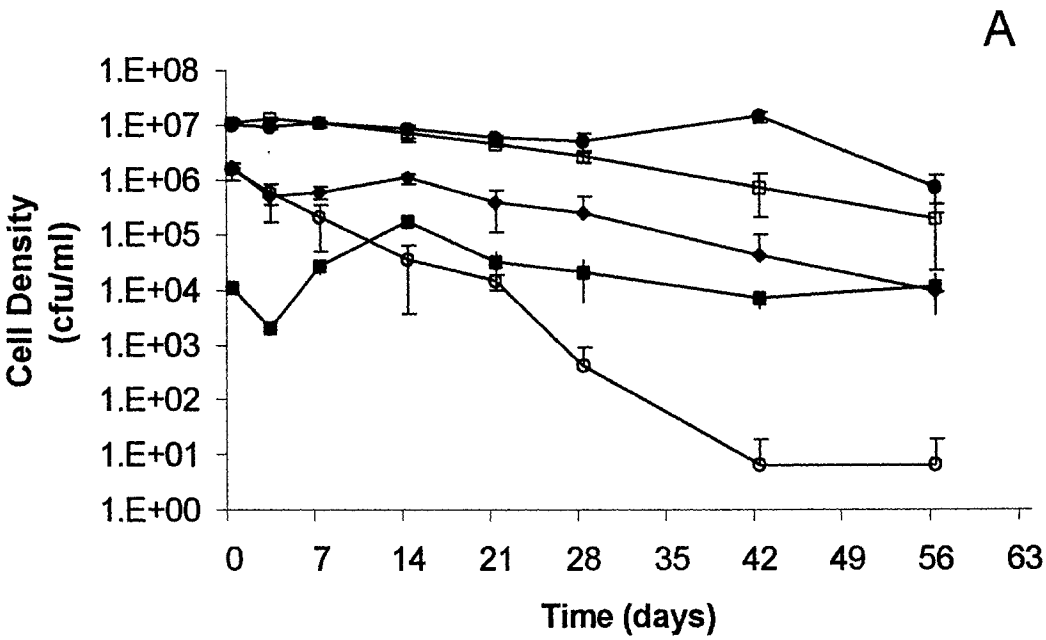
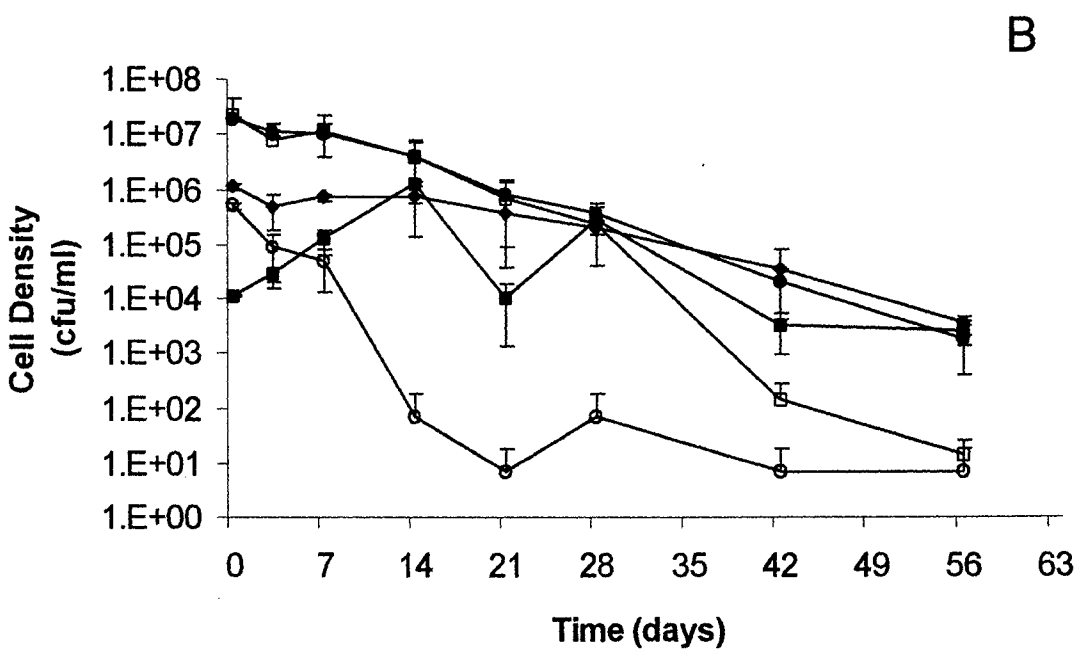


Figure 2.5: Comparison of heterotrophic bacteria counts of untreated well water at (A) 10°C and (B) 22°C with EHEC strains H32 and H22. •, total bacterial counts H32; ◆, total bacterial counts H22; □, *E. coli* counts H32; ○, *E. coli* counts H22; ■ Untreated well water total bacterial counts.





Discussion

A number of factors have been suggested to influence the survival of *E. coli* in water: presence of organic matter (Kerr *et al.*, 1999), differences in water composition, such as heavy metal concentrations (Artz and Killham, 2002), temperature and intrinsic differences in the *E. coli* O157:H7 strains (Wang and Doyle, 1998; Topp *et al.*, 2003). The intrinsic genotypic differences among EHEC are a suspected source of survival variability between strains of *E. coli* (Topp *et al.*, 2003). One aspect of this study attempted to identify a correlation between genotype and survival of pathogenic *E. coli* in untreated well water.

Preliminary work for this study involved determining the genotypes of 91 strains of *E. coli* including EHEC isolates from humans and cows and non-clinical isolates from pigs. The genotypes of these strains were determined using ERIC-PCR. ERIC-PCR is a fingerprinting method that develops an electrophoretic fingerprint pattern from PCR products initiated by primers targeting the enterobacterial repetitive intergenic consensus (ERIC) sequences. The ERIC sequences are 126 bp genomic elements possessing a highly conserved central inverted repeat located in extragenic regions of the genome (Olive and Bean, 1999). Primers are designed to target these sequences resulting in amplification during PCR. Strains with similar genomes will generate PCR products of equal size that will result in similar/identical electrophoretic patterns.

The sixteen *E. coli* strains selected for this study (Table 2.1) were selected based on the genetic variability seen between the strains which fall into genotypes A, B, H, N, R and P that share a phylogenetic similarity of 60 to 100 percent (Figure 2.1). These strains were also selected as they also represent three major EHEC serotypes: O26,

O111 and O157. Survival of the 16 EHEC strains was monitored over a period of 56 days at 10°C and 22°C to determine if the survival of the enterohemorrhagic *E. coli* correlates to their genotype. At both 10°C and 22°C, strains showed varying patterns of survival. At 10°C the EHEC strains showed superior, moderate or poor survival. Strains H32, H8 (Figure 2.2), H15 (Figure 2.3), and H18 (Figure 2.4) demonstrated the greatest level of survival. Strains showing moderate survival were H21, H25, H28 (Figure 2.2), H11, H12 and H38 (Figure 2.4). At the lower temperature strains H22, H24 (Figure 2.2), H5, H4, H35 (Figure 2.3) and H19 (Figure 2.4) showed poor levels of survival. When looking at members of the same genotype and serotype, it can be seen that no correlation can be made between genotype and survival. In Figure 2.2 members of genotype N with serotype O157 (H8, H24, H25, H28, and H32) demonstrates 3 different levels of survival. The lack of correlation is seen amongst member of genotype B of serotype O26 (H4, H5 and H15) (Figure 2.3) and members of genotype A of serotype O111 (H11, H12 and H38) (Figure 2.4).

A study performed by Topp *et al.* (2003) showed that two *E. coli* strains, which belonged to two distinct ERIC-genotypes, were found to lose viability at a comparable rate in an unsupplemented soil. In a manure amended soil, one strain (C279) demonstrated a 10-fold increase in cell density before starting to decline, while the other strain (C278) began to decline immediately. When the two strains were mixed in a ratio of 2.3:1 (C279:C278) and applied to the soil, the ratio increased to only 2.8:1 in soil without manure while in soil with manure the ratio increased to 15:1 after 12 days of incubation. It is thought that the C279 strain survived better, relative to strain C278, in manure amended soil because it possesses the ability to utilize some nutrient that strain

C278 cannot. These findings are of great interest, however, only one representative strain from each ERIC genotype was selected and compared. The behaviour of other members of the ERIC genotypes is not addressed. From the results of our study it is clear that members of the same ERIC genotype show significantly different survivability under the same temperature conditions in untreated well water.

The serotypes of the 16 EHEC strains compared in our study have also been taken into consideration. Serotyping has long been the traditional method of bacterial strain classification. It has been determined, however, those different strains of a bacterial species can be genotypically diverse within a specific serotype grouping (Figure 2.1). As with genotype, it is clear from the results reported in Figures 2.2, 2.3 and 2.4, that no correlation between serotype and strain survival exists. The members of serotype O157 tested for persistence in untreated well water displayed three different survival trends at 10°C (Figure 2.2 A). Strains H8 and H32 decreased in cell density by only approximately 1 log after 56 days of incubation while strains H21, H25 and H28 fell 3-4 log and strains H19, H22 and H24 dropped below the detection limit. The same trend is seen for serotype O26 (Figure 2.3). The cell densities of strains H15 and H18 decrease by only approximately 1 log while strains H4, H5 and H15 decreased to near or below the detection limit by day 56.

A similar study by Fukushima *et al.* (1999) addressed the survival of 3-different *E. coli* serotypes O26 (3 strains), O111 (2 strains) and O157 (5 strains) at 5°C, 15°C and 25°C in cow manure. These researchers combined the 10 strains into a single inoculum and applied it to bovine feces at levels of 10^1, 10^3 or 10^5 cfu/g of feces. In general, strains of O26, O111 and O157 decreased to levels only detectable by enrichment at

week 1 to 3 for low levels of inoculum (10^1 cfu/g), at week 2 to 6 for middle levels of inoculum (10^3 cfu/g) and at week 4 to 10 for the higher level of inoculum (10^5 cfu/g) at 5°, 15° and 25°C. The greatest level of persistence was seen at 15°C with strains continuing to be detected up to week 18 for O26 (inoculum 10^5 cfu/g), O111 (inoculum 10^3 cfu/g) and O157 (inoculum 10^5 cfu/g). The detection of these strains, however, required a specialized enrichment protocol. The method of identification employed by Fukushima *et al.* (1999) involves culturing the strains using a specialized enrichment protocol followed by agglutination screening for serotype. By employing the enrichment step, strains can be detected for a longer period of time relative to our study. This method of identification however, depends heavily on the strains maintaining their serotype. As EHEC strains have been known to lose their antigenicity (Nagano *et al.*, 1998), there is the potential that the numbers of pathogenic strains may be underestimated. By keeping strains separate in our study the survival of individual strains can more easily be compared without fear of misidentification. Although the Fukushima *et al.* (1999) study differed greatly in environment and method, it still showed that E. *coli* strains of different serotypes had varying levels of longevity, supporting the findings of our study.

Temperature showed a great level of influence on the survival of EHEC as 4 patterns of survival were observed when the 10°C and 22°C treatments were compared. These patterns include (1) strong survival at both 10° and 22°C, (2) moderate to strong survival at 10°C with reduced levels of survival at 22°C, (3) poor survival at 10°C with superior survival at 22°C and (4) poor survival rates at both 10°C and 22°C. Strains demonstrating strong survival at both 10°C and 22°C included H32, H8 (Figure 2.2) and

H15 (Figure 2.3). Ten strains fell into the most common pattern of moderate to strong survival at 10°C with poor survival at 22°C: H19, H21, H24, H25, H28 (Figure 2.2), H4, H35 (Figure 2.3), H12, H18 and H38 (Figure 2.4). Two strains, H5 (Figure 2.3) and H11 (Figure 2.4), demonstrated a low level of survival at 10°C while maintaining moderate to superior survival at 22°C. This is an unexpected result as previous studies have shown that most strains show a high level of persistence at lower temperatures (Rice *et al.*, 1992; Wang and Doyle, 1998). The last strain, H22 (Figure 2.2), demonstrated poor survival at both 10°C and 22°C. It can be seen from these varying patterns of survival across the six genotypes, A, B, H, R, N and P, that there is no correlation between the survival of these strains and genotype. However, it can be seen that there is a connection between temperature and survival as it can be seen the majority of strains show greater persistence at 10°C than at 22°C (Figures 2.2, 2.3, and 2.4).

Rice *et al.* (1992) examined the survival of two *E. coli* O157:H7 strains relative to a typical *E. coli* strain in untreated well water at 5°C and 20°C for both the EHEC strains and the typical *E. coli* strain. The study started with initial log cfu/ml values at approximately 6-6.5 log cfu/ml. A rapid decline at 20°C relative to 5°C was seen. The average log decline being approximately 3.5 log by day 70 relative to a 5 log decrease by day 35 at 20°C. The authors (Rice *et al.*, 1992) concluded that their results support the contention that a typical indicator strain of *E. coli* has a survival pattern similar to enterohemorrhagic strains in undisinfected well water and thus standard testing methods for *E. coli* are sufficient for detecting fecal contamination and indicating the possibility of related pathogens in water. However, their study only looked at two *E. coli* O157:H7 strains, an isolate from a patient in Cabool, MO and a strain provided by the

Food Research Institute (University of Wisconsin, Madison, WI), and a typical *E. coli* strain (R1) isolated from river water. The data from our study show that not all EHEC behave similarly within or between different serotypes (Figure 2.2, 2.3, and 2.4). It is, therefore, unrealistic to generalize and predict the survival of EHEC based on one or two representative *E. coli* strains.

Numerous studies have addressed the issue of the persistence of enterohemorrhagic *Escherichia coli* in water and a variety of other environments. Most of those addressing survival in water looked primarily at *E. coli* O157:H7 in potable and recreational water. A study by Warburton *et al.* (1998) investigated the survival of *E. coli* O157:H7 in a variety of bottled waters: spring water, mineral water, distilled water and tap water. Bottled water is any potable water that is manufactured, distributed or offered for sale that is in a sealed, food grade bottle or container intended for human consumption. The study involved inoculating sterilized distilled, mineral, spring and tap water with an inoculum composed of a pool of ten *E. coli* O157:H7 strains, unwashed after culturing. The water samples were stored at 22°C and samples collected at intervals of 5 to 7 days. The *E. coli* strains were found to persist in the sterilized distilled, mineral, spring and tap water for greater than 309, 309, 102 and 303 days, respectively. The researchers attribute the prolonged survival of the contaminating bacteria to a combination of change in oxygen levels, temperature and nutrients in combination with interaction with indigenous competitors in the water (Warburton *et al.*, 1998). The Warburton *et al.* (1998) study does not effectively illustrate how *E. coli* can survive in water for two reasons. Firstly, the comparisons were made between samples of water inoculated with unwashed cells. The culture media in which the cells were suspended

would provide nutrients for the strains inoculated into the water as well as the natural flora of the water. The nutrients would allow bacterial cells to grow and multiply in an environment that would naturally be oligotrophic. Thus potentially elongating the survival time of contaminating strains and elevate competitor stains naturally present in the water thus providing a poor indication of survival of EHEC in water. The second reason being the inoculum was composed of a mixture of 10 different strains of *E. coli* O157:H7. During the course of the study a subset of the original inoculum may persist while others die off thus providing an inaccurate measure of survival. Our study better illustrates how EHEC survive in water as it uses washed cells and single strain inoculums to more accurately determine the longevity of individual strains. This allows comparisons among the different strains under identical conditions and more clearly reveals the influence of genotype on the survival of EHEC.

In conclusion, our findings show that members of various EHEC serogroups are diverse genetically and their persistence in natural well water can be highly variable. For instance, some *E. coli* strains can survive in well water at a level of as high as 5 log cfu/ml in 56 days and some will drop below detection level (< 0.8 log cfu/ml) in less than 42 days at 10°C. In addition, we show that survival of *E. coli* generally decreases at higher water temperature (22°C). In this study, no correlation was found between the ERIC-genotype or serotype of a strain and its survival in water, therefore one cannot predict the survival of an *E. coli* strain in water based on its serotype or ERIC-genotype. This may be due to the low discriminating capability of the ERIC-PCR genotyping method. Other more refined genomic typing methods, such as, amplified fragment length polymorphism (AFLP), RER-PCR genotyping and ribotyping, may show more

promising correlations. In light of the huge variation in persistence of the EHEC strains found in this study, this provides a unique opportunity to study the intrinsic biochemical and/or physiological factors that control the persistence of *E. coli* in the environment.

Chapter 3

Molecular detection of EHEC, ETEC, EPEC and EIEC using Multiplex-PCR and DIG-labeled oligonucleotide probes

Introduction

Pathogenic *E. coli* have become a significant health concern, especially *E. coli* O157:H7, having caused major outbreaks in water and food in the recent past which has increased the demand for a rapid detection method. Pathogenic *Escherichia coli* can be divided in to six virotypes: Enterohemorrhagic (EHEC), Entertoxigenic (ETEC), Enterpathogenic (EPEC), Enteroinvasive (EIEC), Enteroaggregative (EaggEC) and Diffusely Adherent (DAEC) The division is based on the virulence factors they possess which initiate the disease symptoms (Smith, 1992). The four major *E. coli* virotypes used in this study are EHEC, ETEC, EPEC and EIEC. EHEC express numerous virulence factors, two of the most significant being the shiga-like toxins (*slt*) I and II (Nakao and Takeda, 2000). ETEC carry the plasmid-mediated heat-stable (ST) and/or heat-labile (LT) toxins (Rappelli *et al.*, 2001). EPEC rely on plasmid encoded enteroadherence factor (EAF) for the development of localized adherence necessary for infection (Tobe *et al.*, 1999). EIEC infection is mediated by the plasmid encoded invasion associated loci (*ial*) (Rappelli *et al.*, 2001). These virulence factors are the targets for the molecular detection of pathogenic *E. coli* in this study.

In general diarrheagenic *E. coli* can not be identified based on biochemical criteria alone as in most cases they are indistinguishable from non-pathogenic *E. coli*. The differentiation between *E. coli* virotypes requires the use of cell culture assays that are not typically part of a diagnostic routine (Rappelli *et al.*, 2001). The current protocol for the identification of pathogenic *E. coli* O157:H7 is a time consuming process (Okamoto *et al.*, 1999) which involves the use of selective and differential growth media which take into consideration phenotypic traits characteristic of *E. coli* O157:H7 such as

loss of sorbitol fermentation and β-glucuronidase inactivity (Cebula *et al.*, 1995). The use of these characteristics alone is limited as the emergence of *E. coli* O157:H7 strains demonstrating sorbitol fermentation (Ware *et al.*, 2000) and β-glucuronidase activity (Gunzer *et al.*, 1992) have been found. Serotyping is often included in the battery of biochemical tests used to identify *E. coli* O157:H7. This method alone, however, can not identify pathogenic *E. coli* since there is not always a correlation between pathogenicity and the O and H antigens expressed (Rappelli *et al.*, 2001). This test also provides no information on the types of toxins produced and, in the case of anti-O157 sera, lack specificity as the anti-O157 sera cross reacts with a variety of other bacterial species (Cebula *et al.*, 1995). An additional down fall of the protocol is the reliance on culturing. The isolation and detection of pathogens from environmental samples may be difficult if cells have become stressed or injured (Lisle *et al.*, 1998; Wang and Doyle, 1998). For these reasons, methods targeting DNA detection are being pursued.

The polymerase chain reaction (PCR) is a selective and sensitive method that rapidly amplifies specific regions of a gene. Although PCR is rapid and specific the process could become cumbersome when being applied to numerous samples with various potential targets. To minimize time and materials, primers can be combined in a single reaction tube to form a multiplex-PCR (MP-PCR) which can simultaneously detect numerous target genes in a single sample.

The MP-PCR method has been used to identify and differentiate pathogenic *E. coli* strains in a number of studies. A method has been developed which can detect the presence of coliforms and *E. coli* in a sample (Bej *et al.*, 1991b). This method uses PCR to target the *lacZ* and *uidA* genes common in coliforms and *E. coli,* respectively.

Numerous MP-PCR assays have been developed to detect pathogenic *E. coli.* Many of these methods target a single *E. coli* virotype, such as EHEC (Osek, 2002; Pollard *et al.*, 1990; Paton and Paton, 1998; Paton and Paton, 1999; Feng and Monday, 2000) while others target a single serotype, for example the infamous *E. coli* O157:H7 (Cebula *et al.*, 1995; Fratamico *et al.*, 1995; Fratamico *et al.*, 2000, Venkateswaran *et al.*, 1997; Hu *et al.*, 1999; Nagano *et al.*, 1998; Meng *et al.*, 1997; Campbell *et al.*, 2001; Wang *et al.*, 2002). The MP-PCR assay has also been used to differentiate certain pathogenic *E. coli* from other pathogens that cause similar maladies, for example ETEC or EIEC and Shigella (Frankel *et al.*, 1989; Houng *et al.*, 1997). Assays have been developed which attempt to differentiate *E. coli* virotypes by targeting virulence genes and other genes necessary for infection (Tsen and Jian, 1998; Tornieporth *et al.*, 1995; Lang *et al.*, 1994; Reid *et al.*, 1999). Two studies targeted all four of the major *E. coli* virotypes: EHEC, ETEC, EIEC and EPEC. These assays applied three (Rappelli *et al.*, 2001) or four (Pass *et al.*, 2000) different MP-PCR reactions targeting 8 or 11 different genes for the identification of the four different virotypes. The majority of these assays identify their target amplicon by agarose band size alone. It has been acknowledged that MP-PCR can produce non-specific amplification as a product of mixing PCR primers (Pass *et al.*, 2000; Rappelli *et al.*, 2001). Although non-specific amplifications can be confirmed through a process of DNA-DNA hybridization using linear DNA probes (Lang *et al.*, 1994), they were not used in combination with published multiplex-PCR assays targeting four different *E. coli* virotypes (Rappelli *et al.*, 2001; Pass, *et al.*, 2000).

Size differences of the amplification products may serve well as a preliminary identification of target amplicons, however, without confirming the sequence one can

not be certain the product was generated from the target gene as nonspecific amplification can occur. DNA-DNA hybridization has been identified as a reliable method for specific base sequence recognition (Fujiwara and Oishi, 1998). DNA probes, designed to hybridize with the specific sequence within the amplified region, can confirm the specificity of amplification. The use of DIG labeled DNA probes can allow verification of an amplicon sequence resulting in a more sensitive and accurate detection method.

The objective of this study was to develop a set of MP-PCR primers and highly specific DNA probes for the detection and confirmation of pathogenic *E. coli* strains belonging to the four major virulence groups EHEC, ETEC, EIEC and EPEC. The primers and probes were tested against 32 *E. coli* strains and 7 non-*E. coli* enteric and environmental bacterial strains.

Materials and Methods

Bacterial strains and culturing: A total of 31 *Escherichia coli* strains representing the four virotypes EHEC (17 strains), ETEC (8 strains), EPEC (2 strains) and EIEC (4 strains) were included in this study. *E. coli* Sy327 (non-pathogenic) and 7 non-*E. coli* species were included as negative controls (Table 3.1).

All strains were cultured in 10 ml of trypticase soy broth (Becton, Dickinson Company, Sparks, MD) and incubated under aerobic conditions with shaking overnight at 37°C.

DNA extract preparation: DNA extracts used as templates in PCR amplifications were prepared in one of two ways, either by boiling or using a commercially available kit, UltraClean™ Microbial DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA). For the boiling preparation, a one-ml aliquot of each strain was spun at 14,000 rpm for 5-10 minutes using an eppendorf centrifuge 5410 (Brinkman Instruments Inc., Westbury, NY). The supernatant was discarded and the pellet resuspended in 100 µl of sterile double distilled water, boiled in a water bath for 15 and spun again for 5 minutes. The supernatant was removed to a fresh, sterile tube and stored at -20°C and the pellet discarded.

For the UltraClean™ Microbial DNA Isolation Kit bacterial DNA extracts were prepared according to the manufacturer's instructions (MoBio Laboratories, Solana Beach, CA). The procedure involved concentrating cells from a 1.8 ml aliquot of culture by spinning twice for 30 seconds at 14,000 rpm. The pellet was resuspended in 300 µl of MicroBead solution and transferred to a MicroBead tube with the addition of 50 µl of

Solution MD1. The MicroBead tube was vortexed for 10 minutes at maximum speed horizontally using a MoBio Vortex Adapter, spun for 30 seconds and the supernatant transferred to a clean microcentrifuge tube. To the supernatant 100 µl of Solution MD2 was added followed by 5 seconds of vortexing and 5 minutes incubation a 4°C. The tube was then centrifuged and the supernatant transferred to a fresh microcentrifuge tube. A volume of 900 µl of Solution MD3 was added to the supernatant and the tube vortexed for 5 seconds. The mixture was loaded into a spin filter tube and centrifuged. All flow through was discarded and 300 µl of Solution MD4 was applied to the filter, which was centrifuged for one and half minutes. The filter was removed and placed in a clean 1.9-ml microcentrifuge tube. To the center of the filter membrane 50 µl of Solution MD5 was applied and the tube spun for 30 seconds. The spin filter was discarded and the supernatant, now containing the DNA, was stored at -20°C until required.

Primer design: Six primer pairs were designed based on virulence gene sequences retrieved from the GenBank database, a service of the National Center for Biotechnology Information (NCBI), generating amplicons ranging from 170 bp to 390 bp in length (Table 3.2). The virulence gene sequences derived from different *E. coli* strains were selected and aligned using the software program DNAman (Lynnon BioSoft Corporation, Vaudreuil, Quebec) generating consensus sequences to demonstrate homology and identify regions of variability within the genes of interest. The alignment generated a consensus sequence on which the primer sequences were based. Primers were designed, using the same software program, to target the regions showing the highest degree of homology. Primers were prepared by Sigma-Genosys (Oakville, ON)

and hydrated to a stock concentration of 100 μM using sterile double distilled water and then an aliquot of the primer stock solution was diluted to a working concentration of 25 μM using sterile double distilled water. The stock and working primer solutions were stored in 50 μl aliquots at -20°C until required.

PCR and Hexaplex-PCR: PCR was performed in 50 μl reactions in 0.2 ml thin walled PCR tubes. The reaction mixtures contained 5 μl 10x PCR Buffer, 5 μl 25 mM $MgCl_2$, 5 μl 2 mM dNTP mix, 2 μl *Taq* DNA polymerase (MBI Fermentas, Burlington, ON, Canada), 1 μl formamide, 0.8 μl of each forward and reverse primers, 2 μl template DNA and sterile double distilled water to bring the final volume to 50 μl. The PCR procedure was performed in a PCR Sprint Thermocycler (Hybaid Limited, UK). The protocol performed consisted of three stages: Stage 1: one cycle of 95°C for 5 minutes, 55°C for 2 minutes and 72°C for 1 minute; Stage 2: 34 cycles of 95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute; Stage 3: 72°C for 10 minutes and 4°C holding temperature. The PCR amplification products were visualized in 2.5% agarose gels (Top Vision LE GQ Agarose, genetic qualified; MBI Fermentas, Canada) stained with ethidium bromide (0.2 μg/ml).

DNA probe design: Oligonucleotide DNA probes were designed for the purposes of detecting multiplex PCR amplicons using DNAman software (Lynnon BioSoft Corporation, Vaudreuil, Quebec). Preliminary probes were designed based on the consensus sequences generated during the primer design process. To further ensure that the DNA probes would target the amplicons generated, the amplicons generated by

the six primer pairs from the five primary target strains were isolated following separation by agarose gel electrophoresis and sequenced. The DNA sequences obtained were compared with those of the consensus sequence generated using GenBank sequences, resulting in the development of 6 linear DNA probes (Table 3.5). The DNA probes designed were synthesized by Sigma-Genosys (Oakville, ON) and hydrated in sterile double distilled water to a stock concentration of 20 μM.

Chemiluminescent detection: The probes were labeled with a chemiluminescent marker following the protocol for 3' tailing of oligonucleotides with Digoxigenin-11-dUTP/dATP using reagents and protocol from a DIG oligonucleotide Tailing Kit (Boehringer Mannheim, 1995). The protocol involved mixing 4 μl 5x Reaction Buffer (1 M potassium cacodylate, 125 mM Tris-HCl, 1.25 mg/ml bovine serum albumin, pH 6.6), 4 μl $CoCl_2$ solution (25 mM), 1 DIG-dUTP (1 mM Digoxigenin-11-dUTP), 5 μl oligonucleotide (20 pmol/μl), 1 μl dATP (10 mM dATP in Tris buffer, pH 7.5), 1 μl terminal transferase (50 units/μl in 200 mM potassium cacodylate, 1 mM EDTA, 200 mM KCl, 0.2 mg/ml bovine serum albumin; 50% v/v glycerol; pH 6.5) and 4 μl sterile double distilled water. The mixture was incubated for 30 minutes at 37°C then placed on ice. The transferase reaction was terminated with the addition of 1 μl 0.2 M EDTA.

The chemiluminescent oligonucleotide probes were tested for specificity against amplicons generated by the hexaplex-PCR reaction. Six dot blots were prepared with hexaplex-PCR products generated from the 31 strains of pathogenic *E. coli* and 9 non-pathogenic or non-*E. coli* control species. Each strain has been designated a number (Table 3.1) which corresponds to its position on the dot blot (Figure 3.4). Probes were

tested individually for specificity and cross-reaction. PCR products were chemically denatured in a 0.5 M NaOH and 0.5 M NaCl mixture for 10 minutes at room temperature. Ten μl of the denatured PCR product generated from each strain was dotted onto a positively charged nylon membrane (Roche Diagnostics Corporation, Indianapolis, IN) 1 μl at a time allowing membrane to dry between each blot. The membrane was air-dried for 30 minutes and the DNA cross-linked to the membrane with UV light for 5 minutes.

To detect the PCR material on the membrane the blots were prepared for hybridization with linear DNA probes. Membranes were soaked in prehybridization solution (5x SSC (1:4 dilution of stock 20x SSC: 3 M NaCl, 0.3 M sodium citrate, adjusted to pH 7.0), 0.1% N-lauroylsarcosine, 0.02% SDS, 1% blocking reagent) at 55°C for 2 hours. Followed by a 5-7 hour incubation in 20 ml of hybridization solution (equivalent to prehybridization solution plus probe) with a 5 pmol/ml concentration of probe. Following incubation the hybridization solution was reserved and stored at -20°C for future use. The membranes were given two 15-minute washes at room temperature in 2x wash buffer (2x SSC, 0.1% SDS) and two 15-minute washes at room temperature in 0.5x wash buffer (0.5x SSC, 0.1% SDS). The membrane was then equilibrated in detection wash buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5; 0.3% (v/v) Tween20) for 1-5 minute. The membranes were then submerged in 1x blocking solution (0.1 M maleic acid, 0.15 M NaCl, pH 7.5; 1% blocking reagent (w/v)) for 30-60 minutes at room temperature with gentle agitation to allow the blocking reagent to to adhere to the areas of the membrane not bound by DNA to decrease the level of binding of antibody with membrane which results in background. Fresh blocking solution was prepared with anti-

digoxigenin-AP antibody (75 mU/ml) in a ratio1:10,000 and applied to the membrane for 30 minutes at room temperature with agitation. The membranes were washed twice in detection wash buffer for 15 minutes at room temperature. To prepare membranes for detection they were equilibrated for 2 minutes in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5). The chemiluminescent substrate, CSPD (25 mM Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phoshphate) diluted (1:100) in detection buffer, was applied to the damp membranes and once sealed into an airtight bag the membranes were incubated at 37°C for a minimum of 10 minutes to activate the alkaline phosphotase enzyme. Luminescence was detected with a Kodak Image Station 440CF (Perkin Elmer Life Science, Woodbridge, ON)

Results

Multiplex Primer Design

Six primer pairs were successfully designed and tested to use in the multiplex PCR to amplify the six specific virulence genes of the four major *E. coli* virotypes (Table 3.2). Primers were designed to amplify regions showing the highest degree of homology among sequences obtained through GenBank. However, preliminary screening of the primers indicated that not all primers were (i) successful for the amplification of target sequences and/or (ii) specific for the target gene sequences. Unsuccessful primers underwent redesign until eventually six highly specific primer sets were established (Table 3.2). The primers were highly specific in amplifying their corresponding target virulence genes in the 5 representative strains selected (Table 3.1). Amplification with these primers yielded products of expected sizes that were differentiable on an agarose gel (Figure 3.1): 170 bp (*hs*), 232 bp (*sltII*), 293 bp (*eaf*), 322 bp (*hl*), 350 bp (*sltI*) and 390 bp (*ial*).

After the primers were tested against 5 representative strains (Table 3.1) the individual primer sets were tested against 31 *E. coli* strains from 4 virotypes (EHEC, EPEC, EIEC and ETEC), 1 non-pathogenic *E. coli* strain and 7 non-*E. coli* enteric microorganisms and common bacterial species occurring in the environment (Table 3.3). This screening process allowed us to determine which primers initiated amplification of DNA from both target and non-target strains. Some primer sets did show some minor degree of non-specific amplification from non-target strains, however, this amplification tended to be weak and did not fall into the corresponding size range for the primer applied (Table 3.3).

Multiplex PCR

The hexaplex-PCR reaction is a multiplex PCR that contains all six pairs of PCR primers. The primers of the hexaplex-PCR assay were found to specifically amplify the target genes from a mixed DNA sample (Figure 3.2, lane 2) and from individual DNA extracts (Figure 3.2, lanes 3-7). The results of the hexaplex amplification applied to 31 pathogenic *E. coli* strains, 1 non-pathogenic *E. coli* strain and 8 non-*E. coli* enteric microorganisms and common bacterial species occurring in the environment are summarized in Table 3.4. The results indicate that the primers were specific for all strains amplifying products of the expected size, however, in a number of cases additional amplicons of a size falling outside the range of the expected amplicons were seen. Non-*E. coli* control strains also generated some non-specific amplicons falling primarily outside the expected size range for target amplicons.

DNA probe design and application

Oligonucleotide DNA probes were designed for the purpose of detecting multiplex PCR amplicons using DNAman software and their significant properties are summarized in Table 3.5. The oligonucleotide probes designed were labeled with a chemiluminescent signal molecule and tested for specificity against products of the hexaplex-PCR reaction. Figure 3.3 shows the results of the dot blot analysis showing the positive hybridization of the 6 DIG-labeled linear probes with the hexaplex-PCR products generated. The position of each dot corresponds to a bacterial strain listed in Table 3.1. The images seen in Figure 3.3 have been cropped to allow the 6 blots to be on a single page but no visible data has been excluded. The hybridization results

indicate that the current probe designs show specificity for target PCR amplicon sequences and no affinity for the non-specific amplification derived from either *E. coli* or non-*E. coli* strains reported in Table 3.4.

Table 3.6 is a summary of the results for all 6 linear probes applied against 32 strains of pathogenic *E. coli* and 8 non-pathogenic or non-*E. coli* control species. DNA probes were designed to verify that the PCR amplicons generated were derived from the target genes. The results indicate that the probes can effectively detect the PCR products generated by the hexaplex-PCR as hybridization was observed only between the products of the multiplex-PCR from target strains and their corresponding DIG-labeled linear probes. These results indicate the importance of the probes as tools to prevent the reporting of false positives as no hybridization was observed between the DNA probes and non-specific amplification reported in Table 3.4 for control strains.

Table 3.1: Strains of EHEC, EPEC, EIEC and ETEC used to monitor the specificity of hexaplex-PCR and chemiluminescent DNA probes.

Strain	Dot Blot Number	Source
EHEC 920004	A1	a
EHEC 920026	B1	a
EHEC 920115	C1	a
EHEC 920153	D1	a
EHEC 930546*	E1	a
EHEC 961019	F1	a
EHEC 961085	G1	a
EHEC 970112	A2	a
EHEC 930195	B2	a
EHEC 960959	C2	a
EHEC 930529	D2	a
EHEC 920024	E2	a
EHEC 970262	F2	a
EHEC 3069-89	G2	a
EHEC 920029	A3	a
EHEC 920220	B3	a
EHEC 965105	C3	a
EPEC O55:H6*	D3	b
EPEC E2348	E3	b
EIEC O136	F3	b
EIEC O144	G3	b
EIEC O164	A4	b
EIEC 80-3379*	B4	a
ETEC O7*	C4	a
ETEC 505	D4	a
ETEC B44*	E4	a
ETEC PS274	F4	a
ETEC 490	G4	a
ETEC K206	A5	a
ETEC K121	B5	a
ETEC K326	C5	a
E. coli Sy327	D5	d
Serratia marcescens	E5	c
Proteus mirabilis	F5	c
Klebsiella pneumoniae	G5	c
Bacillus subtilis	A6	c
Alcaligenes faecalis	B6	c
Enterobacter aerogenes	C6	c
Enterococcus faecalis	D6	c
Pseudomonas fluorescens	E6	c

* One of 5 primary target strains used in preliminary primer and probe testing
a Dr. C. Gyles, University of Guelph, Guelph, ON
b B. Ciebin, Ministry of Health, Etobicoke, ON
c J. Henderson, Lakehead University, Thunder Bay, ON
d D. Cuppels, Agriculture and Agri-Food Canada, London, ON

Table 3.2: Summary of the primers designed and the sources of sequences on which they are based.

Gene Target	Primer Name	Primer Sequence (5'-3') F/R [a]	Source	Size (bp)	Accession Number
sltI	sltI.3	ACCTCACTGACGCAGTCTGTGG/ TCTGCCGGACACATAGAAGGAAA	Original	350	ab015056
sltII	sltII.4	ACTGTCTGAAACTGCTCCTGTG/ TTATTTTTATAACGGGCCTGTTCGC	Original	262	ab030484
HS	hs.2	TCTTTCCCCTCTTTTAGTCAGTC/ CCAGCACAGGCAGGATTAC	Modified [b]	170	m25607
HL	hi.2	TCTCTATGTGCACACGGAGC/ CCATACTGATTGCCGCAAT	Modified [b]	322	abs60731
EAF	eaf	ACGCTTGGAGTGATCGAACG/ TGCCAACACAGCTTGTCAGAA	Original	293	x76137
ial	ial.2/.3	TTTCTGGATGGTATGGTGAGG/ CACGCTGGTTGTCAATAATGCT	F = Modified [c] R = Original	390	Shigella orf

a F = Forward primer, R = Reverse primer
b Based on modifications of PCR primers designed by Rappelli, P., *et al.* (2001)
c Forward primer based on modification to PCR primer designed by Rappelli, P., *et al.* (2001) while the reverse primer is an original design

Table 3.3: Summary of primer cross-reaction screening for individual primers.

Strain	hs (170 bp)	hl (322 bp)	eaf (293 bp)	sltI (350 bp)	sltII (262 bp)	ial (390 bp)
EHEC 920004	-	-	-	+	+	-
EHEC 920026	-	-	-	+	+	-
EHEC 920115	-	-	-	+	-	-
EHEC 920153	-	-	-	+	-	-
EHEC 930546	-	-	-	+	+	-
EHEC 961019	-	-	-	-	+	-
EHEC 961085	-	-	-	-	+	-
EHEC 970112	-	-	-	+	-	-
EHEC 930195	-	-	-	-	+	-
EHEC 960959	-	-	-	-	+	-
EHEC 930529	-	-	-	+	+	-
EHEC 920024	-	-	-	+	+	-
EHEC 970262	-	-	-	+	-	-
EHEC 3069-89	-	-	-	+	-	-
EHEC 920029	-	-	-	-	+	-
EHEC 920220	-	-	-	-	+	-
EHEC 965105	-	-	-	-	+	-
EPEC O55:H6	-	-	+	-	-	-
EPEC E2348	-	-	+	-	-	-
EIEC O136	-	-	-	-	-	+
EIEC O144	-	-	-	-	-	+
EIEC O164	-	-	-	-	-	+
EIEC 80-3379	-	-	-	-	-	+
ETEC O7	-	+	-	-	-	-
ETEC 505	+	-	-	-	-	-
ETEC B44	+	-	-	-	-	-
ETEC PS274	+	-	-	-	-	-
ETEC 490	+	-	-	-	-	-
ETEC K206	-	+	-	-	-	-
ETEC K121	-	+	-	-	-	-
ETEC K326	+	+	-	-	-	-
E. coli Sy327	-	-	-	-	ns	-
S. marcescens	-	-	-	-	-	-
P.mirabilis	-	-	-	-	-	-
K. pneumoniae	-	-	-	-	-	-
B. subtilis	-	-	-	-	-	-
A. faecalis	-	-	-	-	-	-
E. aerogenes	-	-	-	-	-	-
E. faecalis	-	-	-	-	-	-

+ = amplification equivalent to target band size
- = no amplification with a band equivalent to expected size
ns = non specific amplification from non target strains with a band size equivalent to expected amplicon

Table 3.4. Summary of MP-PCR screening for potential primer cross-reaction.

Strain	hs (170 bp)	hl (322 bp)	eaf (293 bp)	sltI (350 bp)	sltII (262 bp)	ial (390 bp)	Non Specific[a]
EHEC 920004	-	-	-	+	+	-	-
EHEC 920026	-	-	-	+	+	-	-
EHEC 920115	-	-	-	+	-	-	-
EHEC 920153	-	-	-	+	-	-	-
EHEC 930546	-	-	-	+	+	-	-
EHEC 961019	-	-	-	-	+	-	+[a]
EHEC 961085	-	-	-	-	+	-	+[a]
EHEC 970112	-	-	-	+	-	-	+[a]
EHEC 930195	-	-	-	-	+	-	+[a]
EHEC 960959	-	-	-	-	+	-	+[a]
EHEC 930529	-	-	-	+	+	-	+[a]
EHEC 920024	-	-	-	+	+	-	+[a]
EHEC 970262	-	-	-	+	-	-	-
EHEC 3069-89	-	-	-	+	-	-	+[a]
EHEC 920029	-	-	-	-	+	-	-
EHEC 920220	-	-	-	+	+	-	-
EHEC 965105	-	-	-	-	+	-	+[a]
EPEC O55:H6	-	-	+	-	-	-	-
EPEC E2348	-	-	+	-	-	-	-
EIEC O136	-	-	-	-	-	+	+[a]
EIEC O144	-	-	-	-	-	+	-
EIEC O164	-	-	-	-	-	+	-
EIEC 80-3379	-	-	-	-	-	+	+[a]
ETEC O7	-	+	-	-	-	-	-
ETEC 505	+	-	-	-	-	-	-
ETEC B44	+	-	-	-	-	-	-
ETEC PS274	+	-	-	-	-	-	-
ETEC 490	+	-	-	-	-	-	-
ETEC K206	-	+	-	-	-	-	-
ETEC K121	-	+	-	-	-	-	-
ETEC K326	+	+	-	-	-	-	-
E. coli Sy327	-	-	ns	-	-	-	+
S. marscencens	-	-	-	-	-	-	-
P. mirabilis	-	-	-	-	-	-	+[a]
K. pneumoniae	-	-	-	-	-	-	+[a]
B. subtilis	-	-	-	-	-	-	+[a]
A. faecalis	-	-	-	-	-	-	+[a]
E. aerogenes	-	-	-	-	-	-	+[a]
E. faecalis	-	-	-	-	-	-	+[a]
P. fluorescens	-	-	-	-	-	-	+[a]

a = Amplification products are detectable that fail outside expected size range
ns = weak non-specific amplification of a size approximately equal to expected amplicon generated from a non-target strain

Table 3.5: Summary of linear DNA probe sequences and their temperature properties.

Probe	Sequence	Melting temperature °C	Hybridization Temperature °C
hl	5'-AGCGGCGCAACATTTCAGGTCGAAGTCC-3'	77.3	61.9
hs.2	5'-ATCAGAAAATATGAACAACACATTTTACTGCTGTGAAC-3'	69.7	57.1
eaf	5'-CGGCGCTGGTGATTTCGGGTTCGTCA-3'	79.5	62.1
ial	5'-CTTATGTTCAAGGAAATAATTGTTGGCCTCCTTCTC-3'	71.4	59.2
sltI.2	5'-ACAACACTGGATGATCTCAGTGGGCGTTCT-3'	73.5	60.4
sltII	5'-GCGTTCTGTTCGCGCCGTGAATGAAG-3'	76.2	60.5

Table 3.6. Summary of linear probe hybridization screening of MP-PCR product dot blots.

Strain	hs (170 bp)	hl (322 bp)	eaf (293 bp)	sltI (350 bp)	sltII (262 bp)	ial (390 bp)
EHEC 920004	-	-	-	+	+	-
EHEC 920026	-	-	-	+	+	-
EHEC 920115	-	-	-	+	-	-
EHEC 920153	-	-	-	+	-	-
EHEC 930546	-	-	-	+	+	-
EHEC 961019	-	-	-	-	+	-
EHEC 961085	-	-	-	-	+	-
EHEC 970112	-	-	-	+	-	-
EHEC 930195	-	-	-	-	+	-
EHEC 960959	-	-	-	-	+	-
EHEC 930529	-	-	-	+	+	-
EHEC 920024	-	-	-	+	+	-
EHEC 970262	-	-	-	+	-	-
EHEC 3069-89	-	-	-	+	-	-
EHEC 920029	-	-	-	-	+	-
EHEC 920220	-	-	-	+	+	-
EHEC 965105	-	-	-	-	+	-
EPEC O55:H6	-	-	+	-	-	-
EPEC E2348	-	-	+	-	-	-
EIEC O136	-	-	-	-	-	+
EIEC O144	-	-	-	-	-	+
EIEC O164	-	-	-	-	-	+
EIEC 80-3379	-	-	-	-	-	+
ETEC O7	-	+	-	-	-	-
ETEC 505	+	-	-	-	-	-
ETEC B44	+	-	-	-	-	-
ETEC PS274	+	-	-	-	-	-
ETEC 490	+	-	-	-	-	-
ETEC K206	-	+	-	-	-	-
ETEC K121	-	+	-	-	-	-
ETEC K326	+	+	-	-	-	-
E. coli Sy327	-	-	-	-	-	-
S. marcescens	-	-	-	-	-	-
P. mirabilis	-	-	-	-	-	-
K. pneumoniae	-	-	-	-	-	-
B. subtilis	-	-	-	-	-	-
A. faecalis	-	-	-	-	-	-
E. aerogenes	-	-	-	-	-	-
E. faecalis	-	-	-	-	-	-
P. fluorescens	-	-	-	-	-	-

Figure 3.1: Size comparison of PCR amplicons. Lane1, 100 bp DNA ladder; Lane 2, hs (170 bp); Lane 3, *slt*II (253 bp); Lane 4, EAF (293 bp); Lane 5, hl (322 bp), Lane 6, *slt*I (350 bp); Lane 7, *ial* (390 bp).

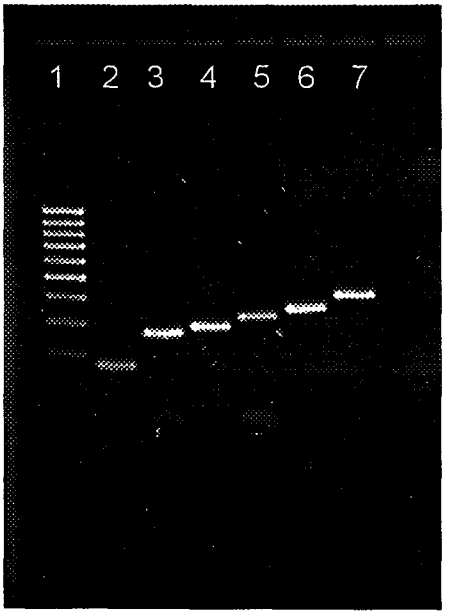


Figure 3.2: Products of Multiplex-PCR. Lane1, 100 bp ladder; Lane 2, six MP-PCR products generated from mixed DNA sample; Lane 3, 170 bp hs MP-PCR product; Lane 4, 350bp *slt*I and 262 bp *slt*II MP-PCR products; Lane 5, 293 bp EAF MP-PCR product; Lane 6, 322 bp hl MP-PCR product; Lane 7, 390bp *ial* MP-PCR product.

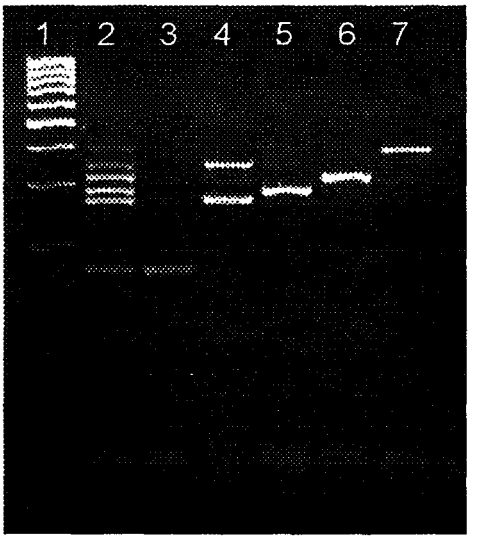
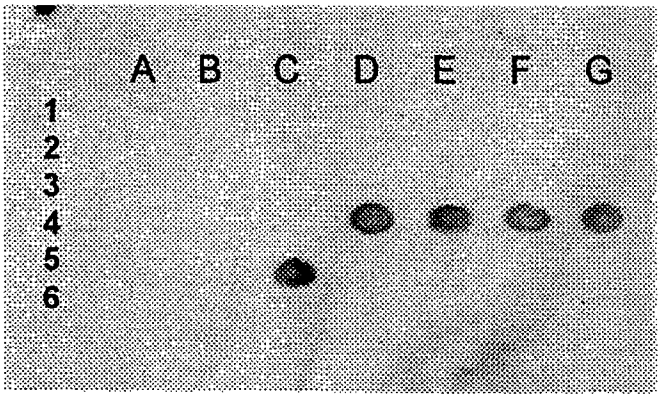
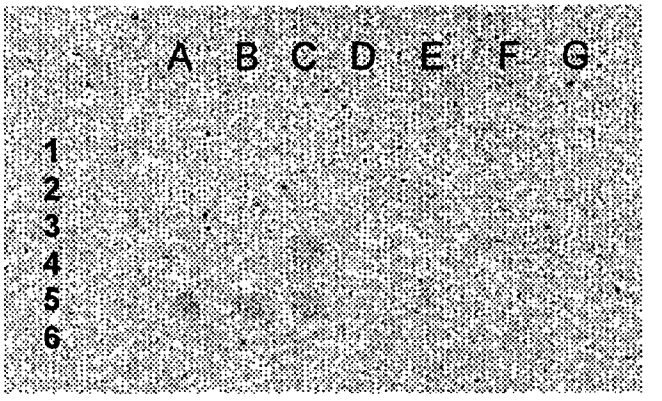


Figure 3.3: Dot blots of multiplex-PCR products generated from 31 pathogenic *E. coli* and 9 control strains and probed with individual DIG-labeled linear probes: (A) hs; (B) hl; (C) *slt*I; (D) *slt*II; (E) EAF; (F) *ial*. Strain identities are defined in Table 3.1.

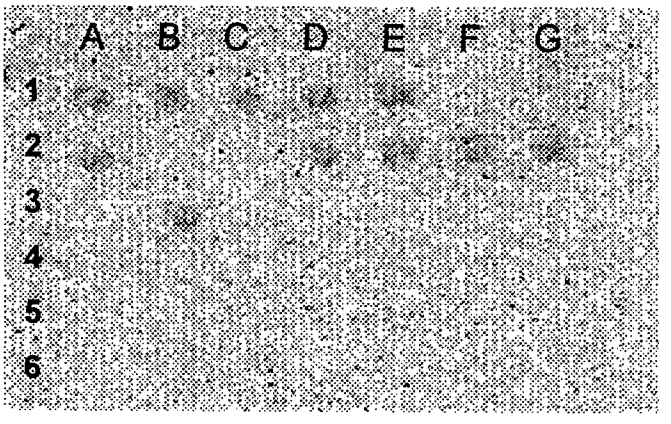
A.



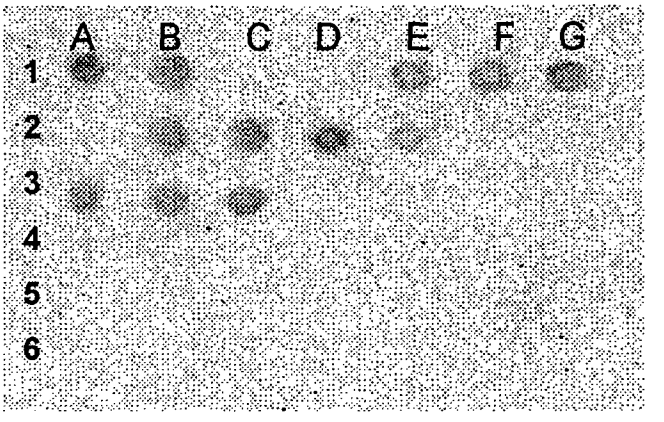
B.



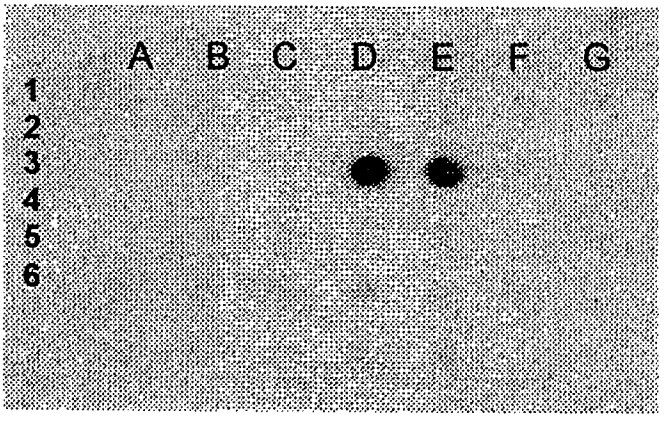
C.



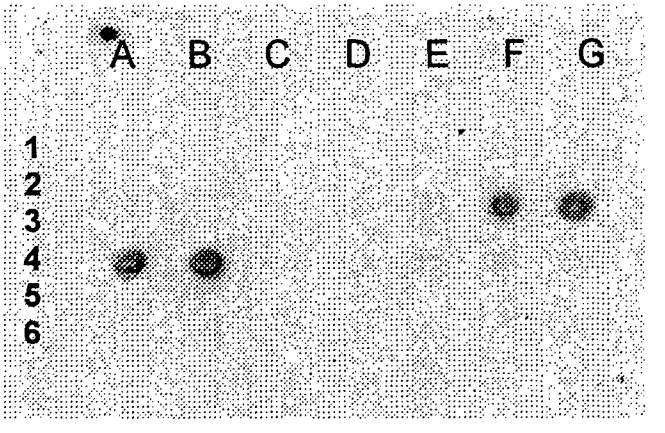
D.



E.



F.



Discussion

The first objective in the development of the multiplex-PCR protocol was to design 6 primer pairs to target 6 major virulence genes (hs, hl, *slt*I, *slt*II, EAF and *ial*) of 4 *E. coli* virotypes (EHEC, EPEC, ETEC, EIEC). Original primers were designed initially rather than using previously published primers because it was necessary to combine primers with similar temperature related properties to ensure they melted and annealed with equal efficiency. It was also necessary to select the primers so that the amplicons generated were close enough in size that amplification efficiency was not hindered but that they differed enough in size that they could be differentiated on an agarose gel. The initial primer design process required revisions to all but one primer pair, the pair targeting EAF. Redesign of primers was necessary. Even though the primers should have been highly specific for their target based on the gene sequences retrieved from GenBank, cross-reactions and low effectiveness were observed. Eventually, six primer pairs were developed (Table 3.2) that were highly specific in amplifying their target virulence genes in 5 representative strains of *E. coli* carrying the 6 target genes selected (Figure 3.1). Although the primers were found to be highly specific for the 5 representative *E. coli* strains (Table 3.1) the primers generated some non-specific amplification when applied to 32 strains of *E. coli* (17 EHEC, 8 ETEC, 2 EPEC, 4 EIEC and 1 non-pathogenic *E. coli*) and 7 non- *E. coli* enteric and environmental control strains in single primer pair driven PCR reactions (Table 3.3). The non-specific amplification observed at this stage of primer screening was observed primarily in the non-target control strains. The non-specific amplification observed fell outside the expected size range for the primers applied and therefore of no concern. There was

some non-specific amplification generated by primers from non-target *E. coli* strains, however, the non-specific amplicons generated also fell outside the size range for the expected amplicons. These non-specific amplifications too are likely due to low levels of non-specific binding between the primers and the bacterial genomic DNA. Following the successful application of the primers individually, they were mixed to produce a multiplex-PCR.

A multiplex PCR (MP-PCR) assay for the simultaneous detection of the four *Escherichia coli* virotypes EHEC, ETEC, EPEC and EIEC was developed by mixing the 6 individual primer pairs in a single reaction, also referred to as a hexaplex-PCR. The primer cocktail was composed of the six primer pairs targeting the virulence and genetic factors *slt*I and *slt*II toxins of EHEC, the hs and hl toxins of ETEC, the EAF factor of EPEC and the invasiveness plasmid of EIEC (Table 3.2). The MP-PCR assay was initially applied against the DNA from the same five representative *E. coli* strains used in Figure 3.1 with the addition of a mixture containing DNA from all five strains (Figure 3.2). The hexaplex mixture was applied against the 32 *E. coli* stains and 8 non-*E. coli* controls to determine if they produced any cross-reaction (Table 3.4). There was a slight increase in the non-specific amplification seen with single primer application (Table 3.3).

This hexaplex-PCR method improves upon those previously developed for the detection of pathogenic *E. coli* as it combines the detection of the four virotypes in a single PCR reaction tube. Numerous multiplex-PCR methods have been developed for the detection of pathogenic *E. coli* though they are limited in comparison as they detect fewer virulence factors and thus fewer virotypes simultaneously. Multiplex-PCR is used to amplify multiple genes simultaneously. Most of the multiplex-PCR assays described

in the literature limit their reactions to amplify two to five genes simultaneously, often being highly focused on a single virotype or serotype, for example *E. coli* O157:H7 (Venkateswaran *et al.*, 1997; Hu *et al.*, 1999; Fratamico *et al.*, 2000; Radu *et al.*, 2001; Campbell *et al.*, 2001; Wang *et al.*, 2002). Two studies addressed the 4 four major *E. coli* virotypes. Pass *et al.* (2000) targeted 11 genes found in 4 four major *E. coli* virotypes. However, certain primer combinations, for example primers targeting CNF1 and CNF2, resulted in spurious PCR products that interfered with the electrophoretic results. The 11 primer pairs were divided into 4 separate MP-PCR reactions. The first MP-PCR combined primers targeting VT1, VT2 and VTe. The second combined primers targeting CNF1 and Eagg. The third MP-PCR combination targeted CNF2 and Einv. The last MP-PCR combination mixed primer targeting LT1, ST1 and ST2. Alternative combinations of primers, to those listed above, could be used but it was found that there was a risk of losing the ability to amplify some genes. Loss of amplification occurred primarily when all of the genes corresponding to the primer pairs included in the primer mixture were present in the DNA sample. In comparison, the hexaplex-PCR developed in my study combines six primer pairs and successfully amplified all six amplicons simultaneously from a DNA mixture containing all 6 gene targets (Figure 3.2). Rappelli *et al.* (2001) took a similar approach targeting 8 genes from the 4 major virotypes in 3 separate reaction mixtures. The first mixture combined primers targeting the *elt* (HL), *sta* (HS) and *uidA* genes. The second mixture combined primers to target the *eae* and *bfp* genes. The final MP-PCR primer mixture targeted the *stx*1, *stx*2 and *ial* genes. The primers were tested both singly and as multiplexes on 190 *E. coli* isolates of unknown pathogenicity, the results from each were complimentary. The primary advantage of the

current study over these two is that all 6 primer pairs successfully targeted 6 genes from the 4 major virotypes EHEC, EPEC, ETEC and EIEC, when mixed in a single reaction tube. This ensures that all primers will experience the same conditions under which to function thereby decreasing the risk of false negatives. Also the mixing of primers in a single tube decreases costs and time and increases the ease of the assay.

Although the detection of pathogenic *E. coli* via the hexaplex-PCR shows a great deal of specificity for the target genes, there is no certainty at this point that the amplicons are actually derived from the intended target. The only thing that can be derived from the agarose gel is a band of expected size was generated. From Table 3.4 it can be seen that the hexaplex-PCR reaction can generate some non-specific amplification. Non-specific amplification can often cause problems in deciphering electrophoretic results (Pass *et al.*, 2000). The development of multiple fragments from different bacterial strains during MP-PCR has been seen in the past. Lang *et al.* (1994) found amplification of some pathogenic *E. coli* strains carrying the LT gene generated two distinct fragments, the expected product of 258bp and an unexpected fragment of 172bp. Southern blotting analysis using a DNA probe designed to target the 258bp fragment confirmed the presence of the desired amplicon (258bp) and that the second was the product of non-specific amplification. The problem of differentiating non-specific amplification from target amplicons has been remedied by the design of 6 linear DNA oligonucleotide probes specific for the hexaplex-PCR amplicons (Table 3.5). These probes were designed based on the amplicon sequences derived from the GenBank sequences used to design the MP-PCR primer and confirmed by sequencing of the PCR products derived from the five representative strains (data not shown). Figure 3.3

provides the dot blots generated from DNA probe application which shows the positive hybridization of linear probes with MP-PCR products generated from relevant *E. coli* strains. During the preliminary screening of the DNA probes against hexaplex-PCR products generated from the 5 representative strains (Table 3.1), minimal cross-reaction was observed. Only the hs probe required redesign due to cross-reaction with hexaplex-PCR amplicons generated from the *ial* positive strain 80-3379. The cross-reaction could not be explained by comparison of the sequences for the hs probe, hs amplicon (170 bp) and the *ial* amplicon (390bp). No clear regions of homology were found. After redesigning the hs probe no further cross-reaction was observed. Once the probe sequences were finalized the probes were screened against available bacterial strains (Table 3.6). The hybridization results indicate that the current probe designs show specificity for target PCR amplicon sequences and no specificity for the non-specific amplification derived from either *E .coli* or non-*E. coli* strains reported in Table 3.4. Like the study performed by Lang *et al.* (1994) DNA probes have been found to effectively identify the presence of PCR amplicons generated from target genes by the highly specific means of DNA-DNA hybridization.

In conclusion, we have developed a simple PCR based method, which is capable of simultaneously detecting the four most important virotypes of *E. coli*. This rapid assay may allow researchers, food inspection agencies and public health laboratories to detect hazardous *E. coli* strains from food and water samples in less than 48 hours. To further improve the assay future work in our laboratory will develop these probe sequences into molecular beacons, single-stranded, hairpin shaped DNA probes (Tyagi and Kramer, 1996). The advantage of such probes is their ability to exist in solution

without detection until hybridization, at which time the hairpin opens allowing a conjugated fluorophore to fluoresce, signaling hybridization.

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