Development of novel seminested polymerase chain reaction assays for detecting toxigenic *Vibrio cholerae* and *Shigella* spp. in water

by

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DECLARATION

I declare that the dissertation, which I hereby submit for the degree Magister Scientiae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at another university.

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SUMMARY

Develpoment of novel seminested polymerase chain reaction assays for detecting toxigenic *Vibrio cholerae* and *Shigella* spp. in water

by

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Rapid and sensitive polymerase chain reaction (PCR)-based assays were developed for the detection of toxigenic *Vibrio cholerae* and virulent *Shigella* spp. in environmental water samples. In contrast to conventional nested and seminested PCR assays, the newly developed pit-stop seminested PCR assays are performed in a total of 30 amplification cycles. Oligonucleotide primers were designed to specifically amplify sequences within the cholera toxin gene (*ctxA*) of *V. cholerae* and the invasion plasmid antigen gene (*ipaH*) of virulent *Shigella* spp. By using agarose gel electrophoresis for detection of the PCR-amplified products, a detection limit of 1.3 x 10^3 cfu of *V.cholerae* and 1.6 x 10^3 cfu *of Shigella flexneri* were obtained from crude cell lysates. However, by coupling the PCR assays with an enrichment culture procedure, the sensitivity of the

assays was improved and as few as 4 cfu and 1.6 cfu of *V. cholerae* and *S. flexneri* organisms, respectively, could be detected in pure culture. Analysis of seeded environmental and drinking water samples yielded detection limits of as few as one *V. cholerae* organism/ml and less than 14 cfu/ml *S. flexneri* in some samples.

The basic PCR detection protocol was modified to incorporate a membrane filtration step in order to concentrate large volumes of water samples. This allowed for the detection off low numbers of viable *V. cholerae* and *Shigella* spp. in naturally contaminated waters. By making use of seeded environmental, and drinking water samples, detection limits of 1 cfu/100 ml of *V. cholerae* and 8 cfu/100 ml of *Shigella* cells were obtained. The detection protocol was successfully applied to the analysis of naturally contaminated environmental water samples. The results obtained in this study indicate that the newly developed pit-stop seminested PCR assays, combined with an enrichment step, fulfills the requirements of simplicity and sensitivity for use in an environmental laboratory and may represent significant tools in monitoring water sources for the presence of *V. cholerae* and *Shigella* spp.

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CHAPTER ONE

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Waterborne microbial diseases, once expected to be eliminated as public health problems, not only remains the leading cause of death worldwide, but the spectrum of disease is expanding and the incidence of many waterborne microbial diseases once thought conquered is increasing (McGinnis and Foege, 1993; Ford, 1999). These infections, which may be transmitted by contaminated recreational waters, surface water and ground water intended for drinking, place entire communities at risk. The most common waterborne microbial disease is a mild to acute gastroenteritis illness. Although for most of the population in developed countries minor gastroenteritis may simply mean several hours of discomfort, in developing countries up to 20 million people die every year as a result of the consumption of contaminated water (Anon, 1996). The symptoms of this condition may be caused by numerous infectious agents including enteric bacteria, viruses and protozoa. In general, the viruses are limited to human hosts, while the bacteria and protozoa (in the form of cysts and oocysts) have a variety of human and nonhuman animal hosts. The agents are transmitted primarily by the faecal-oral route, and as a result, the major source of contamination for water is through contact with human and animal faecal pollution.

Much success has been achieved in controlling the more common forms of waterborne diseases, and infectious diseases like cholera and typhoid fever have been virtually eradicated in the developed world, except for sporadic, imported cases. Progress has been due to the adoption of public health measures as well as the implementation of important water treatment techniques, such as flocculation, filtration, disinfection and sewage treatment. Conventional disinfection of domestic water supplies, accomplished by addition of chlorine, is highly effective against certain Gram-negative and intestinal bacteria, such as the coliform group. The level of chlorination for the conventional contact time is, however, not adequate to control many enteroviruses and is significantly deficient in controlling cyst- and oocyst-forming pathogenic protozoa that are ubiquitous in raw water supplies. Although the practice of flocculation and filtration significantly reduce the numbers of protozoa and enteroviruses, these techniques do not always remove microbial contaminants from drinking water. Thus, failure in the performance of these systems may allow outbreaks of waterborne disease.

Due to the many different infectious agents, reservoirs and asymptomatic infected individuals, complete eradication of waterborne diseases may not be possible. However, it should be possible to control these agents as long as they can be detected and monitored. Coliforms, the traditional indicators of pathogens, are beginning to fail in some cases by giving misleading information and despite advances being made in microbial testing techniques, some pathogens in drinking water remain undetected. The presence of pathogens in water only becomes evident when a number of people become ill with a waterborne illness and a common source of drinking water is identified. Identification of the specific microbial agent that caused the illness may, however, not always be possible. Indeed, no agent is identified as the specific cause in 50% of waterborne disease outbreaks. Hence there is a great need for more appropriate methodologies, both for routine monitoring and for investigating disease outbreaks.

1.2 FACTORS IN THE EMERGENCE AND REEMERGENCE OF WATERBORNE INFECTIOUS DISEASES

In recent years, several "new or emerging" and reemerging pathogens have arisen as problems in drinking water production and distribution. It is therefore important to appreciate the factors that can contribute to the growing problem of waterborne disease, and to understand why these infectious agents are particularly troublesome causes of diseases.

An important factor for the emergence of pathogens is the increasing number of people who are susceptible to infections with specific potential pathogens. These include immunocompromised persons, such as those infected with HIV and patients receiving immunosuppresive therapy for chronic rheumatologic disease, cancer, and solid-organ transplantation, as well as elderly persons whose immune systems are not as active as in healthy young adults (Morris and Potter, 1997; Prier and Solnick, 2000). As a result of diminished immune responses, these persons are subject to infections that do not occur in healthy adults or, if they do occur, are much less severe in healthy adults. What this means, in effect, is that these persons are at increased risk for morbidity and mortality due to diarrheal diseases (Rose *et al.*, 1993). Other groups that may be disproportionately affected by emerging infections include the very young due to their low levels of immunity, persons being cared for in institutional settings, such as the homeless, migrant farm workers, and others of low socioeconomic status.

In many parts of the world, economic conditions are encouraging the mass movement of workers from rural areas to cities. Rural urbanization allows infections arising in isolated rural areas, which may once have remained obscure and localized, to reach larger populations. Once in a city, the newly introduced infection would have the opportunity to spread locally among the population and could also spread further along highways and by airplane (Morse, 1995). Furthermore, urban population growth in many parts of the world has resulted in a decay of some of the basic sanitation practices, such as waste water disposal and insufficient supplies of clean water. The emergence of slum areas and shanty towns and their attendant sanitation problems have also resulted in conditions under which disease-causing agents may grow and thrive. According to a United Nations comprehensive assessment of world water resources, at least 20% of all people worldwide do not have access to safe drinking water, and more than a 50% of all people lack access to adequate sanitation. In South Africa, about 12 million people do not have access to adequate water supply and about 21 million people are without safe sanitation (Tibbets, 2000).

Although classical public health measures have long served to minimize dissemination and human exposure to many pathogens spread by water, the pathogens themselves often still remain, albeit in reduced numbers, in reservoir hosts or in the environment, or in small pockets of infection (Morse, 1995). Thus, they are often able to take advantage of the opportunity to reemerge if there are breakdowns in preventative measures. For example, the rapid spread of cholera in South America may have been abetted by reductions in chlorine levels used to treat water supplies (Glass *et al.*, 1992). Also, the widely publicized U.S. outbreak of waterborne *Cryptosporidium* infection in Milwaukee, Wisconsin, was in part due to a non-functioning water filtration plant (MacKenzie *et al.*, 1994). Limitations in both surveillance and the availability of appropriate diagnostic tests furthermore constrain public health efforts to prevent and control outbreaks.

Microbes are constantly evolving and may include changes in virulence and toxin production. The most prominent example is pathogenic *Escherichia coli* strains that may have taken up virulence genes by horizontal gene transfer, resulting in very potent new pathogens, the enterohemorrhagic *E. coli* (EHEC; Whittam, 1998). Selection for antibiotic-resistant bacteria and drug-resistant protozoa has also become frequent, driven by the wide and sometimes inappropriate use of antimicrobial drugs in a variety of applications (Neu, 1992). Pathogens can acquire new antibiotic resistance genes from other, often nonpathogenic species, in the environment (Davies, 1994). Such

adaptation often results in "new", more deadly strains against which humans have limited resistance.

1.3 ENTERIC PATHOGENS OF CONCERN

Estimates indicate that about 90% of the illness associated with domestic water supplies are related to microbial agents, only about 10% are due to chemical agents (Craun, 1988). The commonly recognized waterborne pathogens consist of several groups of enteric bacteria, viruses and protozoa (Table 1.1). Viruses and protozoa differ from bacterial contaminants in important ways. Because they are environmentally inert, they do not replicate in water and environmental samples. Unlike bacterial pathogens, human enteric viruses and protozoal parasites are environmentally stable (Jaykus *et al.*, 1994), are resistant to many of the traditional methods used to control bacterial pathogens (Jaykus *et al.*, 1994), and have notably low infectious doses (DuPont *et al.*, 1990).

1.3.1 Parasitic protozoa

The most important water-borne human enteric protozoan pathogens are *Giardia lamblia* and *Cryptosporidium parvum* of which the infectious stage is a cyst and oocyst, respectively. In healthy persons, these pathogenic protozoa often cause subclinical infections and self-limiting diarrhea. In infants, immunocompromised persons, or those with underlying illnesses, *C. parvum*, especially, can cause very severe, even fatal diarrhea. Contaminated recreational water (e.g. lakes, rivers or swimming pools) have been frequently associated with waterborne outbreaks of giardiasis (Kramer *et al.*, 1998; Furness *et al.*, 2000) and cryptosporidiosis (Sorvillo *et al.*, 1992; McAnulty *et al.*, 1994; MacKenzie *et al.*, 1995; Furtado *et al.*, 1998). Surveys of raw water supplies indicated that the occurrence of cysts and oocysts are widespread (Rose, 1988; Rose *et al.*, 1991 Furtado *et al.*, 1998) and they are often found in a high percentage of surface waters LeChevallier *et al.*, 1991), especially when these are contaminated by sewage or manure. The occurrence and concentration of the organisms in surface waters are likely to be higher in developing countries in which contamination of water by human and animal waste is more common (Smith *et al.*, 1995). To date, the most prominent recorded public water outbreaks of *Cryptosporidium* have occurred in Carrollton, Georgia, in 1987, involving 13 000 symptomatic cases (Hayes *et al.*, 1989), and in Milwaukee, Wisconsin, in 1993, involving 403 000 symptomatic cases (MacKenzie *et al.*, 1994).

Table 1.1 Examples of microbial pathogens in untreated water and waste waters that may pose a health risk to humans

Microbial organisms	Major disease/s	Infectious dose
Bacteria		
Vibrio cholerae	Cholera	High
Salmonella typhi	Typhoid, Salmonellosis	High
Enteropathogenic *E.coli*	Gastroenteritis	High
Campylobacter jejuni	Gastroenteritis	High
Shigella dysenteriae	Dysentery	Low
Yersinia enterocolytica	Yersiniosis	High
Viruses		
Enteroviruses:Poliovirus,Enterovirus,	Poliomyelitis	Low
Echovirus,Coxsackievirus	Gastroenteritis, menigitis, heart anomalies	Low
Hepatitus A virus	Hepatitis	Low
Adenovirus	Respiratory disease, conjunctivitis	Low
Reovirus	Not clearly established	Low
Norwalk agent	Gastroenteritis, diarrhoea, vomiting, fever	Low
Calicivirus	Not clearly defined	Low
Rotavirus	Gastroenteritis	Low
Astrovirus	Gastroenteritis	Low
Protozoa		
Giardia lamblia	Giardiasis, diarrhoea	Low
Cryptosporidium parvum	Diarrhoea	Low
Entamoeba hystolitica	Amoebic dysentery	Low

1.3.2 Enteric viruses

Human enteric viruses are recognized as important causes of waterborne illness. In addition to causing gastroenteritis, enteric viral infections can also result in meningitis, respiratory disease, and encephalitis. In recent years, it has become clear that many different viruses can be transmitted via drinking water. More than a 100 enteric viruses, all of which are pathogenic to man, have been reported (Payment, 1993). Recently identified pathogens include the enteric adenoviruses, calicivirus, astrovirus and the Norwalk family of agents.

Faecally contaminated water has been frequently identified as a source of viral infections. Cultivable enteric viruses have been detected in surface waters,

groundwaters, and treated drinking waters (Sellwood and Dadswell, 1991; Payment and Franco, 1993). Rotaviruses, some adenoviruses (serotypes 40 and 41), and hepatitis E virus are frequently associated with waterborne disease outbreaks in developing areas in Africa, Asia and Mexico. These outbreaks have been linked to faecally contaminated water and inadequate chlorination (Naik *et al.*, 1992). Norwalk and related small round- structured viruses are the leading cause of epidemic viral gastroenteritis in older children and adults in the United States. Numerous outbreaks linked to drinking water, recreational water, ice and environmental contamination have been documented (Kaplan *et al.*, 1982; Kahn *et al.*, 1994; Brugha *et al.*, 1999; Kukkula *et al.*, 1999; Hafliger *et al.*, 2000).

1.3.3 Enteric bacteria

Many enteric bacteria infect and cause morbidity and mortality in humans via the water route. A considerable number of pathogens from faecal sources (e.g. *Vibrio cholerae*, *Campylobacter*, *E. coli* O157, *Shigella* and *Helicobacter* species) as well as some new pathogens comprising species of environmental bacteria that are able to grow in water distribution systems (e.g. *Yersinia*, *Aeromonas* and *Mycobacterium* species) are being recognized as increasingly important causes of human disease. The risks posed by various bacteria potentially present in drinking water differ among the various genera and species as well as within the same genus and species of a bacterium. Only two of these waterborne pathogenic bacteria, *Vibrio cholerae* and the shigellae, will be discussed in greater detail as they are closely related to the aims of this investigation (Section 1.9).

1.4 *VIBRIO CHOLERAE*

1.4.1 Aetiology

Vibrio cholerae is the type species of the genus *Vibrio* in the family *Vibrionaceae* (Baumann and Schubert, 1984; Farmer and Hickman-Brenner, 1994). *V. cholerae* is a facultatively anaerobic, asporogenous, straight or curved Gram negative rod (0.5 - 0.8 × 1.3 - 2.6 µm). It is highly motile by means of a polar flagellum. Growth occurs at temperatures between 20 and 42°C, and 5 to 15 mM Na^+ is required for optimum growth (Baumann and Schubert, 1984; Falkow and Mekalanos, 1990). *V. cholerae* can grow in alkaline conditions up to a maximum of pH 10, but is inhibited when the pH is below 6.

Currently, 139 O serogroups of *Vibrio cholerae* are recognized. The O serogroups are divided into two main groups, designated the O1 and non-O1 serogroups. Although the results obtained by standard biochemical tests for the O1 and non-O1 serogroups are identical, gene sequencing within the biosynthetic gene cluster of *V. cholerae* (VcRfb) has shown that they differ genetically (Morris, 1994). On the basis of several phenotypic characteristics, isolates of *V. cholerae* serotype O1 can be divided into two biotypes, El Tor and classical. The El Tor and classical biotypes are further differentiated by means of agglutination assays into three serotypes: Inaba, Ogawa and Hikojima (Sakazaki and Balows, 1981; Baumann and Schubert, 1984; Kay *et al.*, 1994). The O139 serogroup can only be identified with specific O139 antiserum. Table 1.2 summarises the *Vibrio cholerae* serogroups, biotypes and serotypes as well as their association with cholera toxin (CT) production and cholera epidemics.

Table 1.2 Characteristics of *Vibrio cholerae*

Characteristic				
Classification	**Serogroups**	**Biotypes**	**Serotypes**	**#CT production**
Epidemic associated	O1, O139	Classical and El Tor (not applicable to O139 strains)	Inaba, Ogawa and Hikojima	Yes*
Not epidemic associated	137 exist	Not applicable to non-O1 strains	The three O1 serotypes are not applicable to non-O1 strains	Usually no, other toxins sometimes produced

*Nontoxigenic O1 strains exist but are not associated with epidemics.

CT=cholera toxin.

1.4.2 Epidemiology

Cholera has been endemic in the Bengal region of India and Bangladesh for centuries. Since the beginning of the 19th century, cholera has been responsible for seven pandemics resulting in death and disease worldwide (Mekalanos *et al.*, 1997). Epidemic and pandemic cholera have until 1992 mostly been associated with only *V. cholerae* serogroup O1. However, from October 1992 to January 1994, more than 700 000 cholera cases were reported from India and Bangladesh, Peru and other Latin American countries with fatalities reaching more than 5 000 in 15 countries (Tauxe *et al.*, 1994; Popovic *et al.*, 1995). This was largely as a result of the seventh pandemic which started in Peru in 1991 and the appearance of a then new cholera serogroup, *V.*

cholerae O139. With the onset of the seventh pandemic, it became apparent that El Tor has become the predominant biotype. It is currently responsible for the majority of cholera cases throughout the world (Alberts, 1994). An increase in cholera cases were reported in The Horn of Africa and this has continued throughout 1998 to spread to the Democratic republic of the Congo, Kenya, Mozambique, Uganda and Tanzania (WHO, 1997). South Africa is currently in the grips of a *Vibrio cholerae* epidemic which started in August 2000. By July 2001, 100 000 cases have been reported resulting in 228 fatalities (National Cholera Status Report, 2001). In contrast to the *Vibrio cholerae* O1 and O139 serogroups, the non-O1 and non-O139 strains of *Vibrio* have not been associated with epidemics, but they can cause sporadic diarrhoea and extraintestinal infections (Saïd *et al.*, 1995).

Outbreaks of cholera has been linked to the consumption of untreated or under-treated drinking water (Glass *et al.*, 1992) as well as feacally contaminated drinking water. Acutely ill cholera patients excrete 10^6 to 10^9 *Vibrio* cells per millilitre of stool which may continue for 5 to 8 days. During epidemics, this shedding of *Vibrio* cells often contaminates water that is used for washing, cooking and drinking. Food plays a lesser but significant role in spreading the disease and a variety of foods, including raw and incompletely cooked seafoods, have been implicated in outbreaks of cholera (Tauxe and Blake, 1992; Feldman, 1992).

1.4.3 Virulence factors of *Vibrio cholerae*

Laboratory studies have shown that 10^4 to 10^6 *V. cholerae* organisms have to be ingested in order to cause disease (Cash *et al.*, 1974). After ingestion, the organisms are directed to the small intestine where it adheres to the intestinal epithelium through filamentous structures such as pili and fimbria. Once the organism has penetrated the mucous layer and have begun to colonise the lining epithelium of the gut, various toxins are produced which stimulate the secretion of salt and water (Greenough, 1985; Kaper *et al.*, 1994). Patients usually experience massive watery diarrhoea, abdominal cramps, fever with nausea and less frequently, vomiting (Morris, 1994).

Of the various extracellular virulence factors produced by *V. cholerae* serogroups O1 and O139, cholera toxin (CT) is the product most responsible for the massive loss of fluid that characterises severe forms of cholera. The cholera toxin is an A-B type ADP-ribosylating toxin and is composed of one A (enzymatic) subunit and five identical B subunits (Spangler, 1992; Salyers and Whitt, 1994; Kaper *et al.*, 1994). Whereas the B subunit is responsible for binding to the epithelial cell surface receptor GM_1, a

ganglioside abundant in the body (King and Heyningen, 1973), the A subunit acts intracellularly through enzymatic functions. The A subunit is transported through the cell membrane and cleaved into two fragments, A_1 and A_2. The A_1 fragment is responsible for activating the enzyme adenylate cyclase of the host's small intestine epithelial cells which then increases the level of cyclic AMP (cAMP), leading to tremendous fluid loss by inducing the secretion of water and salt (Greenough, 1985; Guidolin and Manning, 1987; Olsvik *et al.*,1993; Kaper *et al.*, 1994). The chromosomal genes encoding the A (*ctxA*) and B (*ctxB*) subunits overlap and are expressed as a single transcriptional unit (Mekalanos *et al.*, 1983; Guidolin and Manning, 1987; Shirai *et al.*, 1991).

Another important virulence factor associated only with virulent strains of *V. cholerae* O1 and O139 is the toxin-coregulated pilus (TCP). TCP has rarely been reported among environmental and non-O1 strains of *V. cholerae* (Nair *et al.*,1988). Not only does TCP appear to play a role in the colonisation of the small intestine mucosa of humans and infant mice (Saïd *et al.*, 1995), but it is also known to act as a receptor for CTXΦ, a filamentous bacteriophage. It has been reported that CTXΦ can infect non-toxigenic *Vibrio cholerae* and via horizontal gene transfer lead to the emergence of new toxigenic strains (Waldor and Mekalanos, 1996). Karaolis *et al.* (1999) reported that this phage encodes the cholera toxin as well as a large pathogenicity island called *V. cholerae* pathogenicity island (VPI). The VPI has subsequently been identified on the genome of another filamentous bacteriophage, VPIΦ, which has also been shown to be transferred between *V. cholerae* strains (Karaolis *et al.*, 1999).

The pathogenicity of the non-O1 strains have often been questioned, but there is now compelling evidence that non-O1 strains have toxins that can induce a variety of diseases such as sporadic diarrhoea, wound infections, septicemia and ear infections (Morris, 1994; Dalsgaard *et al.*, 1996). The genetic diversity seen among the non-O1 strains suggests that these strains may cause disease each through a different pathogenic mechanism (Morris, 1994).

1.5 SHIGELLA SPECIES

1.5.1 Aetiology

The genus *Shigella* belongs to the family *Enterobacteriaceae*. Microscopically as well as in stained preparations, shigellae are Gram-negative bacilli (0.3 - 1.5 µm in diameter) indistinguishable from other enterobacteria (Lewis, 1997). Shigellae are non-motile,

non-capsulate, facultative anaerobic and grow optimally at 37°C. The genus *Shigella* is one of the biochemically least reactive members of the *Enterobacteriaceae*. Members of the genus do not produce hydrogen sulphide in triple sugar iron agar; they do not produce urease and they do not produce citrate in Simons' medium or in Christensen's medium. They do not decarboxylate lysine or deaminate phenylalanine. Except for one species, they are all catalase-positive. They ferment only a few carbohydrates mostly without gas production and are sensitive to acidic conditions (Rowe and Gross,1984).

The genus *Shigella* is subdivided into four species according to their biochemical reactions. They are *Shigella dysenteriae* (subgroup A), *Shigella flexneri* (subgroup B), *Shigella boydii* (subgroup C), and *Shigella sonnei* (subgroup D) (Rowe and Gross, 1984). Each species is further subdivided into serotypes based on the O antigens in the outer polysaccharide chains of the lipopolysaccharide (LPS) component of the cell wall (Ewing and Lindberg, 1984; Nikaido and Vaara, 1984; Watanabe and Timmis, 1984; Simmons and Romanowska, 1987). There is, however, extensive sharing of antigenic components among the *Enterobacteriaceae* and some *Shigella* O antigens are identical to *E.coli* O antigens, and many are closely related (Cheasty and Rowe, 1983). It is therefore essential that both serological and biochemical tests be interpreted together in order to accurately identify *Shigella* spp.

1.5.2 Epidemiology

Shigellosis is endemic in tropical areas of the world. It has been estimated that approximately 600 000 people die every year while a further five million cases require hospitalization (Lewis, 1997). Most of these infections are due to *S. dysenteriae* and *S. flexneri* (Bennish, 1991). In contrast, in industrialized countries, infections are primarily due to *S. sonnei* and less frequently *S. dysenteriae. S. sonnei* has become the dominant species in the United Kingdom and USA (Lewis, 1997), accounting for 60 to 80% of cases reported in the USA (Rowe and Gross, 1984). Although shigellosis occurs worldwide, it is most common in areas where sewage treatment and personal hygiene are inadequate. Epidemics, each year increasing in severity in Burundi, Africa, has been reported since 1992 (Engels *et al.*, 1995). KwaZulu-Natal in South Africa, suffered an epidemic during 1995 caused by *S. dysenteriae*. Between the months of February and December during 1995, 158 cases were admitted for treatment of which 13 persons died (Chopra *et al.*, 1997; Pergram *et al.*, 1998).

Waterborne outbreaks of shigellosis are most commonly associated with faecal contamination of non-chlorinated private and community water supplies (Samonis *et al.*, 1994; Pergram *et al.*, 1998). Under-treated water and cross-contamination between wastewater and potable water are the most frequent sources of outbreaks linked to drinking water supplies. Outbreaks have also been associated with recreational waters (Rosenberg *et al.*, 1976; Blostein, 1991; Fleming *et al.*, 2000) as well as to the consumption of raw and improperly cooked fish and shellfish harvested from contaminated waters (Taylor *et al.*, 1991; Maguire *et al.*, 1998; Vantarakis *et al.*, 2000).

1.5.3 Virulence factors of *Shigella*

Infected humans are the only significant reservoir for *Shigella*. The infective dose of *Shigella* cells is low; it has been estimated that ingestion of approximately 10^1 to 10^4 cells can cause human disease (Rowe and Gross, 1984; Dupont, 1990). Once ingested, the organism is directed to the colonic mucosa. The invasive process involves the ability of the organisms to induce endocytosis by epithelial cells with subsequent lysis of the endocytic vacuole. Hereafter, the organisms multiply in the cytoplasm leading to cell destruction, infection of adjacent cells and spread to the connective tissue of the intestinal villus (Sansonetti, 1999). Ingestion of *Shigella* spp. typically produces acute gastroenteritis and dysentry. Ilness can range from mild self-limiting diarrhea to severe toxicity and kidney failure. A further complication of *Shigella* infections is an arthritis which appears after the intestinal infection has passed. The condition, known as Reiter's syndrome, is thought to be the result of an auto-immune response triggered by bacterial antigens that affects joint tissue, leading to inflammation of the joints (Salyers and Whitt, 1994).

All virulent strains of *Shigella* harbour a large 120 to 230 kb plasmid that mediates its virulence properties. This so-called invasion plasmid has been shown to encode genes for ligands that are involved in the adherence of bacteria onto the surface of target epithelial cells. The production of invasion plasmid antigens (*Ipa*; Venkatesan *et al.*, 1989; Dorman and Porter,1998) and about 20 genes (e.g. *mxi* and *spa*) with transport or processing functions that ensure the correct surface expression of the Ipa proteins have a direct role in the *Shigella* invasion process. Furthermore, the invasion plasmid encodes genes for the induction of endocytic uptake of bacteria and disruption of endocytic vacuoles as well as the intra- and intercellular spreading of the organism and genes for the regulation of plasmid-encoded virulence (*vir)* genes (Sansonetti *et al.*, 1982). *S. dysenteriae* also produces an exotoxin known as Shiga toxin. Similar to the toxigenic *V. cholerae* toxin (Section 1.4.3), Shiga toxin consists of one A (enzymatic)

subunit and five B (binding) subunits (Salyers and Whitt, 1994). The genes encoding the respective subunits (*stx*A and *stx*B) are located on the chromosome and comprise a single operon (Sansonetti *et al.*, 1982; Salyers and Whitt, 1994). Shiga toxin is responsible for a serious complication of shigellosis, namely hemolytic uremic syndrome (HUS), a form of acute kidney failure sometimes seen in children after an attack of dysentery. In addition to the production of Shiga toxin, the LPS component of the cell wall has been shown by Lindberg *et al.* (1991) to contribute to the virulence of *Shigella* spp. The lipid A component has endotoxic properties and contributes to the systemic effects of infection, whilst the O antigen polysaccharide provides the bacteria with resistance against opsonization, phagocytosis and intracellular killing (Shears, 1996).

1.6 DETECTION OF ENTERIC MICROBIAL PATHOGENS IN WATER

1.6.1 Use of indicator organisms to detect faecal contamination of water

Problems associated with recovery of pathogens from water have led to the development of methods to detect and enumerate indicators of faecal contamination. These indicator organisms are generally used to establish the potential presence of faecal contamination in raw and drinking water (Grabow, 1996). Not necessarily pathogenic themselves, faecal coliforms, total coliforms, *Escherichia coli*, enterococci, and bacteriophages are all examples of organisms that when present are viewed as predictive of the potential presence of enteric pathogens, since they have the same faecal source as the pathogenic organisms. Tests for coliform bacteria are standardized and relatively easy and inexpensive to perform (APHA, 1998). They are therefore more rapidly administered than tests determining the presence of individual pathogenic microorganisms in water. Despite being successful in predicting possible health risks in many circumstances, total and faecal coliforms have many limitations as predictors of risk of waterborne disease. Because of their shorter survival times in water and their greater susceptibility to water treatment processes, these indicator organisms tend to be poor models for enteric protozoa and viruses (Hellard *et al.*, 1997). Moreover, there are non-faecal sources for these indicator organisms, and in contrast to most enteric pathogens, certain members of the total and faecal coliform group may multiply in aquatic environments with sufficient nutrients and optimal temperatures. Such characteristics may result in false-positive reports of water contamination (Carter *et al.*, 1987; Hegarty *et al.*, 1999). Thus, there is a need to examine newer approaches to monitoring the microbiological quality of water that will lead to a reduction of waterborne disease transmission.

1.6.2 Alternative detection methods

A number of problems are encountered in determining the presence of microbial pathogens in water. For each group of microbes, whether protozoa, viruses or bacteria, the method must cope with a different set of conditions or characteristics that can complicate the task of identifying particular microbes. Various methods have therefore been developed over the last two decades as alternative detection methods for specific microbes. Since the concentrations of enteric organisms are low in water, their detection in water typically starts with some type of concentration process such as filtration. Bacteria can be recovered and concentrated from water by a variety of filtration methods. The most widely used filtration method for recovering bacteria is membrane filtration using microporous membranes typically composed of cellulose esters (APHA, 1998). Following filtration, the cells recovered on the membrane filter can be characterized using conventional methods or they can be directly assayed using nucleic acid-based methods.

1.7 CONVENTIONAL DETECTION TECHNIQUES

1.7.1 Cultivation-based assays

Various approaches for culturing the target bacteria following their concentration by filtration are generally followed. The target bacteria can either be cultured by pre-enrichment and enrichment methods using broth media or the filter can be placed on differential and selective media to allow the development of discrete colonies of the target pathogens. Alternatively, the bacteria can be washed off the filter surface and reconcentrated in a small volume of suspension medium, which is plated on agar media. Quantitative results are preferably obtained by colony counts on the surface of agar media, with or without the presence of the filter used for concentration (APHA, 1998).

Often, organisms may be present in water samples but are unculturable. The bacteria are still viable (exhibit low levels of metabolic activity), but they fail to develop colonies on most traditional solid culture medium. Such a viable but nonculturable (VBNC) state has been described for many pathogenic bacteria including *Helicobacter pylori*, *Campylobacter*, *E. coli*, *Vibrio cholerae*, *Vibrio* spp., *Shigella* spp., and *Legionella pneumophila* (Rozak and Colwell, 1987; Byrd *et al.*, 1991; McKay, 1992; Islam *et al.*, 1993a; Bode *et al.*, 1993; McDougald *et al.*, 1998; Cappelier and Frederighi, 1998). Also, nutrient limitations and environmental stressors, such as disinfectants used during water treatment, can produce unpredictable physiological and morphological changes

in many waterborne bacterial pathogens. This makes their isolation and identification problematic and specialized handling procedures are required for their resuscitation (Ridgway, 1984). By making use of nonselective or less selective media, and other, less stressful culture conditions, the number of culturable cells in a population of VBNC, injured, or stressed bacteria may be increased. To confirm their identity, the cultured bacteria or bacterial colonies can be characterized by making use of a variety of methods, such as subculturing on other differential and selective media, biochemical, metabolic and other phenotype analyses, immunological analyses, and nucleic acid-based analyses.

While conventional culture methods have been found rapid and specific for medical diagnostic applications (when target organisms are usually present in large numbers), the specificity and sensitivity of these methods are not always suitable for the detection of low numbers of bacteria in water. Sensitive, specific and efficient detection techniques and quantification of low levels of bacterial pathogens in water are needed. For some of the recognized enteric bacterial pathogens such as various species of *Salmonella*, *Shigella* and *Vibrio* genera, culture methods for their detection in clinical, food, and water samples have changed little beyond attempts to improve recoveries and provide more distinctive recognition using modified pre-enrichment and enrichment broths and differential and selective agars (Shimada *et al.*, 1990; Stone *et al.*, 1994; Høi *et al.*, 1998). In addition, some waterborne pathogenic bacteria such as *H. pylori* and *Legionella* species are difficult to reliably culture using currently available media and methods because their growth is inefficient, growth rates are slow, and they are often overgrown by other nontarget bacteria (Goodwin *et al.*, 1985; Maiwald *et al.*, 1994).

1.7.2 Immunological assays

Immunofluorescent detection by microscopy or other methods is a specific and potentially powerful way to detect pathogens and other microbes in water. Consequently, many different immunoassay methods have become available for both quantitative and qualitative analysis of pathogenic bacteria in water (Botger *et al.*, 1987; Faude and Hofle, 1997; Buswell *et al.*, 1998). Typically these assays are performed by conjugating monoclonal or polyclonal antibodies, directed against antigens of the target pathogen, with a fluorochrome or fluorescent dye for use in a direct immunofluorescence assay. Alternatively, secondary enzymatically- or fluorescently-labeled antibodies directed against the primary antibodies (now serving as antigens) of the species of animal in which the antibodies against the microbe were raised can be used in an indirect immunofluorescence assay. The antigen-antibody complex is

detected and quantified by the ability of the enzyme to react with a substrate that produces either a coloured product for colorimetry or emits light for luminometry. Enzyme immunoassays are often performed on a solid phase to which the pathogen antigens have been applied, such as a membrane filter or the bottom of a microtitre plate well (De Silva *et al.*, 1992; Hasan *et al.*, 1994; Quadri *et al.*, 1995; Noah *et al.*, 1996).

Studies have, however, repeatedly shown that solid phase enzyme immunoassays generally are too insensitive for direct detection of microbial pathogens in water, as they require a minimum of 10^3 to 10^4 target microbes (or their antigens) for detection. In most situations drinking water and its sources rarely contain high enough levels of most target pathogens for direct immunoenzymatic detection. Furthermore, the antibodies may exhibit cross-reactions which compromise the specificity of the test or they may fail if the original protein target, such as a cell surface protein, undergoes changes under environmental conditions (Vickers *et al.*, 1990), and no information regarding the viability of the organisms are obtained (Kfir and Genthe, 1993). However, by combining microscopic examination with chemical treatments for enzymatic activity, the viability of the concentrated and purified bacterial pathogens may be assayed. For example, an approach combining fluorescent antibody and tetrazolium dye reduction, which measures dehydrogenase activity, has been used to successfully enumerate viable *E. coli* O157:H7 in water (Pyle *et al.*, 1995).

As an alternative to the above immunological assays, agglutination methods can be used to detect pathogens by combining dispersed cells with antibodies (on a slide, for example) and allowing for antigen-antibody reactions to produce agglutination (clumping) that can be scored negative or various degrees of positive (strong, medium or weak) (Colwell *et al.*, 1992; Quadri *et al.*, 1994). As with enzyme immunoassays, agglutination tests are too insensitive to directly detect and quantify most waterborne pathogens in drinking water and other aquatic samples (Ito *et al.*, 1983). The target microbes must first be propagated in order to obtain a sufficient number of them or a sufficient amount of antigen to detect and antigenically characterise them by agglutination methods.

1.8 NUCLEIC ACID-BASED DETECTION METHODS

The discovery of a large number of bacterial toxins and other virulence factors has led

to powerful methods, such as gene probes and PCR, for detecting and identifying pathogenic bacteria in water, as well as bacterial pathogens in a viable but non-culturable state which may not be detected by culture-dependent techniques. The latter is an important additional advantage as bacteria that have entered the VBNC state have the ability to reverse their dormancy to become infectious the moment they enter a favourable environment.

1.8.1 Nucleic acid hybridization techniques

Hybridization techniques rely on the specific binding of nucleic acid probes to complementary DNA or RNA (target nucleic acid). Whole-cell DNA or RNA is extracted from the environmental sample and fixed to a nylon or nitrocellulose membrane or alternatively, bacterial colonies can be replica-plated from agar plates to membranes and their nucleic acids exposed *in situ* following lysis for subsequent hybridization. Single strands of nucleic acids, labelled with enzymes, antigenic substrates, chemiluminescent moieties or radioisotopes, act as probes that are used to detect genes in the bacterial genome (Southern blots) or to detect mRNA or rRNA (Northern blots) (Tenover and Unger, 1993). Two general types of gene probes that have been developed are DNA probes complementary to a single gene or a small region of a gene (Hazen and Jimenez, 1988; Venkatesan *et al.*, 1988; Knight *et al.*, 1991; Anderson *et al.*, 1995) and DNA probes complementary to genus- or species-specific regions of 16S rRNA (Schleifer *et al.*, 1985; Romanuik *et al.*, 1987; Rehnstam *et al.*, 1989) for use in whole cell *in situ* hybridization (FISH). *In situ* hybridization follows the same principles as liquid or filter hybridization, but hybridization occurs within the morphologic context of specific cells (Amman *et al.*, 1995). This hybridization technique allows for great sensitivity and it is possible to detect a single bacterial cell.

The application of direct nucleic acid hybridization using DNA or RNA probes to detect and quantify environmental pathogens is generally regarded as inadequate owing to low sensitivity and large sample volumes that are impractical for most hybridization protocols without further pathogen concentration. Due to sample-related interferences, hybridization failures and nonspecific reactions may occur when samples are analysed directly (Knight *et al.*, 1991).

1.8.2 Polymerase chain reaction and related techniques

PCR amplification-based methods, being more rapid and very sensitive, have become the most used molecular approach for detection of infectious bacterial agents in environmental samples (Steffen and Atlas, 1991; Alvarez *et al.*, 1993, Garrett *et al.*,

1993). A thermostable DNA polymerase, often the *Taq* enzyme from *Thermus aquaticus*, is used to amplify the specific DNA sequence of interest. The target sequence is defined by two sequence-specific oligonucleotide primers that flank the target sequence and which anneal to the complementary strands of the target sequence. During the PCR process, repetitive cycles of DNA denaturation, annealing of the oligonucleotide primers to the target DNA and extension of the primers across the target sequence results in increasingly greater quantities of target sequence (Strachan and Read, 1996). There exist many variations of the basic PCR technique. The sensitivity and specificity of the PCR may be improved by adopting a nested PCR approach (Falklind *et al.*, 1996; Mayer and Palmer, 1996). The amplification is carried out with an initial first pair of primers after which a second round of amplification is conducted with a different primer pair that anneal to an internal area of the amplicon (nested primers). Another variation of the PCR technique, namely multiplex PCR, allows for the simultaneous detection of more than one target organism in a single PCR reaction using multiple primer pairs (Arnheim and Erlich, 1992). RT-PCR has emerged as a sensitive and specific approach for the detection of enteric viruses containing RNA genomes (Sobsey, 1994; Schwab *et al.*, 1996; Huang *et al.*, 2000). RT-PCR is a technique in which reverse transcription (RT) is coupled with PCR amplification of the resulting cDNA using virus-specific primers.

Although the PCR method is both specific and sensitive, such standard PCR reactions are not quantitative (Toze, 1999). To obtain quantitative data from PCR-based analyses, statistical methods based on most probable number (MPN) estimations have been used (Degrange and Bardin, 1995; Wand *et al.*, 1997). In MPN-PCR, DNA extracts are diluted before PCR amplification and limits are set on the number of genes in the sample by reference to known control dilutions. Another way to quantify PCR-amplified products for comparison is to include an internal control in the PCR reaction (Diviacco *et al.*, 1992; Leser *et al.*, 1995). Here, a known amount of target DNA is added to a PCR reaction containing DNA from the mixed microbial population. The known target DNA is complementary to the same primers and thus competes with the target sequences in the extracted DNA sample. By preparing a dilution series of the known and unknown DNA species, it is possible to quantify the amount of product produced from the complementary gene in the extracted DNA.

Other major limitations to using PCR for the detection of pathogenic microbes from environmental samples are the presence of substances inhibitory to PCR (Wilson, 1997) and the possibility of amplification of nonviable cells (Josephson *et al.*, 1993). Inhibition

phenomena have made bacterial DNA purification an important preliminary step for the PCR reacion. Subsequently, numerous techniques for seperating and purifying the DNA have been described, resulting in varying purification levels of the DNA (Tsai and Olson, 1992, Abbaszadegan *et al.*, 1993). In recent years, the use of immunomagnetic beads attached to specific antibodies have become a popular approach in facilitating the capture, concentration and purification of target bacteria prior to DNA extraction (Olsvik *et al.*, 1994).

Several assays based on the direct detection of indicator and pathogenic bacterial cells in environmental water samples by filtration and PCR have been developed (Bej *et al.*, 1991 a; 1991b; Oyofo and Rollins, 1993; Way *et al.,* 1993; Jackson *et al.*, 1996; Covert *et al.*, 1999). The use of combinations of methods such as immunomagnetic capturing of strains, PCR and detection of immobilized amplified nucleic acids by hybridization have been proven to be useful for the detection of waterborne pathogenic bacteria which are difficult to culture from environmental sources, e.g. *Y. enterocolitica* (Kapperud *et al.*, 1993; Sandery *et al.*, 1996) and *H. pylori* (Enroth and Engstrand, 1995; Sasaki *et al.*, 1999). A disadvantage of the above approaches is that they may detect dead as well as viable bacteria. An indirect approach is thus usually adopted for assaying the viability of bacteria from water samples by first culturing bacteria prior to PCR detection (Dorsch *et al.*, 1994; Waage *et al.*, 1999). Not only does an enrichment procedure dilute any sample-related inhibitors present, but dead bacteria are diluted as well, thus reducing the probability of detecting them by the subsequent PCR assay. It thus becomes possible to relate detection to pathogen infectivity. In addition, allowing pathogens to multiply amplifies target nucleic acids thereby facilitating their detection.

1.9 AIMS OF THIS STUDY

Various demographic and other changes combined with complacency about the role of infectious diseases in general have brought about a resurgence in waterborne infectious diseases, and with it, challenges that are unprecedented in recent times. A World Health Organization report states that about 80% of all diseases and over one-third of deaths in developing countries are the result of people consuming contaminated water. Not only are the diseases debilitating, but they also consume valuable time, with about one-tenth of each person's productive time sacrificed to water-related diseases (WHO, 1997). While waterborne diseases may be considered to be problems of underdeveloped countries with inadequate sanitary practices, there is increasing

recognition that industrialized, developed countries also have significant public health problems caused by the use of untreated or inadequately treated domestic water supplies.

The potential public health threat posed by waterborne microbial pathogens have thus attracted renewed attention, both within the scientific community and among the public. Once thought to be under control, they are now referred to as "emerging or reemerging" pathogens. Not only are many of these responsible for recent waterborne epidemics, but more familiar waterborne pathogens are reemerging to cause significant disease. Amongst these are toxigenic *Vibrio cholerae* and *Shigella* spp. which have been responsible for two recent epidemics in South Africa resulting in 288 and 13 fatalities, respectively. The detection of these microbial contaminants in drinking water supplies should, therefore, be viewed as a high priority.

Therefore, the aims of this investigation were the following:

(a) To develop rapid, sensitive and specific PCR-based detection methods for toxigenic *Vibrio cholerae* and *Shigella* spp. in various types of water samples.

(b) To adapt and apply the newly developed methods for detection of toxigenic *Vibrio cholerae* and *Shigella* spp. in naturally contaminated environmental water samples.

CHAPTER TWO*

DEVELOPMENT OF AN ENRICHMENT BROTH CULTIVATION-PIT-STOP SEMINESTED PCR PROCEDURE FOR THE DETECTION OF TOXIGENIC *VIBRIO CHOLERAE* IN VARIOUS TYPES OF WATERS

2.1 INTRODUCTION

Cholera is a highly epidemic diarrhoeal disease which continues to devastate many developing countries where socio-economic conditions are poor, sanitary systems and public hygiene are rudimentary, and access to safe drinking water is limited. In 1992 to 1994, 700 000 cholera cases were reported by 21 countries in the Western Hemisphere, mostly in coastal areas, with more than 5 000 cases resulting in death (Tauxe *et al.*, 1994; Popovic *et al.*, 1995). In July 1994, 14 000 deaths from cholera were reported in refugee camps in Rwanda (Siddique *et al.,* 1995) while in April 1997, a total of 1 521 deaths were recorded during a cholera outbreak among 90 000 Rwandan refugees residing in temporary camps in the Democratic Republic of Congo (Nabeth *et al.,* 1997). During a recent outbreak in South Africa, a 100 000 cholera cases were reported and the outbreak resulted in 228 fatalities (National Cholera Status Report, 2001). Cholera is usually transmitted to humans by ingestion of contaminated water and foods. It has been determined on the basis of human volunteer trials that, depending on the health of a given individual, the ingestion of approximately 10^4 to 10^6 *V. cholerae* O1 organisms is likely to produce clinical cholera (Cash *et al.,* 1974).

The major virulence factor produced by *V. cholerae* is the cholera enterotoxin (CT). Cholera toxin belongs to enterotoxins that consist of two subunits (Spangler, 1992). Subunit A is responsible for adenylate cyclase activation, inducing tremendous loss of fluids during illness. The B subunit is involved in binding the toxin to the epithelial cell surface receptors in the small intestine (Guidolin and Manning, 1987). The chromosomal

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genes encoding the A and B subunits are designated *ctxA* and *ctxB*, and are expressed as a single transcriptional unit (Mekalanos *et al.*, 1983; Guidolin and Manning, 1987). Toxin production, however, does not correlate with serotype, as some strains of *V. cholerae* O1 may not produce CT (Kaper *et al.*, 1981; Kay *et al.*, 1994).

Conventional microbiological methods for identifying *V. cholerae* involve cultural, biochemical and immunological assays which often take several days to complete (Farmer and Hickman-Brenner, 1992). Nucleic acid-based methods, such as the polymerase chain reaction (PCR), have the potential to improve these detection times. It has been shown that PCR can detect microbial species by amplification of gene sequences unique to them, and the sensitivity of PCR is such that theoretically only a single intact nucleic acid template is needed to amplify the target sequence sufficiently for visualization by electrophoresis (Saiki *et al.*, 1988). PCR assays based on amplification of target DNA sequences in the *ctx* gene of *V. cholerae* have been reported (Kobayashi *et al.*, 1990; Shirai *et al.*, 1991; Fields *et al.*, 1992; Varela *et al.*, 1993). These investigators used PCR to identify toxigenic *V. cholerae* in stools of patients with cholera or as an alternative to other *V. cholerae* diagnostic methods, such as enzyme-linked immunosorbent assay or DNA colony hybridization. PCR assays to determine the presence of enterotoxigenic *V. cholerae* in foods have been described by Koch *et al.* (1993) and Falklind *et al.* (1996), while Keasler and Hall (1993) and Shangkuan *et al.* (1995) used multiplex PCR to detect and biotype *V. cholerae* O1.

The aims of this part of the research were (i) to develop a rapid and specific PCR-based detection protocol for low levels of toxigenic *V. cholerae* in water, and (ii) to determine the specificity and sensitivity of the newly developed PCR procedure using small volumes of seeded environmental water samples collected from natural sources.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains

Vibrio cholerae, strain NCTC 5941, obtained from the National Collection of Type

Cultures, UK, was used as the reference strain. This strain was reconfirmed by cultural, morphological and biochemical tests according to standard procedures (Farmer and Hickman-Brenner, 1992). Various other bacterial strains used to test the specificity of the PCR are listed in Table 2.1. The strains were cultivated and maintained in nutrient broth (Difco) at 37˚C, unless the Culture Collection instructions specified otherwise.

2.2.2 Preparation of DNA for PCR

For specificity determination, DNA was extracted from bacterial strains (Table 2.1) by a rapid boiled-lysate technique. Briefly, bacterial colonies from an overnight cultivation on nutrient agar plates were suspended in 1 ml of sterile distilled water to a concentration of approximately 10^6 organisms per ml. The bacteria were lysed by heating for 10 min at 100˚C. After centrifugation at 10 000 × *g* for 5 min to remove the cellular debris, the supernatant containing the bacterial lysate was used in the PCR immediately or following storage at -20˚C. For sensitivity determinations, serial 10-fold dilutions of the culture suspension were prepared and the cell suspensions were lysed by boiling at 100˚C for 10 min as described above. Viable plate counts to determine the cfu/ml were performed in triplicate by plating each of the 10-fold dilutions onto nutrient agar plates and incubating for 18 h at 37˚C before counting colonies.

2.2.3 Enrichment of *Vibrio cholerae* in samples

Serially diluted bacterial cells were recovered from each dilution tube by centrifugation at 10 000 × *g* for 5 min. The bacterial cells were then resuspended in 1 ml CDC medium (1% (w/v) peptone, 0.5% (w/v) NaCl, pH 8.4) (Farmer and Hickman-Brenner, 1992) and the tubes were incubated at 37°C in a shaking incubator. At time 0, 2, 4 and 6 h after seeding, the numbers of cells per milliter were assessed by viable plate counts and template DNA was prepared from each tube. To obtain template DNA for the PCR assays, the bacterial cells were collected by centrifugation at 10 000 × *g* for 5 min, washed twice in distilled water and then resuspended in 1 ml sterile distilled water before heating in a water bath at 100˚C for 10 min. After centrifugation in a microcentrifuge for 5 min, 10 µl of the supernatant fluid was used in the pit-stop seminested PCR method as described below.

Table 2.1 Bacterial strains examined

Microorganism	Source or strain*	No. of strains
Aeromonas hydrophila	RW	1
Bacillus cereus	CSIR	1
Citrobacter freundii	CSIR	1
Enterobacter aerogenes	ATCC 49469	1
Escherichia coli	CSIR	3
Escherichia coli	ATCC 13086	1
Escherichia coli	ATCC 14824	1
Escherichia coli	ATCC 25922	1
Klebsiella pneumoniae	ATCC 49472	1
Legionella pneumophila	ATCC 33153	1
Proteus mirabilis	ATCC 12453	1
Pseudomonas aeruginosa	ATCC 27853	1
Pseudomonas diminuta	CSIR	1
Salmonella typhi	ATCC 49469	1
Salmonella typhimurium	SAIMR	1
Shigella boydii	SAIMR	1
Shigella dysenteriae	NCTC 1311	1
Shigella flexneri	CCRC 10772	1
Shigella sonnei	ATCC 8574	1
Vibrio cholerae	NCTC 5941	1
Vibrio cholerae	ATCC 25870	1
Vibrio metschnikovii	CSIR	1
Vibrio parahaemolyticus	SAIMR	1
Vibrio cholerae (non-O1)	RW	16

*NCTC = National Collection of Type Cultures, Public Health Laboratory Service, London, UK
SAIMR = South African Institute for Medical Research, Johannesburg, South Africa
ATCC = American Type Culture Collection, Rockville, MD, USA
CCRC = Culture Collection and Research Center, Hsinchu, Taiwan
RW = Rand Water, Johannesburg, South Africa
CSIR = Council for Scientific and Industrial Research, Pretoria, South Africa

2.2.4 Preparation of seeded environmental water samples for PCR

Treated sewage, surface, ground as well as drinking water samples were collected from various localities in and near Pretoria, South Africa. The water samples were pretested for the presence of amplifiable *Vibrio* spp. DNA by PCR and found to be negative. A suspension of *V. cholerae* cells was prepared in the respective sterile environmental water samples (autoclaving at 121°C for 15 min at a pressure of 15 psi) and then used to seed two dilution series' consisting of sterile and nonsterile treated sewage, lake, river, ground and tap water as diluent. The bacterial cells from 1 ml of each dilution were recovered by centrifugation at 10 000 × *g* for 5 min and then resuspended in 1 ml of CDC enrichment medium. Following incubation at 37°C for 6 h, template DNA for pit-stop seminested PCR analysis was prepared as described above for enrichment samples. Positive as well as negative, uninoculated controls were included in each experiment.

2.2.5 DNA amplification

The PCR was carried out by performing different experiments. Boiled cultures of bacterial species were amplified to test the specificity of the seminested PCR primer, while the sensitivity of the pit-stop seminested PCR assay was determined and compared to a single step PCR by amplifying a serially diluted culture of *V. cholerae*. Also, the sensitivity for detecting *V. cholerae* cells, in small volumes of seeded environmental water samples, was determined.

2.2.5.1 Selection and synthesis of primers

Oligonucleotide primers CTX2, CTX3 and CTX15 from *V. cholerae ctxA* sequences were used in a pit-stop seminested PCR assay. Primers CTX2 (5' - CGGGCAGATTCTAGACCTCCTG - 3') and CTX3 (5' - CGATGATCTTGGAGCATTCCCAC - 3') have been described previously by Fields *et al.* (1992). An internal primer, CTX15 (5' - GAGTATGGAATCCCACCTAAAGC - 3'), was designed on the basis of the published sequence of the *ctxAB* operon from *V. cholerae* 2125 (Mekalanos *et al.*, 1983). The first PCR step, performed with primers CTX2 and CTX3, amplified a 564 bp region of the *V. cholerae ctxA* gene, while the size of the final PCR product obtained with primers CTX2 and CTX15 was 347 bp. The

primers were synthesized by MWG Biotech (Germany) with automatic DNA synthesizers.

2.2.5.2 Pit-stop seminested PCR

The reaction mixtures used for both PCR steps contained 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5% (vol/vol) Triton X-100), $MgCl_2$ at 1.5 mM, each deoxynucleoside triphosphate at a concentration of 0.1 mM, and 1 U of Taq DNA polymerase (all these were purchased from Promega) per 50 µl of reaction mixture. For the first PCR step, 22 pmol of primer CTX2, 32 pmol of primer CTX3, and a sample volume of 10 µl was used. The reaction tubes were placed in a GeneAmp 2400 thermal cycler (Perkin-Elmer). The following conditions were used: heat denaturation at 94°C for 3 min, followed by 10 cycles consisting of heat denaturation at 94°C for 1 min, primer annealing at 61°C for 1 min, and DNA extension at 72°C for 1 min. The second PCR step was performed by using a total volume of 50 µl. A 1 µl aliquot of the first PCR product was used as the template. Primer concentrations of 22 pmol of primer CTX2 and 32 pmol of primer CTX15 were used. The cycle profile consisted of the same heat denaturation, primer annealing, and DNA extension conditions as those used for the first PCR step, but the number of cycles was 20. After the last cycle, the samples were kept at 72°C for 7 min to complete synthesis of all strands. For control purposes, reaction mixtures containing distilled water and all other reagents but no template were included.

2.2.5.3 Single-step PCR reactions

PCR reactions were performed by using 10 µl of bacterial lysate as template DNA, buffer, deoxynucleoside triphosphate mixture, primers CTX2 and CTX3 or primers CTX2 and CTX15, at the above-mentioned primer concentrations, and Taq DNA polymerase in a final reaction volume of 50 µl. The reactions were then subjected to 30 cycles of amplification under the cycle conditions as described above.

2.2.6 Electrophoretic detection of amplicons

The amplification products were analysed by agarose gel electrophoresis and sized according to their migration in the gel as compared to that of a standard molecular weight marker (100 bp DNA ladder; Gibco BRL, Life Technologies). For this purpose,

horizontal 2% (w/v) agarose slab gels were cast and electrophoresed at 100 V in 1 × TAE buffer (40 mM Tris-HCl, 20 mM Na.acetate, 1 mM EDTA; pH 8.5) using a BioRad Mini Sub™ electrophoresis unit. The agarose gels were stained with ethidium bromide (0.5 µg/ml) and the DNA fragments visualised by UV fluorescence.

2.2.7 Nucleic acid sequencing of amplicons

2.2.7.1 DNA purification

The amplicons were concentrated and purified by ethanol precipitation. Briefly, the DNA was precipitated by the addition of 2 volumes 96% ethanol and Na. acetate (pH 7.0), to a final concentration of 0.3 M. Following incubation for 30 min at -70˚C, the DNA was recovered by centrifugation at 10 000 × *g* for 15 min, washed twice with 70% ethanol and dried under vacuum before being suspended in 20 µl UHQ water.

2.2.7.2 Nucleotide sequencing and sequence analysis

Sequencing of the amplified DNA was performed using an ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Perkin-Elmer). Each reaction mixture contained 100 ng of template DNA, 3.2 pmol sequencing primer (CTX2 or CTX3), 2 µl Terminator Ready Reaction Mix, 2 µl of 5 × buffer and UHQ water in a final reaction volume of 10 µl. Cycle sequencing was performed using the following program for 25 cycles: denaturation at 96˚C for 30 s, annealing at 50˚C for 15 s and extension at 60˚C for 4 min. Following brief centrifugation, the extension products were precipitated by the addition of 8 µl UHQ water and 32 µl 95% ethanol (final concentration 60%). The tubes were incubated at room temperature for 15 min, centrifuged at 10 000 × *g* for 15 min and the supernatant carefully aspirated. The pellets were washed twice with 50 µl 70% ethanol, vacuum-dried for 10 min and then stored at -20˚C. Prior to electrophoresis the purified extension products were resuspended in 3.5 µl Blue dextran/EDTA loading buffer (PE Applied Biosystems), denatured for 2 min at 90˚C and loaded onto the ABI PRISM model 377 DNA sequencer. The obtained nucleotide sequences were edited with the ABI PRISM Sequencing Analysis 3.1 and the ABI PRISM Sequencing Navigator 1.0.1 software programs (PE Applied Biosystems). Sequence comparison was subsequently carried

out using the BLAST 2.1 program and the GenBank non-redundant database.

2.3 RESULTS

2.3.1 Specificity of PCR

A novel protocol for detection of low numbers of *V. cholerae* in water samples was developed based on the technique of seminested PCR. Any nonspecific amplicons produced during the first PCR step should not be able to function as target DNA during the second PCR step due to a lack of complementarity with the inner primer sequence, thereby making confirmation of the product by other procedures such as hybridization unnecessary. The specificity of the oligonucleotide primer pair CTX2 and CTX3 had been previously demonstrated by Fields *et al.* (1992). In the present study, however, specificity testing was performed due to the development of a novel assay as well as due to modifications of the amplification conditions, compared to those described by Fields *et al.* (1992). Thus, to confirm and to validate amplicon integrity in the present study, sequencing reactions were performed on aliquots of the purified amplicon generated from toxigenic *Vibrio cholerae* culture and primers CTX2-CTX3. The sequence was identical to nucleotide sequences of the targeted area of the *ctxA* gene (GenBank, accession no. AF 175708). In order to study the specificity of the seminested CTX15 primer, crude lysates of a panel of different bacterial strains (Table 2.1) were subjected to PCR using the CTX2 and CTX15 primers. Only toxin-producing *Vibrio* strains yielded a single amplicon of the expected length corresponding to 347 bp. All other strains tested were negative by testing with this primer pair, including non-toxigenic *Vibrio* strains. Pit-stop seminested PCR on these bacterial extracts also resulted in the amplification of a 347 bp amplicon from only toxigenic *Vibrio* strains.

2.3.2 Sensitivity of PCR with pure cultures

To determine the sensitivity of the PCR detection systems, *V. cholerae* NCTC 5497 cells were serially diluted 10-fold in sterile distilled water and the DNA was extracted by the boiling method described in Materials and Methods (Section 2.2.2). The bacteria present in the 10-fold dilutions were enumerated by dilutional plating. Lysate supernatants were subjected to PCR amplification, and the products were visualised on

2% agarose gels stained with ethidium bromide. With the single primer pair, CTX2 and CTX3, the 564 bp fragment could be visualized in ethidium bromide-stained gels in reaction mixtures that contained 1.3×10^7 cfu/ml of *V. cholerae*, which corresponds to 1.3×10^5 cfu per PCR reaction. The sensitivity was improved by the pit-stop seminested PCR. In this assay, samples were subjected to a 10-cycle PCR amplification with primers CTX2 and CTX3, and 1 µl of the first round PCR product was subjected to another 20 cycles of amplification with oligonucleotides CTX2 and CTX15. The application of this procedure rendered possible the detection of 1.3×10^5 cfu/ml without loss of specificity (1 300 cfu per reaction). The results of gel electrophoresis analysis following CTX2-CTX3 PCR, and after pit-stop seminested PCR are presented in Fig. 2.1.

The sensitivity following enrichment of pure cultures was also investigated. Serially-diluted and previously enumerated *V. cholerae* cells were recovered by centrifugation, resuspended in CDC enrichment broth and incubated at 37°C. Pit-stop seminested PCR reactions were subsequently performed on crude lysates after 0, 2, 4, and 6 h of enrichment. Although *ctx*-positive signals were produced after 2 h, the detection limit was greatly enhanced after an 6 h enrichment. The obtained results demonstrated that 390 cfu/ml (3.9 cfu per reaction) could be detected after 6 h of incubation.

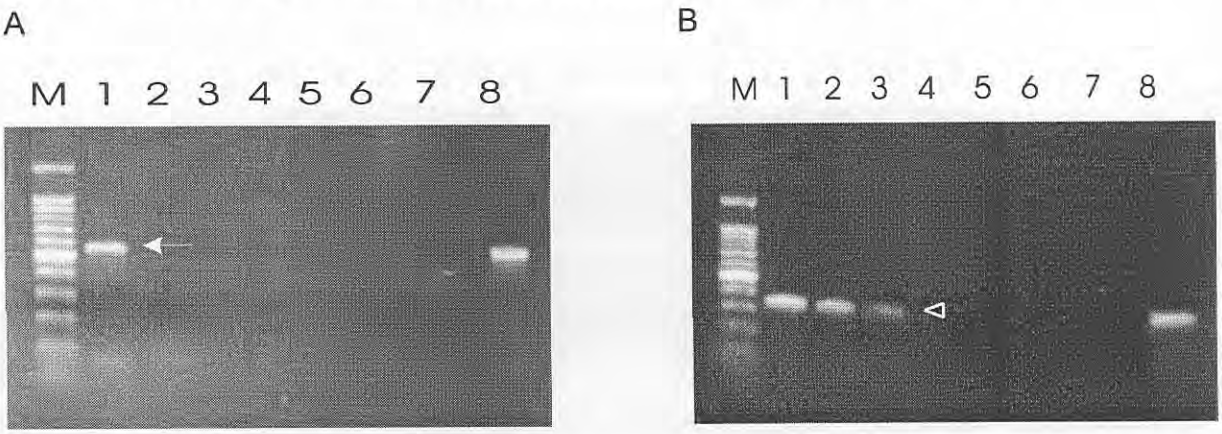

Fig. 2.1 Sensitivity of the PCR assay for the detection of toxigenic *V. cholerae* by a single (A) and with a pit-stop seminested (B) PCR, evaluated with serially diluted whole cells from toxigenic *Vibrio cholerae*. Lanes: 1, 1.3×10^5 cfu; 2, 1.3×10^4 cfu; 3, 1.3×10^3 cfu; 4, 1.3×10^2 cfu; 5, 1.3×10^1 cfu; 6, 1.3×10^0 cfu; 7, DNA negative control; lane 8, positive toxigenic *V. cholerae* control. Lanes M, 100 bp ladder as a molecular size standard. The 564 bp fragment of the single and the 347 bp fragment of the pit-stop protocol are indicated by closed and open arrows, respectively.

2.3.4 Examination of small volumes of seeded environmental water samples

To test the applicability of the proposed detection method to different types of water samples, treated sewage as well as surface, ground and drinking water samples were artificially inoculated with toxigenic *V. cholerae*. Following enrichment for 6 h in CDC broth, bacterial lysates were prepared and subjected to the pit-stop seminested PCR.

Application of this PCR detection assay to these diverse environmental water samples (Fig. 2.2) yielded the following detection limits. While as few as 1 cfu/ml (0.01 cfu) of toxigenic *V. cholerae* organisms could be detected in seeded ground water, 92 cfu/ml (0.92 cfu) and 510 cfu/ml (5.1 cfu) could be detected in seeded lake and river water samples, respectively. In the case of seeded tap water and treated sewage samples, 960 cfu/ml (9.6 cfu) and 13 cfu/ml (0.13 cfu) toxigenic *V. cholerae* organisms could be detected, respectively. No amplified products were detected in unseeded water samples. All seeded control environmental water samples resulted in positive amplification, indicating that humic acids, microorganisms and other interfering substances which may be present in the water samples did not greatly influence the PCR assay. When the detection limits were determined using the duplicate sterile water samples, the detection limits were found to be either the same as determined for the nonsterile samples, or showed a 10-fold increase.

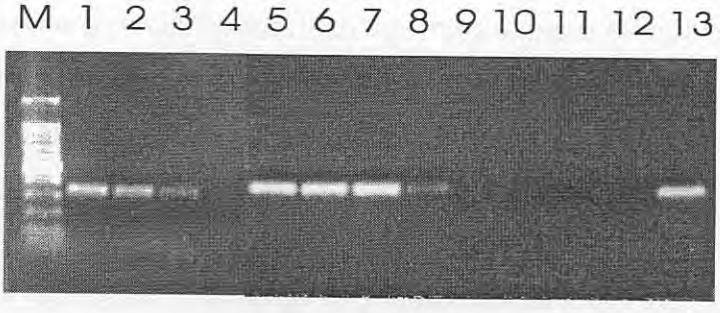

Fig. 2.2 Analysis of the limit of detection upon seeding environmental water samples with toxigenic *V. cholerae* following enrichment in CDC broth using the pit-stop seminested PCR protocol. Lanes: 1 through 4, well water seeded with 1×10^2 (lane 1), 1×10^1 (lane 2), 1×10^0 (lane 3), 1×10^{-1} (lane 4) cfu/ml; 5 through 10, tap water seeded with 9.6×10^5 (lane 5), 9.6×10^4 (lane 6), 9.6×10^3 (lane 7), 9.6×10^2 (lane 8), 9.6×10^1 (lane 9), 9.6×10^0 (lane 10) cfu/ml; lane 11, DNA negative control; lane 12, water negative control; lane 13, positive toxigenic *V. cholerae* control. Lane M, 100 bp ladder as a molecular size standard.

2.4 DISCUSSION

In order to evaluate the public health threat posed by toxigenic *Vibrio cholerae* in source and drinking water, a rapid, accurate method for the detection of these organisms within large populations of other bacteria is essential. The standard method for *Vibrio* detection involves isolation on a selective TCBS medium followed by a battery of biochemical and physiological tests. However, several problems are encountered with culturing methods, including the presence of viable but nonculturable cells, loss of viability of bacteria after collection, difficulties in isolation from biocontaminated samples and the time required for culture and confirmation, which can be several days (Wright *et al.*, 1993). To avoid these problems, different methods based on molecular biology techniques have been developed, with those based on DNA amplification being the most rapid and sensitive (Garret *et al.,* 1993; Ramamurthy *et al.,* 1993). Amplified DNA produced using these methods are, however, seldom detected by direct visualization in ethidium bromide-stained agarose gels, but rather by Southern blot or dot-blot hybridization (Koch *et al.*, 1993; Wright *et al.*, 1993; Nair *et al.*, 1995). Although membrane hybridization is useful in research because it provides excellent sensitivity, these methods are generally time-consuming and labour-intensive. These detection methods are therefore considered impractical for routine laboratory use.

The PCR has become a powerful tool with which to explore microbial activities and identities in environmental microbiology (Mahbubani *et al.,* 1990; Bej and Mahbubani, 1992; Pillai *et al.,* 1991; Koenraad *et al.,* 1995; Juck *et al.,* 1996; Sandery *et al.,* 1996; Catalan *et al.,* 1997). Due to the ability of PCR to amplify specifically a gene or a segment of gene directly from a sample, an important factor in evaluating any DNA-based test is the specificity of the DNA sequence chosen for the genes and strains of interest. The ability to produce cholera toxin is an important step in the diagnosis of cholera, because only toxin-producing strains have been associated with severe, watery diarrhoea and epidemics (Finkelstein, 1988). Various cholera toxin gene PCR assays, using primers that amplify regions of either *ctxA* or regions covering both *ctxA* and *ctxB*, have been described (Kobayashi *et al.,* 1990; Shirai *et al.,* 1991; Fields *et al.,* 1992; Keasler and Hall, 1993; Koch *et al.,* 1993;

Varela *et al.,* 1993). The respective PCR

assays were applied to the identification of toxigenic *V. cholerae* from bacterial colonies, fecal and food samples.

In this part of the investigation, a modified seminested PCR assay (pit-stop seminested PCR) for the detection of toxigenic *V. cholerae* in small volumes of environmental water was developed and evaluated. The pit-stop seminested PCR, based on amplification of the *ctxA* sequence, was used with a rapid and simple DNA preparation procedure to detect small numbers of toxigenic *V. cholerae* cells. In contrast to conventional nested and seminested PCR procedures (Arnheim and Erlich, 1992) which are performed using two rounds of successive amplification consisting of 30 cycles each with an outer and an inner primer pair, respectively, this pit-stop seminested PCR is performed in a total of 30 cycles. Not only did the pit-stop seminested PCR assay increase the specificity of the assay, but also the sensitivity. Only toxigenic *Vibrio* spp. strains screened by the PCR assay resulted in visualization of the predicted 347 bp amplified product in ethidium bromide-stained gels. Direct detection of *V. cholerae* by a single, first round PCR assay was possible with 130 000 bacteria. However, the modified pit-stop seminested PCR system yielded a positive signal from 1 300 bacteria.

The use of PCR for identifying specific organisms obtained from environmental samples has been problematic. Detection of low copy number targets may require a large number of PCR cycles. Increasing cycles often leads to the formation of nonspecific amplification products that result from mispriming within nontarget sequences encountered in the sample or by "primer-dimer" formation (Starnbach *et al.*, 1989; Koch *et al.,* 1993; Juck *et al.*, 1996). In addition, the presence of various interfering substances, such as humic acids, metal ions, and organic matter (Rossen *et al.*, 1992) may lead to false-negative results occurring. To overcome these problems, methods to separate DNA from extracts containing humic acid substances (Tsai and Olssen, 1992) and filtration through chelating ion exchange resins to eliminate metal ions (Abbaszadegan *et al.*, 1993) have been developed. Immunomagnetic beads attached to specific antibodies to assist in capturing and

concentrating organisms prior to DNA extraction have also been used (Islam *et al.,* 1993b). However, such extraction methods greatly increase the cost and time required to identify pathogenic organisms. Furthermore, there is a risk of losing target DNA in each purification step and certain chemicals used for extraction of nucleic acids inhibit PCR (Rossen *et al.,* 1992). Assays based on direct detection of bacterial cells in environmental water samples by filtration and without an enrichment procedure have been developed (Starnbach *et al.,* 1989; Bej and Mahbubani, 1992; McDonald *et al.,* 1995; Sinigalliano *et al.,* 1995), but a disadvantage of such methods, however, is that they may detect dead bacteria as well as viable bacteria.

To minimize these problems, the present study utilized PCR technology coupled with an enrichment procedure that not only diluted PCR inhibitors but also resulted in increased numbers of *V. cholerae* organisms in reaction mixtures. Not only does an enrichment procedure dilute any inhibitors present, but dead bacteria are diluted as well, thus reducing the probability of detecting them by the subsequent PCR assay. By allowing exponential bacterial growth to amplify target copy number rather than to use increased numbers of amplification cycles to detect less target, the chance that false-positives might be generated during extended PCR cycling, as in the case of conventional nested and seminested PCR assays (Starnbach *et al.,* 1989; Lindqvest, 1999; Waage *et al.,* 1999), is thus minimized. In this investigation, enrichment in CDC broth for as short as 6 h of incubation before amplification enhances the limit of detection considerably (at least 300-fold) and as few as 4 cfu of *Vibrio* organisms were detectable in the assay. Furthermore, this combined procedure requires minimal sample manipulation, but is still applicable to most diagnostic laboratories for rapid detection of toxigenic *Vibrio* species.

The sensitivity of the pit-stop seminested PCR assay was also determined using small volumes of environmental water samples from various sources inoculated with *V. cholerae*. In these seeding experiments, different numbers of toxigenic *V. cholerae* could be detected and depending on the water sample examined, as few as 1 cfu/ml could be detected by this method. The obtained results indicated that inhibitory substances did not interfere significantly with the PCR when the protocol

described above was used. The sensitivity of detection of *V. cholerae* organisms in prepared environmental samples was in some instances lower than that in pure cultures. Others have reported similar results of sensitivities with clinical and environmental samples (Hermans *et al.,* 1990; Shawar *et al.,* 1993; Stone *et al.,* 1994). It should be noted, however, that the bacteria used to seed the samples were freshly cultured. The apparent lower sensitivity observed for the tap water may be due to initial stress on cells inoculated into this environment of decreased osmotic pressure, and subsequent slower recovery and growth in the enrichment broth (Farmer and Hickman-Brenner, 1992). Detection of sublethally damaged *V. cholerae* cells in naturally contaminated water with this assay should depend initially on the ability of the bacteria to recover from injury and enter the growth phase and subsequently on their capacity to compete with the background flora.

In conclusion, the newly developed method which includes a combination of enrichment, rapid sample preparation and pit-stop seminested PCR, is specific for detection of toxigenic *V. cholerae* and can be used for detection of these pathogens in small volumes of environmental water samples. The assay can detect low numbers of *V. cholerae* cells in contaminated samples when preparations are incubated in an enrichment medium prior to bacterial lysis and pit-stop seminested PCR. The analysis can be completed in 10 h, which is a considerably shorter period of time than is needed for traditional culturing and subsequent bacterial identification. The method described here should be a significant tool in monitoring environmental water and drinking water sources, including sources suspected to be involved in outbreaks of cholera, for the presence of toxigenic *V. cholerae* cells.

CHAPTER THREE*

A SENSITIVE SEMINESTED PCR METHOD FOR THE DETECTION OF *SHIGELLA* IN SPIKED ENVIRONMENTAL WATER SAMPLES

3.1 INTRODUCTION

The genus *Shigella* is composed of Gram-negative facultative anaerobes of four species: *Shigella dysenteriae*, *S. boydii*, *S. sonnei* and *S. flexneri*. All are pathogens of humans and are usually transmitted from person to person as well as by ingestion of contaminated water and foods. The infective dose is very low, varying from 10^1 to 10^4 organisms (Rowe and Gross, 1984; DuPont, 1990). Virulent *Shigella* organisms cause the human illness known as bacillary dysentery, as do enteroinvasive *Escherichia coli* (EIEC) strains. Clinical features of bacillary dysentery (shigellosis) include diarrhoea, fever, dysentery, and even death in some cases if effective intervention strategies are not used. Epidemiological studies of shigellosis in Bangladesh have shown that various water sources, e.g. ponds, lakes, wells, and rivers, can act as sources of infection (Islam *et al.*, 1993a). In the United States, outbreaks of shigellosis have also been attributed to swimming in contaminated water (Rosenberg *et al.*, 1976; Blostein, 1991; Fleming *et al.*, 2000). In South Africa, children under five years of age living in settlements with rudimentary access to water supply and sanitation are the most susceptible to diarrhoea whereas adults often become symptomless carriers (Pergram *et al.*, 1998). The difficulty in detecting carriers by culture techniques makes them a potential source of environmental contamination.

All of the virulent *Shigella* spp. and EIEC strains harbor a 120 to 230 kb plasmid named the virulence plasmid, which was first described for *S. flexneri* 2a. It was established that the invasion plasmid antigen gene (*ipaH*), unique to shigellae and EIEC and

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implicated in virulent functions, is present in multiple copies on the invasion plasmid and the chromosome (Venkatesan *et al.*, 1989; Hartman *et al.*, 1990; Hale, 1991; Venkatesan *et al.*, 1991).

The standard procedure for *Shigella* spp. detection is based on isolation of *Shigella* by selective culture media followed by identification by biochemical tests and agglutination assays (Frankel *et al.*, 1989; June *et al.*, 1993). This process may take 48 to 72 h or even longer to obtain results. Since shigellae are very fastidious organisms, appropriate collection, rapid transport to the laboratory and rapid plating of the sample are important for isolation. Such conditions are often difficult to attain in developing countries. Thus, rapid, highly sensitive and specific techniques based on genetic characteristics have been developed. DNA hybridization (Venkatesan *et al.*, 1988) and PCR are the best known of these techniques and are often used as tests for the detection and identification of pathogenic microorganisms. Several PCR protocols for detection of *Shigella* in faeces (Frankel *et al.*, 1989; Sethabutr *et al.*, 1993; Yavzori *et al.*, 1994), food (Rafii *et al.*, 1995; Lindqvist, 1999), and some in water (Bej *et al.*, 1991a; Bej *et al.*, 1991b) have been published. These protocols use primers directed at sequences located on the invasion plasmid of *Shigella* spp. and EIEC (Frankel *et al.*, 1989; Yavzori *et al.*, 1994; Lindqvist, 1999), or on both the plasmid and the chromosome (Sethabutr *et al.*, 1993). The major obstacle to using PCR for the detection and identification of pathogenic organisms from clinical samples or environmental water samples is the presence of substances that are inhibitory to PCR (Wilde *et al.*, 1990; Rossen *et al.*, 1992). This obstacle can be minimized by direct chemical extraction of nucleic acids (Frankel *et al.*, 1989; Rafii *et al.*, 1995), immunomagnetic separation (IMS) of bacteria with antibodies (Islam and Lindberg 1992), or buoyant density gradient centrifugation (Lindqvist, 1999), but these procedures are laborious and expensive.

The aims of this part of the research were (i) to develop a highly sensitive and specific detection method for virulent *Shigella* organisms and EIEC by seminested PCR combined with a short enrichment step, and (ii) to determine the sensitivity of the procedure by using artificially seeded ground and surface water samples collected from different natural sources.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains

Shigella flexneri, strain CCRC 10772, obtained from the Taiwanese Culture Collection, Taiwan was used as the test organism in this study. This strain was reconfirmed by cultural, morphological and biochemical tests according to standard procedures (June *et al.*, 1993). Numerous bacterial strains were tested to determine the specificity of the detection protocol (Table 3.1). The organisms were cultivated on MacConkey agar plates and maintained in LB broth (10 % (w/v) peptone, 5 % (w/v) yeast extract, 10 % (w/v) NaCl, 1 % (w/v) glucose) at 37˚C, unless the Culture Collection instructions specified otherwise.

3.2.2 Preparation of lysates for PCR

For specificity determination, a direct lysis method was used for isolation of DNA from bacteria. Bacterial colonies were suspended in 1 ml of sterile water to a concentration of 10^6 organisms per ml. The bacteria were lysed by heating for 10 min at 100˚C and then immediately placed on ice for 5 min. Particulate material present after processing was removed by centrifugation at 10 000 × *g* for 5 min. The lysate supernatant was removed and 10 µl used as the template in the PCR assays immediately or following storage at -20˚C.

3.2.3 Enrichment and enumeration of *Shigella*

S. flexneri CCRC 10772, which was used to seed water samples and to determine the sensitivity of the seminested PCR assay, was grown in LB medium to mid-exponential phase (A_{600} = 0.35), corresponding to 10^8 cfu/ml. Appropriate bacterial concentrations were obtained by preparing serial 10-fold dilutions in sterile distilled water. To enumerate the bacteria, aliquots were spread onto nutrient agar plates and incubated at 37˚C overnight and the bacterial concentration was estimated by calculating the average colony count on plates containing between 30 and 300 colonies. For enrichment of the bacterial cultures, serially diluted bacterial cells were recovered by centrifugation at 10 000 × *g* for 5 min. The cells were then resuspended in 1 ml of Gram-negative broth (GN broth; Difco) and the tubes were incubated at 37°C with

shaking. At time 0, 2, 4 and 6 h after seeding, the numbers of cells per milliter were assessed by viable plate counts and template DNA was prepared from each tube as described above, except that the collected bacterial cells were washed twice in distilled water prior to heating in a water bath.

3.2.4 Preparation of seeded environmental samples prior to PCR

Mid-exponential phase *S. flexneri* cells were serially diluted (10^{-1} to 10^{-9}); 100 µl of the serial dilutions were seeded into 900 µl of both sterile and nonsterilized environmental water samples (treated sewage, lake, river, well and tap water) and enumerated by plate counting. The respective water samples were pretested for the presence of amplifiable *Shigella* DNA by PCR and found to be negative. The bacterial cells from each dilution were recovered by centrifugation at 10 000 × *g* for 5 min and then resuspended in 1 ml of GN broth. Following incubation at 37°C for 6 h, template DNA for seminested PCR analysis was prepared as described above for enrichment samples. Positive as well as negative, uninoculated controls were included in each experiment.

3.2.5 DNA amplification

3.2.5.1 Selection of primers

Oligonucleotide primers H8, H15 and H10 from the multicopy invasion plasmid antigen gene, *ipaH*, were used in a seminested PCR assay. Primers H8 (5' - GTTCCTTGACCGCCTTTCCGATAC - 3') and H15 (5' - GCCGGTCAGCCACCCTC - 3') have been described previously by Islam *et al.* (1993a). An internal primer, H10 (5' - CATTTCCTTCACGGCAGTGGA - 3'), was designed based on the *ipaH* gene sequence reported by Hartman *et al.* (1990). The first PCR step, performed with primers H8 and H15, amplified a 620 bp region of the *ipaH* gene, while the size of the final PCR product obtained with primers H8 and H10 was 401 bp.

3.2.5.2 Seminested PCR

The reaction mixtures used in the PCR steps contained 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5% (vol/vol) Triton X-100), 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 0.1 mM, 24 pmol of primer H8, 34

Table 3.1 Bacterial strains examined

Microorganism	Amplification (Seminested PCR)	*Source or strain
Aeromonas hydrophila	-	RW
Bacillus cereus	-	CSIR
Citrobacter freundii	-	CSIR
Escherichia coli HB101	-	DSM
Escherichia coli	-	ATCC 25922
Escherichia coli	-	CCRC 13086
Escherichia coli	-	CCRC 14824
Escherichia coli O:112	+	OVI
Escherichia coli O:102	+	OVI
Enterobacter aerogenes	-	ATCC 25922
Klebsiella pneumoniae	-	ATCC 49472
Legionella pneumophila	-	ATCC 33153
Proteus mirabilis	-	ATCC 49469
Pseudomonas diminuta	-	CSIR
Pseudomonas aeruginosa	-	ATCC 27853
Salmonella enteritidis	-	ATCC 13076
Salmonella typhi	-	ATCC 49469
Salmonella dublin	-	CCRC 13852
Salmonella heidelberg	-	CCRC 123437
Shigella dysenteriae	+	NCTC 1311
Shigella flexneri	+	CCRC 10772
Shigella sonnei	+	ATCC 8574
Shigella boydii	+	SAIMR
Vibrio cholerae	-	ATCC 25870

*CTC = National Collection of Type Cultures, Public Health Laboratory Service, London, UK
ATCC = American Type Culture Collection, Rockville, MD, USA
DSM = Deutsche Sammlung von Mikroorganism und Zelkulturen, Braunschweig, Germany
CCRC = Culture Collection and Research Center, Hsinchu, Taiwan
OVI = Culture Collection, Onderstepoort Veterinary Research Institute, Onderstepoort, South Africa
RW = Rand Water, Johannesburg, South Africa
CSIR = Council for Scientific and Industrial Research, Pretoria, South Africa

pmol of primer H15, and 1 U of *Taq* DNA polymerase (Promega) per 50 µl of reaction mixture. The sample volume was 10 µl. The reaction tubes were placed in a Model 2400 GeneAmp thermal cycler (Perkin-Elmer). The following conditions were used: heat denaturation at 94°C for 3 min, followed by 10 cycles consisting of heat denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and DNA extension at 72°C for 1 min. The second PCR step was performed by using a total volume of 50 µl. A 1 µl aliquot of the first PCR was used as the template and 24 pmol H8 primer and 31 pmol H10, as the seminested primer, were used. The cycle profile consisted of the same heat denaturation, primer annealing, and DNA extension conditions as those used for the first PCR step, but the number of cycles was 20. After the last cycle, the samples were kept at 72°C for 7 min to complete synthesis of all strands.

3.2.5.3 Single-step PCR reactions

PCR reactions were performed by using 10 µl of bacterial lysate as template DNA, buffer, deoxynucleoside triphosphate mixture, primers H8 and H15 or primers H8 and H10, and Taq DNA polymerase in a final reaction volume of 50 µl. The reactions were then subjected to 30 cycles of amplification under the cycle conditions as described above. Control reaction mixtures containing distilled water and all other reagents but no template were amplified along with the test samples throughout the amplification reaction. A positive control consisting of a *S. flexneri* suspension was included.

3.2.6 Electrophoretic detection

The amplicons were resolved on a 2% (w/v) agarose gel in 1 × TAE (40 mM Tris-HCl, 20 mM Na.acetate, 1 mM EDTA, pH 8.5), as described in Section 2.2.6, and visualized by UV-induced fluorescence after staining with 0.5 µg of ethidium bromide per ml. A 100 bp DNA ladder (Gibco BRL, Life Technologies) was included on each gel as a molecular size standard.

3.2.7 Restriction enzyme digestion and polyacrylamide gel electrophoresis

3.2.7.1 Purification of the amplified DNA

The 620 bp H8-H15 amplified DNA fragment was purified by phenol-chloroform

extraction. The volume of the sample was adjusted to 400 µl by the addition of 1 × TE (1 mM Tris.HCl, 0.1 mM EDTA; pH 7.6). An equal volume of a phenol:chloroform:isoamyl alcohol mixture (25:24:1) was added to the samples, mixed and the organic and aqueous phases separated by centrifugation at 10 000 × *g* for 5 min. The upper aqueous phase was recovered and extracted twice with an equal volume of chloroform. The DNA was precipitated from the aqueous phase by the addition of 2.5 volumes 96% ethanol and Na.acetate (pH 7.0), to a final concentration of 0.3 M. The DNA was recovered by centrifugation at 10 000 × *g* for 10 min, washed with 70% ethanol and dried under vacuum before being suspended in a suitable volume of 1 × TE.

3.2.7.2 Restriction endonuclease digestion of DNA

Samples (10 µl) of the purified amplicon were subsequently digested with 5 U *Hae* III (Roche Diagnostics) in the appropriate restriction endonuclease buffer. Following incubation at 37°C for 1.5 h, the fragments were separated on a polyacrylamide gel. *Hae* III was predicted to digest the 620 bp *ipaH* amplicon into 273, 215 and 135 bp fragments. The sizes of the restriction fragments were estimated by comparison with a molecular weight marker (pBR 328 DNA cleaved with *Bgl* I and *Hinf* I, Roche Diagnostics).

3.2.7.3 Polyacrylamide gel electrophoresis

An aliquot of the restriction enzyme digestion reaction mixture was separated by vertical 8% polyacrylamide gel electrophoresis at 9.0 V/cm for 2-3 h in 1 × TAE buffer (Sambrook *et al.*, 1989). Following electrophoresis, the gel was stained in ethidium bromide solution for 5 to 10 min and visualized with a UV transilluminator.

3.3 RESULTS

3.3.1 Specificity of PCR

The oligonucleotide primer pair H8 and H15 has previously been described by Islam *et al.* (1993a) and was used in a PCR to detect viable but nonculturable *S. dysenteriae* in

laboratory microcosms. However, no primer specificity data were provided. In the absence of such data, specificity testing of four strains of *Shigella* and 21 strains belonging to other genera was performed. To investigate the specificity of the H8-H15 PCR, samples of all strains listed in Table 3.1 were subjected to 30 cycles of amplification. All *Shigella* and EIEC strains produced an intense band of 620 bp. With all other strains tested, no PCR product was detectable. To confirm and to validate amplicon integrity, restriction enzyme digestions were performed with *Hae* III on aliquots of the purified 620 bp amplicons generated in PCR using crude cell lysates from the *Shigella* spp. The polymorphism patterns of the restriction fragments obtained experimentally were identical to those predicted from published nucleotide sequences of the targeted area of the *ipaH* gene (Fig. 3.1).

To investigate the specificity of the seminested PCR, cell lysates prepared of all strains listed in Table 3.1 were subjected to a 10-cycle PCR amplification with primers H8 and H15, and 1 µl of this PCR was subjected to another 20 cycles of amplification with oligonucleotides H8 and the nested primer, H10. The PCR amplified not only DNA from *S. flexneri*, but also from all the other *Shigella* and EIEC strains tested. All amplification-generated products were of the expected size (approximately 401 bp) on agarose gel electrophoresis. No amplification product was observed from microorganisms other than

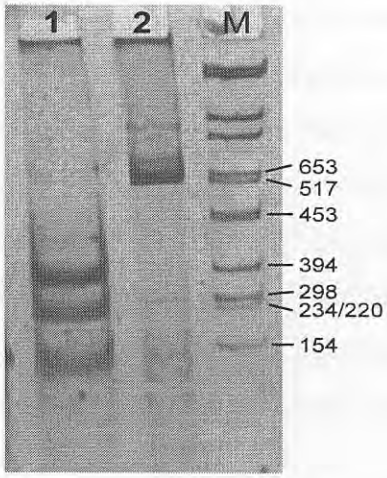

Fig 3.1. Characterisation of the amplicon generated by the H8-H10 single step PCR by resctriction enzyme digestion. The identity of the amplicon (lane 2) was confirmed by restriction with *Hae* III (lane 1), prior to poylacrylamide gel electrophoresis. The sizes of the molecular weight marker (lane M) are indicated to the right of the figure.

Shigella and EIEC, even though there was sufficient DNA to detect a single copy sequence of *ipaH*. In addition to the 401 bp fragment, amplification of the DNA of the *Shigella* and EIEC strains occasionally did produce larger amplicons. The size of these amplified fragments was identical to those obtained in the single step PCR using primers H8 and H15 (620 bp). These amplicons may be the amplified products of the first PCR step, which is used as template for the second round PCR amplification, resulting in amplification of the expected 401 bp DNA fragment. Raising the primer annealing temperature to 61°C during the first PCR step decreased this problem to a minimum and did not influence the sensitivity of the assay. Since no amplification was observed when DNAs from other bacterial strains were used as targets for PCR, we concluded that the seminested PCR assay can be used to specifically detect virulent *Shigella* spp. and
EIEC strains.

3.3.2 Sensitivity of PCR

In order to evaluate the minimal detectable number of *S. flexneri* organisms, viable CCRC 10772 cells were diluted 10-fold in sterile distilled water, enumerated by dilutional plating and template DNA was prepared by the boiling method described in Materials and Methods. Aliquots of the lysate supernatants were first assayed for sensitivity with the single primer pair H8 and H15. The detection limit was at least 1.6×10^4 cfu of *S. flexneri* per assay, corresponding to 1.6×10^6 cfu/ml of lysate. To reach a lower detection limit with simultaneous confirmation of the reaction product, the samples were subjected to the seminested PCR assay. After seminested PCR, the last dilution step amplified corresponded to approximately 1.6×10^3 cfu per assay (1.6×10^5 cfu/ml of lysate). Thus, detection levels were increased 10-fold in seminested PCR assays. The sensitivity of the PCR assays following enrichment of pure cultures in GN broth was also investigated. PCR reactions were performed on crude lysates after 0, 2, 4, and 6 h of enrichment. The obtained results demonstrated that 1.6 cfu (160 cfu/ml) *S. flexneri* organisms could be detected in the seminested PCR assay after 6 h of incubation. In contrast, the limit of detection in the single-step PCR reaction using primers H8 and H10 was 16 cfu (1.6×10^3 cfu/ml), which gave a faintly stained band (Fig. 3.2).

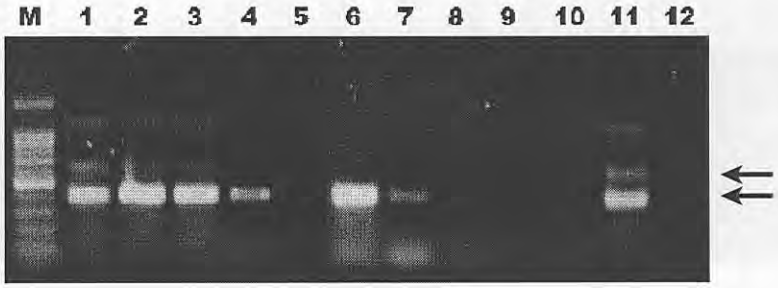

Fig. 3.2 Sensitivity of the PCR assay by a seminested (lanes 1 - 5) and single-step H8-H10 (lanes 6 - 10) PCR following enrichment in GN broth for 6 h. Lane 11, positive control; lane 12, negative virulent *S. flexneri* control. Lanes 1 and 6, 1.6×10^5; 2 and 7, 1.6×10^4; 3 and 8, 1.6×10^3; 4 and 9, 1.6×10^2; and 5 and 10, 16 cfu/ml. Lane M, 100-base pair ladder as a molecular size standard. The bottom arrow indicates the expected seminested PCR-amplified fragment length of 401 bp. The top arrow indicates the first round 620 bp *S. flexneri* fragment occasionally observed during seminested PCR amplification.

3.3.3 Examination of seeded environmental water samples

To test the efficacy of the seminested PCR assay for monitoring small volumes of environmental water samples, serial dilutions of artificially contaminated treated sewage, lake, river, well and tap water samples were prepared for seminested PCR as described in Materials and Methods, and 1/100 of the lysed cells were amplified by the seminested PCR system. Application of this PCR detection assay to these diverse environmental water samples (Fig. 3.3) yielded the following detection limits. While 2×10^3 cfu/ml (20 cfu per reaction) of virulent *S. flexneri* organisms could be detected in seeded well water, 14 cfu/ml (0.14 cfu per reaction) and 580 cfu/ml (5.8 cfu per reaction) could be detected in seeded lake and river water samples, respectively. In the case of seeded sewage and tap water samples, 610 cfu/ml (6.1 cfu per reaction) and 11 cfu/ml (0.11 cfu per reaction) virulent *S. flexneri* organisms could be detected, respectively. No amplified products were detected in sterile and nonsterile unseeded water samples. All seeded control environmental water samples did give positive amplification, indicating that humic acids, microorganisms and other interfering substances which may be present in the water samples did not greatly influence the seminested PCR assay. When the detection limits were determined using the duplicate sterile water samples, the detection limits were found to be either the same as determined for the nonsterile samples, or showed a 10-fold increase in sensitivity.

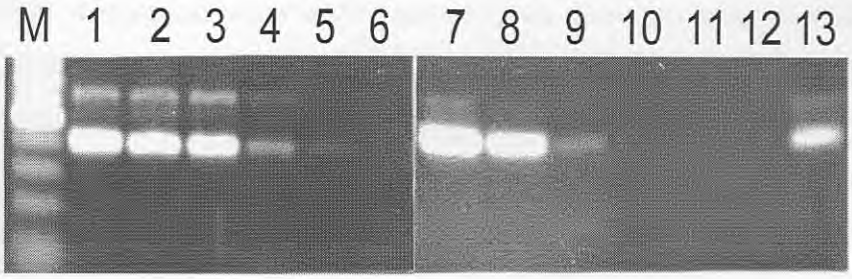

Fig. 3.3. Analysis of the limit of detection upon seeding environmental water samples with virulent *S. flexneri* following enrichment in GN broth using the seminested PCR protocol. Lanes 1 through 6, lake water seeded with 1.4×10^5 (lane 1), 1.4×10^4 (lane 2), 1.4×10^3 (lane 3), 1.4×10^2 (lane 4), 1.4×10^1 (lane 5), 1.4×10^0 (lane 6) cfu/ml; lanes 7 through 11, river water seeded with 5.8×10^4 (lane 7), 5.8×10^3 (lane 8), 5.8×10^2 (lane 9), 5.8×10^1 (lane 10), 5.8×10^0 (lane 11) cfu/ml; lane 12, negative control; lane 13, positive virulent *S. flexneri* control. Lane M, 100-base pair ladder as a molecular size standard.

3.4 DISCUSSION

Detection and identification of *Shigella* from clinical samples has traditionally involved microbiological cultures, biochemical analyses and in some cases, serological methods. The same methods are used to identify suspected *Shigella* colonies isolated from water, food and other environmental samples. However, these methods are not well-suited to the unique situations associated with environmental water samples, where many of the organisms present are stressed and do not perform as expected in clinical testing methods. In the case of *Shigella*, testing problems arise due to the instability of some biochemical characteristics. Studies have also shown that shigellae fail to grow in conventional culture media but remain viable when grown in laboratory microcosms (Islam *et al.*, 1993a). The potential health hazard presented by such *Shigella* species existing in the nonculturable state may therefore be significant. One difficulty in elucidating the potential hazard of viable but nonculturable pathogenic bacteria is the inability to detect such cells in the natural environment by employing routine bacteriological methods. Any detection method that is employed must therefore be capable of detecting low numbers of shigellae against a large background of other cells and of organic material which may be present in the sample. Previous studies have

described PCR-hybridization approaches for the detection of *Shigella* spp. (Islam and Lindberg, 1992). However, most testing procedures describe laborious DNA extraction procedures which are necessary to eliminate substances in samples that can inhibit PCR. To minimize these problems, the present study utilized seminested PCR technology coupled with an enrichment procedure that not only diluted PCR inhibitors, but also resulted in increased numbers of *S. flexneri* organisms in reaction mixtures. This combined procedure requires minimal sample manipulation, but is still applicable to most diagnostic laboratories for detection of small numbers of *Shigella* spp. and EIEC cells in environmental water samples.

The primers selected for this study were based on the sequences of the *Shigella ipaH* gene whose protein product is necessary for invasion of colonic epithelial cells (June *et al.*, 1993). All virulent *Shigella* strains as well as enteroinvasive *E. coli* (EIEC) screened by the seminested PCR assay resulted in visualization of the predicted 401 bp amplified product in ethidium bromide-stained gels. There was no amplification of DNA from samples not inoculated with either the *Shigella* or EIEC. However, amplification of a 620 bp fragment from *Shigella* spp. and EIEC was occasionally observed. Because this fragment was amplified only in the presence of *Shigella* spp. and EIEC, but was not observed when other bacterial strains were used, we attribute its presence to amplification of this fragment from these organisms rather than amplification of DNA from any other bacteria. This may be the result of further amplification of the 620 bp first round amplification product, together with the expected 401 bp product, during the second round of amplification in the seminested PCR assay. Detection of *Shigella flexneri* by a single-step PCR assay was possible with 1.6×10^4 bacteria per reaction. Under the tested parameters, 1.6×10^3 bacteria per reaction were detectable with the seminested PCR system. A low detection limit is, however, indispensable for analysis of environmental water samples, with the infectious dose of shigellae being very low. Enrichment in GN broth for as short as 6 h of incubation before seminested PCR amplification enhanced the limit of detection considerably (at least 1000-fold), and 1.6 cfu of *Shigella flexneri* organisms were detectable in this assay. These results indicate that a high level of sensitivity can be obtained by including an enrichment process.

Methods which could directly detect *Shigella* cells in environmental water samples without an enrichment step would be preferable, especially when viable but nonculturable cells are present. The major obstacle to the development of such methods is the presence of PCR inhibitors, such as humic substances. Insoluble fractions of these substances are concentrated along with bacteria on membrane filters, and extensive extraction procedures may be required to eliminate the inhibitors prior to PCR. Extraction of total DNA by a proteinase K and phenol-chloroform treatment (Frankel, 1989; Bej *et al.*, 1991b; Rafii *et al.*, 1995) or by use of immunomagnetic beads attached to specific antibodies to assist in capturing and concentrating organisms prior to DNA extraction have also been attempted (Islam and Lindberg, 1992; Islam *et al.*, 1993b). These procedures greatly increase the cost and time required to identify pathogenic organisms and certain chemicals used for extraction of nucleic acids inhibit PCR (Rossen *et al.*, 1992).

In this study, use of an enrichment procedure prior to the seminested PCR analysis sufficiently diluted PCR inhibitory substances, while presumably increasing the sensitivity caused by multiplication of the organisms. Furthermore, collection of bacterial cells from the enrichment broth by centrifugation followed by subsequent boiling to lyse the bacteria is a simple and rapid method for preparing DNA for PCR and does not involve any of the aforementioned costly or laborious extraction or purification steps. Minimizing the number of manipulations reduces the risk of contamination and loss of target DNA in each purification step. The sensitivity obtained for boiled *S. flexneri* lysate was 1.6 cfu, which corresponds to the detection sensitivity of 10 cfu obtained by Islam and Lindberg (1992) using an IMS-PCR assay. In addition, small volumes of a variety of different environmental samples were inoculated with *S. flexneri* and analysed using the seminested PCR assay to determine the sensitivity of the assay. The detectable numbers of cells, to some degree, depended on the type of water sample that was seeded. As few as 11 to 14 cfu of *Shigella flexneri* per ml could be detected in some samples.

In conclusion, the PCR system described can permit a rapid and reliable means of assessing the bacteriological safety of water and should provide an effective alternative

methodology to the conventional viable culture methods. Cultures as well as environmental samples can be tested for the occurrence of virulent *Shigella* spp. and EIEC by a seminested PCR assay and ethidium bromide visualization of the PCR products in agarose gels following an enrichment step. The method is easy to perform, sensitive, requires little specialized equipment or training, and provides same day results necessary for rapid action in the case of potential disease outbreaks.

CHAPTER FOUR

DETECTION OF VIABLE TOXIGENIC *VIBRIO CHOLERAE* AND VIRULENT *SHIGELLA* SPP. IN NATURALLY CONTAMINATED ENVIRONMENTAL WATERS BY PIT-STOP SEMINESTED PCR ASSAYS AND COMPARISON TO THE CULTURE TECHNIQUE

4.1 INTRODUCTION

Most pathogens occur in relatively low numbers in environmental waters and because adequate protection of public health requires their rapid detection, the range of available detection methods for pathogenic microorganisms is rapidly increasing, both with regard to the types of pathogens that can be detected as well as the methodology involved. Some form of concentration procedure will usually be the first step in a method for pathogen detection (Oyofo and Rollins, 1993; Gerhardt and Drew, 1994; Shepard and Wyn-Jones, 1995; Graczyk *et al.*, 1997; Vilaginès *et al.*, 1997). Following concentration of the sample, bacteria retained on the filter can be detected by culturing in or on selective media (Toro *et al.*, 1995; Høi *et al.*, 1998; Cerdà-Cuéllar *et al.*, 2000). Some methods may give reliable results in one single step, but usually additional steps are necessary such as resuscitation to allow detection of sublethally injured cells, selective incubation, and confirmatory tests (Kang and Siragusa,1999; Reissbrodt *et al.*, 2000). The sensitivity and specificity of these methods depend strongly on the number of confirmatory tests performed and such tests may take several days to complete.

Given these drawbacks of culture techniques, molecular biology methods appear as a primary alternative for detecting pathogenic bacteria in water samples. Unlike traditional techniques, these methods are based on the detection of a fraction of the genetic material of the targeted bacteria. By using such techniques, the selectivity and sensitivity problems associated with culture techniques can be overcome. Various detection protocols have thus been developed based on cell filtration and PCR amplification of target sequences. Detection limits of 5×10^3 spiked cells/ml in water samples were demonstrated by Pepper *et al.* (1991) using a two-stage filtration and

concentration process, PCR amplification of the target gene, and ethidium bromide staining of gels. Bej *et al.* (1990; 1991a) used a single stage filtration method combined with PCR and Southern hybridization and observed a detection limit of 1 cell/100 ml.

A disadvantage of the above direct detection assays is that inhibitors may hamper the PCR reaction. Such inhibition phenomena have often been described for different types of matrixes, including water concentrates (Kreader, 1996; Wilson, 1997). In addition, it is not possible to assess the viability of the detected bacteria (Josephson *et al.*, 1993). Yet, the viability concept is fundamental for interpreting the result in terms of public health when dealing with water samples. The PCR technique must consequently be associated with a viability test. In the preceding two chapters, the development of protocols consisting of an enrichment step followed by a novel pit-stop seminested PCR for the detection of viable toxigenic *V. cholerae* and entero-invasive *Shigella* spp. in environmental water has been described. Although these methods were developed using 1-ml volumes of seeded water samples, the optimized PCR methods nevertheless showed excellent specificity with a high level of sensitivity and the results could be obtained rapidly. However, the physiological state of the pure cultures used to artificially seed the water samples does not reflect the physiological state of naturally occurring cells, which may have been exposed to a variety of unfavourable conditions or suffered some degree of sublethal injury.

Thus, to establish the seminested pit-stop PCR as a practical tool for routine laboratory use, its performance with regards to the detection of toxigenic *V. cholerae* and enteroinvasive *Shigella* spp. in large volumes of naturally contaminated environmental waters would have to be evaluated. Since conventional culture-based detection methods require the analysis of 100-ml volumes of the water samples, analysis of similar volumes of water would align the pit-stop seminested PCR method with these conventional methods and standards for water quality monitoring. The aims of this part of the research were thus (i) to determine the sensitivity of the pit-stop seminested PCR assays using large volumes (100 ml) of seeded environmental water samples, (ii) to compare the sensitivity of PCR assays with that of conventional culture methods, and (iii) to use the developed detection protocols to analyse naturally contaminated

environmental water samples for the presence of toxigenic *V. cholerae* and enteroinvasive *Shigella* spp.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains

Vibrio cholerae strain NCTC 5941, obtained from the National Collection of Type Cultures, London, UK, and *Shigella flexneri* strain CCRC 1077, obtained from the Taiwanese Culture Collection, were used for seeding of environmental water samples. The bacterial strains were cultivated and maintained on nutrient agar or in nutrient broth at 37˚C.

4.2.2 Preparation of bacterial lysates for PCR

DNA was extracted from the bacterial cells by a rapid boiled-lysate method, as previously described (Sections 2.2.2 and 3.2.2). Bacterial cells were concentrated by centrifugation, the pellets suspended in 20 µl sterile Milli-Q water by vortexing and then lysed by boiling. Following removal of the bacterial debris by centrifugation, 10 µl of the supernatant was immediately used as template in the PCR assays.

4.2.3 Sensitivity of the pit-stop seminested PCR assays

The bacterial strains were streaked onto nutrient agar and after overnight incubation of the plates, a single colony was suspended in test tubes containing sterile saline (0.8% (w/v) NaCl in distilled water). For sensitivity determinations, serial 10-fold dilutions of the *V. cholerae* and *S. flexneri* suspensions were prepared in 9 ml sterile saline as diluent. Enumeration of the bacteria to determine the cfu/ml were performed in triplicate by plating each of the 10-fold dilutions onto nutrient agar plates and incubating at 37˚C for 18 h before counting colonies. One ml of the prepared 10-fold dilution series' was subsequently used to seed 99-ml volumes of different environmental water samples (surface water, tap water and treated effluent). The serially-diluted *V. cholerae* and *S. flexneri* cells in the 100-ml water samples were then recovered by filtering through 50-mm cellulose nitrate filters with a pore size of 0.45 µm (Millipore Corp.) using a vacuum

pump (APHA, 1998). The filter membranes and trapped cells were rolled and aseptically transferred to 50-ml Schott bottles containing 4 ml of either CDC or GN broth for enrichment of *V. cholerae* and *S. flexneri* cells, respectively. The filters were vortexed for 5-10 s to release the cells from the filter surface to the liquid phase. Following incubation at 37˚C for 6 h with shaking, 1 ml of the broth was removed and used to prepare template DNA for pit-stop seminested PCR analysis. Negative, uninoculated water samples were included as controls in each of the experiments.

4.2.4 Analysis of water samples by comparison of culturing and PCR methods

To compare the sensitivity of the seminested PCR assays to culturing, 30 naturally contaminated environmental water samples, as indicated in Table 4.2, were analyzed. The water samples were collected in KwaZulu-Natal and were supplied by the CSIR, Durban after the following had been performed. The microorganisms from 50 lt of the environmental water samples were concentrated by making use of Moore swabs, prepared by folding a 100 mm × 8 ply gauze swab into three. The Moore swabs were placed in funnels and after the water samples had been filtered through the swabs, they were removed and transferred into 100 ml CDC broth and incubated at room temperature for 12 h. These samples were subsequently transported to the CSIR, Pretoria by airfreight in cooler bags with ice-packs. The respective samples were immediately analysed on arrival, which was within 20 h after collection of the water samples. The presence of *V. cholerae* and *S. flexneri* in the samples was determined by bacteriological culturing methods as well as by pit-stop seminested PCR assays. In addition, 10 of the original water samples collected, were included for analysis by seminested PCR assays following membrane filtration and enrichment.

4.2.4.1 Isolation of *V. cholerae* by culture methods

(i) Isolation media

For the isolation of *Vibrio* spp., a loopful of growth was obtained from the area just below the culture surface (pellicle), inoculated into *Vibrio* enrichment broth (Biolab) and incubated at 37˚C for 18 h. Freshly prepared thiosulfate-citrate-bile salt-sucrose agar plates (TCBS; Difco) were streaked with bacterial growth before and after culturing in

the *Vibrio* enrichment broth. The agar plates were incubated at 37°C for 18 h and presumptive *Vibrio* isolates were selected based on a yellow (sucrose-fermenting) colony phenotype.

(ii) Biochemical identification

The yellow sucrose-fermenting colonies were streaked on the slant and stabbed into the butt of triple sugar iron agar (TSI; Oxoid) slants and the tubes incubated at 37°C for 18 h. Isolates of which the reactions in TSI agar medium were acid butt and acid slope without production of gas and hydrogen sulphide (H_2S) were selected for further analysis. A colony of each isolate was subsequently inoculated into tryptone water after which a loopful of the cell suspension was streaked onto MacConkey agar (Oxoid) and brain heart infusion (BHI) agar (Oxoid), while SIM agar (Oxoid) slants were stab-inoculated to within a few centimetres from the bottom of the tube. The agar plates and tubes were incubated at 37°C for 18 h. The individual colonies growing on BHI agar plates were analysed for oxidase activity by using strips of filter paper impregnated with a 1% (w/v) tetramethyl-*p*-phenylenediamine solution and 0.1% (w/v) ascorbic acid, to retard auto-oxidation. The strips were moistened with sterile water just prior to use. A colour change to purple in less than 10 s was regarded as a positive reaction, but if it took longer than 60 s it was considered to be negative. To determine indole production, 0.5 ml of Kovac's reagent (Merck) was added to the cell suspensions prepared in tryptone water. Indole production was indicated by the development of a red colour. Only isolates which displayed motility, grew on MacConkey agar and were postive for oxidase and indole, but negative for H_2S production, were retained. These isolates were confirmed as belonging to the genus *Vibrio* by the API 20E system (bioMérieux) according to the manufacturer's instructions.

(iii) Serological identification

Toxigenic *V. cholerae* isolates were identified by agglutination assays using O1-polyvalent antiserum (Murex). For each isolate, a single colony was emulsified in duplicate in a drop of sterile saline to uniform turbidity. A drop of the polyvalent antiserum was added to one of the emulsions, while sterile saline was added to the duplicate emulsion which served as a negative control. Mixing of the reagents were

performed by tilting the slide back and forth for 60 s. Agglutination was investigated microscopically by viewing under indirect light against a dark background.

4.2.4.2 Isolation of *S. flexneri* by culture methods

(i) Isolation media

For the isolation of *Shigella* isolates, a loopful of growth was streaked onto xylose-lysine-deoxycholate (XLD; Biolab) agar and the plates incubated at 37°C for 24 h. Presumptive *Shigella* isolates were identified as colourless (non-lactose fermenting) colonies on the XLD agar plates.

(ii) Biochemical identification

Non-lactose fermenting colonies were streaked on the slant and stabbed into the butt of triple sugar iron agar (TSI; Oxoid) and lysine iron agar (LIA; Oxoid). The colonies were also stabbed into the butt of urea agar (Biolab) slants. All of the tubes were incubated at 37°C for 18 h. Isolates which were urease negative (urea agar medium remained yellow) and the reactions in TSI and LIA agar media were acid butt and alkaline slant without production of gas, and without production of H_2S in TSI agar medium, were retained. These presumptive *Shigella* isolates were then investigated for catalase activity as follow. Following overnight incubation of inoculated nutrient agar slants, a drop of 3% (v/v) hydrogen peroxide was added to the culture on the agar slant. The formation of oxygen bubbles was regarded as a positive test. Oxidase activity was determined as described in Section 4.2.4.1. Only the oxidase-negative, but catalase-positive isolates were retained and characterized serologically.

(iii) Serological identification

Presumptive *Shigella* isolates were identified to the species level by latex agglutination assays using a commercial kit, Wellcolex Colour Shigella (Murex), according to the manufacturer's instructions. Briefly, one drop of each latex reagent was dispensed onto a disposable reaction card supplied by the manufacturer. One drop (40 µl) of the bacterial suspension, prepared in 200 µl sterile saline, was added to the respective latex reagents and mixed thoroughly. The card was placed on a flat-bed orbital shaker and

incubated for 2 min at 150±5 rpm. The cards were observed for agglutination without removing from the shaker. A positive and negative control provided by the manufacturer were included in each test. A summary of the results that can be obtained, is indicated in Table 4.1.

Table 4.1 Wellcolex Colour Shigella identification of *Shigella* spp.

Latex reagent*	Particle colour	Species	Serotype covered
1	Red Blue	*S sonnei* *S flexneri*	Forms I and II 1 to 6, X, Y
2	Red Blue	*S. dysentarieae* *S. boydii*	1 to 12 1 to 15

*Wellcolex Colour Shigella uses the principle of coloured latex particle mixtures. It is a two colour system: Red and blue latex particles coated with antibodies to the four different *Shigella* spp. are mixed together to make two different (purple) test reagents. In the presence of homologous antigen, one of the colours will agglutinate. The identity of the antigen is determined by the colour of the agglutination and the resulting change in the background colour.

4.2.5 Pit-stop seminested PCR

For detection of *V. cholerae*, the pit-stop seminested PCR consisted of 10 cycles with primers CTX2 and CTX3, and 1 µl of product thus obtained was subjected to 20 cycles of amplification with primers CTX2 and CTX15, as described in Section 2.2.5.2. In the case of *S. flexneri*, pit-stop seminested PCR was similarly performed with H8 and H15 as the outer primer pair and H8 and H10 as the seminested primer pair, as described in Section 3.2.5.2. Following PCR amplification, the amplicons were analyzed with 2% (w/v) agarose gels (Section 2.2.6), and the sizes of the amplicons were estimated by comparison with a molecular weight marker (pBR 328 DNA cleaved with *Bgl* I and *Hinf* I, Roche Diagnostics).

4.2.6 Analysis of naturally contaminated environmental water samples

A total of 84 water samples were analyzed for the presence of *V. cholerae*, while 48 water samples were analyzed for the presence of *S. flexneri*. Aliquots of the samples (100 ml) were concentrated by membrane filtration after which the membranes were transferred to Schott bottles containing 4 ml of either CDC or GN broth for enrichment of *V. cholerae* and *Shigella* spp., respectively, and then incubated at 37°C for 6 h.

Positive control samples were prepared by seeding duplicate water samples with *V. cholerae* and *S. flexneri* cell suspensions at approximately 10^2-10^3 cells/100 ml and 10^3-10^4 cells/100 ml, respectively. The control samples were processed by the same procedures used for the test samples. Following incubation, 1 ml of the broth was removed from both the test and control samples and used to prepare bacterial lysates for pit-stop seminested PCR analysis.

4.3 RESULTS

4.3.1 Sensitivity of the pit-stop seminested PCR assays following concentration of large volumes of seeded environmental water samples

For monitoring purposes, PCR-based detection of indicator and pathogenic organisms requires not only specificity, but also sufficient sensitivity to ensure the safety of the various water users. For monitoring of potable water quality, 100-ml volumes are typically tested for the presence of indicator and/or pathogenic microorganisms. Protocols based on PCR have previously been developed in this study for the detection of viable *V. cholerae* and *Shigella* spp. In order to assess the feasibility of the overall protocol and to evaluate its sensitivity and robustness with regards to water samples from diverse sources, 100-ml aliquots of different water samples were analyzed after seeding with serially-diluted *V. cholerae* and *Shigella flexneri*, respectively. Following concentration of the samples by membrane filtration and incubation of the membranes in the appropriate enrichment broths for 6 h at 37˚C, the cells from 1 ml of the enrichment broth of each dilution were recovered, lysed and subjected to the pit-stop seminested PCR protocols. Water samples without seeding were analyzed as controls.

The results obtained for toxigenic *V. cholerae* indicated that the minimum number of cells that could be detected in seeded drinking water and seeded treated effluent, were 15 cfu/100 ml and 3 cfu/100 ml, respectively. However, in the case of seeded surface water, 1 cfu/100 ml toxigenic *V. cholerae* could be detected. In contrast, the detection limits obtained for virulent *S. flexneri* in the same types of water samples were slightly lower. While 8 cfu/100 ml of virulent *S. flexneri* could be detected in seeded treated

effluent, 21 cfu/100 ml could be detected in seeded drinking water. A detection limit of 28 cfu/100 ml was obtained in seeded surface water. No amplification products were observed in unseeded water samples, indicating an absence of *V. cholerae* and *Shigella* spp. from the water samples prior to spiking.

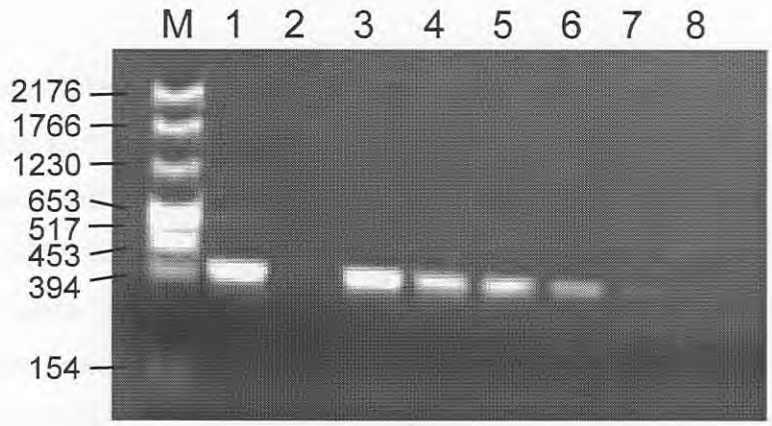

Fig: 4.1 Analysis of the limit of detection upon seeding a surface water sample with serially diluted toxigenic *V. cholerae* following enrichment in CDC broth using the pit-stop seminested PCR protocol for large volume samples. Lane 1, positive toxigenic *V. cholerae* control; Lane 2, negative control. Lanes 3 through 8, dam water seeded with 3.9×10^5 (lane 3), 3.9×10^4 (lane 4), 3.9×10^3 (lane 5), 3.9×10^2 (lane 6), 3.9×10^1 (lane 7), 3.9×10^0 (lane 8) cfu/100 ml. The sizes of the molecular weight marker (lane M) are indicated to the left of the figure.

4.3.2 Examination of environmental samples by culture-based methods and pit-stop seminested PCR assay

A total of 30 water samples were tested in parallel for the presence of toxigenic *V. cholerae* and virulent *Shigella* spp., and the results obtained by bacteriological culturing were compared to those obtained by pit-stop seminested PCR. Although many different conventional culture media and enrichment regimes have been proposed for isolating *V. cholerae* and *Shigella* spp., with some having been reported to be superior to others, no single standardized method exists as yet (Gonzalez, 1995; Donovan and van Netten, 1995; Shears, 1996; APHA, 1998; Lindqvist, 1999). The bacteriological culturing procedures used in this investigation for the isolation of *V. cholerae* and *Shigella* spp. were identical to those routinely used by laboratories in South Africa. Prior to analysis, the water samples were filtered through Moore swabs which were then recovered and incubated in CDC enrichment broth for 12 h at room temperature. These samples were

transported from the CSIR laboratories in Durban to those in Pretoria for subsequent analysis. For 10 of the above samples, the original water samples collected were also supplied. These samples were investigated by pit-stop seminested PCR following concentration of 100 ml of the samples by membrane filtration and enrichment in CDC broth for 6 h.

All of the samples tested negative for *Shigella* spp. in both the bacteriological culturing as well as pit-stop seminested PCR assays (data not shown). In contrast, toxigenic *V. cholerae* could be detected in several of the water samples (Table 4.2). Analysis of the water samples supplied as enrichment cultures, indicated that only 2 of the 30 (7%) water samples were positive for toxigenic *V. cholerae* in culture, as confirmed by biochemical and serological analysis. However, 4 of the water samples tested positive by pit-stop seminested PCR. Analysis of 10 water samples concentrated by membrane filtration prior to enrichment and PCR (Table 4.3) revealed 3 positive samples. Two of these samples were positive in culture, but PCR-negative in instances where the water samples had been concentrated by making use of Moore swabs (Table 4.2). This may have been due to the ineffective concentration capability of the Moore swabs which consisted merely of folded surgical gauze through which samples were poured. In contrast, filtration of 100 ml water samples through 0.45 µm membrane is considerably more effective in trapping the bacterial cells present in the sample. Thus, of the 30 water samples analyzed, using PCR and culturing, a total of 7 samples (23%), tested positive for *V. cholerae*. Of the 7 positive samples, 5 were negative for *V. cholerae* in culture and only one sample was positive for both culture and PCR. Conversely, 1 of the 24 PCR-negative samples was found to be positive for *V. cholerae* in culture. No amplification products could be obtained from two control, seeded water samples. The lack of PCR amplification in these control samples may be due to substances that inhibited the PCR. However, both samples tested negative for *V. cholerae* in culture.

Table 4.2 **Comparison of culture based and pit-stop seminested PCR methods for detection of *V. cholerae* from environmental water samples following filtration through Moore swabs and enrichment**

No	Sample name	Culturing	Seminested PCR	Seeded control samples
1	Gezinsila effluent	-	-	+
2	Gezinsila pond effluent	-	-	-
3	Nyezane river	-	-	+
4	Mnanda river	-	-	+
5	Bumba river	-	-	+
6	Judea river	-	-	+
7	Mpushin river	-	-	+
8	Mabhokweni river	-	-	+
9	Kwamfana river	-	-	+
10	Ithole river	-	-	+
11	Ngwelezana effluent	-	-	-
12	Vondlo river	-	+	+
13	Ofasimba river	-	-	+
14	Mpangeni river	-	-	+
15	Nseleni river	-	-	+
16	Ndabayake river	-	-	+
17	Mhlatuze river	-	+	+
18	Mhlatuze estuary	-	+	+
19	Makwensi foutain	-	-	+
20	Ntuthunga foutain	-	-	+
21	Guncu river	-	-	+
22	Mduki river	-	+	+
23	Mkhiwana river	-	-	+
24	Mvuzana river	-	-	+
25	Mzumbi river	+	-	+
26	Mnambithi river	+	-	+
27	Dikwe river	-	-	+
28	Kwakhomo fountain	-	-	+
29	Mgolomi river	-	-	+
30	Umgeni river	-	-	+

4.3.3 Examination of naturally contaminated environmental water samples

From the above results, it was concluded that the pit-stop seminested PCR assays may be sufficiently sensitive for monitoring purposes. Additionally, results were obtained more rapidly with the PCR assays than with culture and subsequent biochemical and serological assays. Thus, to test the efficacy of the pit-stop seminested PCR assays for monitoring environmental water samples, samples from various sources in South Africa were examined. Aliquots (100 ml) of the samples were filtered through cellulose nitrate filters and following enrichment in CDC broth for 6 h, cell lysates were prepared and subjected to PCR analysis. Control, seeded samples were performed to confirm that chemicals and particulates from the water did not interfere with PCR amplification.

Analysis of 84 environmental water samples for *V. cholerae* by the enrichment broth pit-stop seminested PCR detection protocol, resulted in amplification of amplicons of the expected size from only 5 samples (6%) (Table 4.4). Of the 84 duplicate control seeded water samples, nine samples did not result in an amplification product. These samples consisted of six highly contaminated sewage effluents and three drinking water samples, six showed no amplification product. Three samples consisted of chlorinated tap water, while the other samples were highly contaminated sewage effluents. For all of the test samples that yielded positive results, the duplicate seeded control samples lso yielded amplification products of the expected sizes. The lack of PCR amplification in some of the control samples therefore indicated that these water samples probably contained substances that inhibited the PCR and proves the necessity of including internal controls in order to interpret the negative results.

Table 4.3 Detection of *V. cholerae* in selected water samples by pit-stop seminested PCR following filtration through cellulose nitrate filters and enrichment

No	Sample	Seminested PCR	Seeded control sample
21	Guncu river	-	+
22	Mduki river	+	+
23	Mkhiwana river	-	+
24	Mvuzana river	-	+
25	Mzumbi river	-	+
26	Mnambithi river	+	+
27	Dikwe river	-	+
28	Kwakhomo fountain	-	+
29	Mgolomi river	-	+
30	Umgeni river	+	+

Table 4.4 Detection of *V. cholerae* and *Shigella* spp. in naturally contaminated samples by pit-stop seminested PCR assays

	Tap		River		Dam		Treated sewage effluents	
V. cholerae	No of samples	PCR results	No of samples	PCR results	No of samples	PCR results	No of samples	PCR result
	9	0/9	6	2/6	20	1/20	49	2/49
Shigella spp.	4	0/4	4	0/4	9	2/9	31	1/31

4.4. DISCUSSION

Health risks associated with the waterborne transmission of disease make the detection of pathogenic organisms critical for water quality monitoring. Since many pathogens are present in the natural environment only at low cell densities, a technique to detect low

levels of cells is essential. Filtration methods are typically used to concentrate microorganisms for analysis requiring low detection levels. The analysis is often completed by placing the filter directly on a selective medium and performing a series of biochemical and/or serological tests for specific identification of an indicator microbe or microbial pathogen (Bobb *et al.*, 1981; Lewis and Mak, 1989; De Ryck *et al.*, 1994; Barrett and Feeley, 1994; Lewis, 1997). Alternatively, bacteria can be washed off the filter surface and reconcentrated in a small volume of suspension medium, which is either plated on agar media or prepared for DNA extraction. This procedure allows pre-treatment of samples in order to enhance suppression of background flora and PCR inhibitory substances (Knight *et al.* 1991; Rafii and Lunsford, 1997). However, there can be problems associated with these methods. Enrichment involves competition with other, possibly faster growing organisms in a mixed population, and may result in overgrowth of unwanted bacteria (Arroyo and Arroyo, 1995; Huang *et al.*, 1999). The use of selective media containing inhibitory compounds to eliminate background bacteria may also be inhibitory to environmentally stressed isolates (Arroyo and Arroyo,1996). Biochemical tests and/or serological assays require tedious subculture of numerous individual isolates, and the strain variability for many biochemical assays makes identification questionable, unless a sufficient number of assays are done. In addition, the ability of microorganisms to enter a viable but nonculturable state due to starvation and physical stress may result in failure to isolate these organisms from contaminated water samples by culture techniques, thus usually leading to an underestimation of their numbers (Colwell *et al.*, 1985; Morinigo *et al.*, 1989; Colwell *et al.*, 1990; Byrd et al., 1991; Nilsson *et al.*, 1991; Warner and Oliver, 1998; Dixon,1998).

Since the cost and labour involved in this type of assessment can be prohibitive for many laboratories, methods which could directly detect pathogenic microorganisms in environmental water samples would be preferable. Molecular-based methods, particularly the PCR, are able to achieve the desired sensitivity and reliability without the need to first culture the organism (Bej *et al.*, 1990; 1991a; Oyofo and Rollins, 1993; Toranzos *et al.*, 1993; Juck *et al.*, 1996). By combining filtration and PCR methods, samples contaminated with targeted pathogens and especially viable but nonculturable organisms can be detected within hours, instead of the days required for traditional

biochemical methods (Knight *et al.*, 1991; Palmer *et al.*, 1993; Way *et al.*, 1993; McDonald *et al.*, 1995; Sinigalliano *et al.*, 1995). The most commonly used filters for the filtration of water samples are cellulose nitrate, cellulose acetate and polycarbonate filters (APHA, 1998). However, such filters have been reported to be inhibitory to PCR DNA amplification as DNA amplification is inconsistent in the presence of these filters (Bej *et al.*, 1991a; Oyofo and Rollins, 1993; Juck *et al.*, 1996). The combination of filtration, enrichment and PCR offers the advantage of reducing the negative influence of the membrane filters and complex water matrix on DNA amplification by dilution of the potential inhibitory substances present in the water sample. Also, with this procedure, nonviable cells will not be able to grow during the enrichment step, thereby reducing the risk of false-positives (Maiwald *et al.*, 1994; Dupray *et al.*, 1997; Waage *et al.*, 1999).

The development and evaluation of methods for pathogen detection have focused largely on the use of serially diluted pure culture cells or lysates. Although amplification from pure culture is relatively simple, the true test of a PCR-based method for detection of waterborne pathogens is its robustness and sensitivity in terms of its application to water samples from diverse sources. The sensitivity of the pit-stop seminested PCR was determined with 100-ml volumes of seeded environmental water samples. Depending on the type of water sample, detection limits of 1 cfu/100 ml and 8 cfu/100 ml for toxigenic *V. cholerae* and virulent S. *flexneri*, respectively, could be obtained. The results proved that the protocol developed allows for sensitive detection of the targeted bacteria in different types of water matrixes.

Comparative studies regarding detection of *V. cholerae* and *Shigella* spp. in 30 environmental water samples by bacteriological culturing methods and by pit-stop seminested PCR was undertaken. Analysis of the samples for the presence of *Shigella* spp. yielded no positive results for either of the methods. Since the corresponding control seeded samples yielded amplification products, the lack of these products in the test samples was thought not to be the result of failures in the reactions due to the presence of inhibitors and/or the unavailibility of the DNA, e.g. no bacterial lysis. In contrast, analysis of the samples for *V. cholerae* yielded 6 positive samples by pit-stop

seminested PCR and only 2 positive samples by culturing methods. Thus, culturing appears to be less sensitive than PCR for detection of *V. cholerae* in environmental samples. This may be due to large numbers of other organisms that can outcompete the toxigenic *V. cholerae* on the media, the inability of injured or nonculturable *V. cholerae* to form colonies on the media, low concentrations of viable cells that were below the level of detection by culture, or inhibition owing to bactericidal products produced by other microorganisms. The higher sensitivity of PCR-based methods, in comparison to culture-based methods, has also been reported for other pathogenic microorganisms, including *Legionella* spp. (Frahm and Obst, 1995) and *Shigella* spp. (Islam *et al.*, 2001).

Application of the pit-stop seminested PCR to environmental water samples for the detection of *V. cholerae* and *Shigellae* spp. indicated that these pathogens could be detected in 5 of 84 and 3 of 48 samples, respectively. The low incidence of *Shigella* spp. is in agreement with the current low prevalence of *Shigella* infections (Personal communication, L. Taylor, Addington Hospital, Durban). Some of the water samples exhibited inhibition of the PCR, most notably drinking water and sewage effluent. The inhibition observed for the tap water samples may have been due to the presence of residual chlorine which was not neutralized by sodium thiosulphate prior to analysis. The inhibition observed for the heavily contaminated effluent may have been due to volatile acid by-products derived from the growth of a large number of antagonistic and competing bacteria present in these samples. However, the sensitivity of the PCR for the detection of pathogens in these types water samples may be enhanced by further dilution of sample prior to PCR analysis. This may result in dilution of the PCR-inhibitory substances to a point where they no longer interfere with the assay (DePaola and Hwang, 1995; Weaver and Rowe, 1997).

Rapid tests for identification of *V. cholerae* and *Shigella* spp., such as those described here, will probably complement, not replace, bacterial culture techniques as there will always be a need to culture the organism for serotyping and epidemiological purposes. However, when combined with a cultivation procedure, these pit-stop seminested PCR assays may significantly increase the number of positive results, while reducing the

number of false-negative results. In addition, the methodology may also allow for the processing of a large number of samples in a relatively short period of time.

CHAPTER FIVE

CONCLUDING REMARKS

Despite drinking water being a precious resource and necessary to human health, well-being and socio-economic development, nearly one-half of the world's population suffers from diseases contracted by drinking water of inadequate quality (WHO, 1997). Due to the large number of infectious agents, reservoirs and asymptomatic infected individuals, it may not be possible to eradicate waterborne diseases. However, it may be possible to control these agents as long as they can be detected and monitored. Monitoring of microbial contaminants typically involves filtration and cultivation of indicator bacteria on selective media, followed by colony counting. These traditional methods are laborious, time-consuming and not always sensitive enough to exclude risk of non-specific contamination. In addition, coliforms, the traditional indicators of pathogens, are beginning to fail in some cases by giving misleading information. There is thus a great need for more appropriate methodologies, both for routine monitoring and for investigating disease outbreaks. Due to the rapid advances in biotechnological research, a wide range of new molecular methods have become available. The use of PCR, especially, as a means whereby specific microbial strains in a mixed microbial population can be detected, has become a primary alternative for the detection of pathogens in different types of environmental samples. In this study, PCR technology was used to develop novel approaches aimed at detecting toxigenic *Vibrio cholerae* and *Shigella* spp. with a high degree of sensitivity and specificity. Furthermore, the newly developed detection protocols were modified and their applicability for the detection of toxigenic *Vibrio cholerae* and *Shigella* spp. in environmental water samples was investigated.

Rapid sensitive pit-stop seminested PCR assays for the detection of toxigenic *V. cholerae* (Chapter 2) and virulent *Shigella* spp. (Chapter 3) in pure culture were developed. The performance of these assays were subsequently evaluated using small volumes (1 ml) of environmental water samples seeded with the respective bacterial

pathogens. The *V. cholerae*-specific PCR amplifies sequences within the cholera toxin operon. The PCR procedure coupled with an enrichment culture procedure detected as few as four *V. cholerae* organisms in pure culture. Treated sewage, surface, ground and drinking water samples were seeded with *V. cholerae* and following enrichment, a detection limit of as few as one *V. cholerae* cfu/ml was obtained with amplification reactions from crude bacterial lysates. Similarly, a set of primers specific for the invasion plasmid antigen gene (*ipaH*) of virulent *Shigella* spp. and enteroinvasive *E. coli* produced a 620 bp fragment that was used as template for the seminested primer pair delineating a 401 bp fragment. By using agarose gel electrophoresis for detection of the seminested PCR-amplified products, a detection limit of 1.6×10^3 cfu *S. flexneri* was obtained with amplification reactions from crude bacterial lysates. The PCR procedure coupled with an enrichment culture incubated for 6 h detected as few as 1.6 organisms in pure culture. Small volumes (1 ml) treated sewage, ground, surface and drinking water samples collected from various sources were seeded with *S. flexneri* and incubated in GN broth for 6 h before detection by seminested pit-stop PCR. A detection limit lower than 14 cfu/ml was achieved in some water samples.

To establish the seminested pit-stop PCR assay as a practical tool for routine laboratory use, its performance with regards to the detection of toxigenic *V. cholerae* and entero-invasive *Shigella* spp. in large volumes (100 ml) of seeded and environmental waters was evaluated (Chapter 4). The basic PCR detection protocol was modified to incorporate a membrane filtration step in order to concentrate large volumes of water samples. By making use of seeded environmental water samples, detection limits of as few as 1 cfu/100 ml and 8 cfu/100 ml of toxigenic *V. cholerae* and *S. flexneri*, respectively, were obtained. Comparative analysis of environmental samples using bacteriological culturing methods and PCR indicated that the pit-stop seminested PCR assay was more sensitive for the detection *V. cholerae*. The higher sensitivity of the PCR-based detection methods may be due to its ability to amplify a fraction of the genetic material of the targeted bacteria, even in the presence of mixed microbial populations. In contrast culture-based methods usually rely on the use of selective media which may inhibit growth of the targeted bacteria and these methods may also

yield false-negative results due to the inability of injured or viable, but non-culturable organisms, to form colonies on the media. In the case of *Shigella* spp. the results obtained by seminested PCR and culturing were in agreement, despite the fact that no bacterial cells could be detected in the tested water samples. Inhibiting substances hampered PCR detection only in a very limited number of samples. These consisted mainly of drinking water and heavily contaminated effluents. These problems may be overcome by extracting the DNA prior to analysis or by further dilution of the sample prior to analysis. Application of the pit-stop seminested PCR for the detection of *V. cholerae* and *Shigella* spp. in environmental water samples yielded positive results for 6% of the samples analyzed, indicating that the methods may be suitable for use in the analysis of environmental water samples. In instances where the invasive plasmid antigen gene, *ipa*H, are used as the targeted gene in seminested pit-stop PCR, a positive amplification result maybe due to the presence of shigellae and/or EIEC bacteria as *ipa*H, occurs in both virulent shigellae and EIEC. However, both organisms cause bacillary dysentery and their presence in water is highly undesirable.

Although molecular technologies, such as those developed in this study, present potential new tools for assessing microbial quality of water, their widespread application to water may depend on several factors. For example, the detection costs must be low and the benefits must outweigh the continued use of conventional methods; the molecular methods must be specific for the microorganisms of concern, which means specifically being able to detect live organisms capable of causing disease; and the sensitivity must be adequate to provide protection against waterborne disease, which means being able to concentrate targets for detection from large volumes of water and to overcome interfering factors that may be present so as to detect very low numbers of microorganisms. The results obtained during the course of this investigation have shown that it is possible to detect pathogenic bacteria from the water samples within 12 hours. The combination of membrane filtration, an enrichment procedure and the pit-stop seminested PCR provided a sensitive, specific and easy method for the detection of vaible *V. cholerae* and *Shigella* spp. in environmental water samples.

CHAPTER SIX

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Papers published and congress contributions during the course of this study

Papers published:

Detection of toxigenic *Vibrio cholerae* from environmental water samples by an enrichment broth cultivation-pit-stop semi-nested PCR procedure. 2000. Theron, J., Celliers, J., du Preez, M., Brözel, V.S. and Venter, S.N. *J. Appl.Microbiol.* 89:539-546.

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