

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 immunosuppressive 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		
<i>Vibrio cholerae</i>	Cholera	High
<i>Salmonella typhi</i>	Typhoid, Salmonellosis	High
Enteropathogenic <i>E.coli</i>	Gastroenteritis	High
<i>Campylobacter jejuni</i>	Gastroenteritis	High
<i>Shigella dysenteriae</i>	Dysentery	Low
<i>Yersinia enterocolytica</i>	Yersiniosis	High
Viruses		
Enteroviruses: Poliovirus, Enterovirus,	Poliomyelitis	Low
Echovirus, Coxsackievirus	Gastroenteritis, meningitis, heart anomalies	Low
Hepatitis 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		
<i>Giardia lamblia</i>	Giardiasis, diarrhoea	Low
<i>Cryptosporidium parvum</i>	Diarrhoea	Low
<i>Entamoeba histolytica</i>	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 CHOLERAЕ

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

*Nontoxicogenic 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 GM₁, 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, A₁ and A₂. The A₁ 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 (Sansoneetti, 1999). Ingestion of *Shigella* spp. typically produces acute gastroenteritis and dysentery. Illness 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 (*lpa*; 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 *lpa* 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 (Sansoneetti *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 (*stxA* and *stxB*) are located on the chromosome and comprise a single operon (Sansone *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 reaction. Subsequently, numerous techniques for separating 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; Oyofe 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.