THE OCCURRENCE OF EMERGING VIRAL, BACTERIAL AND PARASITIC PATHOGENS IN SOURCE AND TREATED WATER IN SOUTH AFRICA

SN Venter

WRC Report No. 1031/1/03



Water Research Commission



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Report to the

Water Research Commission

by

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WRC No.:1031/1/03
ISBN No.: 1-86845-990-X

February 2003

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This report emanates from a project financed by the Water Research Commission (WRC) and is approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the WRC or the members of the project steering committee, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Printed by Silowa Printers

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EXECUTIVE SUMMARY

Background and motivation

At the end of the 20th century the scientific community has, after years of complacency, again come to appreciate the significance and extent of infectious diseases. Apart from an increase in the number of cases of diseases such as malaria, rabies, diphtheria, tuberculosis and streptococcal infections, infections caused by emerging pathogens are also of great concern. Among the most prominent emerging infectious diseases are AIDS, the hemorrhagic fevers, Hanta and West Nile virus infections, cryptosporidiosis, and infections by bacteria such as *E. coli* O157:H7, *Vibrio choleae* O139 and *Bartonella*..

Emerging water-borne diseases are particularly serious because it could place an entire community at risk. In 1993, *Cryptosporidium*, caused one of the largest recognized waterborne disease outbreaks in Milwaukee, USA with an estimated 403 000 persons affected (MacKenzie *et al.*,1994). Clinical and epidemiological data on the occurrence and transmission of these diseases are often insufficient. Less or even no information is available on the presence of these pathogens in the source and treated water utilized by both developed and developing communities. An urgent need, therefore, exists for the detection and quantification of emerging pathogens in water in order to be able to determine the role played by water-borne pathogens and to control and minimize the human health risk.

Research Objectives

The broad objectives of this project were to select specific examples of emerging viral, bacterial and parasitic pathogens, to establish techniques for their detection in water and to monitor a number of source and treated water samples from different localities in South Africa for their presence.

The specific aims as described in the original proposal was:

1) To verify the selection of emerging pathogens chosen for this study,

2) To establish techniques for the specific detection and possible quantification of the following emerging pathogens in source and treated water:

Campylobacter spp and *Helicobacter pylori*;

Cyclospora cayetanensis; (*The microsporidia)*

Hepatitis E and Astroviruses;

3) To compare environmental isolates of *Campylobacter* with clinical isolates in order to establish the importance of water in the transmission of these pathogens.

4) To establish the developed detection techniques in the laboratories of participating organizations and other historically disadvantaged institutions.

5) To monitor various types of source and treated water for the presence of the above-mentioned emerging pathogens. Areas to be monitored will include the Western Cape, Free State, Gauteng, and Kwazulu-Natal.

6) To formulate future research needs and management strategies with regard to the above-mentioned emerging water-borne pathogens should it be found that these organisms pose a possible health risk to water users.

Summary of major results and conclusions

From the results of this study it can be concluded that the emerging pathogens are most likely to be found in surface and waste waters. During this study none of the pathogens were detected in drinking and ground water. Although the results of this study suggest a low incidence of the selected emerging pathogens in various water sources care should be taken when interpreting the results. The sensitivity and reliability of a number of these techniques were highly unsatisfactory. At present the only method suitable for routine monitoring of water samples would be the one developed for the detection of the human Astroviruses.

In this study a HAstV-specific RT-PCR oligonucleotide probe assay was used for the detection of HAstVs in water samples. This method which combines a molecular assays and cell culture propagation was EM and EIA normally used for the detection of HAstVs in environmental water samples.HAstVs were only detected in surface and waste water once from each geographical region during the late winter sampling period. The low prevalence of HAstVs

noted in this study corresponds with recent reports of HAstVs in river and dam waters in Gauteng.

The development of a reliable technique for the detection of HEV is still under investigation as the nested-PCR still needs to be optimized for the testing of water samples. Seroprevalence studies however suggest that HEV is endemic in the swine populations in South Africa and that zoonotic transfer of the viruses might be one of the routes of infection.

The enrichment approach used for the detection of *Campylobacter* was much more efficient than the optimized modified Cape Town Protocol for use with environmental waters. Of the 22 potential *Campylobacter* isolates obtained after enrichment only 3 true *Campylobacter* species were isolated, the rest of the isolates belonged to the genus *Arcobacter*, which forms part of the broader *Campylobacter* group. The majority of the isolates were from surface water with only six isolated from sewage. No isolates were obtained from ground and drinking water.

A novel seminested PCR assay for the detection of *H. pylori* in environmental water samples has been developed .Under the tested parameters, the equivalent to 5×10^2 bacteria, were detectable by the seminested PCR assay. *H. pylori* was not detected in any of the samples tested but this does not necessarily indicate that they were absent from the tested samples.

Staining procedures used for diagnosis of microsporidian spores in clinical specimens are not specific or sensitive enough for environmental water samples. Inhibition of the PCR method by some of the surface water samples had a negative impact on the on the sensitivity of the method. Cultivation of microsporidia in cell culture as a means of detection is at present too labourious and lengthy and is prone to failure due to contamination with specimens from non-sterile sources.

Achievement of objectives and recommendations for future research

The objectives were achieved with varying levels of success, depending on the specific pathogen targeted. Although detection techniques were established for all of the selected pathogens apart from Hepatitis E virus, the sensitivity of a number of these techniques were highly unsatisfactory.

Due to the lack of reliable detection methods, the objective to transfer the technology to participating and interested laboratories could not be achieved. At present the only method suitable for routine monitoring of water samples would be the one developed for the detection of the human Astroviruses, but few labs have the infrastructure and capacity to perform this type of analysis.

If detected, most of the pathogens were only present in a limited number of samples. The significance of these results is difficult to assess and further studies are required to elucidate the actual risk of infection and to develop suitable management strategies.

This study has shown that a great need still exists for reliable and effective methods for the detection of emerging pathogens in water. It has clearly shown that the methods developed for clinical use are often not able to deal with the complexities of environmental samples. Although progress has been made in the adaptation of a number of detection methods for environmental use, this study has highlighted a number of areas where further research is still required. Detection, cultivation, and identification techniques for Hepatitis E virus, *Helicobacter pylori*, *Campylobacter* and *Arcobacter* species as well as the microsporidia still need further development.

Due to the above-mentioned lack of reliable methods for the detection of Hepatitis E virus, *Helicobacter pylori* as well as the microsporidia the occurrence of these pathogens could not be determined. In terms of human Astroviruses the current information suggests a sporadic occurrence mainly associated with winter months. The information also suggests that *Campylobacter* spp. are less abundant in environmental waters than *Arcobacter* spp. *Arcobacter* spp. have been detected at levels as high as 10^4 organisms per ml of water but the health implications of these finding still need to be established. It is, however, encouraging to notice that in all cases where emerging pathogens were detected, faecal coliform data already indicated serious water quality problems. This suggested that current water quality guidelines and management procedures based on normal microbial water quality indicators should provide the necessary protection.

1 RECOMMENDATIONS FOR FUTURE RESEARCH

- As HAstVs were detected in only one sample from each geographical region the significance of these results is difficult to assess and further studies are required to elucidate the actual risk of infection.
- Molecular techniques for the detection and comparison of Hepatitis E virus in the stool and water samples still need to be developed.
- More work has to be done to refine the use of enrichment media for testing environmental samples for the presence of *Campylobacter*.
- A molecular method for identifying all *Campylobacter* and *Arcobacter* species from environmental waters is needed.
- There is a great need for further research into and the development of an effective isolation method for *H. pylori* based on culturing procedures.
- There is still a need for an effective method for the preparation of environmental samples to be analysed for the presence of microsporidia spores using tissue culture.

2 PUBLICATIONS

Technology transfer, scientific outputs and data availability

Based on the research of this project the following presentations were made at local and international conferences:

S. M. Diergaardt, S. N. Venter, J. Theron, P. A. Gouws and V. S. Brözel Detection of campylobacters from environmental water in South Africa. Campylobacter, Helicobacter and Arcobacter. Summer conference of the Society for Applied Microbiology, Glasgow, Scotland, 11 - 13 July 2000.

S. M. Diergaardt, M. Chalmers, S.N. Venter, J. Theron, P.A. Gouws and V. S. Brözel. Development of a method for the detection and recovery of campylobacters from water. BioY2K combined millenium meeting, Grahamstown, 23 – 28 January 2000.

S. M. Diergaardt, A. Spreeth, S. N. Venter, J. Theron, P. A. Gouws and V. S. Brözel Adaptation of the Cape Town protocol for environmental isolation of Campylobacter. *Campylobacter* 2000. University of the Western Cape. Bellville, South Africa, August 2000.

The original data are available from the following institutions:

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ACKNOWLEDGEMENTS

The project team would like to acknowledge the contribution of the following people and institutions:

The Water Research Commission for funding this project.

The Steering Committee for their involvement and advice:

Ms A P M Moolman, Water Research Commission (Chairperson)

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Dr M L van der Walt, Water Research Commission, (Committee Secretary)

Mr M C Nel, Magalies Water

Prof O L Obi, University of Venda

Everyone who has helped in the execution of the project and the preparation of the report. A special word of thanks should go to all those who help with the collection and transport of the samples.

Department of Finance, University of Pretoria, who had to manage a number of sub-contracts.

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
Biochem ID	Biochemical identification
bp	base pairs
cfu	colony forming units
CLO	*Campylobacter*-like organisms
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleic-5'-triphosphate
HAstVs	Human Astroviruses
HEV	Hepatitis E Virus
HIV	Human immunodeficiency virus
ISO	International Organization for Standardization
KZN	Kwazulu Natal
LB	Luria Bertani broth/ agar
mCCD	modified charcoal cefoperazone deoxycholate
mCCDA	modified charcoal cefoperazone deoxycholate agar
mFC	membrane filtration method for fecal coliforms
MM	molecular mass
NCBI	National Centre for Biotechnology Information
ND	not detected
NT	not tested
PCR	Polymerase Chain Reaction
rDNA	ribosomal deoxyribonucleic acid
RK	Rabbit Kidney
TBA	Tryptose Blood Agar
TCA	tricarboxylic acid cycle
TNTC	too numerous to count
TSI	Triple Sugar Iron agar
UHQ	ultra high quality water
UVB	ultraviolet B rays

INTRODUCTION

CHAPTER 1

EMERGING WATER BORNE-DISEASES

S N Venter

1.1 EMERGING INFECTIOUS DISEASES

At the end of the 20th century the scientific community has, after years of complacency, again come to appreciate the significance and extent of infectious diseases which remain the leading cause of deaths worldwide (NIAID, 1992). Apart from an increase in the number of cases of diseases such as malaria, rabies, diphtheria, tuberculosis and streptococcal infections, infections caused by emerging pathogens are also of great concern. According to the Institute of Medicine, emerging diseases are diseases of infectious origin whose incidence have increased within the last two decades or threaten to increase in the near future (Institute of Medicine, 1992). Among the most prominent emerging infectious diseases are AIDS, the hemorrhagic fevers, Hanta and West Nile virus infections, cryptosporidiosis, and infections by bacteria such as *E. coli* O157:H7, *Vibrio choleae* O139 and *Bartonella.* (Centers for Disease Control and Prevention, 1994). Although not restricted to immuno-compromised individuals, these diseases often have serious consequences for persons with lowered immunity such as people infected with HIV, small or malnourished children, the elderly as well as persons under treatment for cancer.

The dramatic growth in the world's population has directly or indirectly been one of the main factors leading to the emergence or re-emergence of these diseases. Most of the population growth has occurred in developing countries and has resulted in unplanned and uncontrolled expansion of urban areas. Millions of people ended up living in crowded shanty towns without access to basic services. It is therefore not surprising that apart from person to person contact, a number of the emerging pathogens can be linked to transmission by contaminated water or food. The increasing demand for food has lead to changes and intensification of agricultural practices, resulting in an increase in the importance of zoonotic and vector-borne diseases. Improved travel

and food exports and the displacement of people by conflicts have also increased the risk of these diseases spreading rapidly between countries and continents (Lauria, 1998, Gubler 1999).

Scientists are only beginning to appreciate the importance of other factors in the emergence of infectious diseases. Climatic factors such as global warming and the El Nino phenomena have been suggested of playing an important role. The ability of the microorganisms to adapt to changing environments should also not be underestimated as is clearly illustrated by their ability to development of antibiotic resistance (Lauria, 1998, Gubler 1999).

Water-borne diseases are particularly serious because it could place an entire community at risk. In 1993, *Cryptosporidium*, caused one of the largest recognized waterborne disease outbreaks in Milwaukee, USA with an estimated 403 000 persons affected (MacKenzie *et al.*,1994). In South Africa, as in many other parts of the world, less than half of the cases of gastroenteritis can be attributed to a specific microorganism, toxin or chemical (Schaefer, 1997). It is believed that a substantial number of the unattributable case could be linked to emerging infectious disease agents. Clinical and epidemiological data on the occurrence and transmission of these diseases are often insufficient. Less or even no information is available on the presence of these pathogens in the source and treated water utilized by both developed and developing communities. An urgent need, therefore, exists for the detection and quantification of emerging pathogens in water in order to be able to determine the role played by water-borne pathogens and to control and minimize the human health risk (Grabow, 1996).

1.2 MOTIVATION FOR THE SELECTION OF PATHOGENS

1.2.1 Project aim

The aim of this project was to select specific examples of emerging viral, bacterial and parasitic pathogens, to establish techniques for their detection in water and to monitor a number of source and treated water samples from different localities in South Africa for their presence.

The selection of emerging pathogens relevant to South Africa and its social situation and

environmental conditions was one of the challenges of this study. During the initial project proposal the following pathogens were proposed for inclusion in this study:

Astro and Hepatitis E virus;

Campylobacter spp. and *Helicobacter pylori* ; and

Cyclospora cayetanensis.

A short motivation for the selection of these pathogens and reasons for the substitution of *Cyclospora cayetanensis* for the microsporidia are described in the following three sections.

1.2.2 Viruses

Some of the enteric viruses are among the more recently recognized water-borne pathogens. Rota, Norwalk (and related viruses) and Astroviruses were all first described in the 1970s. Astroviruses have been associated with acute gastroenteritis, mainly in children and the elderly and have a worldwide distribution. The transmission of the viruses is mostly limited to humans and animal to human transmission is believed to be uncommon. In contrast Hepatitis E virus has only been associated with large outbreaks in developing countries in Africa, Asia and in Mexico (Moe, 1997). Recent reports have suggested that the virus could infect a number of animals such as swine, goats and sheep and there might be a zoonotic element to the transmission of the diseases (Meng *et al.*, 1998). Astro and Hepititis E virus have been selected for inclusion in this study because the Department of Medical Virology, University of Pretoria has already done some preliminary work to develop methods for the detection of these two viruses.

1.2.3 Bacteria

During the last two decades a number of bacterial pathogens have gained greater importance as the cause of possible water-borne infections. Included in this group are the pathogenic E. coli, Listeria monocytogenes, Mycobacteria spp, Yersinia enterocolitica, Aeromonas spp, Campylobacter spp, Helicobacter pylori and Plesiomonas shigelloides. Of these Campylobacter has gained considerable importance and Campylobacter enteritis is considered to be one of the major bacterial diseases transmitted by water in the developed world (Skirrow and Blaser, 1992).

Campylobacter enteritis is caused by a number of Campylobacter species, including the better known thermophilic species such as C. jejuni, C. coli and C. upsaliensis. Asymptomatic and multiple infections are common and the incidence peaks in young adults and children. A number of the species are found in the normal intestinal flora of many domestic and wild animals and birds, but they are also associated with diseases of animals. Poultry is still considered to be the main source of human infections (Lior, 1996) but the direct and indirect role played by water during transmission are also of importance.

Members of the genus Arcobacter share the same general features with Campylobacter and have been isolated from poultry as well as water samples. Their ability to grow aerobically and at temperatures lower than those most suitable for Campylobacter allow for better survival of these bacteria in the environment (Wesley et al., 2000). Arcobacter butzleri has been associated with diarrheal illnesses in humans and primates (Lenner et al., 1994)

It is estimated that the incidence of Campylobacter enteritis in developing countries could be several orders of magnitude higher than what is reported for developed countries (Taylor, 1992). Campylobacter enteritis occurs frequently in South African infants (le Roux and Lastovica, 1998) but whether water or food constitute the major source of infection is still unclear and Campylobacter was therefore selected as the bacterial pathogen to be investigated during this study.

Helicobacter pylori, another bacterium closely related to the genus Campylobacter was also selected for inclusion in this study. This organism is strongly associated with gastritis, peptic and duodenal ulcers and gastric cancer (Graham, 1991; Dunn et al., 1997). Its mode of transmission is still unresolved but observations support among others a possible fecal-oral route of infection. (Feldman et al., 1997; Stone, 1999).

1.2.4 Parasites

In recent years, especially after the onset of the AIDS epidemic, a number of formerly unknown parasites have been linked to human diseases (Ortega, et al 1992; Casemore et al., 1985). Although primarily associated with immuno-compromised individuals many of these parasites

have also been diagnosed in healthy children and adults. Among the emerging parasites the sporeforming protozoa eg. Cryptosporidium, Isospora, Cyclospora and the microsporidia are among the most prominent genera or groups of parasites. The first reports of human cases of intestinal cryptosporidiosis were reported about 25 years ago and the documentation of Cyclopsora and the microsporidia followed even later (Nime et al, 1976; Sterling and Ortega, 1993; Desportes et al, 1985).

Apart from the abovementioned emerging parasites, a number of other parasites have also been linked with water-borne transmission. Included in this group is another of the sporozoa Toxoplasma, the flagellate Giardia, the amoeba Entamoeba and Naegleria and the ciliate Balantidium. Blastocystis hominis have also been implicated in gastrointestinal infections but some researchers have however, expressed their doubts as to whether this parasite can really be classified as a pathogen (Schaefer, 1997).

After careful consideration it was decided that the one of the spore forming parasites should be selected for this study as their spores or oocysts are able to resist stressful environmental conditions. Some of these parasites even have the ability to survive a number of the common disinfection processes used for drinking water treatment.

During the initial phase of the study contact has been made with a number of South African pathologists or parasitologists to establish whether these parasites have frequently been reported in clinical samples. According to them, the highest incidence has been noted for Cryptosporidium. This parasite has already been fairly well studied in South Africa during the last decade and it was decided to focus on another of these parasites. According to the scientists Isospora has been noted infrequently and Cyclospora has only been reported once in South Africa. Although there have been overseas reports on water-borne transmission of toxoplasmosis, the primary route of Toxoplasma gondii infections in humans is still through infected meat or contact with pets (Tenter et al., 200). No information was available on the presence of the microsporidia but everybody agreed that due to their small dimensions they could have been easily overlooked.

Taking all the above-mentioned factors into account, as well as the inability to propagate Cyclospora cayetanensis or even find positive stool samples it was decided to rather focus on the occurrence of the microsporidia. The microsporidia are eukaryotic obligate intracellular protozoan parasites, associated with infections of every major animal group, especially insects, fish and mammals (Weiss and Vossbrinck, 1998). In humans the majority of cases have been attributed to infection by Enterocytozoon bieneusi but the precence of Encephalitozoon (Septata) intestinales has also been reported. Most of these infections have been linked to HIV infected patients, but a few cases have been noted where the patient was not infected with HIV. In most of these cases the affected patients lived in or had traveled to tropical or sub-tropical areas (Franzen and Müller, 1999).

At present there is no clear evidence that microsporidiosis in humans could be a zoonosis but the presence of some of these pathogens has been noted in other mammals such as dogs, pigs and cows. Person to person transmission still seems to be the main route of infection. Enterocytozoon bieneusi and Encephalitozoon intestinales have also been detected in a variety of environmental water samples and there are strong suggestions that the transmission could also be water-borne (Dowd et al., 1998).

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VIRUSES

CHAPTER 2

THE DETECTION OF HUMAN ASTROVIRUSES IN WATER SAMPLES

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2.1 INTRODUCTION

Astroviruses (AstVs) are small, non-enveloped RNA viruses and are one of the increasing number of viruses aetiologically associated with gastroenteritis in humans (Bern and Glass, 1994; Kurtz, 1994). Human astrovirus (HAstV) infection has been reported in all age groups with the very young, the elderly and the immunocompromised at greatest risk (Glass *et al.*, 1996). The clinical relevance of HAstV infection has come to the fore with reports that, next to rotavirus, HAstVs are the second most common cause of viral gastroenteritis in children in Thailand (Matsui and Greenberg, 1996), France (Bon *et al.*, 1999) and in selected settings in South Africa (Marx *et al.*, 1998a; Steele *et al.*, 1998). AstVs are typically transmitted via the faecal-oral route, and outbreaks have been associated with the consumption of sewage-polluted shellfish and ingestion of water from contaminated streams (Cubitt, 1991; Kurtz, 1994).

Until recently, direct electron microscopy (EM) and immune EM (IEM) were the only methods of detection available to many laboratories. The limit of detection of EM is a viral concentration of about 10^6 particles/ml (Bennet *et al.*, 1995; Metcalf *et al.*, 1995), and EM detection may fail because only 10% of the particles have the characteristic stellate structure (Willcocks *et al.*, 1992). The rapid and accurate detection of HAstVs in faecal specimens was enhanced by the development of enzyme immunoassays (EIA) (Herrmann *et al.*, 1990; Moe *et al.*, 1991), but these are not sensitive enough for the detection of low titres of HAstVs present in environmental waters. In addition HAstVs cannot readily be isolated in conventional cell cultures (Kurtz, 1994). However, the human primary liver carcinoma cell line (PLC/PRF/5) and human colonic carcinoma cell line (CaCo-2) have been shown to support productive growth in the presence of trypsin (Willcocks *et al.*, 1990; Marx *et al.*, 1995; Taylor *et al.*, 1997). The detection of astroviruses, even at very low concentrations (10 ng) (Cook and Myint, 1995), was further improved with the advent of molecular technology, specifically the reverse transcriptase-

polymerase chain reaction (RT-PCR) (Jonassen *et al.*, 1993, 1995; Mitchell *et al.*, 1995; Saito *et al.*, 1995) and hybridisation (Pintò *et al.*, 1995, 1996). These molecular assays have facilitated the accurate detection and characterisation of astroviruses from both human faecal specimens (Mitchell *et al.*, 1995; Marx *et al.*, 1998a) and water samples (Pintò *et al.*, 1995, 1996; Marx *et al.*, 1997, 1998b; Taylor *et al.*, 2001). Using a glass wool adsorption-elution recovery technique (Grabow and Taylor, 1993) and a set of primers which reportedly detected only HAstV, HAstVs have also been detected, by RT-PCR, in selected environmental water samples in the Gauteng region, South Africa (Marx *et al.*, 1998b; Taylor *et al.*, 2001). Prior amplification of the virus in cell culture was found to enhance the detection of HAstVs with the added benefit of detecting viable viruses (Marx *et al.*, 1995, 1998b; Taylor *et al.*, 2001).

The purpose of this investigation was firstly to establish the techniques for the recovery and detection of HAstVs from different water sources, and secondly to apply these techniques to the monitoring of source and treated waters from four different regions in South Africa.

2.2 SAMPLING SITES

The sampling sites selected for the investigation and their laboratory codes are presented in Table 1. Ten litre samples were drawn at each site and forwarded to the Dept of Medical Virology, University of Pretoria, for astroviral analysis. There were three sampling periods, namely late March to early May, July to August and October to November, to cover any possible seasonal variation in the occurrence of HAstVs in the water.

2.3 METHODS AND MATERIALS

2.3.1 Viral Recovery

HAstVs are recovered from ±10 ℓ water samples by a glass wool adsorption-elution procedure (Grabow and Taylor, 1993). Viruses from the 100 mℓ eluate were recovered using a polyethylene-glycol/sodium chloride technique as described by Minor (1995). The final 15 ml concentrate was treated with antibiotics (PSN Antibiotic Mixture [100x] and Nyasatin

Table 2.1: Samplings sites and laboratory codes for the different geographical regions.

REGION	SAMPLING SITE	LAB. CODE
Western Cape	Surface water/Kuilsrivier (off N2)	K1
	Surface water/Kuilsrivier (Baden-Powell Drive)	K2
	Drinking water/Stellenbosch (Gouws household)	K3
	Drinking water/Stellenbosch (UWC Food Micro. Dept)	K4
	Surface water/Kuilsrivier (Monwabizi)	K5
	Surface water//Stellenbosch (Plankenberg River)	K6
	Waste water/Athlone	K7
	Surface water/Kuilsrivier (Stellenbosch Aterial)	K8
Kwazulu/Natal	Surface water/Slangspruit river 61	U1
	Surface water/Dusi river above Darvill 66.2	U2
	Surface water/Darvill STW Final Effluent 626	U3
	Waste water/Darvill STW Raw 700	U4
	Saline surface water/Umgeni Estuary 28.1	U5
	Drinking water/Craigieburn WW final	U6
	Ground water/Dairy Top borehole	U7
	Surface water/Mngeweni river (below abbatoir	U8
	discharge) 22	U9
	Waste water/Hammarsdale STW Raw 598	U10
	Surface water/Hammarsdale STW final effluent 599	
Gauteng	Surface water/Rietspruit RV2	R1
	Drinking water/Klipwater (treated, distribution endpoint)	R2
	Surface water/Klipriver K25	R3
	Surface water/Blesbokspruit B10	R4
	Surface water/Vaal River (Riviera)	R5
	Drinking water/Sandton (treated, distribution endpoint)	R6
	Surface water/Klip River K19	R7
	Surface water/Vaal Dam A18	R8
Free State	Surface water/Urban Runoff (polluted stream)	S1
	Treated wastewater effluent/poorly treated	S2
	Surface water/downstream of urban stream (S1) inflow	S3
	Surface water/below recreational dam wall (downstrean	S4
	from Botshabelo)	
	Surface water/Urban runoff (outside Bloemfontein,	S5
	receiving all city effluent and runoff)	
	Treated wastewater/well treated in new facility	S6
	Drinking water/storage container (contains biofilm	S7
	residue)	S8
	Tap water/communal stand pipe (chlorinated municipal	
	water - sample has been dechlorinated)	S9
	Ground water/unprotected borehole (arterial)	S10
	Ground water/unprotected borehole (arterial)	

Suspension [100x]; GIBCO BRL, Life Technologies Ltd., Paisley, Scotland) and 10µg/ml trypsin (Trypsin 250: Difco, Detroit, MI) prior to inoculation onto cell culture.

2.3.2 Cell culture amplification

Cultures of PLC/PRF/5 (ATCC CRL 8024) and CaCo-2 (ATCC HTB 37) cells are used for the propagation of HAstVs. Using standard cell culture techniques the PLC/PRF/5 cells are propagated in Earle's minimal essential medium with Earle's salts, L-glutamine and containing non essential amino acids (Highveld Biological [Pty] Ltd., Kelvin, South Africa), 60µg/ml tylocine (anti-PPLO, GIBCO BRL), penicillin (100U/ml) and streptomycin (100µg/ml)(GIBCO BRL)(MEM) and supplemented with 5-7% foetal calf serum (FCS)(Highveld Biological). The same medium, but without the addition of tylocine and supplemented with 10-15% FCS, was used for the CaCo-2 cells. For infection of the cells, 25cm^2 flasks are seeded with 4 ml of 10^5cells/ml and incubated at 37°C overnight. The propagation media is then withdrawn from the semi-confluent monolayers which are washed once with phosphate buffered saline before starving for 60 min at 37°C in serum-free MEM. After withdrawal of the serum-free MEM the starved cells are inoculated with 1 ml of the treated water sample concentrate. After adsorption for 60 min, with rotation every 15 min, at 37°C 5 ml serum-free MEM containing 10µg/ml trypsin (Trypsin 250: Difco) is added to each flask and the infected cells.

2.3.3 RNA extraction

Total RNA was extracted from 140 µl of the concentrated water samples using QIAamp spin columns (QIAamp viral RNA extraction kit; QIAGEN, Hilden, Germany) or Trizol™ reagent (GibcoBRL-Life Technologies Ltd.) according to manufacturer's instructions. Harvests of cell culture suspensions (1.5–2 mℓ) were concentrated by ultracentrifugation and the cell culture concentrates homogenised using a biopolymer shredding system (QIAshredder™, QIAGEN) prior to RNA extraction using QIAamp spin columns (QIAamp viral RNA extraction kit; QIAGEN). The extracted RNA was resuspended in a final volume of 20 µl sterile water.

2.3.4 RT-PCR amplification

To exclude the possibility of cross-contamination, all reagents used for RT-PCR were prepared in a laminar flow cabinet. The RT-PCR reactions were carried out in rooms separate from those used for the isolation of the viruses and processing of the specimens. Unless stated to the contrary all reagents used for RT-PCR were from Roche Molecular Biochemicals, Mannheim, Germany.

Primers: A published set of primers, with a predicted size of 89 base pairs (bp), are used (Mitchell *et al.*, 1995). These primers, designed to amplify regions in the highly conserved 3' end of the HAstV genome, reportedly detect all known HAstV serotypes.

Reverse transcription (RT): RT was performed in a 30 µl reaction mix containing 5 µl extracted RNA, 1 X avian myeloblastosis virus (AMV) buffer (50mM Tris-HCl [pH8.3], 8.3mM $MgCl_2$, 30mM KCl, 1mM dithiothreitol); 0.5mM dNTPs; 0.5µM reverse primer; 40 units RNase inhibitor and 10 units AMV reverse transcriptase. The reaction mix was incubated at 42°C for 1 h.

Polymerase chain reaction (PCR): The amplification reaction mixture and cycling programme for the primers have been described previously (Marx *et al.*, 1997; Taylor *et al.*, 2001)

2.3.5 Polyacrylamide gel electrophoresis (PAGE) and hybridisation analysis

PAGE: Twenty microlitres of the RT-PCR products were analysed by PAGE and visualised by ethidium bromide staining and UV illumination. The cDNA amplicons which co-migrated with that of the HAstV-1 control were considered to be positive for HAstV.

Probe preparation: A 30 bp positive sense oligonucleotide probe, designed by Marx *et al.* (1998), was non-radioactively labelled with digoxigenin (DIG)-dUTP using the DIG oligonucleotide 3'-end labelling kit (Roche Molecular Biochemicals).

Dot-blot hybridisation: Five microlitres (6.25%) of the total RT-PCR product was denatured at 100°C for 10 minutes and maintained on ice before application to a nylon membrane (Hybond$^+$) by dot-blot microfiltration (Bio-Dot Apparatus, Bio-Rad Laboratories, Hercules, Ca). The membranes were air-dried and the cDNA was fixed to the membrane by UV cross-linking for 5 min followed by baking at 80°C for 15 min. Pre-hybridisation was carried out for at least 2 h at 55°C in 50 µl hybridisation solution/cm^2 membrane (hybridisation solution: 6 X saline sodium citrate [SSC], 5 X Denhardt's reagent [Sambrook *et al.*, 1989], 16mM Tris-HCl [pH 8.3]; 10 µg/ml denatured salmon sperm DNA and 0.1% sodium dodecyl sulphate [SDS]). Hybridisation was done overnight at 55°C in the same pre-hybridisation solution with the addition of 30 pMol DIG-labelled probe. Hybridised membranes were washed twice with 6 X SSC and 0.01% SDS for 15 min each at 65°C. Immunological and chemiluminescent detection of the PCR amplicon-oligonucleotide probe hybrids are subsequently done using the DIG nucleic acid detection kit (Roche Molecular Biochemicals) and DIG wash and block buffer set (Roche Molecular Biochemicals) according to the manufacturer's instructions. All RT-PCR DNA amplicons not detected by dot-blot hybridisation were considered to be negative for HAstV.

2.4 RESULTS

The AstV RT-PCR results for the water samples from the different geographical regions are summarised in Table 2. From these results it is evident that AstVs were only detected in a limited number of samples, i.e. one from each geographical region, and then only during the late winter (July to August) sampling period.

2.5 DISCUSSION

Faecally contaminated water has been implicated in the transmission of HAstVs (Cubitt, 1991) but the actual role and impact of waterborne transmission of HAstVs still needs to elucidated (Glass *et al.*, 1996; Taylor *et al*, 2001). In this investigation a sensitive HAstV-specific RT-PCR oligonucleotide probe assay (Marx *et al.*, 1998b; Taylor *et al.*, 2001) was used for the detection of HAstVs in a number of different water sources from different geographical regions in South Africa. This combination of molecular assays and cell culture propagation has been shown to be more sensitive than EM and EIA for the detection of HAstVs in environmental water samples (Marx *et al.*, 1998b). As HAstVs were detected in only one sample from each geographical region the significance of these results is difficult to assess. These results, i.e. the low prevalence of HAstVs in environmental water samples, are however similar to those recently reported for river and dam waters in Gauteng (Taylor *et al.*, 2001), and further studies are required to elucidate the actual risk of infection.

Table 2.2: Results on the astroviral analysis on the water samples from the different sampling sites in the four geographical regions during the three sampling periods.

ASTROVIRUS POSITIVE SAMPLES			
REGION and SAMPLING SITE	SAMPLING PERIOD : 2000 LATE MARCH - MAY	JULY - AUGUST	OCTOBER – NOVEMBER
Western Cape			
Surface water K1	-	-	Nd
Surface water K2	-	+	Nd
Surface water K5	-	-	Nd
Surface water K6	-	-	Nd
Surface water K8	-	-	Nd
Drinking water K3	-	-	Nd
Drinking water K4	-	-	Nd
Waste water K7	-	-	Nd
Kwazulu/Natal			
Surface water U1	-	-	-
Surface water U2	-	-	-
Surface water U3	-	-	-
Surface water* U5	-	-	-
Surface water U8	-	-	-
Surface water U10	-	-	-
Drinking water U6	-	-	-
Ground water U7	-	-	-
Waste water U4	-	+	-
Waste water U9	-	-	-
Gauteng			
Surface water R1	-	+	-
Surface water R3	-	-	-
Surface water R4	-	-	-
Surface water R5	-	-	-
Surface water R7	-	-	-
Surface water R8	-	-	-
Drinking water R2	-	-	-
Drinking water R6	-	-	-
Free State			
Surface water S1	-	-	-
Surface water S2	-	-	-
Surface water S3	-	-	-
Surface water S4	-	+	-
Surface water S5	-	-	-
Surface water S6	-	-	-
Drinking water S7	-	-	-
Drinking water S8	-	-	-
Ground water S9	-	-	-
Waste water S10	-	-	-

Surface water* Saline surface water, nd = not done as no specimens were received

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CHAPTER 3

HEPATITIS E VIRUS

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3.1 INTRODUCTION

Hepatitis E virus (HEV) is an important cause of faecal-orally transmitted acute hepatitis with an uneven global distribution (Aggarwal *et al.*, 1999). Initially, hepatitis E was referred to as enterically transmitted non-A, non-B hepatitis virus (Scharschmidt *et al.*,1995). HEV shares many characteristics with hepatitis A virus (HAV) (Grabow *et al.*, 1994). The predominant mode of transmission of both viruses is via the faecal-oral route and large waterborne outbreaks have been recorded, particularly in areas with low standards of living, inadequate sanitation and poor drinking water supplies (Tucker *et al.*, 1996). However, hepatitis E virus occurs more often in young adults many of whom are already immune to hepatitis A (Grabow *et al.*, 1994). In areas where the disease is endemic, HEV is an important cause of death due to liver failure, especially in pregnant women, for whom the fatality rates can reach as high as 20% (Grabow *et al.*, 1994).

Secondary transmission of HEV via person-to-person contact is rare, which suggests that faecally polluted water plays a more important role in the spread of HEV than HAV (Jotikumar *et al.*, 1993). The important role of water in the transmission of HEV may offer opportunities for the virus to be transmitted from animals to humans via water sources polluted with animal wastes.

Hepatitis E virus is an important cause of sporadic or epidemic viral hepatitis in many developing countries in Asia, The Middle East and north Africa (Scharschmidt *et al.*, 1995). The epidemiology of HEV seems to include a geographic element (Grabow *et al.*, 1996). Although sero-epidemiological studies have indicated that HEV is endemic in South Africa (Grabow *et al.*, 1994; Grabow *et al.*,1996; Tucker *et al.*, 1996,), and conditions in many

communities should favour the spread of the virus, very few clinical cases have been recorded. In most instances, the clinical infections were imported, having been acquired in high risk areas (Grabow *et al.*, 1996).

The absence of clinically evident hepatitis E in SA is surprising as outbreaks have been recorded in neighbouring countries such as Namibia and Botswana (Byskov *et al.*, 1989). It has to be borne in mind that SA imports animals from Namibia and Botswana, both of which are countries where outbreaks of HEV have occurred (Byskov *et al.*, 1989). This phenomenon of a relatively high prevalence of anti-HEV in communities where few clinical cases are seen has also been demonstrated in many parts of the world, including the USA (Bernal *et al.*, 1996; Favarov *et al.*, 1998; Hsieh *et al.*, 1999; Kabrane-Lazizi *et al.*, 1999; Wu *et al.*, 2000)

In a number of recent studies, evidence presented suggests that hepatitis E may, in some cases, be a rare zoonotic infection (Meng *et al.*, 1998). It has been possible to transmit HEV to non-human primates (Bradley *et al.*, 1987; Ticehurst *et al.*, 1992; Nanda *et al.*, 1994) as well as other animals namely swine (Balayan, *et al.*, 1990; Hsiesh, *et al.*, 2000), rats (Maneerat *et al.*, 1996; Tsarev *et al.*, 1998; Favorov *et al.* In preparation; Kabrane-Lazizi *et al.*, 1999), cows (Meng *et al.*, 1999), goats (Favorov *et al.*, 1998) and sheep (Favorov *et al.*, 1998; Meng *et al.*, 1998).

Molecular epidemiological studies have shown swine and human HEV to be closely related (Meng *et al.*, 1997), with swine HEV being able to cross the species barrier to infect non-human primates (Meng *et al.*, 1998). In an investigation conducted in China and Thailand, over 71% of pig handlers tested were found to have anti-HEV, suggesting that HEV is possibly enzootic in pigs (Meng *et al.*, 1999). However anti-HEV is evident in populations not in contact with pigs, suggesting another reservoir(s) of infection (Favorov *et al.*, 1998; Favorov *et al.*, In preparation). A high prevalence of antibodies to HEV has been detected in wild rats in areas of the USA where the human population had a relatively high level of anti-HEV and was not in contact with swine (Kabrane-Lazizi *et al.*, 1999 Favorov *et al.*, In preparation).

The purpose of this investigation is to determine the prevalence of anti-HEV in selected swine serum samples from certain rural and urban districts in SA and to detect and sequence the virus found in swine and human stool samples and water samples. This data will provide valuable

information with regard to possible animal reservoirs of HEV in SA and how wide spread the virus is. Depending on results obtained, it is hoped that the relatedness of the viruses found in human and swine can be determined, and enough data analysed to provide the basis for further seroepidemiological studies in related human communities.

3.2 RESEARCH PROGRESS

The seroprevalence studies on animals were started using modified enzyme-linked immunosorbant assay (ELISA) intended for detecting human anti-HEV. Control sera and related reagents appropriately designed for detecting and evaluating pig anti-HEV have been supplied by colleagues from the Hepatitis Branch of the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, USA. Arrangements are being made to obtain appropriately selected animal sera from rural areas in the South African provinces of Gauteng, Mpumulanga and Northern Province.

The molecular detection and comparison of hepatitis E virus in the stool and water samples is still in progress. The nested-PCR technique for the detection of HEV must still be optimised.

Sera were collected from swine from the Agricultural Research Council (ARC) in Irene (samples I1-I22), the experimental farm of the University of Pretoria (samples P1-P20) and from the Onderstepoort Veterinary Institute (samples O1-O100). These last serum samples had been obtained from swine from a wide variety of different areas throughout the country.

A total of five ELISAs have been performed testing a total of 142 swine serum samples. A total of 14 of the 142 serum samples were shown to be positive for anti-HEV. The positive samples were all retested to ensure accuracy and they are listed in Table 3.1.

Table 3.1 Results of the ELISA tests detailing the swine anti-HEV IgG seropositive samples from various regions of South Africa

Sample Number	Sample detail	Magisterial district	Province
114	F584	Pretoria	Gauteng
119	W98218	Pretoria	Gauteng
016	JTR26/2	Pretoria	Gauteng
020	JTR29/2	Warmbaths	N. Province
028	LA12	Greylingstad	Mpumulanga
048	125	Jeffreys Bay	E. Cape
052	GB17	Paarl	W. Cape
056	GB66	Somerset West	W/Cape
058	GB107	Durbanville	W.Cape
080	50	Kliprivier	KZN
081	3	George	W.Cape
086	12	Malmesbury	W.Cape
092	GFZ67	Middelburg	Mpumulanga
095	GFZ56	Kroondal	North West

Additional swine serum samples have been collected and tested for anti-HEV IgG. The provines of Gauteng, Northern Province and Mpumulanga became the main focal points of the study to facilitate further specimen collection of serum and stool samples of swine from these areas for use in the molecular aspects of this research project. The results of the additional ELISA tests are shown in Table 3.2.

3.2.1 Molecular Study

The nested-PCR to be used in the molecular study is currently underway. The lack of positive control for hepatitis E virus has so far prevented the advancement of this study .

3.3 CONCLUSIONS

We can conclude that 11.3% of the 124 samples tested were positive for anti-HEV, suggesting that HEV is endemic in the swine populations in South Africa. The additional

Table 3.2 Results of the ELISA tests showing the percentage of anti-HEV IgG positive Cases from Gauteng, Northern Province and Mpumulanga

Province	District	Number of samples tested	Number of samples positive for IgG anti-HEV (%)
Gauteng	Pyramid	10	2 (20)
	Bronkhorstspruit	12	1 (8)
	Heidelburg	3	2 (67)
Northern Province	Warmbaths	79	13 (16.5)
	Pietersburg	24	1 (4)
Mpumulanga	Middelburg	18	7 (39)
	Roossenekal	3	2 (67)

3.3 CONCLUSIONS

We can conclude that 11.3% of the 124 samples tested were positive for anti-HEV, suggesting that HEV is endemic in the swine populations in South Africa. The additional seroprevalence tests seem to confirm this result. A working nested-PCR procedure will allow the molecular detection of HEV in water for the determination of the presence of the virus in environmental, sewage and drinking water samples.

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BACTERIA

CHAPTER 4

DEVELOPMENT OF THE MODIFIED CAPE TOWN PROTOCOL FOR THE ISOLATION OF *Campylobacter* FROM WATER

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4.1 BACKGROUND

Campylobacter is recognized as one of the major causes of gastroenteritis worldwide (Allos, 2001; Griffiths and Park, 1990; Lior, 1996). *Campylobacter jejuni, C. coli* and *C. lari* are amongst the species known to be pathogenic to humans, but *C. jejuni* is the most commonly isolated species from patients (Engberg, 2000; Stanley *et al.*, 1998; Tauxe, 1997; Thomas *et al.*, 1999b). For example, in a South African study, *C. jejuni* has been isolated in 33% of 2351 stool samples, but a range of other species, including non-thermophilic *Campylobacter,* have also been isolated (Le Roux and Lastovica, 1998).

Poultry still remains the primary source implicated in infections of humans, mainly because this organism forms part of the chicken's commensal intestinal microflora. In addition to poultry, unpasteurized milk and water, especially untreated surface water has also been implicated as a possible source of infection (Beumer *et al.*, 1992; Jones *et al.*, 1991; Koenraad *et al.*, 1995). According to studies by Obiri-Danso and Jones (1999a; 1999b) in the United Kingdom, water was found to be contaminated with *Campylobacter* by the introduction of sewage runoff. In another study this group found that *Campylobacter* is destroyed by extended exposure to UVB rays and that this might be the reason for low numbers or the absence of organisms if tested at different times of the day (Obiri-Danso *et al.*, 2001).

Methods for the detection and isolation of this fastidious organism have been developed with specific application in the medical field. In most cases, selective culture media containing combinations of various different antibiotics, that inhibit the growth of competitor microflora, are used for the culturing of Campylobacters.

Examples of such selective media include Skirrow, Cary-Blair, modified charcoal cefoperazone deoxycholate agar (mCCDA) and Preston medium (Bridson, 1995; Griffiths and Park, 1990). Detection from the environment is based mainly on molecular methods such as PCR (Kirk and Rowe, 1994; Koenraad *et al.*, 1995; Lawson *et al.*, 1998; Oyofo and Rollins, 1993; Waage *et al.*, 1999) and nucleic acid hybridizations (Buswell *et al.*, 1998). All of these methods have been designed to detect or isolate only the thermophilic *Campylobacter* species, *i.e. C. jejuni, C. coli* and in some cases *C. lari.* The only method reported to yield isolates of most of the known members of the genus is based on the ability of *Campylobacter* to swim through 0.6µm pores in mixed ester membrane filters (Steele and McDermott, 1984). This method has been refined by Dr A. Lastovica and his team at the Red Cross Children's Hospital in Cape Town and is commonly referred to as the Cape Town Protocol. Since no selective agents are used in this method, it is possible to isolate virtually all of the known *Campylobacter* species (Le Roux and Lastovica, 1998).

Experimental data obtained by the Red Cross Children's Hospital has shown that *C. jejuni* and *C. coli* are not the dominant *Campylobacter* species found in South African patients over the last 4 to 5 years. It was therefore argued that it would be appropriate to develop a method facilitating the recovery of all the known species of *Campylobacter.*

The aim of this section of the work was thus firstly, to develop a filtering method for the detection of *Campylobacter* species from environmental water, based on the Cape Town Protocol, and secondly, to evaluate the newly developed method using artificially contaminated and environmental water samples.

4.2 MATERIALS

Campylobacter jejuni and *C. coli* strains were obtained from the Red Cross Children's Hospital (Rondebosch, Cape Town). *Escherichia coli* JM105, *Pseudomonas aeruginosa* PAO1 and *Vibrio cholera* were obtained from the laboratory culture collection.

The *Campylobacter* species were grown on Blood Agar Base No.2 (Oxoid CM271) supplemented with 10% (v/v) sterile horse blood (South African Institute for Medical Research, Johannesburg, Republic of South Africa). For the second round of sampling, Tryptose Blood Agar (Oxoid CM233) was used instead of Blood Agar Base No. 2, because it yielded better results than the latter. (Lastovica, personal communication). All *Campylobacter* cultures were incubated at 39°C in atmosphere generated by an Oxoid Gaspack BR038B in order to provide a hydrogen-rich atmosphere. Cells were subcultured every 48 to 74 h. For liquid culture, strains were inoculated into Luria Bertaini (LB) Broth (Sambrook *et al.*, 1989). *P. aeruginosa* and *E. coli* were cultured on Luria Bertaini (LB) Agar under normal atmospheric conditions at 37°C. All other chemicals were of analar grade or the highest purity available.

Filtrations were done through presterilized nitrocellulose 0.45µm and 0.22µm membrane filters (Millipore). Cellulose nitrate and cellulose acetate membrane filters (0.60µm pore size, Scheicher and Schuell, ME 26) and *Campylobacter* blood-free selective agar base (modified CCDA) (Oxoid CM739) with CCDA selective supplement (Oxoid SR155) were also used. Fecal coliform detection was performed on mFC agar (Biolab C92).

4.3 METHODS

4.3.1 Preparation of water samples artificially contaminated with *Campylobacter*

LB broth was inoculated with *C. coli* and *C. jejuni*, respectively, and incubated in a H_2-rich atmosphere for 24 h, as described above. Serial dilutions (up to 10^{-6}) were prepared in 9ml ¼ strength Ringer's solution (Merck). One hundred microliter aliquots of these dilutions were spread onto blood agar plates to determine the direct culturable counts. Various different water samples (99ml) (sterile distilled, tap, borehole water as well as sewage effluent) were then artificially contaminated with 1ml of each of the different dilution series. Traces of residual chlorine in the water were inactivated prior to spiking by the addition of sodium thiosulphate to a final concentration of 0.01% (m/v) (Le Chavelier *et al.*, 1988).

4.3.2 The development modified Cape Town Protocol

The Cape Town Protocol was modified by the inclusion of an initial filtering step of the artificially contaminated water samples through 0.45µm membrane filters by applying a vacuum. The filters were then aseptically inverted onto a 0.6µm membrane filter on blood agar plates (Fig 4.1). The plates were incubated under 5% CO_2 at 39°C

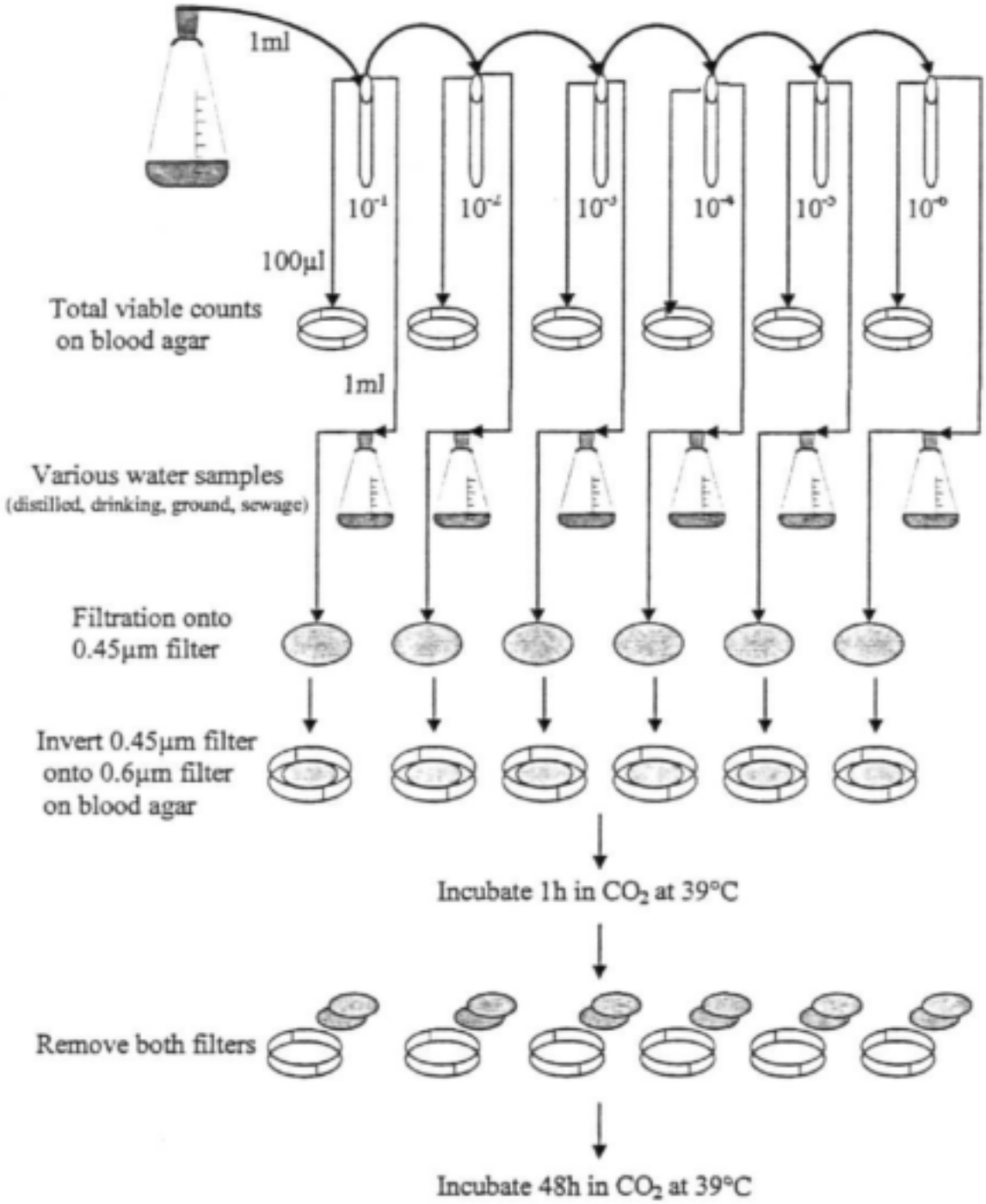


Figure 4.1 **Diagrammatic representation of the modified Cape Town Protocol.**

for 15 min, whereafter both filters were removed and the plates incubated for a further 24 to 48 h. Henceforth, we will refer to this method as the modified Cape Town Protocol.

4.3.3 Evaluation of the modified Cape Town Protocol under laboratory condition

The modified Cape Town Protocol was evaluated for its ability to recover *Campylobacter* cells from artificially contaminated water samples by performing various experiments. The efficacy of the various steps was evaluated as outlined below.

In order to determine whether the *Campylobacter* cells moved through the 0.45µm membrane filters during filtration, one milliliter of the filtrate was concentrated by centrifugation at 15 000×*g* for 10 min. The pellet was resuspended in 100µl sterile dH_2O and the suspension was placed onto a 0.6µm membrane filter on blood agar. In addition, 200µl of the filtrate was placed directly onto a separate 0.6µm membrane filter. The filters were removed after one hour and the plates were subsequently incubated as described in Section 4.3.2 and Figure 4.1.

In order to determine whether 0.22µm membrane filters would be more effective in retaining the *Campylobacter* cells than 0.45µm membrane filters, the entire procedure, as described in Section 4.3.2 and Figure 4.1, was performed using both 0.22 and 0.45µm membrane filters in parallel.

To determine whether the 0.45µm membrane filters retained the *Campylobacter* cells, therefore preventing passage through the 0.6µm membrane filter, the 0.45µm membrane filters were inverted directly onto the blood agar, and then removed after 15 min. The plates were incubated, after which the number of colonies were counted.

The influence of time on passage of *Campylobacter* cells from the 0.45µm membrane filter through the 0.6µm membrane filter onto the agar was determined by incubating plates with both filters for 15 min and 60 min, respectively, in 5% CO_2 before removing the filters.

To ascertain whether movement through the 0.6µm membrane filter was hampered by the lack of fluid, 500µl, 250µl and 100µl of sterile distilled water were added to the inverted 0.45µm membrane filters and processed further as described in Section 4.3.2 and Figure 4.1.

To ascertain whether movement through the 0.6µm membrane filter was limited by cellular energy, glycine and citric acid (pH 7) were added to the artificially contaminated water samples to a final concentration of 0.5% (w/v), prior to filtration.

To determine whether *Campylobacter* cells lost culturability or died during the various treatments before the actual filtration took place, the experiment was performed as described in Section 4.3.2 and Figure 4.1. Prior to filtration, 100µl of the contaminated water and of the serial dilution used for preparing artificially contaminated water samples were taken and used to determine total culturable counts on blood agar.

To determine whether cells moved from the 0.45µm membrane filter through the 0.6µm membrane filter onto the blood agar, the number of cells recovered after filtration was compared to the total culturable count obtained for the artificially contaminated water samples prior to filtration.

Recovery of starved *Campylobacter* cells

To determine whether starved *Campylobacter* cells moved through the 0.22 and 0.45µm membrane filters, *Campylobacter* cells were cultured in LB broth for 24 h. The bacterial cells were collected by centrifugation at 10 000×*g* at 9°C for 10 min. The pellet was resuspended in sterile phosphate buffer (pH 7.2) and centrifuged as above. The washed pellet was then resuspended in 100µl sterile dH_2O and placed under 5% CO_2 at 37°C. The bacteria cells were observed microscopically on a regular basis for morphological changes from the typical spirilloid rods to coccoid form (Thomas *et al.*, 1999b). Once coccoidal cells were obtained, 1ml of the cell suspension was added to 99ml sterile dH_2O and filtered through 0.22 and 0.45µm membrane filters. One milliliter of the filtrate was removed and the bacterial cells

concentrated by centrifugation at 10 000×*g* for 5 min. The pellets were resuspended in 100µl sterile distilled water and placed onto a 0.6µm membrane filter on blood agar. After 1 h of incubation under 5% CO_2 at 37°C, the filters were removed and the plates incubated for a further 48 h. In addition, 200µl of the filtrate was pipetted directly onto a separate 0.6µm membrane filter and incubated as above.

To determine whether starved cells could be recovered on blood agar, starved cells, prepared as described above, were plated directly onto blood agar plates and incubated as described previously (Section 4.3.2 and Figure 4.1).

Passage of small, motile rods through the system

To determine whether other small motile rods would pass through the 0.6µm membrane filter, the standard procedure (Section 4.3.2 and Figure 4.1) was performed using *P. aeruginosa* and *E. coli* as inoculants, with *C. coli* and *C. jejuni* as controls.

Evaluation of a blood-free selective agar (mCCDA)

To determine the degree of recovery of *Campylobacter* cells using a blood-free selective agar (mCCDA, Oxoid), artificially contaminated water was filtered through 0.45µm membrane filters and these were inverted directly onto mCCDA plates. The standard procedure (Section 4.3.2 and Figure 4.1) was performed in parallel and the results compared.

Determination of fecal coliforms

Detection of fecal coliforms was performed by placing 0.45µm membrane filters, bearing the filtrated sample, onto mFC agar and incubating at 44.5°C. After 22 to 24 h of incubation, all the dark blue colonies were counted.

4.3.4 The optimized modified Cape Town Protocol used in the analysis of environmental water samples

Serial dilutions were prepared from various different water samples to determine the total culturable counts. Different volumes of the water were filtered through 0.45µm membrane filters, which were inverted onto 0.6µm membrane filters on blood agar, and directly onto mCCDA plates. These were incubated for 1 h in 5% CO_2 at 37°C

after which the filters were removed and the plates incubated further for 48 h. The procedure is represented diagrammatically in Fig 4.2. We have decided to lower the incubation temperature to 37°C to allow for growth of the non-thermophilic *Campylobacter* species that might be present in the environmental waters. It was also more convenient, since only one incubator at 37°C was required, for both the adapted modified Cape Town Protocol and for the mCCDA plates.

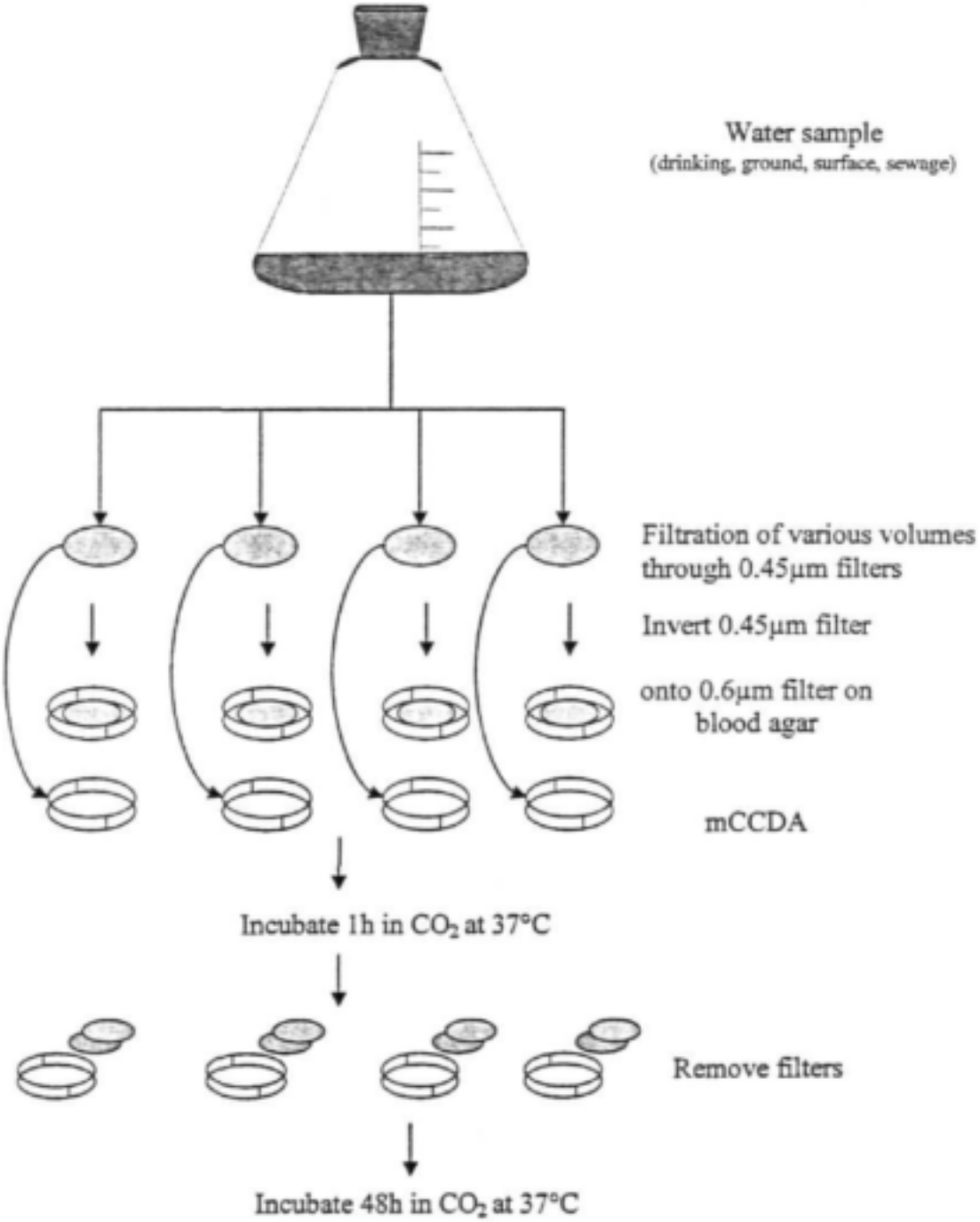


Figure 4.2 **Diagrammatic representation of the optimized modified Cape Town Protocol as used during environmental sampling.**

4.3.5 Evaluation of the modified Cape Town Protocol using environmental water samples

To detect and isolate any possible *Campylobacter* species, water samples (10 samples each) were received from tap, surface, ground and sewage sources in four provinces of South Africa (Table 4.1). Samples were taken by representatives of Rand Water Board (Gauteng), Umgeni Water Board (Kwazulu-Natal), the Freestate Technikon (Freestate) and University of the Western Cape (Western Cape). All samples were transported to the laboratory on ice and analyzed on the day of receipt. Samples were processed as described in Section 4.3.4. and Fig 4.2. The fecal coliform count of each sample was determined as described in Section 4.3.3. *Campylobacter*-like colonies were randomly selected from those isolated on both blood and mCCDA agar plates. These colonies were round, smooth-edged and about 0.5 to 1mm in diameter after 48 h of incubation.

Table 4.1 **Water samples from the four regions and the different sources that were sampled during March and June 2000 (see Table 8.1)**

	Gauteng (Rand water)	Kwazulu Natal (Umgeni water)	Freestate (Freestate Technikon)	Western Cape (University of the Western Cape)
Tap water	Sandton (R6)	Graigburn WW (U6)	Storage container (S7)	Stellenbosch (K3)
	Klipwater (R2)		Communal stand pipe (S8)	Bellville (K4)
Ground water	Roodeplaat dam	Dairy Top bore hole (U7)	Unprotected bore hole (S9)	Sacks Circle
	Pretoria North		Unprotected borehole (S10)	
Surface water	A18 (R8)	Slangspruit River (U1)	Urban run off, polluted stream (S1)	Kuilsriver-N2 (K1)
	B10 (R4)	Mngeweni River (U8)	S1 after it received effluent (S3)	Kuilsriver- Baden Powell Drive (K2)
	K19 (R7)	Dusi River (above Darvill) (U2)	Below recreational dam wall (S4)	Kuilsriver-Stellenbosch Arterial (K8)
	K25 (R3)	Umgeni Estuary (U5)	Urban runoff, outside Bloemfontein (S5)	Kuilsriver-Monwabizi (K6)
	RV2 (R1)			Plankenberg River (K6)
	Riviera (R5)			
Sewage		Hammersdale Effluent (U10)	Raw, poorly treated (S2)	Athlone Effluent (K7)
		Hammersdale Raw (U9)	Effluent, well treated, new facility (S6)	Athlone Raw (??)
		Darvill Effluent (U3)		
		Darvill Raw (U4)		

Table 4.2 **Labeling key for all presumptive *Campylobacter* isolates**

Example	1SanDC3	
1	Sampling round	1- first round (March 2000)
		2- second round (July 2000)
		3- third round (October 2000)
San	Sampling site	First three letters/numbers of sampling site
D	Sampling source	S- surface water
		D- drinking water
		G- ground water
		F- final sewage effluent/ treated sewage
		R- raw/ untreated sewage
C	Media from which isolated	C- mCCDA
		B- Blood agar
3	Number of isolate	

4.3.6 Phenotypic identification of presumptive *Campylobacter* species

Five colonies were selected from each plate corresponding to the highest dilution from which growth was observed (see Table 4.2 for labeling system). The selected isolates were first screened by performing Gram stains. Only gram negative spirilloid rods were selected, subcultured to attain pure culture and then stored on Microbank™ beads (Pro-lab Diagnostics) at -70°C for later identification. Due to an unanticipated delay in access to a training session with Mrs E. Le Roux (retired expert on *Campylobacter*), identification by phenotypic traits was delayed until July 2000, *i.e.* after completion of the second sampling round. All presumptive *Campylobacter* isolates were recultivated from frozen stocks, subjected to seven biochemical tests, as indicated below, and identified using the key of Lastovica (personal communication).

The sensitivity to atmospheric oxygen was determined by streaking all of the presumptive *Campylobacter* isolates onto blood agar and incubating aerobically at 37°C for 24 to 48 h. Only those isolates that did not grow aerobically were characterized further.

The cytochrome oxidase test was performed by adding two to three drops of tetramethyl-*p*-phenylenediamine (1% w/v in DMSO) to the culture. A color change to purple in less than 10 s was regarded as a positive reaction, but if it took longer than than 60 s it was considered to be negative. A delayed positive reaction was one in which a colour change occurred between 10 to 60 s.

The ability to reduce nitrate was determined by spreading a large loopful of culture onto blood agar containing 1% (m/v) KNO_3 and incubating overnight in a H_2-rich atmosphere. Plates were exposed to the atmosphere for 5 to 10 min on the workbench before analysis. A dark green-brown zone indicated a positive reaction. This meant that the KNO_3 had been reduced to KNO_2 resulting in oxidation of the haemoglobin in the blood.

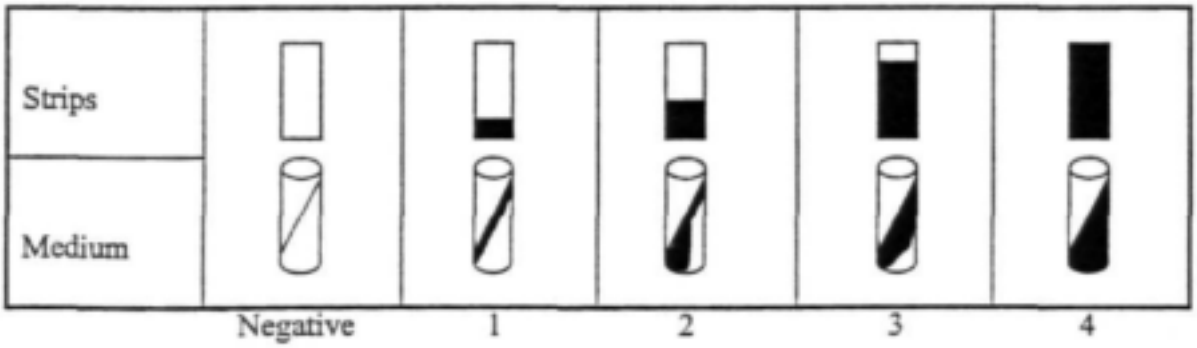
The presence of catalase was determined by adding a drop of 30% (v/v) H_2O_2 to a loopful of culture taken carefully from the blood agar. The formation of oxygen bubbles was regarded as a positive reaction as catalase hydrolyse hydrogen peroxide to water and oxygen.

Pyrazinamide activity was determined by inoculating pyrazinamide semi-solid media (Lastovica, personal communication) with an overnight culture. The culture and top third of the medium in the tube were mixed, thereby leading to emulsification. The tubes were incubated in a H_2-rich atmosphere for 48 h after which 1ml of freshly prepared 10% (w/v) ferrous sulphate ($FeSO_4$) was added to the tube, and left to stand for a few minutes. Development of a brown ring indicated a positive reaction (Burnens and Nicolet, 1993).

Hippurate activity was determined by inoculating hippurate broth (Penner, 1988) with the isolates. Following overnight incubation in 7% CO_2 at 37°C, approximately 0.5ml ninhydrin solution was added to the tubes, without shaking. The reaction was read within 10 min. A color change to dark purple was regarded as a positive reaction, whereas light purple to colorless indicated a negative reaction.

Hydrogen Sulphide (H_2S) production from thiosulphate was determined by inoculating Triple Sugar Iron (TSI) agar slants with cultures grown under CO_2. A lead acetate strip was suspended over the slant, without touching the medium, and incubated for 48 h under 7% CO_2. Blackening of the medium or strip indicated a positive reaction. The scale depicted in Figure 4.3 was used to score both the medium and the strip.

Figure 4.3 **Scale used for scoring H_2S production on TSI slants and lead acetate strips**



4.3.7 *Genotypic characterization of the presumptive Campylobacter species*

4.3.7.1 Extraction of genomic DNA for Polymerase Chain Reaction (PCR)

Genomic DNA was extracted using the boiling method as described by Lemanceau *et al.* (1995). Bacterial cell suspensions of the respective isolates were prepared by scraping off the growth on blood agar plates and suspending in 5ml sterile dH_2O. The suspension was mixed by vortexing for a few seconds and its optical density was measured at 620 nm, using distilled water as a blank. The volume of cells to be treated further was calculated using the following formula: Volume (in ml) = $0.2/OD_{620}$. Following centrifugation of the appropriate volume of cells at 15 000×*g* for 5 min, the harvested cells were resuspended in 100µl sterile UHQ water. Then, 100µl of 10mM Tris-HCl (pH 8.3) and 13µl of Proteinase K (1mg/ml in water) were added to the cell suspension and the mixture was incubated overnight at 55°C. Proteinase K was inactivated by incubating the mixture for 10 min at 100°C.

4.3.7.2 Verification of members of the genus *Campylobacter* by PCR

Primers CG12F and CG1507R, reportedly specific to two regions of the *Campylobacter* 16S rDNA, were used for PCR to yield a 1495bp amplicon (Lawson *et al.*, 1998). Amplification was carried out as follows on a Perkin Elmer thermal OCR cycler. Five microliters of DNA was used in a 50µl PCR reaction containing 2µl (2.5 mM) of each dNTP (Promega), 1µl 50 pmol/µl of each primer (forward and reverse) (Roche), 4µl 25 mM $MgCl_2$, 5µl 10× Reaction Buffer and 0.02U/µl *Taq* Polymerase (Southern Cross Biotechnologies). The cycling conditions were: denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extention at 72°C for 1 min. These were repeated for 30 cycles and then concluded by a final extention step at 72°C for 5 min. Five microliters of the PCR reaction was analyzed by electrophoresis in a 1% (w/v) agarose gel (Promega). A 100bp DNA ladder (Promega) was included on each gel as a molecular size standard. The fidelity of the method was evaluated using *C. coli, C. jejuni, C. fetus* and *C. upsaliensis* as positive controls, and *P. aeruginosa* PAO1 and *V. cholera* as negative controls.

4.3.7.3 Identification of the isolates by sequencing of their 16S rDNA genes

A 1500bp region of the 16S rDNA gene of all isolates selected in Section 4.3.6 was amplified using the universal 16S rDNA primers, fD1 and rP2, as described by Weisburg *et al.* (1991). Prior to sequencing, these amplicons were purified with a Geneclean™ Kit (Bio 101, Inc) as per manufacturer's instructions, but using silica instead of glassmilk (Boyle and Lew, 1995). Sequencing was performed on an ABI PRISM 377 Sequencer (Perkin Elmer), using BigDye™ Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems). Sequences were edited by Sequence Navigator and the identities were determined by searching known sequences in GenBank using a basic BLAST search of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/).

4.4 RESULTS

The number of colony forming units was compared to the total culturable count as performed on the serial dilutions used to artificially inoculate different water samples. The modified Cape Town Protocol yielded a recovery of less than 0.1% of the total number of *Campylobacter* cells introduced into various environmental water samples. This held true for both *C. jejuni* and *C. coli* in all the different water samples (tap, ground, surface water and sewage) tested, and therefore necessitated detailed investigation of the reasons underlying the dismal recovery rate.

4.4.1 Evaluation of the modified Cape Town Protocol under laboratory conditions

In the modified Cape Town Protocol, the use of a 0.45µm membrane filter prior to placing onto a 0.6µm membrane filter serves to concentrate the *Campylobacter* cells from suspension.

Campylobacter did not pass through the 0.45µm membrane filters during filtration as no growth was achieved on blood agar, neither when the filtrate was first concentrated before passing it through the 0.6µm membrane filter, nor when it was placed directly onto the 0.6µm membrane filter.

By comparison, the use of 0.22µm membrane filters did not retain more cells than the 0.45µm membrane filter, supporting the above finding that no cells passed through the 0.45µm pores. It was therefore decided to use the 0.45µm membrane filter in subsequent experiments as it did not become blocked with other waterborne particles as easily as the 0.22µm membrane filters.

The culturable count of cells transferred from the 0.45µm membrane filter to blood agar was only slightly lower (*ca.* 10%) than that of the artificially contaminated water. This indicated that few, if any, *Campylobacter* cells adhered to the filter material, thereby not being released to the next phase. It was therefore decided to continue using the 0.45µm membrane filters.

By leaving the 0.45µm membrane filter on the 0.6µm membrane filter for 60 min instead of 15 min, a ten-fold increase in the culturable count was observed. Longer exposure did not, however, lead to a further improvement in recovery. The protocol was therefore modified by increasing the filter exposure time to 60 min, improving overall recovery to *ca.* 1%. This low degree of recovery indicated that the bulk of the *Campylobacter* cells were probably retained in some way by the 0.6µm membrane filter.

In an attempt to improve the low level of passage of cells through the 0.6µm membrane filter, 100µl of sterile water was pipetted onto the filter to enhance transport by possible capillary action. This treatment did not contribute to improved recovery. The addition of 250 and 500µl of water to the filters caused flooding, resulting in growth all over the agar surface, and was therefore terminated. The results showed that passage of *Campylobacter* through the 0.6µm membrane filter was not limited by fluid availability.

In order to compensate for a possible drop in the cellular adenylate energy charge, neutralized citrate and glycine were added to contaminated water samples. *Campylobacter* does not utilize the full Embden Meyerhoff Pathway, but does incorporate organic substrates from the tricarboxylic acid (TCA) cycle (Hoffman and Goodman, 1982; Hugdahl, *et al.*, 1988; Penner, 1988). Citric acid is an intermediate of the TCA cycle and glycine is incorporated via glyoxylate (Michal, 1999). The

culturable count of the artificially contaminated water was, however, not affected by the addition of glycine. Contrary to expectation, the recovery by the modified Cape Town Protocol decreased by a factor of $\log_{10}$=1 with the addition of citric acid. This indicated that, whilst glycine did not affect culturability of cells, citric acid did deleteriously affect their ability to move through the membrane onto the agar (Leach *et al.*, 1997).

The culturable counts of the dilutions prepared for spiking and of the artificially contaminated water were not significantly different, indicating that the processing did not affect the culturability of the cells.

Comparison of the recovery after filtration with the total culturable count from the artificially contaminated water samples indicated a $\log_{10}$=2.5 decrease in cell numbers. Passage though the 0.6µm membrane filter was therefore regarded as the transfer-limiting step.

Recovery of starved *Campylobacter*

The filtrate obtained after filtering the starved *Campylobacter* cells through 0.2 and 0.45µm membrane filters did not yield any growth, indicating that no culturable cells passed through the pores. Inversion of the above filters onto 0.6µm membrane filters also did not yield any growth on blood agar. Similarly, aliquots of aged cultures plated directly onto blood agar failed to yield any growth. These results show that non-culturable cells can not be detected using this protocol.

Passage of small motile rods through the system

Both *P. aeruginosa* and *E. coli* are motile (Prescott *et al.*, 1990) but both failed to pass through the 0.6µm membrane filter. *P. aeruginosa* is both a small cell, and highly motile and its failure to pass through the filters indicated that few, if any, bacteria other than *Campylobacter* would be isolated with the modified Cape Town Protocol.

Evaluation of the blood-free selective agar (mCCDA)

As an alternative approach, artificially seeded water samples were filtered through 0.45µm membrane filters and then inverted directly onto mCCDA agar. The selective agar mCCDA yielded a recovery of 10% or a loss in culturability of $\log_{10}$=1. The

artificially contaminated water contained actively growing pure cultures of *C. coli* and *C. jejuni*, both reported to grow on mCCDA (Engberg *et al.*, 2000; Korhonen and Martikainen, 1990; Koenraad *et al.*, 1995). The poor recovery is therefore cause for concern, indicating that the selective agents are too stringent, suppressing even the growth of desired species. An additional drawback of mCCDA for environmental isolation is that it reportedly supports growth of only three species of *Campylobacter*, *viz. C. coli, C. jejuni* and *C. lari* (Engberg *et al.*, 2000; Korhonen and Martikainen, 1990; Koenraad *et al.*, 1995). Despite the above, mCCDA was far superior to the optimized modified Cape Town Protocol with its loss in culturability of $\log_{10}$=2.5.

4.4.2 Quantitative isolation of *Campylobacter* from environmental water samples

Both the optimized modified Cape Town Protocol and the use of mCCDA selective agar were found to have serious drawbacks with regard to *Campylobacter* recovery when evaluated in the laboratory. The results of these analyses are indicated in Tables 4.3 to 4.6. Note: the third round (October 2000) of sampling for the Western Cape was done using the optimized modified Cape Town Protocol.

Table 4.3 Results of analysis of water samples obtained from various sources in Gauteng (values represent cfu/100ml). Counts on blood agar and mCCDA reflect the presumptive *Campylobacter* counts before confirmation

		mFC*		Blood agar#		mCCDA†	
Source	**Site**	**March**	**July**	**March**	**July**	**March**	**July**
Drinking water	Sandton	ND‡	ND	7.5×10^{0}	ND	1.5×10^{1}	ND
	Klipwater	1.0×10^{1}	ND	2.5×10^{0}	ND	1.1×10^{3}	ND
Ground water	Roodeplaat dam	2.0×10^{1}	NT§	9.5×10^{0}	NT	2.3×10^{1}	NT
	Pretoria North	1.8×10^{1}	2.5×10^{0}	1.0×10^{0}	1.0×10^{1}	4.3×10^{2}	1.6×10^{3}
Surface water	A18	4.5×10^{2}	ND	1.5×10^{2}	ND	7.0×10^{1}	1.2×10^{2}
	B10	5.0×10^{0}	3.0×10^{1}	1.5×10^{1}	1.2×10^{3}	6.0×10^{2}	5.3×10^{3}
	K19	7.5×10^{2}	8.0×10^{1}	3.0×10^{1}	1.8×10^{5}	8.3×10^{2}	1.5×10^{5}
	K25	9.3×10^{4}	1.9×10^{2}	1.3×10^{2}	3.0×10^{2}	2.9×10^{3}	9.4×10^{3}
	RV2	3.1×10^{4}	3.8×10^{4}	1.1×10^{2}	3.9×10^{4}	1.4×10^{3}	1.0×10^{6}
	Riviera	3.0×10^{3}	2.5×10^{1}	4.5×10^{1}	3.5×10^{1}	7.1×10^{2}	2.7×10^{3}

*- Fecal coliform count, #- modified Cape Town Protocol, †- modified Charcoal Cefoperozone Deoxycholate Agar, ‡- not detected, §- not tested

Table 4.4 Results of analysis of water samples obtained from various sources in Kwazulu Natal (values represent cfu/100ml). Counts on blood agar and mCCDA reflect the presumptive *Campylobacter* counts before confirmation

		mFC*		Blood agar#		mCCDA†	
Source	**Site**	**March**	**July**	**March**	**July**	**March**	**July**
Drinking water	Graigburn	ND‡	ND	1.2×10^{4}	1.5×10^{0}	6.6×10^{4}	5.0×10^{0}
Ground water	Dairy Top borehole	ND	NT§	2.5×10^{2}	NT	2.4×10^{3}	NT
Surface water	Slangspruit River	6.5×10^{5}	5.0×10^{0}	1.5×10^{4}	1.0×10^{1}	1.5×10^{7}	1.7×10^{2}
	Mngeweni River	7.0×10^{2}	2.0×10^{2}	5.0×10^{5}	1.4×10^{5}	8.2×10^{4}	4.0×10^{2}
	Dusi River	4.0×10^{5}	1.6×10^{2}	6.0×10^{5}	1.5×10^{5}	1.0×10^{4}	8.7×10^{5}
	Umgeni Estuary	2.5×10^{5}	1.4×10^{2}	5.5×10^{5}	1.5×10^{1}	3.9×10^{5}	1.6×10^{5}
Sewage Effluent	Darvill	1.0×10^{1}	5.0×10^{0}	4.5×10^{4}	3.0×10^{1}	2.4×10^{4}	3.6×10^{2}
	Hammersdale	1.0×10^{5}	3.3×10^{2}	1.0×10^{5}	2.3×10^{2}	9.8×10^{6}	1.4×10^{5}
Sewage Untreated	Darvill	1.9×10^{7}	5.0×10^{6}	4.5×10^{6}	1.7×10^{7}	2.5×10^{7}	1.8×10^{7}
	Hammersdale	8.5×10^{7}	1.3×10^{8}	2.3×10^{5}	9.9×10^{5}	2.4×10^{7}	1.4×10^{6}

*- Fecal coliform count, #- modified Cape Town Protocol, †- modified Charcoal Cefoperozone Deoxycholate Agar, ‡- not detected, §- not tested

Table 4.5 Results of analysis of water samples obtained from various sources in Freestate (values represent cfu/100ml). Counts on blood agar and mCCDA reflect the presumptive *Campylobacter* counts before confirmation

		mFC*		Blood agar#		mCCDA†	
Source	**Site**	**March**	**July**	**March**	**July**	**March**	**July**
Drinking water	S7	6.5×10^{5}	ND‡	2.0×10^{2}	ND	1.3×10^{4}	5.0×10^{1}
	S8	6.0×10^{4}	ND	ND	ND	ND	ND
Ground water	S9	ND	2.0×10^{2}	5.0×10^{-1}	ND	5.0×10^{0}	2.5×10^{0}
	S10	ND	ND	1.0×10^{1}	5.0×10^{-1}	1.1×10^{3}	ND
Surface water	S1	1.1×10^{2}	2.0×10^{1}	ND	ND	1.5×10^{2}	5.0×10^{0}
	S3	1.0×10^{4}	1.2×10^{2}	2.0×10^{1}	3.0×10^{1}	5.3×10^{4}	1.6×10^{4}
	S4	ND	ND	5.0×10^{0}	9.0×10^{1}	1.5×10^{1}	1.0×10^{1}
	S5	ND	6.3×10^{4}	1.5×10^{1}	4.9×10^{5}	8.0×10^{1}	2.7×10^{5}
Sewage Effluent	S2	4.5×10^{4}	1.3×10^{3}	1.5×10^{5}	1.5×10^{2}	1.4×10^{2}	7.7×10^{4}
	S6	5.0×10^{0}	1.3×10^{6}	ND	3.8×10^{5}	ND	5.7×10^{6}

*- Fecal coliform count, #- modified Cape Town Protocol, †- modified Charcoal Cefoperozone Deoxycholate Agar, ‡- not detected, §- not tested

Table 4.6 Results of analysis of water samples obtained from various sources in Western Cape (values represent cfu/100ml). Counts on blood agar and mCCDA reflect the presumptive *Campylobacter* counts before confirmation

		MFC*			Blood agar#			mCCDA†		
Source	**Site**	**March**	**July**	**October**	**March**	**July**	**October**	**March**	**July**	**October**
Drinking water	Stellenbosch	ND‡	ND	ND	ND	ND	ND	1.5×10^{0}	ND	ND
	Bellville	ND	ND	ND	ND	ND	ND	1.5×10^{0}	ND	ND
Ground water	Sacks Circle	4.0×10^{2}	NT§	NT	3.2×10^{2}	NT	NT	3.2×10^{5}	NT	NT
Surface water	Kuilsriver-N2	5.2×10^{1}	8.6×10^{4}	9.4×10^{4}	5.0×10^{0}	1.4×10^{3}	1.7×10^{3}	1.4×10^{4}	3.2×10^{3}	2.1×10^{3}
	Kuilsriver-Baden Powell Drive	8.8×10^{5}	2.2×10^{2}	3.4×10^{2}	8.0×10^{1}	7.4×10^{1}	1.0×10^{2}	1.6×10^{6}	1.6×10^{2}	1.3×10^{2}
	Kuilsriver-Stellenbosch Arterial	5.2×10^{4}	1.3×10^{5}	1.5×10^{5}	3.0×10^{1}	2.9×10^{4}	2.4×10^{4}	2.4×10^{4}	3.5×10^{4}	3.8×10^{4}
	Kuilsriver-Monwabizi	6.1×10^{5}	3.6×10^{3}	4.9×10^{3}	3.0×10^{3}	2.1×10^{3}	1.1×10^{3}	5.1×10^{4}	2.3×10^{3}	1.8×10^{3}
Sewage Effluent	Athlone	8.4×10^{4}	3.2×10^{5}	2.5×10^{5}	2.4×10^{3}	2.7×10^{4}	2.4×10^{4}	1.6×10^{4}	3.1×10^{4}	2.9×10^{4}
Sewage Untreated	Athlone	2.4×10^{7}	NT	NT	3.4×10^{4}	NT	NT	7.9×10^{4}	NT	NT

*- Fecal coliform count, #- modified Cape Town Protocol, †- modified Charcoal Cefoperozone Deoxycholate Agar, ‡- not detected, §- not tested

With the optimized modified Cape Town Protocol, 221 *Campylobacter*-like organisms, *i.e.* based on their morphology being gram negative, spirilloid rods and growing in 5% CO_2 were isolated. Counts obtained by the optimized modified Cape Town Protocol for drinking and ground water ranged from zero to 1.2×10^4 cfu/100ml, for surface water and treated sewage from not detected to 1.8×10^5 cfu/100ml, and for raw sewage above $2,3\times10^5$ cfu/100ml. Counts obtained by the mCCDA method were generally slightly higher (*ca.* $\log_{10}=1$) than with the optimized modified Cape Town Protocol. Furthermore, there was no correlation between fecal coliform counts and the optimized modified Cape Town Protocol or mCCDA methods.

4.4.3 Identification of *Campylobacter* by biochemical tests

Of the 221 *Campylobacter*-like organisms, only eight failed to grow aerobically, fundamentally questioning the validity of the filter-based method for isolation of *Campylobacter* cells from environmental samples (these numbers reflect only data from Gauteng, Kwazulu Natal and the Freestate). Five isolates were from surface waters, only two originated from sewage and one from a drinking water container. All these gram negative spiralloid rods that failed to grow aerobically were subjected to seven biochemical tests for identification to species level (Table 4.7). None of the isolates from the Western Cape were confirmed and identified as *Campylobacter*.

Table 4.7 **Properties and identity of the eight candidate Campylobacters isolated during the March and July 2000 samplings**

Location	Source	Isolate	Identity by 16S rDNA Sequences	Gram	CG PCR‡	Catalase	Oxidase	NO_3	Hippurate	Pyrazinamide	TSI		Biochem ID
											strip	agar	
Gauteng	Surface water	1A18SC3	*Acinetobacter* sp.	- rods	+	+	+	+	+	+	3	1	*C. jejuni jejuni 1 & 2*
Gauteng	Surface water	1K25SB6	*Bacillus* sp.	- rods	+	-	+	+	-	-	-	-	*A. butzleri*
Gauteng	Surface water	1K25SB9	*Arcobacter butzleri*	- rods	-	-	+	-	-	-	1	-	*C. jejuni doylei A. butzleri*
Gauteng	Surface water	1RV2SC2	*Arcobacter butzleri*	- rods	-	+	+	+	+	-	1	-	*A. skirrowi A. cryaerophila C. fetus*
KZN	Surface water	1MngSB2	*Arcobacter butzleri*	- rods	-	+	+	-	-	+	1	-	*A. butzleri C. upsaliensis C. jejuni doylei*
Freestate	Drinking water	1S7DC7	*Microvirgula aerodenitrificans*	-rods	+	-	-	-	-	+	2	-	*C. jejuni doylei*
Freestate	Treated Sewage	2S6FC6	*Acinetobacter* sp.	- rods	+	-	Delayed +	+	-	-	1	-	*A. butzleri*
KZN	Raw Sewage	1DarRB3	*Arcobacter butzleri*	-rods	-	-	+	+	-	-	-	2	*A. butzleri*

‡ - PCR method as described by Lawson *et al.*, 1998. (see Section 4.3.7.2 for details)

4.4.4 Confirmation to genus level using CG12F/CG1507R Primers

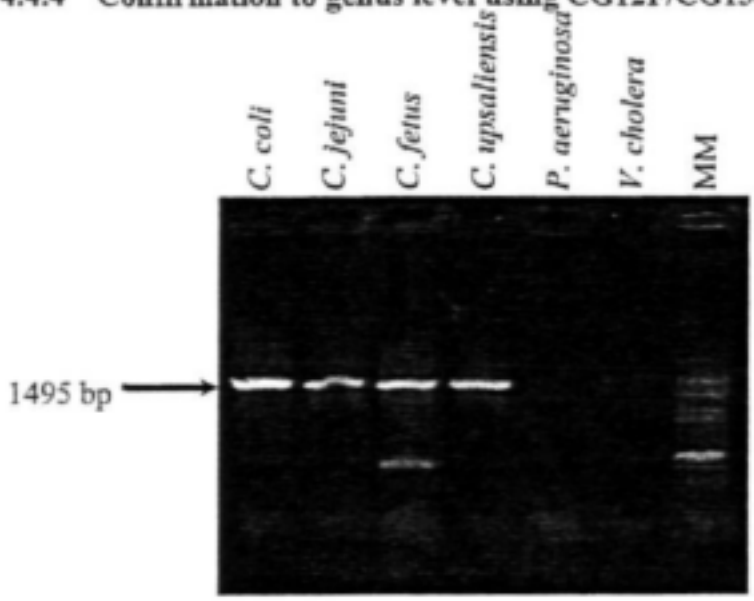


Figure 4.4 **Ethidium bromide-stained 1% agarose gel displaying amplicons of positive and negative controls for primers CG12F and CG1507R. MM:100bp DNA marker as molecular weight indicator**

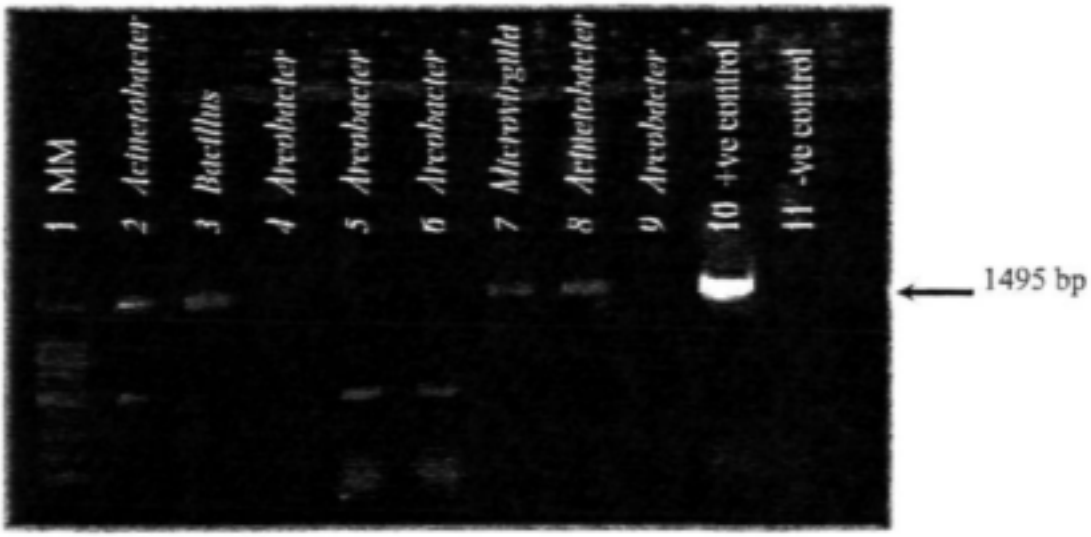


Figure 4.5 **Ethidium bromide-stained 1% agarose gel displaying amplicons of isolates not growing in atmospheric oxygen using primers CG12F and CG1507R.**

Lane 1- 100bp molecular marker (Promega), 2- 1A18SC3, 3- 1K25SB6, 4- 1K25SB9, 5- 1RV2SC2, 6- 1MngSB2, 7- 1S7DC7, 8- 2S6FC6, 9- 1DarRB3

All the positive controls yielded the correct sized amplicon of 1495bp. *C. fetus* yielded an additional smaller sized amplicon (*ca.* 500bp) typically found for those

environmental isolates that were not *Campylobacter* or *Arcobacter* (Fig 4.4). The *Campylobacter* species-specific CG12F/CG1507R primers amplified the expected sized amplicon (1495bp) in 4 of the 8 isolates that did not grow aerobically, and were shown by their 16S rDNA sequence not to be *Campylobacter* or *Arcobacter* (Fig 4.5). In addition to this product, a second non-specific band, smaller than 500bp, was also amplified. This product was also present in 2 of the other reactions that did not amplify the 1495bp band.

4.4.5 16S rDNA sequence analysis

To identify the isolates, universal primers were used to amplify a portion of the 16S rDNA gene, purified and then sequenced. None of the isolates were identified as *Campylobacter* (Table 4.7). Four of the eight isolates were identified as *A. butzleri* based on their 16S rDNA sequences. This data showed that the CG12F/CG1507R primers were not specific for only the *Campylobacter* species as mentioned in Lawson *et al.* (1998). This primer pair also yielded a 1495bp amplicon of various other bacteria, including *Bacillus, Acinetobacter* and *Microvirgula*. Only *A. butzleri* did not react positively. A closer look at the published data revealed that the primers were not tested against non-Campylobacters (Lawson *et al.*, 1998), which could possibly explain why some of the isolates produced bands when amplified with the primers.

Seven randomly selected isolates that did grow aerobically were also sequenced to determine whether possible Campylobacters which could grow aerobically could have been lost. None of these isolates were identified as *Campylobacter* or as a close relative. The identity of these isolates is summarized in Table 4.8. All seven were isolated initially from mCCDA, highlighting that this selective medium is not sufficiently stringent to exclude growth of non-campylobacters.

Table 4.8 **Identity of randomly selected isolates that did not grow aerobically**

ISOLATE	IDENTITY
1DarRC7	*Acinetobacter* spp.
1DusSC3	*Burkholderia* spp.
1DusSC7	*Cellulomonas* spp.
1HamRC5	*Acinetobacter* spp.
1HamRC7	*Paracraurococcus* spp.
1KLWDC5	*Paracraurococcus* spp.
1SanDC4	*Paracraurococcus* spp.

4.5 DISCUSSION AND CONCLUSIONS

Based on a filter method, which has been proven to be very successful in the isolation of Campylobacters from medical specimens by Le Roux and Lastovica (1998), the development of the modified Cape Town Protocol appeared very promising in the initial stages of this study. Even during evaluation of the method in the laboratory, neither *P. aeruginosa* nor *E. coli* were recovered after passage through the 0.6µm membrane filter. All colonies isolated from environmental water samples showed the typical *Campylobacter*-like morphology, *i.e.* they were curved gram negative rods. Based on the number of cfu's obtained with this method, a large number of these isolates were ecpected to fall within the genus *Campylobacter*. Only 4 of the isolates obtained by the optimized modified Cape Town Protocol were aero-sensitive. The 16S rDNA sequencing data revealed that none of these isolates were *Campylobacter*. Three of the four isolates were identified as *Arcobacter butzleri*. The other one was a *Bacillus*. With the mCCDA, only four of the isolates were aero-sensitive, and 75% were not related to *Campylobacter* while one was identified as *Arcobacter butzleri*. From all this it can be concluded that the modified Cape Town Protocol, despite being a useful method for isolation and detection of *Campylobacter* in clinical samples, cannot be used for their isolation from aquatic samples. This might be due to the presence of a large number of a more diverse group of organisms, many of which pass through the purportedly selective 0.6µm membrane filter, whereas in clinical samples, a significantly lower diversity of species may be found at a specific time. The number of organisms in the water were also much less concentrated than in the clinical samples and the presence of other inhibitory organisms might also decrease the

chance of recovering *Campylobacter*. Even with all the modifications to the original Cape Town Protocol, the number of *Campylobacter* isolated could not be increase.

Another factor that has to be considered, is the warm climate that is experienced in South Africa. In addition to this, South Africa also has long daylight hours. Previous studies have shown that *Campylobacter* does not survive in water at high temperature for very long periods, and that cells are destroyed by exposure to UVB rays (Korhonen and Martikainen, 1991; Obiri-Danso *et al.*, 2001). Looking at the counts obtained with both the modified Cape Town Protocol and mCCDA, higher counts were expected during July (winter in the southern hemisphere), but it was rarely the case in any of the four provinces. Only one of the eight isolates that passed the aerobic test (not growing) was from the second round of sampling (in July). It was isolated from treated sewage in the Freestate and was identified as *Acinetobacter*, which showed that no *Campylobacter* was identified during the colder winter months. Obiri-Danso and Jones (1999a; 1999b) were able to isolate *Campylobacter* from environmental water in the UK with mCCDA, a method very similar to the one used in parallel to the optimized modified Cape Town Protocol in this study. No *Campylobacter* was however isolated, only a few *Arcobacter*, from environmental water in South Africa. The results necessitated investigation of an alternative isolation procedure. As the only current alternatives employ various selective media, it was decided to explore this route for further analysis.

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CHAPTER 5

COMPARISON OF AN ENRICHMENT MEDIA FOR THE ISOLATION OF *Campylobacter* FROM WATER

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5.1 BACKGROUND

Gastroenteritis resulting from infection with *Campylobacter* continues to be the leading cause of foodborne infections in the United States of America and the United Kingdom (Engberg *et al.*, 2000; Madden *et al.*, 2000; Stanley *et al.*, 1998; Thunberg *et al.*, 2000). Although the primary source of infection remains poultry, water has also been implicated. Various *Campylobacter* species have been isolated from surface and sea water in the UK (Obiri-Danso *et al.*, 2001; Obiri-Danso and Jones, 1999a; Obiri-Danso and Jones, 1999b). This group has also found that the numbers of thermophilic Campylobacters are higher in the colder winter months than during summer. *Campylobacter* does not multiply in water but is able to survive for extended periods of time at low temperatures (Hazeleger *et al.*, 1998). According to Obiri-Danso *et al.* (2001), *Campylobacter* are introduced into the water by sewage effluent and also by bird and animal droppings.

Detection and isolation methods for *Campylobacter* have been developed predominantly for use in the medical and veterinary fields. Methods incorporate both selective and non-selective media, as well as molecular methods such as Polymerase Chain Reactions (PCR). The filtration method (Steele and McDermott, 1984) does not use any selective agents and is therefore able to isolate almost all the known Campylobacters. This was the method used during the previous two rounds of environmental water testing, and found that it is not very useful for analysis of environmental water samples (Chapter 4).

Selective media for the isolation of *Campylobacter* contain various combinations of different antibiotics that suppress the growth of other organisms. Skirrow's medium

was one of the first to be described (Griffiths and Park, 1990; Tenover *et al.*, 1992; Bridson, 1995) and contains vancomycin, polymyxin and trimethoprim. For best results incubation is at 42°C. Modified charcoal cefoperazone deoxycholate agar (mCCDA) is described to be excellent for the isolation of *C. jejuni, C. coli* and *C. lari* from stool samples. mCCDA contains cefoperazone and amphotericin. It also has the advantage that it can be incubated at 37°C and is a blood free medium. It contains charcoal, ferrous sulphate and sodium pyruvate to bind toxic oxygen derivatives (Corry and Atabay, 1997; Bridson, 1995). Comparing these two selective media, Skirrow and mCCDA, Engberg *et al.* (2000) found mCCDA to be more effective in recovery of thermophilic Campylobacters. In some cases, such as where samples may contain low numbers of cells, or where cells might be injured, it is advisable to first have a period of enrichment (Griffiths and Park, 1990; Koenraad *et al.*, 1995; Tenover *et al.*, 1992). mCCD, Preston and Bolton enrichment broths are only a few of the enrichment broths commercially available (Bridson, 1995). A study conducted by Madden *et al.* (2000) found Preston to give lower recovery than mCCD, but it allows the isolation of minor species. They also found that extended enrichment periods reduce the number of Campylobacters recovered, depending on the species and the media used.

After it was found that the filtration method (Chapter 4) was not useful for the recovery of *Campylobacter* from the environment, selective media was explored. In the work reported in this chapter, two enrichment media were tested in order to see which one give the best results for environmental samples, and employed Bolton enrichment broth in combination with mCCDA and the optimized modified Cape Town Protocol for the isolation of Campylobacters from environmental water samples.

5.2 MATERIALS

Campylobacter jejuni jejuni, Campylobacter coli, Campylobacter lari, Campylobacter upsaliensis, Campylobacter fetus and *Campylobacter jejuni doylei* were obtained form Dr. A. Lastovica (Red Cross Children's Hospital), and *Pseudomonas aeruginosa* PAO1 and *Escherichia coli* JM105 were taken from the laboratory culture collection. All strains were maintained and cultured as described in

Section 4.2. The enrichment media, Preston *Campylobacter* Selective Supplement (Oxoid SR117E) in Nutrient broth No. 2 (Oxoid CM67), and Bolton broth Selective Supplement (Oxoid SR183E) in Bolton Broth (CM983), were prepared according to manufacturer's instructions and supplemented with 5% lysed horse blood. Lysed blood was prepared by freezing the blood overnight (*ca.* 12h) at -20°C (Anon, 2000). Recovery of organisms was performed on Tryptose Blood Agar Base (Oxoid CM233), supplemented with 5% horse blood and on *Campylobacter* Blood-free Selective Medium (mCCDA) (Oxoid CM739) supplemented with CCDA Selective Supplement (Oxoid SR155). Plates were incubated in hydrogen rich atmosphere as produced by the Gas Generating Kit (Oxoid BR038B). Cellulose nitrate and cellulose acetate membrane filters (0.6µm pore size, Scheicher & Schuell, ME26) were used with the blood agar for selective isolation of *Campylobacter*. The detection of fecal coliforms was performed by filtration of samples through pre-sterilized 0.45µm nitrocellulose membrane filters (Millipore), and placing on mFC Agar (Biolab C92). All other chemicals used were of the highest purity available.

5.3 METHODS

The enrichment method was based on the procedure described by the International Organization for Standardization (ISO) (Anon, 2000). In short it involved the concentration of cells by filtration of various volumes of water through 0.45µm membrane filters. The filters were then placed in the enrichment broth and incubated in H_2- rich atmosphere at 37°C for 24h. After incubation, a loopful (*ca.* 10µl) was drawn from just beneath the surface, streaked onto mCCDA and incubated as above.

5.3.1 Laboratory evaluation of enrichment broths

Preston and Bolton enrichment broths were prepared as described in Section 5.2. and aliquoted in 9ml volumes in test tubes. Twenty isolates from the first sampling round (March 2000, Chapter 4, 1A18SB5, 1B10SB1, 1DaiGC4, 1DarRB2, 1DarRB3, 1DusSB2, 1GraDB1, 1HamRC6, 1K25SB8, 1KLWDC4, 1MngSB2, 1RivSC2, 1RV2SC1, 1S2RC6, 1S5SC3, 1S7DC7, 1S10GB1, 1SanDB1, 1SlaSB1, 1UmgSB1), six positive controls (*C. coli*, *C. jejuni doylei*, *C. jejuni jejuni* 1, *C. lari*, *C. upsaliensis* and *C. fetus*) and one negative control (*E. coli*) were used to evaluate the enrichment

method. Only three of the isolates from the first round used in this evaluation did not grow aerobically and their 16S rDNA genes were thus sequenced. These were 1DarRB3, 1MngSB2 and 1S7DC7 These isolates were grown in LB broth until an A_{600}=0.1 was reached and dilution series were prepared in ¼ strength Ringer's solution. Enrichment broths were then inoculated with 1ml from each dilution series of the different cultures and incubated for 48h as described in Section 5.3. The culturable count was determined by plating 100µl of each dilution series onto blood agar plates and incubating as described in Section 4.2. After 24 and 48h, a loopful of each broth was removed from just beneath the media surface and streaked onto mCCDA agar. Enrichment media were mixed by vortexing and the above procedure repeated. This was done to compare the different time intervals necessary for enrichment and the effect of mixing the broth.

5.3.2 Final Protocol (as used during the October sampling)

Bolton enrichment media was aliquoted in 9ml volumes in test tubes. Water samples were either concentrated by filtration through a 0.45µm membrane filter, or placed directly in the enrichment broth, depending on the source.

One hundred milliliters of drinking and ground water sources were filtered and the filter was placed in the broth. For surface water and treated sewage effluent, 10ml of the water was filtered and the filter placed in the broth. Water was also diluted up to 10^{-1} and 1ml of this dilution and 1ml from the undiluted water sample were added directly to the enrichment broth. Untreated or raw sewage was diluted up to the 10^{-2} dilution. One milliliter of each dilution and 1ml of the sample, were added to enrichment broth.

The inoculated enrichment broth with the added sample was incubated for 24 h at 37°C in H_2 rich atmosphere. After 24h, 100µl of the broth was removed from just beneath the media surface, spread onto mCCDA agar and also placed onto a 0.6µm membrane filter on blood agar. Plates were incubated for 48 h at 37°C in H_2 rich atmosphere.

5.3.3 Determination of fecal coliforms

The same volumes used in Section 5.3.2 were used for the detection of fecal coliforms (see Section 4.3.3) in the various water sources.

Table 5.1 **Different water sources sampled in the 3 provinces of South Africa**

	Gauteng (Rand water)	**Kwazulu Natal** (Umgeni water)	**Freestate** (Freestate Technikon)
Tap water	Sandton (R6)	Graigburn WW (U6)	Storage container (S7)
	Klipwater (R2)		Communal stand pipe (S8)
Ground water	Pretoria North	Dairy Top bore hole (U7)	Unprotected bore hole (S9)
			Unprotected borehole (S10)
Surface water	A18 (R8)	Mngeweni River (U8)	Urban run off, polluted stream (S1)
	B10 (R4)		S1 after it received effluent (S3)
	K19 (R7)		Below recreational dam wall (S4)
	K25 (R3)		Urban runoff, outside Bloemfontein (S5)
	RV2 (R1)		
	Riviera (R5)		
Sewage		Hammersdale Effluent (U10)	Raw, poorly treated (S2)
		Hammersdale Raw (U9)	Effluent, well treated, new facility (S6)

5.3.4 Evaluation of the enrichment procedure using environmental sample

Various water samples were taken in 3 regions of South Africa by representatives of Rand Water Board (Gauteng), Umgeni Water Board (Kwazulu Natal) and Technikon Freestate (Freestate Province) (Table 5.1). All samples were transported to the laboratory on ice and processed on the day of arrival according to Section 5.3.2 and 5.3.3.

5.3.5 Identification of presumptive *Campylobacter*

Isolated colonies were subjected to the aerobic test (Section 4.3.6). Gram stains and biochemical tests (Section 4.3.6) were performed only on those isolates that did not grow aerobically. Genomic DNA was extracted (Lemanceau *et al.*, 1995) from these isolates for PCR and primers CG12f and CG1597r were used to verify the isolates as *Campylobacter* (Lawson *et al.*, 1998). A 1.5kb region of the 16S rDNA gene of these isolates was also amplified using the universal primers, fD1 and rP2 (Weisburg *et al.*,

1991). These amplicons were purified as described in Section 4.3.7.3, subsequently sequenced on an ABI PRISM 377 sequencer (Perkin Elmer) and the sequence data edited using Sequence Navigator. Sequence identity was determined by searching known sequences in GenBank using a basic BLAST search of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/).

5.4 RESULTS

5.4.1 Evaluation of enrichment media

Recovery of *Campylobacter* using Bolton broth was better than with Preston broth (Table 5.2). There was no difference whether samples were taken from below the surface, or from mixed enrichment broth, and the number of colonies recovered was lower after 48h than 24h of enrichment. Madden *et al.* (2000) also observed a reduction in recovery of *Campylobacter* if the incubation period for the enrichment broth was prolonged.

Preston and Bolton enrichment broths are both selective for only *C. coli* and *C. jejuni* and with mCCDA, which was selective for the same two species and *C. lari*, it was therefore expected to find only these three species to grow after passage through the enrichment broths. It was however found that *C. fetus* was growing as well, whereas *C. lari* did not, placing a question mark on the real selectivity of the enrichment broth. The only non-*Campylobacter* isolate that grew after passage through the Preston broth (1KLWDC4) grew very well aerobically. All isolates that were able to grow aerobically were regarded as non-*Campylobacter*. Two *Arcobacter* species isolated by the modified Cape Town Protocol also failed to survive the enrichment procedure and grow on the selective agars (Table 5.2).

Table 5.2 **Evaluation of Preston and Bolton enrichment broths for the selective isolation of *Campylobacter* on mCCDA using isolates obtained by the modified Cape Town Protocol - March 2000**

	ISOLATE	PRESTON	BOLTON	AEROBIC TEST	NOTES
Positive controls	*C. coli*	+	+	-	
	C. jejuni doylei	+	+	-	
	C. jejuni jejuni 1	+	+	-	
	C. upsaliensis	-	-	-	
	C. fetus	+	+	-	
	C. lari	-	-	-	
Negative control	*E. coli*	-	-	+	
Isolates	1A18SB5	-	-	+	
	1B10SB1	-	-	+	
	1DaiGC4	-	-	+	
	1DarRB2	-	-	+	
	1DarRB3	-	-	-	*A. butzleri*
	1DusSB2	-	-	+	
	1GraDB1	-	-	+	
	1HamRC6	-	-	+	
	1K25SB8	-	-	+	
	1KLWDC4	+	-	+	
	1MngSB2	-	-	-	*A. butzleri*
	1RivSC2	-	-	+	
	1RV2SC1	-	-	+	
	1S2RC6	-	-	+	
	1S5SC3	-	-	+	
	1S7DC7	-	-	-	*Microvirgula* spp.
	1S10GB1	-	-	+	
	1SanDB1	-	-	+	
	1SlaSB1	-	-	+	
	1UmgSB1	-	-	+	

5.4.2 Evaluation using environmental samples

5.4.2.1 Fecal coliform counts

Table 5.3 **Fecal coliform counts from the different water sources received from three South African provinces (data given as cfu/100ml)**

	Drinking water		Ground water		Surface water		Raw Sewage	
Gauteng	Sandton	ND‡	Pretoria North	ND	A18	ND		
	Klipwater	ND			B10	ND		
					K19	1.3×10^{2}		
					K25	2.9×10^{2}		
					RV2	7.0×10^{2}		
					Riviera	5.0×10^{2}		
Kwazulu Natal	Craigburn	ND	Dairy top borehole	ND	Mngeweni River	ND	Hammers-dale Raw	TNTC¶
					Hammers-dale Effluent	ND		
Freestate	S7	ND	S9	2.5×10^{1}	S1	5.0×10^{2}	S2	7.0×10^{2}
	S8	ND	S10	ND	S3	3.0×10^{2}		
					S4	1.4×10^{1}		
					S5	ND		
					S6	2.1×10^{4}		

‡- not detected ¶- too numerous to count/ more than 300 colony forming units

5.4.2.2 Enrichment method

Gauteng

Table 5.4 (a) **Recovery of putative *Campylobacter*, after enrichment in Bolton broth, on blood agar for samples from Gauteng. Counts reflect the presumptive *Campylobacter* counts before confirmation**

SOURCE	SITE	SAMPLE VOLUMES TESTED:				RESULTS (cfu/100ml)
		100ml	10ml	1ml	10^{-1}	
Drinking water	Sandton	-	NT§	NT	NT	<1
	Klipwater	-	NT	NT	NT	<1
Ground water	Pretoria North	-	NT	NT	NT	<1
Surface water	A18	NT	-	-	-	<10
	B10	NT	-	-	-	<10
	K19	NT	+	+	-	>100 <1000
	K25	NT	+	+	+	>1000
	RV2	NT	+	+	-	>100 <1000
	Riviera	NT	-	-	-	<10

§- not tested

Table 5.4 (b) **Recovery of putative *Campylobacter*, after enrichment in Bolton broth, on mCCDA† for samples from Gauteng. Counts reflect the presumptive *Campylobacter* counts before confirmation**

SOURCE	SITE	SAMPLE VOLUMES TESTED:				RESULTS (cfu/100ml)
		100ml	10ml	1ml	10^{-1}	
Drinking water	Sandton	-	NT§	NT	NT	<1
	Klipwater	-	NT	NT	NT	<1
Ground water	Pretoria North	+	NT	NT	NT	>1
Surface water	A18	NT	+	-	-	>10 <100
	B10	NT	+	-	-	>10 <100
	K19	NT	+	-	-	>10 <100
	K25	NT	+	-	-	>10 <100
	RV2	NT	+	-	-	>10 <100
	Riviera	NT	-	-	-	<10

†- modified Charcoal Cefoperozone Deoxycholate Agar §- not tested

Kwazulu Natal

Table 5.5 (a) **Recovery of putative *Campylobacter*, after enrichment in Bolton broth, on blood for samples from Kwazulu Natal. Counts reflect the presumptive *Campylobacter* counts before confirmation**

SOURCE	SITE	SAMPLE VOLUMES TESTED:					RESULTS (cfu/100ml)
		100ml	10ml	1ml	10^{-1}	10^{-2}	
Drinking water	Graigburn	-	NT§	NT	NT	NT	<1
Ground water	Dairy Top borehole	-	NT	NT	NT	NT	<1
Surface water	Mngeweni river	NT	-	-	-	NT	<10
Sewage	Hammers dale effluent	NT	-	-	-	NT	<10
	Hammers dale raw	NT	NT	+	+	+	>10000

†- modified Charcoal Cefoperozone Deoxycholate Agar §- not tested

Table 5.5 (b) **Recovery of putative *Campylobacter*, after enrichment in Bolton broth, on mCCDA† for samples from Kwazulu Natal. Counts reflect the presumptive *Campylobacter* counts before confirmation**

SOURCE	SITE	SAMPLE VOLUMES TESTED:					RESULTS (cfu/100ml)
		100ml	10ml	1ml	10^{-1}	10^{-2}	
Drinking water	Graigburn	-	NT§	NT	NT	NT	<1
Ground water	Dairy Top borehole	-	NT	NT	NT	NT	<1
Surface water	Mngeweni river	NT	-	-	-	NT	<10
Sewage	Hammers dale effluent	NT	-	-	-	NT	<10
	Hammers dale raw	NT	NT	+	+	+	>10000

†- modified Charcoal Cefoperozone Deoxycholate Agar §- not tested

Freestate

Table 5.6 (a) **Recovery of putative *Campylobacter*, after enrichment in Bolton broth, on blood agar for samples from the Freestate. Counts reflect the presumptive *Campylobacter* counts before confirmation**

SOURCE	SITE	SAMPLE VOLUMES TESTED:					RESULTS (cfu/100ml)
		100ml	10ml	1ml	10^{-1}	10^{-2}	
Drinking water	S7	-	NT§	NT	NT	NT	<1
	S8	-	NT	NT	NT	NT	<1
Ground water	S9	-	NT	NT	NT	NT	<1
	S10	-	NT	NT	NT	NT	<1
Surface water	S1	NT	-	-	-	NT	<10
	S3	NT	+	+	+	NT	>1000
	S4	NT	+	+	-	NT	>100 <1000
	S5	NT	-	-	-	NT	<10
	S6	NT	+	+	+	NT	>1000
Sewage	S2	NT	NT	+	+	-	>1000 <10000

†- modified Charcoal Cefoperozone Deoxycholate Agar §- not tested

Table 5.6 (b) **Recovery of putative *Campylobacter*, after enrichment in Bolton broth, on mCCDA† for samples from the Freestate. Counts reflect the presumptive *Campylobacter* counts before confirmation**

SOURCE	SITE	SAMPLE VOLUMES TESTED:					RESULTS (cfu/100ml)
		100ml	10ml	1ml	10^{-1}	10^{-2}	
Drinking water	S7	-	NT§	NT	NT	NT	<1
	S8	-	NT	NT	NT	NT	<1
Ground water	S9	+	NT	NT	NT	NT	>1
	S10	-	NT	NT	NT	NT	<1
Surface water	S1	NT	+	-	-	NT	>10 <100
	S3	NT	+	+	-	NT	>100 <1000
	S4	NT	+	+	-	NT	>100 <1000
	S5	NT	-	-	-	NT	<10
	S6	NT	+	+	+	NT	>1000
Sewage	S2	NT	NT	+	+	+	>10000

†- modified Charcoal Cefoperozone Deoxycholate Agar §- not tested

As with the modified Cape Town Protocol (Chapter 4), no clear correlation were found between the presence of fecal coliforms and *Campylobacter* like organisms (CLO). For both Gauteng and Freestate samples there were sites that tested positive for CLO's but negative for fecal coliforms and vice versa. For KZN all sites but the raw sewage tested negative for both CLO's and fecal coliforms on both mCCDA and blood agar. A possible reason for this might be prolonged exposure to sunlight pr unfavourable temperature during transport. The organisms might have survived in the raw sewage sample because of the very high numbers that were present. Obiri-Danso *et al.* (2001) have found that UVB rays can reduce or even kill off *Campylobacter* cells. The same might be true for fecal coliforms. There were cases where samples tested negative on blood agar but positive on mCCDA (Pretoria North, A18, B10, S1 and S9). This was very strange since the blood agar was thought to be less selective than the mCCDA, which should only isolate 3 species.

5.4.3 Biochemical tests

Five colonies were selected from the highest dilution showing growth from each water sample from both blood agar and mCCDA respectively, and subjected to the aerobic test. Only 22 of these did not grow aerobically and were further characterized according to the key of Lastovica (personal communication). Ten of these isolates were from Gauteng, 5 from Kwazulu Natal and 7 from the Freestate. The results of all

biochemical tests can be seen in Table 5.7. Nine of the 22 isolates could not be identified to a specific species with this key. The rest were identified as *C. upsaliensis*, *C. jejuni* and *A. butzleri*.

5.4.4 Identification of *Campylobacter*-like organisms

5.4.4.1 Polymerase Chain Reaction with CG12F/CG1507R Primers

The CG12f/CG1507r primer set yielded amplicons for only 6 of the 22 isolates (Figure 5.1). According to the biochemical tests, 5 of these 6 isolates were *Campylobacter* and the other was *A. butzleri*.

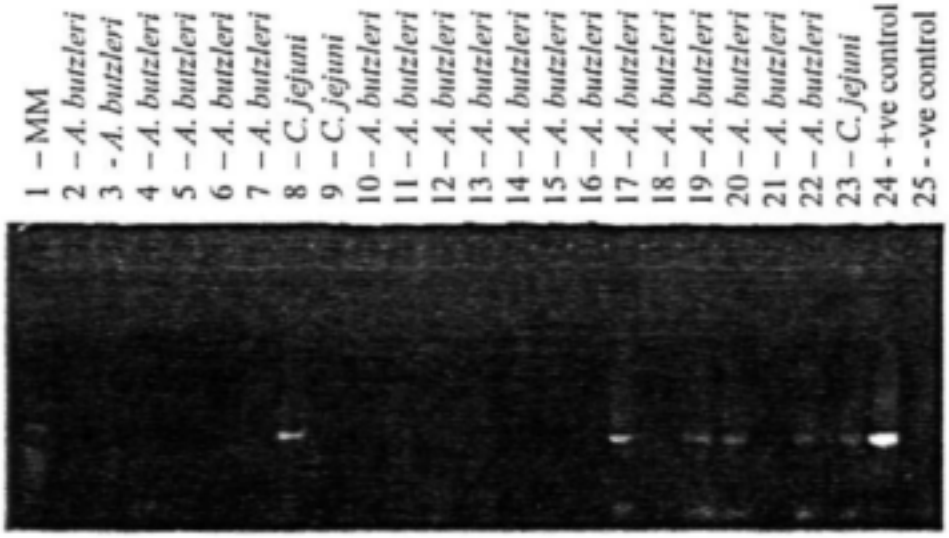


Figure 5.1 **Ethidium bromide stained 1% agarose gel displaying amplicons of isolates not growing in oxygen using primers CG12f and CG1507.**
Lane 1 – 100bp marker (Promega), 2 - 3A18SC1, 3 - 3A18SC2, 4 - 3B10SC3, 5 - 3B10SC4, 6 - 3BI0SC5, 7 - 3K25SB1, 8 -3K25SB2, 9 - 3K25SB3, 10 - 3RV2SC3, 11 -3RV2SC4, 12 -3HamRB1, 13 - 3HamRB2, 14 - 3HamRB2 (48hrs), 15 - 3HamRB3, 16 -3HamRC3, 17 - 3S1SC1, 18 - 3S1SC2, 19 - 3S3SC1, 20 - 3S3SC2, 21 - 3S4SC1, 22 - 3S4SC2, 23 - 3S6EB3. Species names reflect identities determined by the respective 16S rDNA sequences.

5.4.4.2 Sequencing of the partial 16S rDNA gene

The results from the partial 16S rDNA sequencing of the isolates can be seen in Table 5.7. From this data it is clear that the CG12f/CG1507r primer set was not as specific as described (Lawson *et al.*, 1998). Four of the 6 isolates that were amplified, were

identified as *A. butzleri* according to their 16S rDNA sequence analysis. Three of the 22 isolates were identified as *C. jejuni*, all 3 isolated from blood agar, and only one, 3K25SB2, corresponded with the biochemical identification. The rest of the isolates were all identified as *A. butzleri*. All the colonies isolated from the mCCDA were identified as *A. butzleri*. This was very surprising since both the enrichment broth and the mCCDA are believed to be selective against *A. butzleri*. Contrary to what was expected, only 5 of the isolates came from raw sewage and the rest from surface water samples. Obiri-Danso and Jones (1999a) suggested and proved that sewage runoff and animal droppings polluted surface and seawater. Thomas *et al.* (1999c) reported the same phenomena. Unfortunately that conclusion can not be made from this study since the raw sewage sample was the only sample from KZN that tested positive for CLO's. For the Freestate samples, the effluent isolate was identified as *C. jejuni* while the surface water isolates were all identified as *A. butzleri* thus making it highly unlikely to be the source of pollution for these surface water sites.

5.1.1 Summary of the results

Table 5.7 Summary of results of isolates in the third round of sampling

Location	Source	Isolate	16S rDNA Sequences	Gram	GC primer[1]	Catalase	Oxidase	NO_3	Hippurate	Pyrazinamide	TSI		Biochem ID
											strip	agar	
Gauteng	3A18SC1	Surface water	*A. butzleri*	-rods	-	-	+	+	-	+	1	2	*C. upsaliensis*
Gauteng	3A18SC2	Surface water	*A. butzleri*	-rods	-	+	+	+	-	+	2	1	*C. lari,C. coli C. hyoilei*
Gauteng	3B10SC3	Surface water	*A. butzleri*	-rods	-	-	+	+	-	+	2	-	*C. upsaliensis*
Gauteng	3B10SC4	Surface water	*A. butzleri*	-rods	-	-	+	+	-	+	2	-	*C. upsaliensis*
Gauteng	3B10SC5	Surface water	*A. butzleri*	-rods	-	-	+	+	-	+	2	-	*C. upsaliensis*
Gauteng	3K25SB1	Surface water	*A. butzleri*	-rods	-	+	+	+	-	+	3	-	*C. lari, C. coli C. hyoilei*
Gauteng	3K25SB2	Surface water	*C. jejuni*	-rods	+	+	+	+	+	+	-	-	*C. jejuni jejuni* 1 & 2
Gauteng	3K25SB3	Surface water	*C. jejuni*	-rods	-	+	+	+	-	+	1	-	*C. lari, C. coli C. hyoilei*
Gauteng	3RV2SC3	Surface water	*A. butzleri*	-rods	-	+	+	+	-	+	2	-	*C. lari, C. coli C. hyoilei*
Gauteng	3RV2SC4	Surface water	*A. butzleri*	-rods	-	+	+	+	-	+	3	-	*C. lari, C. coli C. hyoilei*
KZN	3HamRB1	Raw sewage	*A. butzleri*	-rods	-	+	+	+	-	+	-	-	*C. lari, C. coli C. hyoilei*

Table 5.7 (cont.)

Location	Source	Isolate	16S rDNA Sequences	Gram	GC primer‡	Catal ase	Oxid ase	NO_3	Hipp urate	Pyrazina mide	TSI		Biochem ID
											strip	agar	
KZN	3HamRB2	Raw sewage	*A. butzleri*	-rods	-	-	+	+	-	+	-	-	*C. upsaliensis*
KZN	3HamRB2 (48hrs)	Raw sewage	*A. butzleri*	- rods	-	-	+	+	-	-	-	-	*A. butzleri*
KZN	3HamRB3	Raw sewage	*A. butzleri*	- rods	-	-	+	-	-	+	3	-	*C. jejuni doylei*
KZN	3HamRC3	Raw sewage	*A. butzleri*	- rods	-	+	-	-	-	+	1	-	*C. jejuni doylei*
Freestate	3S1SC1	Surface water	*A. butzleri*	- rods	+	+	+	+	-	+	2	-	*C. lari, C. coli*
Freestate	3S1SC2	Surface water	*A. butzleri*	- rods	-	-	+	+	-	+	1	-	*C. jejuni doylei*
Freestate	3S3SC1	Surface water	*A. butzleri*	- rods	+	+	+	+	-	+	2	-	*C. lari, C. coli*
Freestate	3S3SC2	Surface water	*A. butzleri*	- rods	+	+	+	+	-	+	-	-	*C. lari, C. coli*
Freestate	3S4SC1	Surface water	*A. butzleri*	- rods	-	-	+	+	-	+	2	-	*C. upsaliensis*
Freestate	3S4SC2	Surface water	*A. butzleri*	- rods	+	-	+	+	-	-	2	-	*A. butzleri*
Freestate	3S6EB3	Sewage effluent	*Campylobacter jejuni*	- rods	+	-	+	+	-	+	2	1	*C. upsaliensis*

‡ - PCR method as described by Lawson *et al.*, 1998. (see section 4.3.7.2 for details)

5.5 DISCUSSION AND CONCLUSIONS

The enrichment approach was much more efficient than the optimized modified Cape Town Protocol for the isolation of Campylobacters from environmental waters. Although only 3 true *Campylobacter* species were isolated, the rest of the isolates belonged to the genus *Arcobacter*, which forms part of the *Campylobacter* group. *Arcobacter* was previously referred to as the aerotolerant *Campylobacter* until it was renamed (Vandamme *et al.*, 1991). Their primary phenotypic characteristics are their ability to grow in the presence of atmospheric oxygen and at temperatures as low as 15°C but not at 42°C (Vandamme *et al.*, 1991; Rice *et al.*, 1999). These are also the main features that distinguish them from *Campylobacter*, especially the thermophilic species. *A. butzleri* and *A. cryaerophilus* have been associated with human disease, with *A. butzleri* being isolated in most of the cases (Lerner *et al.*, 1994). Symptoms for *Arcobacter* illnesses are very similar to *Campylobacter* illnesses (Lerner *et al.*, 1994; Wesley *et al.*, 1995). Water is implicated as the major risk in acquiring *Arcobacter* associated diarrhoeal illnesses (Rice *et al.*, 1999; Vandamme *et al.*, 1992) and the organism has been isolated from raw and drinking water (Jacob *et al.*, 1998; Lerner *et al.*, 1994).

In this study, *A. butzleri* was isolated and identified in 19 out of 22 (86%) of the isolates that did not grow aerobically. This was not strange as *Arcobacter* is able to grow microaerophilically. A previous study has shown that it grows microaerophilically during initial isolation, but becomes more aerotolerant on subculturing (Wesley *et al.*, 1995). What strange however, was that all isolates from mCCDA were identified as *Arcobacter* according to their 16S rDNA identity. The use of the Bolton enrichment media and mCCDA were supposed to eliminate all non-*Campylobacter* species (Bridson, 1995). The majority of the isolates were from surface water with only six isolated from sewage.

The use of enrichment media, although all developed for the medical field, looked promising for use in the environment, but more work has to be done to refine the procedure. The primers used to verify all the presumptive *Campylobacter* isolates, were again proven not to be specific to the 9 species as claimed by Lawson *et al.* (1998). Four of the 6 isolates that produced the 1495bp amplicon with these primers,

were identified as *A. butzleri* by their 16S rDNA sequence, whereas one of the three *Campylobacter* were not detected.

This data showed that virtually no *Campylobacter* was isolated from South African waters. This might be due to it not being recovered with all the selective agents or it not being present at all. It could be said that the latter is true since no Campylobacters could be isolated with the optimized modified Cape Town Protocol (Chapter 4), where no antibiotics were used. Another possible reason for its absence or low prevalence may be the warm South African climate, concominant with a higher and longer UV exposure period. Ultraviolet rays and warmer temperatures have been shown to destroy *Campylobacter* cells (Obiri-Danso *et al.*, 2001).

In conclusion, there is no need for routine testing of environmental waters in South Africa for *Campylobacter*, at least based on cultural methods. There is, however a need to develop a proper molecular method for identifying all *Campylobacter* and *Arcobacter* species from environmental waters and also to compare them with those isolated from persons suffering from illnesses associated with these organisms.

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CHAPTER 6

DEVELOPMENT AND EVALUATION OF A NOVEL POLYMERASE CHAIN REACTION DETECTION METHOD OF *HELICOBACTER PYLORI* IN ENVIRONMENTAL WATER SAMPLES

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6.1. INTRODUCTION

Helicobacter pylori is a microaerophilic, spiral-shaped, motile gram negative bacterium and is strongly associated with gastroduodenal disease, including chronic active gastritis, peptic and duodenal ulcer disease and gastric cancer (Graham, 1991; Dunn *et al.*, 1997). Although *H. pylori* infection is widespread throughout the world (Goodwin *et al.*, 1997), the mode of transmission, the natural history and other aspects of the epidemiology of *H. pylori* infection are still unclear. Reported observations in general support a person-to-person mode of transmission via the fecal-oral, oral-oral and gastro-oral route (Feldman *et al.*, 1997; Stone, 1999). Various *H. pylori* disease outbreaks have been associated with the consumption of untreated well or spring water by children (Goodman and Correa, 1995) and the pathogen has also been found to occur in sewage water (Valentine *et al.*, 1991). Increased risk factors for infection have also been associated with poor sanitation practices and the consumption of uncooked vegetables irrigated with untreated sewage water (Johnson *et al.*, 1997). Reports have also indicated no significant correlation between the occurrence of total coliforms or *E. coli* in the water and the presence of *H. pylori* (Hegarty *et al.*, 1999).

Several methods for detecting *H. pylori* are used at present. Most of these diagnostic tests are performed on gastric biopsy samples and the bacterium can be identified in these specimens by a urea hydrolysis test, staining techniques and culturing (Coudron and Kirby, 1989; Schnell and Schubert, 1989). Despite the various different culture media and enrichment regimes that

have been proposed for isolating *Helicobacter* species from clinical samples, these organisms are still difficult to culture. Furthermore, conventional plate counting is tedious, time-consuming and usually underestimates the number of these organisms (Bickley *et al.*, 1993). This may be due to the ability of *Helicobacter* species to enter a viable but nonculturable state characterized by coccoid *H. pylori* organisms (Bode *et al.*, 1993). Laboratory studies (Bode *et al.*, 1993; Shahamat *et al.*, 1993, Fan *et al.*, 1998) have demonstrated that *H. pylori* can survive in freshwater and sterile, distilled water (7▒C) for 10 and 14 days, respectively. These studies also found evidence that the viable, nonculturable coccoid form of *H. pylori* can survive for up to 10 days at 4▒C in tap water. Low levels (10^2 cfu) of the coccoid *H. pylori* forms are needed to induce an acute inflammatory reaction in the stomach mucosa of mice, but higher levels (10^4 - 10^6 cfu) of the vegetative rod form are needed to induce a similar reaction (Aleljung *et al.*, 1996; Wang *et al.*, 1997).

Based on the difficulty of culturing *H. pylori* from sites other than the gastric mucosa (Goodwin *et al.*, 1985), greater interest has grown in the use of molecular techniques for detection of this species. The use of gene-specific probes have been described for the detection of *H. pylori* (Van den Berg *et al.*, 1989; Jiang *et al.*, 1998), and progress has been made by use of the polymerase chain reaction (PCR), which provides a specific and highly sensitive means of detecting microbial pathogens. PCR assays have detected *H. pylori* DNA in clinical specimens such as fresh gastric biopsy samples (Clayton *et al.*, 1992; Hammar *et al.*, 1992; Bickley *et al.*, 1993; Fabre *et al.* 1994; Li *et al.*, 1995) and in faeces (Makristathis *et al.*, 1998; Watanabe *et al.*, 1998). *H. pylori* has not yet been isolated from environmental water samples by traditional culture techniques, but its presence in such samples has been shown using techniques such as filtration, immuno-concentration, polymerase chain reaction (PCR) and hybridization with specific probes (Enroth and Engstrand, 1995; Hultén *et al.*, 1996; Hultén *et al.*, 1998).

The goal of this research was to develop a rapid and sensitive detection protocol for low levels of *H. pylori* and to evaluate the method for use in environmental water sources. We have subsequently developed such a protocol based on the technique of seminested PCR following isolation of the organisms in a selective medium.

6.2. MATERIALS AND METHODS

6.2.1. Bacterial strains

Helicobacter pylori, obtained from the Culture Collection at the South African Institute for Medical Research (SAIMR), Johannesburg, South Africa, was used as the reference strain throughout this study. *H. pylori* was cultured on Colombia agar (Oxoid) supplemented with 7% (vol/vol) laked horse blood and a *H. pylori* selective supplement (Dent, Code SR147E), consisting of vancomycin, trimethroprim lactate, cefsulodin and amphotericin B. The inoculated agar plates were incubated at 37°C under microaerophilic conditions for up to 7 days (Hachem *et al.*, 1995).

6.2.2. Preparation of DNA for PCR

Genomic DNA was extracted from bacterial strains by a rapid boiled-lysate technique. Briefly, bacterial colonies from an overnight cultivation on 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.

6.2.3. Selection and synthesis of primers

Oligonucleotide primers glmMF, glmMR and glmMI from the phosphoglucosamine mutase gene, *glmM*, were used in a seminested PCR assay.

Primers glmMF (5' - CGCGAGCCACAACCCTTTTGAAG - 3),

glmMR (5 - CGCGCTCACTTGCAAAGCGCACAC - 3')

as well as an internal primer, glmMI (5' - GCTTATCCCCATGCACGATATTC - 3'), were designed based on the *glmM* gene sequence reported by Labigne *et al.* (1991).

The first PCR step, performed with primers glmMF and glmMR, amplified a 765-bp region of the *H. pylori glmM* gene, while the size of the final PCR product obtained with primers glmMF and glmMI was 496-bp.

6.2.4. Primer specificity

Oligonucleotide primers glmMF, glmMR and glmMI were tested for amplification specificity using DNA extracted from the following panel of bacterial strains: *Helicobacter pylori* ATCC 700392, *Enterobacter aerogenes* ATCC 49469, *Escherichia coli* ATCC 13086, *Escherichia coli* ATCC 14824, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 49472, *Proteus mirabilis* ATCC 12453, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi* ATCC 49469, *Salmonella heidelberg* CCRC 123437, *Salmonella dublin* CCRC 13852, *Shigella dysenteriae* NCTC 1311, *Shigella flexneri* CCRC 10772, *Shigella sonnei* ATCC 8574, *Vibrio cholerae* NCTC 5941, and *Vibrio cholerae* ATCC 25870. *Salmonella typhimurium, Shigella boydii, Vibrio parahaemolyticus, Staphylococcus aureus,* and a clinical isolate of *Helicobacter pylori* were obtained from the Culture Collection at the South African Institute for Medical Research (SAIMR), Johannesburg, South Africa. *Bacillus cereus* was obtained from the Culture Collection at the Council for Scientific and Industrial Research (CSIR), Pretoria, South Africa. The strains were cultivated and maintained in nutrient broth (Difco) at 37°C, unless the Culture Collection instructions specified otherwise. DNA was extracted from the bacterial strains as described above.

6.2.5. PCR amplification

(i) Seminested PCR: The template DNA (5 µl) was added to 50 µl of a reaction mixture containing 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5% [vol/vol] Triton X-100), 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 µM, 50 pmol each primer (glmMF and glmMR), and 1 U of *Taq* DNA polymerase (Promega, Madison, Wisconsin, USA). The reaction mixtures were placed in a thermal cycler (Perkin Elmer, Model 2400). The following conditions were used: initial heat denaturation at 94°C for 1 min, followed by 10 cycles consisting of heat denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and DNA extension at 72°C for 1 min. The second PCR step was performed as above, except that a 1 µl aliquot of the first PCR was used as the template and 50 pmol of each glmMF and glmMI, 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 5 min to complete synthesis of all strands. At each amplification event, standard reaction mixtures containing *H. pylori* genomic DNA from type

strain ATCC 700392 was used as a positive control, whilst the negative control contained double-distilled water instead of sample DNA.

(ii) Single-step PCR reactions: PCR reactions were performed by using 5 µl of template DNA, buffer, deoxynucleoside triphosphate mixture, primers glmMF and glmMR, and *Taq* DNA polymerase in a final reaction volume of 50 µl. For primer pair glmMF and glmMR, the reactions were subjected to 30 cycles of amplification under the cycle conditions as described above.

6.2.6. Electrophoretic detection of amplicons

The amplified products were resolved on a 1% (wt/vol) agarose gel in 1 × TAE (40 mM Tris-HCl, 20 mM Na.acetate, 1 mM EDTA, pH 8.5) and visualized by UV-induced fluorescence after staining with 0.5 µg of ethidium bromide per ml. A 100-bp DNA ladder (Promega) was included on each gel as a molecular size standard.

6.2.7. Analysis of environmental water samples

Water samples (10 samples each) were received from tap, surface, ground and sewage sources in three provinces of South Africa (Table 6.1). The samples were taken by representatives of Rand Water Board (Gauteng), Umgeni Water Board (Kwazulu-Natal) and the Freestate Technikon (Freestate). All of these samples were transported to the laboratory on ice and analyzed on the day of receipt. The respective water samples were processed by first preparing a 10-fold dilution series in quarter-strength Ringer's solution and then plating of the relevant dilutions directly onto Colombia blood agar plates. In the case of drinking and surface water samples, 100 µl aliquots of the 10^{-1} to 10^{-3} dilutions were plated. In the case of raw sewage samples, 100 µl aliquots of the 10^{-2} to 10^{-4} dilutions were plated. The agar plates were subsequently incubated under microaerophilic conditions, as described above. Following incubation, presumptive *H. pylori* isolates were primarily identified by a translucent colony appearance and morphology in Gram stains. To confirm their identity, additional biochemical tests such as the urease, catalase and oxidase tests were also performed on selected isolates.

TABLE 6.1.: Water samples from three South African provinces indicating the different water sources that were sampled during July and October 2000

	GAUTENG (Rand water)	KWAZULU NATAL (Umgeni water)	FREESTATE (Freestate Technikon)
Tap water	Sandton	Graigburn WW	S7-Storage container
	Klipwater		S8-Communal stand pipe
Ground water	Roodeplaat dam	Dairy Top borehole	S9-Unprotected borehole
	Pretoria North		S10-Unprotected borehole
Surface water	A18	Slangspruit River 61	S1-urban run off, polluted stream
	B10	Mngeweni River	S3-S1 after it received effluent
	K19	Dusi River (above Darvill)	S4-below recreational damwall
	K25	Umgeni Estuary	S5-urban runoff, outside Bloemfontein
	RV2		
	Riviera		
Sewage		Hammersdale	S2-raw, poorly treated
		Hammersdale Raw	S6-effluent, well treated, new facility
		Darville	
		Darville Raw	

6.2.8. Amplification and sequencing of 16S rRNA genes obtained from environmental isolates

Selected isolates that could not be amplified by the *H. pylori*-specific PCR method, was subjected to further characterization by determining their 16S rRNA gene sequence as follow. DNA of these isolates were extracted according to the method described by Marmur and Doty (1962). The 16S rRNA genes were amplified by PCR by using the universal primers FD1 (5' - AGAGTTTGATCCTGGCTCAG - 3') and RP2 (5' - ACGGCTACCTTGTTACGACTT - 3') (Weisburg *et al.*, 1991). Amplification reactions were performed in a Perkin Elmer Model 2400 thermocycler. The PCR reaction mixtures (50 µl) contained 1 × PCR buffer, 1.5 mM $MgCl_2$, 25 ng of genomic DNA, 50 pmol of each primer, 50 mM of each deoxynucleoside triphosphate, and 2.5 U of Taq polymerase (Promega, Madison, Wisconsin, USA). After initial heating to 94˚C for 1 min, 30 cycles consisting of denaturation (93˚C, 1 min), annealing (54˚C, 1 min) and extension (72˚C, 1 min) were performed. Cycling was completed by a final elongation step (72˚C, 5 min). Positive controls along with negative controls (no DNA added) were also included. The presence and size of the amplification products were determined by agarose (1%) gel electrophoresis of the reaction products. The amplified DNA fragments were purified from the gel and 1 µl of the purified PCR product was used as template for sequencing. The sequencing reactions were performed using primer RP2. Nucleotide sequences were determined on an Applied Biosystems 377 DNA sequencer with the BigDye terminator cycle sequencing ready reaction kit as per the manufacturer's instructions (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA). The sequence data was edited with the programmes Sequencing Analysis 3.1 and Sequencing Navigator 1.0.1 included in the ABI PRISM software package (Perkin-Elmer, Applied Biosystems). Sequence comparison was subsequently carried out using the BLAST 2.1 software program and the GenBank databases.

6.3. RESULTS

6.3.1. Specificity of the PCR

To investigate the specificity of the glmMF-glmMR PCR, DNA samples of all strains listed in Materials and Methods were subjected to 30 cycles of amplification. Only the *H. pylori* strains yielded a single intense band of the expected length corresponding to 765-bp. With all other

strains tested, no PCR product was detectable. To confirm and to validate amplicon integrity, we performed sequencing reactions on aliquots of the purified 765-bp amplicons generated in PCR. The obtained sequence of the amplicon was almost identical to that of the targeted area of the *glmM* gene.

To investigate the specificity of the seminested PCR, the bacterial extracts were subjected to a 10-cycle PCR amplification with primers glmMF and glmMR, and 1 μl of the first round PCR product was subjected to another 20 cycles of amplification with oligonucleotides glmMF and the nested primer, glmMI. Only the *H. pylori* strains yielded a single amplicon of the expected size (approximately 496-bp) on agarose gel electrophoresis. No amplification product was observed from microorganisms other than *H. pylori*, even though there was sufficient DNA to detect a single copy sequence of *glmM*. We therefore concluded that both the single-step and seminested PCR assays can be used to specifically detect *H. pylori* DNA sequences. The results following a typical single-step and seminested PCR are indicated in Fig.6.1.

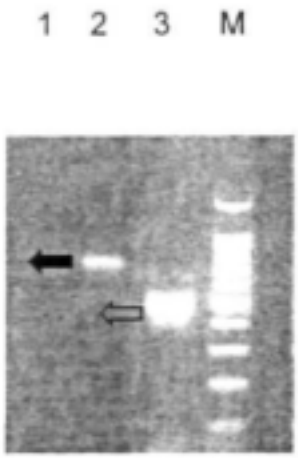


Figure 6.1: Detection of *H. pylori* DNA by a single-step PCR with primers glmMF and glmMR (Lane 2) and seminested PCR (lane 3). Lane 1, negative control (no DNA template). Lane M, 100-bp ladder as a molecular size standard. The 765-bp fragment of the single-step PCR and the 496-bp fragment of the seminested PCR are indicated by closed and open arrows, respectively.

6.3.2. Sensitivity of the PCR The sensitivity of the PCR assays for detection of *H. pylori* sequences was investigated by preparing serial dilutions of genomic DNA from *H. pylori* type strain ATCC 700392 in sterile double-distilled water.

Each dilution was subjected to PCR amplification and the products were visualized on 1% agarose gels stained with ethidium bromide. With the single primer pair, glmMF and glmMR, the 765-bp fragment could be visualized in ethidium bromide-stained gels in reaction mixtures that contained 10 pg of total chromosomal DNA. This level of detection is equivalent to the detection of 5×10^4 *H. pylori* cells. To reach a lower detection limit with simultaneous confirmation of the reaction product, the samples were subjected to the hemi-nested PCR assay. The application of this procedure rendered possible the detection of 0.1 pg DNA (5×10^2 cells) without loss of specificity. Thus, detection levels were increased 100-fold in the seminested PCR assays (not shown).

6.3.3. Detection of *H. pylori* in environmental samples

In order to evaluate the feasibility of the newly developed PCR amplification method for application for the detection of *H. pylori* in water as well as to possibly determine the prevalence of this pathogen in South African source waters, various environmental water samples were studied by culturing on Columbia blood agar plates and by the seminested PCR procedure. It was noteworthy that although the culture medium used in this study is being marketed as being selective for *H. pylori*, the plates were often found to be overgrown by competing microflora (Table 6.3). Nevertheless, in total 12 presumptive *H. pylori* isolates were selected based on colony morphology and appearance in Gram stains. These corresponded to 4 isolates from the first round of sampling in July 2000, and a further 8 isolates from the second round of sampling in October 2000. Isolates which were therefore gram negative and appeared to be spiral-shaped were subjected to the seminested PCR procedure. The control sample did give positive amplification, but all of the environmental isolates were negative after PCR amplification. To confirm these results, the isolates were further characterized by three biochemical tests, i.e. urease, oxidase and catalase tests. None of the selected isolates tested positive for all three these tests, as would have been expected if these isolates were indeed *H. pylori*. It thus appeared that *H. pylori* was either not present in

the tested water samples or it could not be successfully cultured from the environmental water samples. We furthermore concluded that the *H. pylori*-specific seminested PCR could be a potentially valuable diagnostic tool, as no false positive results were obtained in this analysis. The identity of 8 of the 12 biochemically characterized environmental isolates were subsequently determined by sequence analysis of the respective PCR-amplified 16S rRNA genes. The obtained results indicated that the four isolates isolated during the July sampling period were *Arcobacter* spp. In contrast, the identity of the remaining four isolates, which were obtained during the October sampling period, was confirmed to be *Salmonella enterica* serovar *Typhimurium*, *Serratia liquefaciens* and *Edwardsiella ictaluri*. One of the isolates, however, did not yield any significant homology to known bacterial species when compared to the sequences contained in the database. All of the above results are summarized in Table 6.2.

TABLE 6.2: **Identity of selected environmental isolates following PCR characterization, biochemical assays and nucleic acid sequencing**

SAMPLING PERIOD (2000)	ISOLATE#	PCR*	BIOCHEMICAL TESTS			IDENTITY¶
			Catalase	Oxidase	Urease	
JULY						
	S5-1	-	+	-	-	*Arcobacter cryaerophilus*
	S5-2	-	+	-	-	*Arcobacter butzleri*
	S5-3	-	-	-	-	*Arcobacter butzleri*
	S5-4	-	+	-	-	*Arcobacter cryaerophilus*
OCTOBER						
	S598-1	-	+	-	-	No significant homology
	S598-2	-	+	-	-	*Salmonella enterica* serovar *Typhimurium*
	S598-3	-	+	-	-	*Serratia liquefaciens*
	S598-4	-	+	-	-	*Edwardsiella ictaluri*
	S3-1	-	-	-	-	ND§
	S6-1	-	-	+	+	ND
	S6-2	-	-	-	-	ND
	S6-3	-	+	-	-	ND

#: Isolates numbered according to the key indicated in Table 4.2.

*: *H. pylori*-specific seminested PCR procedure, as developed in this study

§: Not determined

¶: Identity of isolates were determined by automated sequencing of the PCR-amplified 16S rRNA gene

TABLE 6.3: Concentration of bacteria (cfu/ml) in water samples, obtained from various water sources in three South African provinces, for which growth was observed on Colombia blood agar plates

SAMPLING PERIOD (2000)	PROVINCE	NATURE OF SAMPLES	CONCENTRATION* (CFU/ML)
JULY	GAUTENG	RV2 (surface water)	ND¶
		K25 (surface water)	1×10^4
		Pretoria North (surface water)	1.3×10^4
	KWAZULU NATAL	Umgeni (estuary)	1×10^2
		Darvil (final effluent)	1×10^4
		Darvill (raw sewage)	1.6×10^6
		Dusi (river)	1×10^3
		Dairytop (borehole)	1×10^2
		Mngeweni (river)	3×10^2
		Hammersdale (final effluent)	2×10^4
		Hammersdale (raw sewage)	ND
	FREESTATE	S2 (raw sewage)	1×10^3
		S3 (surface water)	3×10^2
		S5 (surface water)	2.7×10^5
		S6 (final effluent)	ND
OCTOBER			
	KWAZULU NATAL	Sample 598	ND
	FREESTATE	S2 (raw sewage)	ND
		S3 (surface water)	3×10^2
		S6 (final effluent)	5×10^4

*: Only samples for which growth on selective Colombia blood agar plates was observed, are indicated

¶ND: Not determined as colonies were too numerous to count

6.4. DISCUSSION

In recent years diagnostic laboratories have been concerned with reducing the time required for diagnosis of *H. pylori* infections. Different methods based on molecular biology techniques have therefore been developed for identification of *H. pylori*, with those based on DNA amplification being the most rapid and sensitive. Nevertheless, amplified products are seldom detected by direct visualization in ethidium bromide-stained agarose gels, but rather by Southern blot or dot-blot hybridization (Bickley *et al.*, 1993; Li *et al.*, 1995; Song *et al.*, 2000). 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. 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 primers selected for this study were based on the sequences of the *H. pylori glmM* gene. Investigation of sixty *H. pylori* isolates by PCR analysis and DNA sequencing indicated that the gene was present in every isolate (Kansau *et al.*, 1996). Furthermore, the *glmM* gene has recently been shown to be essential for *H. pylori* cell growth (De Reuse *et al.*, 1997). PCR assays based on the *glmM* (*ureC*) gene have been described (Bickley *et al.*, 1993; Bamford *et al.*, 1998) and were applied to the identification of *H. pylori* from bacterial colonies, gastric biopsies and faeces.

In this part of the study, a novel seminested PCR assay for the detection of *H. pylori* in environmental water samples has been developed and evaluated. 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 seminested PCR, based on amplification of the *glmM* sequence, is performed in a total of 30 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). Not only did the seminested PCR assay increase the specificity of the assay, but also the sensitivity. Only *H. pylori* strains screened by the PCR assay resulted in visualization of the predicted 496-bp amplified product in ethidium bromide-stained gels. Under the tested parameters, as little as 10 pg of purified chromosomal DNA, equivalent to 5×10^4 bacteria, were detectable by a single, first round PCR assay. However, seminested PCR amplification enhanced the limit of detection considerably (at least 100-fold), and 0.1 pg of *H. pylori* DNA (5×10^2 organisms) were detectable in this assay. This is an improvement over an earlier study in which 100 pg of purified chromosomal DNA was needed for detection of *H. pylori* by PCR on the *glmM* gene (Bickley *et al.*, 1993).

The applicability of the seminested PCR procedure for screening environmental water samples for the occurrence of *H. pylori* was also investigated. The water samples were obtained from various sources in three South African provinces and tested by seminested PCR following culturing on a *H. pylori* selective medium. None of the 12 presumptive *H. pylori* isolates tested positive by means of the newly developed *H. pylori*-specific PCR method. These results were subsequently verified by performing biochemical tests on the respective isolates as well as by sequence analysis of their 16S rRNA gene. The sequence data indicated the presence of various different bacterial spp. as indicated in Table 6.2. Two of the bacterial species identified in this study are of minor human concern. *Serratia* species are gram negative rods which are causes of nosocomial infection and are only rarely associated with community-acquired infections in normal hosts. Most human disease is caused by *S. marcescens*, whilst *S. liquefaciens* has rarely been isolated in serious infections (Hejazi and Falkiner, 1997). *Edwardsiella ictaluri* is the causative agent of septicemia of catfish (ESC) and is responsible for causing substantial financial losses to catfish farmers (Klesius and Shoemaker, 1999). However, the other bacterial species, identified as *Arcobacter* spp. and *Salmonella enterica* serovar *Typhimurium*, may be of greater importance. Arcobacters are gram negative, slightly curved, curved or helical shaped. They are microaerobic and may grow aerobically at 30°C and anaerobically at 35 to 37°C. Two of the four known *Arcobacter* species have been associated with human infection. Both *A. butzleri* and *A. cryaerophilus* have been isolated from patients with bacteremia and diarrhea (Kiehlbauch *et al.*, 1991; Hsueh *et al.*, 1997). In the case of *S. enterica* serovar *Typhimurium*, this pathogen usually

causes an intestinal infection (accompanied by diarrhea, fever and abdominal cramps). *Salmonella* is ubiquitous in animal populations, and human illness is usually linked to foods of animal origin. Salmonellosis also is transmitted by direct contact with animals, by non-animal foods and by water (Miller *et al.*, 1995; Tauxe, 1996). Each year an estimated 1 to 4 million cases of illness and 1 000 deaths are caused by nontyphoidal salmonellosis in the United States (Tauxe, 1996). Although *H. pylori* was not detected, these results indicated firstly, that the supposed *H. pylori* selective medium is not selective when used for the isolation of *H. pylori* from environmental water samples, and secondly, that the newly developed *H. pylori*-specific PCR method could have a potential value for further epidemiological studies for *H. pylori*-related research as no false positive results were observed.

Although *H. pylori* could not be detected in the environmental water samples tested in this study, some care should be taken in interpreting the results. To conclude that the failure in detecting *H. pylori* in the environmental samples as being indicative of it not being of concern may be too simplistic. While conventional culture methods have often been suitable for medical diagnostic microbiology applications, these methods are not always suitable for application to the detection of bacteria in water. This is because of the need for sensitive, specific and efficient detection techniques and quantification of low levels of bacterial pathogens in water. 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 (Hoffner, 1994; Kusnetsov *et al.*, 1994; Stone *et al.*, 1994). 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).

Furthermore, 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 state (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 Collwell, 1987; Byrd *et al.*, 1991; McKay, 1992; Islam *et al.*, 1993; Bode *et al.*, 1993; McDougald *et al.*, 1998; Cappelier and Frederighi, 1998). This makes their isolation and identification problematic and specialized handling procedures are required for their resuscitation. 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 subculturing on other differential and selective media or by making use of biochemical, immunological and nucleic acid-based analyses. However, as *H. pylori* is fastidious in its growth requirements and takes several days to grow, these nonselective isolation procedures are impractical and may be of little value. This is due to the plates being overgrown by unwanted, faster growing bacteria and which may aid in providing false negative results. There is thus a great need for further research into and the development of an effective isolation method for *H. pylori* based on culturing procedures. Consequently, is possible that in this study a greater degree of positivity may have been obtained if DNA was directly isolated from the samples and then used in the seminested PCR procedure.

To conclude, evidence for waterborne transmission routes have been provided by a number of investigators. Despite epidemiological evidence supporting a waterborne route of infection, there is little information available regarding the occurrence of the organism in environmental waters, since detection of *H. pylori* in these sources is confounded by the lack of standard procedure for the detection of this organism. Attempts to culture *H. pylori* directly from water samples have been unsuccessful, as was also the case in this study. This is probably due to its conversion into a coccoid, nonculturable form. Future research in this field should focus on optimizing culture conditions for *H. pylori* in environmental samples and studies of transmission pathways of *H. pylori* infection may also provide valuable information for the understanding of this important pathogen.

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PARASITES

CHAPTER 7

THE DEVELOPMENT OF METHODS FOR THE DETECTION OF MICROSPORIDIA IN DIFFERENT WATER SAMPLES.

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7.1 INTRODUCTION

Gastrointestinal parasitology was, until recently, limited to a few endemic protozoa (*Giardia* and amoeba) and roundworm infections (hookworm, *Ascaris*, *Trichuris* and *Strongyloides*). Since the onset of the immunodeficiency syndrome (AIDS) epidemic, the number of parasitic pathogens recognized and the frequency with which they are encountered in clinical practice have increased. Patients with AIDS are increasingly diagnosed with *Crypotosporidium*, microsporidia, *Isospora* and *Cyclospora* (Goodgame, 1996).

The microsporidium group is a large group of obligately intracellular parasites that form environmentally resistant spores. Approximately 143 genera and 1 200 species are known. They infect every major animal group, especially insects, fish and mammals (Sprague and Vavra, 1977; Weiss and Vossbrinck, 1998). They are important opportunistic agents of infection seen in immunological healthy persons (the first well-documented case was described by Matsubayashi *et al.*, 1959) and human immunodeficiency virus (HIV)-infected patients with AIDS. They are associated with a variety of illnesses including diarrhoea (Raynaud, *et al.*, 1998), keratoconjunctivitis, myositis, peritonitis, hepatitis, sinusitis (Visvesvara *et al.*, 1995 (b)) and dissemminated illness (Wittner *et al.*, 1993; Doultree *et al.*, 1995).

"Microsporidia" is a nontaxonomic name used to describe protozoan parasites belonging to the phylum Microspora. Seven species have been shown to be etiological agents of disease in humans. *Enterocytozoon bieneusi* and *Encephalatizoon (Septata) intestinales* are very common intestinal pathogens in AIDS patients (Dowd *et al*, 1998;Weber *et al*, 1994; Brian and Weber,

1993). Disseminated infections with *Encephalitozoon cuniculi*, *Encephalitozoon hellem* and *Encephalitozoon* (*Septata*) *intestinales* are also increasingly being recognised (Govinda *et al* 1995; Doultree *et al* , 1995, Kock *et al*, 1997). A recent study showed that up to 40% of patients with AIDS-associated diarrheas were shedding microsporidia (Kotler, 1995). Although person to person transmission has been recognised as one of the main routes of infection it is not yet clear whether microsporidiosis in humans could be a zoonosis. Dowd *et al.* (1998) confirmed the presence of *Enterocytozoon bieneusi*, *Encephalitozoon intestinales* and *Vittaforma corneae* in environmental water indicating that contaminated water may be a vehicle for spreading these organisms in the environment. Human pathogenic microsporidia therefore could be classified as waterborne.

Methods for the detection of microsporidia present in clinical samples (Chioralla *et al.*, 1998; Aldras *et al.*, 1994) and insects (Avery and Undeen, 1987) are available but methods for the concentration and detection from environmental water samples are only at the developing stage.

The size of the microsporidium spores (1.5x0.9 μm. *E. intestinales* spores may be slightly bigger (3.5x 2.0 μm) is one of the main factors contributing to difficulties experienced with identification of these organisms from environmental waters. It is virtually impossible to positively identify isolates visually without the use of electron microscopy. The presence of other genera belonging to the microsporidia, not associated with human pathogenicity and fungal spores complicates detection of these organisms in environmental water samples. Because the spores are small, and might be present in low numbers, large volumes of environmental samples have to be concentrated. Debris which accumulates during the concentration process further hamper the identification of these organisms.

The question of viability of human-pathogenic microsporidia in environmental water has in part been addressed through propagation of some species in cell culture (Wolk *et al.*, 2000). However *E. bieneusi*, the most commonly identified human -pathogen has not yet been propagated in cell culture.

The main objective of this part of the study was to establish techniques for the detection of

microsporidia spores in source and treated water using tissue culture techniques. To achieve this objective the following procedures had to be established:

- The propagation of *Encephalatizoon intestinales* spores in cell culture.
- Recovery of *Encephalatizoon intestinales* spores from seeded environmental water samples.
- Various clean-up and disinfection procedures of seeded water samples which have been concentrated prior to inoculation into cell culture.
- Stains to visualise microsporidia spores microscopically.
- A polymerase chain reaction (PCR) technique to confirm the presence of spores in seeded and unseeded water samples.

7.2 MATERIALS AND METHODOLOGY

7.2.1 In vitro growth of *Encephalatizoon intestinales* spores using RK13 cell line

Encephalatizoon intestinales spores (ATCC number 50603) were provided by the University of Arizona, Department of Veterinary Science and Microbiology, Tucson, Arizona. Spores were propagated in Rabbit Kidney (RK) 13 cell line.

7.2.1.1 Maintenance of cell line used for propagation of *E. Intestinales* spores

Rabbit Kidney (RK-13) cells, ATCC number CCL-37 were cultured in plastic 75 cm^2 flasks (Costar). Cells were maintained in DMEM/Ham's F10 (1:1) mixture with L-glutamine and 20 mM HEPES supplemented with 5% foetel calf serum (FCS). Flasks were incubated at 37°C.

Propagation of the cell line was as follows:

- Maintenance medium was removed by aspiration.
- 5 ml trypsin-EDTA were added and swirled over the cells to remove the remaining serum and divalent calcium and magnesium.
- Trypsin-EDTA was aspirated.
- 10 ml trypsin solution (1:1 trypsin and diluent, Appendix 1) were added.
- Flasks was incubated for 3-4 minutes at 37°C for cells to be released.

- 20 ml DMEM F10 medium supplemented with 5% FCS were added to the cell suspension.
- The cell suspension was transferred into two flasks, each containing 20 ml of the cell suspension .
- Flasks were incubated at 37°C.
- Cells were maintained by replacing used medium weekly with fresh DMEM F10 supplemented with 5% FCS.

RK-13 cells were grown for one week to confluency in DMEM F10 with 5% FCS in 75cm^2 flasks and then inoculated with spores.

7.2.1.2 Inoculation of RK13 cells with *E. intestinales* spores

DMEM was aspirated from a flask with confluent cell growth. Spores (200 µl of a 10^9 spores per mℓ suspension) diluted in 5ml DMEM F10 with 5% serum were added to the flask . The flask was incubated at 37°C for 10 minutes where after an additional 20 ml DMEM F10 with 5% was added. Flasks were incubated at 37°C.

7.2.1.3 Harvesting spores

Spores were harvested weekly. Cell culture medium of inoculated flasks, containing the spores, were collected into a centrifuge tube and spun for 10 min at 10 000 rpm. The supernatant was discarded and the pellet resuspended in 2ml DMEM F10. The suspended cells were reintroduced into the same flasks with fresh medium containing 5% FCS. After four weeks all spores were harvested from the flasks by centrifugation and stored in 1 ml DMEM F10 at 4°C.

Concentration of the harvested spores was determined using a haemacytometer. Counts obtained were 10^7 to 10^8 spores per mℓ.

7.2.2 Concentration and seeding of water samples

Water samples were collected by Technikon Free State, Water, Rand Water, Umgeni Water and the University of the Western Cape. Samples consisted of surface water, ground water, drinking water and waste water effluents.

7.2.2.1 Concentration of water samples

Water samples were put into an Amicon 40M stainless steel tank and passed through a flat membrane (Sartorius pore size 0.45 µm) using a Sartorius filter holder and air pressure. Very turbid samples were pre-filtered using glass fibre pre-filters prior to seeding and membrane concentration. After filtration the membrane was transferred to a crystalising dish. All residual liquid in the tank was collected and added to the crystalising dish. The liquid was used to wash the membrane with the help of a rubber policeman. The "wash" liquid was collected and stored at 4°C. These will be referred to as water sample concentrates.

7.2.2.2 Seeding water samples concentrates

To determine the feasibility of PCR detection of spores in drinking water (10ℓ), ground water (10ℓ) and surface and waste water (5ℓ) concentrates were seeded with one millilitre of a spore suspension containing 10^8 spores per mℓ prior to PCR.

To determine the sensitivity of the PCR concentrated samples of drinking water, ground water, surface water and waste water samples were inoculated with concentrations of 10^3, 10^4 and 10^6 spores per mℓ respectively.

7.2.3 Clean-up procedures of water sample concentrates

The concentrated volume of each sample was approximately 20 ml. Concentrates contained debris, bacteria, yeasts and fungal spores. The presence of these organisms not only complicated the identification of spores but caused massive contamination and loss of cell cultures when introduced into flasks. Various procedures were applied in an attempt to obtain a cleaner concentrate.

7.2.3.1 Sucrose flotation purification

Twenty millilitre sample concentrates were pelleted by centrifugation at 10 000 rpm for 10 min. The pellets were resuspended in 10 mℓ distilled water and carefully underlayed in round bottomed 50 mℓ tubes containing 30 mℓ 1.0 M sucrose. Tubes were centrifuged in a swinging bucket rotor at 2 800 rpm for ten minutes. After centrifugation the upper aqueous layers containing the spores were aspirated with an additional 5 to 8 mℓof the interface. These samples

were washed with sterile distilled water (30mℓ) and pelleted by centrifugation at 10 000 rpm. for 10 min. The pellet was resuspended 5 mℓ saline or DMEM and stored at 4 °C.

7.2.3.2 Differential density gradient centrifugation.

Debris were removed from concentrated water samples using differential density gradient centrifugation with an isopycnic gradient of Percoll, an inert substance unlikely to interfere with spore infectivity.

Sample concentrates were further concentrated by centrifugation at 10 000 rpm. Percoll was mixed with concentrated sample pellets. Equal volumes of the Percol/pellet mixture and sterile Milli-Q water were transferred to a 15 mℓ conical centrifuge tube. The resulting mixture had a specific gravity of 1.067 suitable for separating debris from in tact spores. Tubes were centrifuged at room temperature at 2 300x*g* for 30 min. The pellet was collected and washed in sterile Milli-Q water and centrifuged again and stored in saline.

7.2.4 Decontamination of water sample concentrates prior to inoculation into cell culture

Concentrated water sample pellets contained considerable amounts of debris even after preliminary clean-up procedures described above. The following procedures were applied to obtain sterile pellet preparations for inoculation into cell culture flasks.

7.2.4.1 Antibiotics

Researchers have used different antibiotic mixtures to disinfect clinical samples before inoculating cell culture flasks (Van Gool *et al.*, 1994; Doultree *et al.*, 1995). In this study 100 units/mℓ each of Penicillin, Gentamycin, Kanamycin and 100 µg/mℓ Streptomycin were added to concentrated water samples (5 to 10 mℓ volumes of sample concentrate). Samples were left to stand for 18 h. After 18 h samples were centrifuged at 10 000 rpm for 10 min. The pellet was washed once with sterile water, pelleted by centrifugation, resuspended in DMEM and used as inocula for cell cultures.

7.2.4.2 Chlorine disinfection

A 1mℓ inoculum of 10^6 spores per mℓ was added to 100 mℓ 0.05 M phosphate buffer. The mixture

was chlorinated to a concentration of 2 mg/ℓ. The contact time was 4 min after which chlorine was neutralised with 1mℓ 10% w/v sodium thiosulphate ($Na_2S_2O_3$). Chlorine concentrations were adjusted to compensate for any chlorine demand exerted by the spores. Chlorine concentrations were measured using the N,N-dimethyl-p-phenylenediamine colorimetric method.
After neutralisation with thiosulphate spores were concentrated by centrifugation to obtain a small pellet for inoculation into tissue culture flasks.

7.2.4.3 Chloroform

Three mℓ chloroform were added to 20mℓ of a sample concentrate. The chloroform was mixed with the sample by shaking and then left to stand at room temperature. After 20 min the chloroform was removed by centrifugation at 2000 rpm for 20 min. The supernatant, which contained the spores, was removed and stored at 4 °C.

7.2.5 Detection methods for microsporidium spores

Microscopic detection of spores in seeded and unseeded sample concentrates and spores in tissue culture was conducted using different staining techniques commonly used for detection of microsporidia in clinical samples.

Tissue culture chambers and an in vitro micro-well plate method were used for the microscopic detection of parasitophorus vacuoles containing spores visible after infection with viable microsporidium spores.
Tissue culture were also used to demonstrate viability and infectious capabilities of the spores. Spores were visible under 400X magnification using a green FS 550 exciter filter after four weeks of incubation.

Microsporidia spores were stained using fluorochrome Calcofluor white stain, modified Trichrome blue stain (Ryan *et al.*, 1993) and Giemsa stain during this study.

A polymerase chain reaction (PCR) procedure was used for confirmation of the presence of spores in water samples and cell culture flasks.

7.2.5.1 Staining with Fluorochrome Calcofluor white stain

A small drop (10-15 µl) was placed on a glass microscope slide.

The slide was air dried and fixed in 100% methanol for 5 min.

Fluorochrome calcofluor white stain was prepared as follows:

Solution 1: A 0.1% solution of fluorescent brightner 28 (Calcofluor white M2R) was prepared in distilled water and store in the dark.

Solution 2: A 10% potassium hydroxide solution was prepared in distilled water.

Solutions 1 and 2 were combined 1:1 just before staining.

Staining:

The slide was flooded with the 1:1 mixture described above and incubated in a humid container at room temperature for 30 min.

The slide was rinsed with distilled water and air dried.

Visualisation was conducted under oil immersion, 100X magnification using a epifluorescent microscope fitted with a 100W halogen lamp, G365 excitor and LP420 barrier filter.

7.2.5.2 Staining with Trichrome blue stain

Microscope slides were prepared as described in 7.2.5.1.

Staining was as follows:

Trichrome blue was heated to 50 °C.

Slides were immersed in the stain for 10-30 min. at 50°C.

Slides were rinsed first with acid alcohol (0.45% acetic acid in 90% ethanol) for 10 sec and then with 95% ethanol for 10 sec.

A first dehydration in 95% ethanol for 5 min. was followed by a second dehydration in 100% ethanol for 10 min.

Slides were cleared in xylene for 10 min and then air dried.

Visualisation was conducted using 100X oil immersion and a light microscope.

7.2.5.3 Staining with Giemsa

The slide was air dried and fixed in methanol for 5 min.

Giemsa powder (0.5 g) was dissolved in 33 mℓ glycerol at 60 °C for 1.5 to 2 h. Absolute methanol (33 mℓ) was added to the mixture allowed to stand and then decanted. The sediment free stock solution was kept at room temperature. One part stock solution was diluted with 40 parts distilled water before use.

Staining was as follows:

The microscope slide was covered with freshly diluted Giemsa for 1 h.

The slide was rinsed with 95% (vol/vol) ethanol to remove any excess dye and air dried.

The slides were examined under oil immersion using a 100X magnification and a light microscope.

7.2.5.4 In vitro micro-well plate viability assay

A twenty four well plate format was used to determine the viability and infectivity of spores. Cells were removed from cell culture flasks with trypsin-EDTA. Cells were counted using a haemacytometer. Cells were diluted in cell culture medium to a concentration of 10^5 cells /mℓ.

Twenty four well plates were seeded by placing 1 mℓ aliquotes of the cell suspension on glass cover slips placed inside each well. Plates were incubated for three days. The monolayers on the glass cover slips were then inoculated with microsporidium spores (concentration 10^3 per mℓ) and replenished with fresh DMEM and 5% FCS. Negative control wells (not inoculated with microsporidia spores) were included. The plates were incubated for 6 days. After incubation the cover slips were fixed with methanol and stained with Giemsa or Trichrome blue. The cover slips were mounted on glass microscope slides and examined with a light microscope at a magnification of 400X to determine whether parasitophorous vacuoles containing mature microsporidium spores developed in the cystoplasm of the RK13 cells.

7.2.5.5 Tissue culture chambers

The method described in section 7.2.5.4 was used to grow monolayers directly onto a microscope slides divided into eight chambers. After inoculation and incubation the whole slide was stained with Trichrome or Giemsa to visualise parasitophorus vacuoles.

7.2.5.6 Polymerase chain reaction (PCR)

Primers targeting the small-subunit ribosomal DNA were used for the detection of the microsporidia. Based on the amplicon size a distinction could be made between *E. cuniculi* (268 bp), *E bieneusi* (250 bp) *E. intestinales* (270 bp) and *E. hellem.*(Dowd *et al.*, 1998). DNA extraction: DNA from seeded and unseeded samples were extracted by pelleting concentrated water samples. Pellets were exposed to Proteinase K to lyze spores and the DNA was then extracted using the Dneasy kit (Qiagen). After elution of the DNA from the column, it was used as template for the PCR reaction.

The PCR was performed as initially described by Dowd *et al.* (1998). Amplicons were visualized after separation on agarose gels and visualization with ethidium bromide.

7.2.6 Determination of the effect of chloroform on the viability of the spores in environmental samples

Environmental sample concentrates were divided into five equal volumes. One volume served as an unseeded control. The remaining three volumes were seeded with 10^3, 10^4 and 10^5 spores. The samples containing spores were decontaminated with chloroform as described in section 7.2.4.3. The decontaminated supernatants were concentrated by centrifugation at 10 000 rpm. Pellets were resuspended in 5 mℓ DMEM supplemented with 5% FCS and inoculated into 25cm^2 tissue culture flasks with confluent monolayers. Flasks were incubated at 37°C. Controls included one tissue culture flask inoculated with spores not exposed to chloroform and one tissue culture flask with uninoculated cells. After four weeks cells were observed using a light microscope, 400X magnification and a green FS 550 filter to determine whether cells were infected. Results were confirmed by the PCR method.

7.3 RESULTS

7.3.1 Propagation of *E. intestinales* spores in RK 13 tissue culture cells

Spores were successfully propagated in RK 13 cells. Cyst yields varied between 10^7 to 10^8 per mℓ. Presence of spores in cell culture medium was confirmed by PCR method. Harvested spores were accumulated and used during seeding experiments.

7.3.2 Concentration of water samples

Ten litre water samples with low turbidity could easily be concentrated using membrane filtration. Turbid samples however clogged membranes and it was necessary to remove some of the debris and particles using glass fibre pre-filters prior to final concentration using the 0.45 µm membranes. It was not possible to concentrate more than 5 ℓ of some of the more turbid samples.

7.3.3 Cleanup procedures

The sucrose flotation method did not remove debris efficiently. Because the spores are very small in size they are separated to a certain extent but mud particles bacteria and other debris were also concentrated. These particles seriously interfered with the detection and identification of spores.

Percol density gradient flotation resulted in a cleaner pellet although not sufficiently clean for inoculation into tissue culture. Further disinfection was still necessary before inoculation into tissue culture flasks.

7.3.4 Results obtained using different decontamination methods

7.3.4.1 Antibiotics

Adding antibiotics to very turbid water sample concentrates did not result in sufficient disinfection of the samples. Contamination of the tissue culture appeared after some days of incubation and resulted in the loss of the cells. Doubling the concentrates of the antibiotics given in section 7.3.4.1 also did not prevent contamination of the cell line. "Clean" samples such as drinking water samples and ground water samples could be decontaminated successfully using antibiotics.

7.3.4.2 Chlorinated concentrates

Chlorinated concentrates were not inoculated into tissue culture flasks as all cultures were lost to contamination. It since has not been possible to obtain healthy tissue culture to complete the experiments.

7.3.4.3 The effect of chloroform decontamination on the viability of microsporidia spores

Initial results using chloroform as a method for decontamination looked promising. Observing the spores using light microscopy did not indicate any change in the physical appearance of the

spores after chloroform treatment. It was not possible to determine whether or not their viability was affected relying on light microscopy alone. Spores were therefore inoculated onto tissue culture. Tissue culture flasks inoculated with 10^5 spores, after chloroform treatment, were successfully infected. Spores could be visualised by light microscopy at 400X magnification using a green FS 550 filter after four weeks incubation. PCR results confirmed the presence of the spores.

7.3.5 Results obtained using various detection methods

7.3.5.1 Staining techniques

Fluorescence staining (Calcuflour), Trichrome blue stain and Giemsa stain produced poor and confusing results for visualisation of both "clean" spores and spores in environmental samples. Although fluorescence staining was found to be quick and easy it was impossible to distinguish microsporidian spores from other fluorescing bodies and fungal spores of different sizes. The Trichrome staining method is commonly used for clinical specimens. Stained spores should display a characteristic refractile ovoid shape with a clear vacuole-like zone. The spore contents should remain either transparent or demonstrate a pinkish red belt-like or equatorial structure. During this study spores from a pure culture showed the vacuole like zone but the stained belt-like structure was never visible.

Staining environmental water concentrates using Trichrome resulted in a confusing red background from which it was impossible to distinguish any spores. Staining tissue culture cells to visualise the parasitophorous vacuoles which form inside the cells after infection was also not very satisfactory but some vacuoles were visible.

Giemsa stain used for staining infected cells resulted in visible parasitophorous vacuoles. Generally detection of spores using stains and light microscopy was very unsatisfactory. Interfering debris made it impossible to make any worthwhile distinction in environmental samples

7.3.5.2 In vitro micro-well plate and tissue culture chamber viability assays

The in vitro micro-well plate assay failed. Extreme difficulties were experienced removing the glass cover slips from the wells and mounting them on a microscope slide to apply the Trichrome

blue stain. It was not possible to identify any parasitophorus vacuoles after staining with Trichrome blue stain.

The tissue culture chamber slides were found more practical and easier than the in vitro micro-well plate assay. Monolayers infected with pure microsporidian spores were stained with Giemsa. Some vacuoles were visible under 400X magnification using a light microscope.

7.3.5.3 Determining the presence of microsporidian spores in unseeded environmental water samples

One drinking water sample, one ground water sample, four surface water samples and four waste water samples were concentrated as described in section 7.2.2.2. DNA was extracted from the concentrates and used as templates for the PCR. Results are shown in Table 7.1 .

The results either indicate that samples did not contain any microsporidian spores or that the concentration of the spores in the samples was too low to be detected by the PCR.

Table 7.1: PCR results for unseeded concentrated water samples

Type of water sample	PCR Result
Positive control	Positive
Negative control	Negative
Drinking water(one sample)	Negative
Ground water (one sample)	Negative
Surface water (4 samples)	Negative
Waste water (4 samples)	Negative

7.3.5.4 PCR results for seeded samples

A total of 34 samples were seeded and analysed using the PCR method in order to determine the degree to which the PCR would be inhibited by chemical and humic substances in the environmental samples. Results are shown in Table 7.2. Eight of the seeded samples produced negative results. These consisted of surface waters samples from three sites which possibly contained some inhibitory substance which influenced the outcome of the PCR. The remaining samples were positive indicating little interference from inhibitory substances in drinking water, ground water, and waste water

The sensitivity of the PCR was determined by seeding drinking water, ground water, surface water and waste water with 10^3, 10^4 and 10^6 spores respectively. Results were positive for all samples containing 10^4 and 10^6 spores except surface water. Inhibitory substances may have prevented the amplification of the DNA.

Table 7.2: PCR results for seeded concentrated water samples

Number of samples tested	Sample name and type	PCR result
DRINKING WATER		
2	Bloemfontein- standpipe	Positive
2	Rand Water Klipwater	Positive
2	Umgeni-Craigburn final	Positive
2	Gouws Household	Positive
GROUND WATER		
2	Bloemfontein-unprotected borehole(S9)	Positive
2	Bloemfontein-unprotected borehole(S10)	Positive
2	Umgeni- borehole at dairy	Positive
SURFACE WATER		
3	Bloemfontein urban runoff (S1)	Negative
2	Bloemfontein urban runoff (S5)	Negative
2	Rand Water K25	Negative
2	Rand Water Riviera	Positive
1	Umgeni Estuary	Positve
1	Umgeni Slangspruit	Positive
2	Kuilsrivier- Baden Powell drive	Positive
2	Stellenbosch-Kuilsrivier	Positive
WASTE WATER		
3	Bloemfontein treated waste water (S2)	Positive
1	Bloemfontein treated waste water (S6)	Positive
1	Athlone waste water	Positive

7.3.5.5 Sensitivity of the PCR for detection of spores in chloroform decontaminated environmental samples

Six samples consisting of one drinking water, one ground water, one surface water and three waste waters were inoculated with 10^3 , 10^4 and 10^6 spores and treated with chloroform as described in section 7.2.4.3.

After four weeks of incubation no spores were visible by light microscopy at 400X magnification using a green FS 550 filter. PCR results were also negative. Incubation was continued for another two weeks but results remained negative. The experiment was repeated but visual and PCR results were found negative.

7.4 DISCUSSION

This study was conducted with the aim to investigate methods for the detection of microsporidian spores in environmental water samples. Existing methods, normally used for the detection of spores in clinical specimens, have been adapted and applied in an attempt to detect microsporidian spores in drinking water, ground water, surface water and waste water samples. *E. intestinales* spores were used to assess the procedures conducted during this study. Polymerase chain reaction (PCR) methodology has been used to confirm the presence of spores.

An in vitro system for the production of microsporidian spores was established for the purpose of producing sufficient spores to be used in assessments for infectivity and viability. Because of a limited number of animal models for microsporidian growth and the low spore concentrations that can be produced in these models (Accoceberry *et al* 1977; Tzipori *et al.*, 1997) in vitro models are the method of choice. Microsporidia grow well in a variety of cell lines such as monkey kidney cell line E6 and human embryonic lung cells (Visvesvara *et al.*, 1995 (a); Doultree *et al.*, 1995). *E.bieneusi*, the most common microsporidium that infects humans, have only been cultivated in vitro on a short term basis (Visvesvara *et al.*,1995 (b)). Successful cultivation and confirmation of the presence of spores in the RK13 cell line established in our laboratory served as a source of spores for investigating the infectivity and viability of the spores

after exposure to different purification and decontamination procedures.

The most limiting factor for detection of microsporidian spores in environmental samples is their small size. *E. bienuesi* spores measure between 1 and 1.5 µm and *Encephalitozoon* spp. measure between 2 and 3 µm (Weiss and Vossbrinck, 1998) These measurements are the same as those of most bacteria. Because microsporidia are usually present in environmental water in lower numbers than in clinical samples large volumes of water has to concentrated to detect these tiny parasites. During concentration, bacterial cells, fungal spores, and bacterial spores and other debris are also concentrated. The resultant concentrate, especially in the case of turbid waters has to be treated with flotation purification procedures and decontamination procedures. This is necessary to minimize interference when spores are stained and viewed by microscopy, inoculated into tissue cell culture or analysed using PCR. Water samples were concentrated using a flatbed membrane filter holder, air pressure and nitrocellulose membranes (pore size of 0.45 µm). The membrane pore size limited the volumes of water that could be concentrated. In some cases only 5 litres of the more turbid water could be concentrated. Pre-filtration using glass fibre membranes alleviated this problem somewhat. Twenty litre volumes of drinking water and borehole water could be concentrated with ease. Other researchers have applied the USEPA Information Collection Rule (ICR) concentration method (Dowd *et al*, 1998) and continuous flow centrifugation concentration (Avery and Undeen, 1987).

Debris, fungal spores, other parasitic and bacterial spores which were concentrated using membrane filtration proved to be the main obstacle to successful identification and cultivation of microsporidian spores. Flotation purification procedures (sucrose purification flotation and density gradient flotation) proved unsatisfactory in the removal of contaminating particles. Decontamination of these samples had to be conducted in an attempt to disinfect samples prior to inoculation into cell culture. Three decontaminating methods were applied during this study, namely, antibiotics, chlorination and chloroform treatment. Although antibiotics have been used with seemingly great success for decontamination of clinical specimens (Van Gool et al., 1994; Doultree,1995), findings of this study showed that antibiotics could not decontaminate environmental samples sufficiently for inoculation into tissue culture cells without resulting in contamination. Antibiotics suppressed growth of other bacteria and fungi only for a short period of time after which cultures were lost to contamination.

Chloroform treatment is a well known method used to decontaminate samples that are inoculated into tissue culture for analysis for viruses (Idema, 1992). The viability and infectivity of chloroform treated spores were evaluated using tissue culture. Initial experiments included exposure of "clean" spores, harvested from tissue culture, to chloroform treatment and inoculation into tissue culture. The presence of spores in the culture medium after incubation confirmed the development of parasitophorus vacuoles, indicating that cells were indeed infected and that spores were viable. The presence of the spores were further confirmed with the PCR method.

A number of environmental water sample concentrates, (drinking water, ground water, surface water and waste water) seeded with high concentrations of spores and treated with chloroform also resulted in infection of tissue culture cells indicating spore infectivity and viability. Results were again confirmed by PCR. Initial results using chloroform treatment looked promising. However, inoculation of chloroform-treated sample concentrates, seeded with low numbers of spores, did not give positive results for either tissue culture infection or the PCR. This may indicate that when low numbers of spores are present, none survive chloroform treatment. On the other hand, the presence of high numbers of spores in an inoculum may provide some spores with a better chance of remaining infectious and viable. The exact reason for the negative results could not be determined because the study was plagued with interfering contamination in both the tissue culture and the spore source at the time.

Effective morphologic demonstration of microsporidia by light microscopy can be established by staining methods that produce differential contrast between spores of the microsporidia and other cells and debris in samples such as stool. Diagnosis of microsporidia spores in clinical specimens by light microscopy has been known to be a challenge for parasitologists. So much more the diagnosis of microsporidial spores in environmental water concentrates. This proved virtually impossible, using either a chemifluorescent method (Calcufluor white M2R), Trichrome blue or Giemsa during this study. This was mainly due to staining characteristics of the spores, which are similar to those of enteric bacteria and fungi, the small size of the spores and interfering debris.

Calcufluor white, a chemifluorescent brightner, binds to the endospore layer of the microsporidia and allows the spores to be detected in smears of specimens with a fluorescent microscope (Weber and Bryan, 1994). It has been reported as a very quick assay for clinical specimens (Chioralla, *et al.*, 1998) even though fluorescing fungal spores may complicate identification. Using this stain for environmental samples, in this study, resulted in a confusing number of fluorescing spores of different sizes which made specific identification of microsporidia spores impossible. Chiorolla *et al*, (1998) concluded that *Clostridium* spores also take up the fluorescent dye. The relevance of this false-positive artifact is high since infections with *Clostridium* species are increasingly being recognised in AIDS patients (Mastroanni *et al.*, 1997).

Identification using Trichrome blue has been reported to show high specificity for clinical specimens (Chioralla, *et al.*, 1998). With this stain spores should appear bright pinkish-red, and most have a distinctive diagonal or equatorial line that allows microsporidia and bacteria to be easily differentiated. However, evaluation of this stain, using spores harvested from cell culture and seeded environmental water concentrates, showed very poor differentiation and gross non-specificity. Similar results were obtained when using Giemsa.

All stages of the microsporidian's life cycle can be found in infected tissue, but only the spore stage can be observed in bodily fluids and stool. During the in vitro replication cycle, *Encephalitozoon* spp. form cytoplasmic parasitophorous vacuoles filled with mature spores. These vacuoles eventually rupture and release spores into the culture medium. The parasitophorous vacuoles can be visualised in stained infected monolayers. Micro-well plates and tissue culture chambers prepared to determine the formation of parasitophorus vacuoles in the cells and to confirmation of the ability of the spores to infect cells showed poor results. The method was very laborious, staining mostly non-specific and tissue culture monolayers were prone to contamination.

Isolation of spores from environmental water samples can be greatly enhanced with the use of immunomagnetic separation (IMS) technolgy. This technology is routinely used in conjunction with fluorescent antibodies for the detection *Giardia* and *Cryptosporidium* spores present in environmental waters. Although IMS has been applied successfully used for the concentration of

the microsporidia, the antibodies are not yet commercially available and therefore could not be tested as an alternative method during this study (Dowd *et al.*, 1998).

7.5 CONCLUSIONS

Inhibition of the PCR method was evident when some of the seeded surface water samples were analysed. This had a negative impact on the on the sensitivity of the method. To improve the sensitivity of the technique and to test the viability of the spores, the PCR will have to be combined with primary amplification of the microsporidia in cell culture. Amplification of spores making use of cell culture will, however, not be possible until a method for sample preparation, which would ensure effective separation of spores from debris in environmental samples, such as immunomagnetic seperation become available commercially..

In vitro cultivation is a practical and relatively easy way for propagating microsporidian spores. Disadvantages of the method are

- the long period of time it takes (three to four weeks) before spores can be clearly identified and
- the increased possibilities for contamination associated with the handling and incubation of long-term tissue culture.

Purification and decontamination procedures to prevent contamination of tissue culture were mostly inefficient. Initial positive results for decontamination of environmental samples using chloroform treatment could not be verified due to contamination of the spore source and tissue culture.

Staining procedures used for diagnosis of microsporidian spores in clinical specimens are not specific or sensitive enough for environmental water samples. Examination of stained monolayers as a basis for in vitro determination of infectivity and viability is a highly sophisticated method not applicable for routine analysis.

Isolating microsporidia in cell culture as a means of diagnosing infection is too laborious and lengthy and is prone to failure due to contamination with specimens from non-sterile sources.

It is recommended that the chloroform decontamination method for environmental samples is verified. It may provide a simple practical method for the preparation of environmental samples to be analysed for the presence of microsporidia spores using tissue culture.

Indirect methods such as the PCR should be further investigated for identification of microsporidia spores from environmental samples.

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PROJECT SUMMARY

CHAPTER 8

SUMMARY OF MONITORING PROGRAMME AND RESULTS

S N Venter

8.1 INTRODUCTION

One of the aims of the project was to monitor water samples from different localities in South Africa for the presence of the selected emerging pathogens. The detailed description of the methods and approaches used as well as the results obtained are given in the separate chapters. The purpose of the chapter is only to provide a summary of the monitoring programme and results. The data will first be presented according to the various provinces and thereafter according to the type of water sample.

8.2 SAMPLING SITES

Table 8.1 gives a summary of the sampling sites according to geographical regions and Table 8.2 presents the same information according to the type of water sampled. The location of most of the sampling points is also indicated on maps representing the four regions sampled (Figure 8.1 - 8.4).

8.3 MONITORING RESULTS

Tables 8.3 - 8.4 present the water quality data according to the four geographical regions. None of the samples tested were positive for microsporidia or *Helicobacter pylori*. For clarity only the positive values were indicated for the *Campylobacter* and Astroviruses analysis. None of the drinking or ground water samples tested postive for the Astroviruses or Campylobacters. These pathogens were only detected in surface water.

Table 8.1 Sampling sites for the different geographical regions.

REGION	SAMPLING SITE	CODE
Western Cape	Surface water/Kuilsrivier (off N2)	K1
	Surface water/Kuilsrivier (Baden-Powell Drive)	K2
	Drinking water/Stellenbosch (Gouws household)	K3
	Drinking water/Stellenbosch (UWC Food Micro. Dept)	K4
	Surface water/Kuilsrivier (Monwabizi)	K5
	Surface water//Stellenbosch (Plankenberg River)	K6
	Waste water/Athlone	K7
	Surface water/Kuilsrivier (Stellenbosch Aterial)	K8
Kwazulu/Natal	Surface water/Slangspruit river 61	U1
	Surface water/Dusi river above Darvill 66.2	U2
	Surface water/Darvill STW Final Effluent 626	U3
	Waste water/Darvill STW Raw 700	U4
	Saline surface water/Umgeni Estuary 28.1	U5
	Drinking water/Craigieburn WW final	U6
	Ground water/Dairy Top borehole	U7
	Surface water/Mngeweni river (below abbatoir discharge) 22	U8
	Waste water/Hammarsdale STW Raw 598	U9
	Surface water/Hammarsdale STW final effluent 599	U10
Gauteng	Surface water/Rietspruit RV2	R1
	Drinking water/Klipwater (treated, distribution endpoint)	R2
	Surface water/Klipriver K25	R3
	Surface water/Blesbokspruit B10	R4
	Surface water/Vaal River (Riviera)	R5
	Drinking water/Sandton (treated, distribution endpoint)	R6
	Surface water/Klip River K19	R7
	Surface water/Vaal Dam A18	R8
	Ground Water/Pretoria North	R9
Free State	Surface water/Urban Runoff (polluted stream)	S1
	Treated wastewater effluent/poorly treated	S2
	Surface water/downstream of urban stream (S1) inflow	S3
	Surface water/below recreational dam wall (Botshabelo)	S4
	Surface water/Urban runoff (outside Bloemfontein, receiving all city effluent and runoff)	S5
	Treated wastewater/well treated in new facility	S6
	Drinking water/storage container (contains biofilm residue)	S7
	Tap water/communal stand pipe (chlorinated municipal water - sample has been dechlorinated)	S8
	Ground water/unprotected borehole (arterial)	S9
	Ground water/unprotected borehole (arterial)	S10

Table 8.2 Sampling sites grouped according to the different types of water.

WATER TYPE	SAMPLING SITE	CODE
Surface Water	Surface water/Kuilsrivier (off N2)	K1
	Surface water/Kuilsrivier (Baden-Powell Drive)	K2
	Surface water/Kuilsrivier (Monwabizi)	K5
	Surface water//Stellenbosch (Plankenberg River)	K6
	Surface water/Kuilsrivier (Stellenbosch Aterial)	K8
	Surface water/Slangspruit river 61	U1
	Surface water/Dusi river above Darvill 66.2	U2
	Saline surface water/Umgeni Estuary 28.1	U5
	Surface water/Mngeweni river (below abbatoir discharge) 22	U8
	Surface water/Rietspruit RV2	R1
	Surface water/Klipriver K25	R3
	Surface water/Blesbokspruit B10	R4
	Surface water/Vaal River (Riviera)	R5
	Surface water/Klip River K19	R7
	Surface water/Vaal Dam A18	R8
	Surface water/Urban Runoff (polluted stream)	S1
	Surface water/downstream of urban stream (S1) inflow	S3
	Surface water/below recreational dam wall (Botshabelo)	S4
	Surface water/Urban runoff (outside Bloemfontein, receiving all city effluent and runoff)	S5
Drinking water	Drinking water/Stellenbosch (Gouws household)	K3
	Drinking water/Bellville (UWC Food Micro. Dept)	K4
	Drinking water/Craigieburn WW final	U6
	Drinking water/Klipwater (treated, distribution endpoint)	R2
	Drinking water/Sandton (treated, distribution endpoint)	R6
	Drinking water/storage container (contains biofilm residue)	S7
	Drining water /communal stand pipe (chlorinated municipal water - sample has been dechlorinated)	S8
Waste Water	Waste water/Athlone	K7
	Waste water/Darvill STW Raw 700	U4
	Treated wastewater/Darvill STW Final Effluent 626	U3
	Waste water/Hammarsdale STW Raw 598	U9
	Reated wastewater/Hammarsdale STW final effluent 599	U10
	Treated wastewater effluent/poorly treated	S2
	Treated wastewater/well treated in new facility	S6
Ground Water	Ground water/Dairy Top borehole	U7
	Ground Water/Pretoria North	K9
	Ground water/unprotected borehole (arterial)	S9
	Ground water/unprotected borehole (arterial)	S1

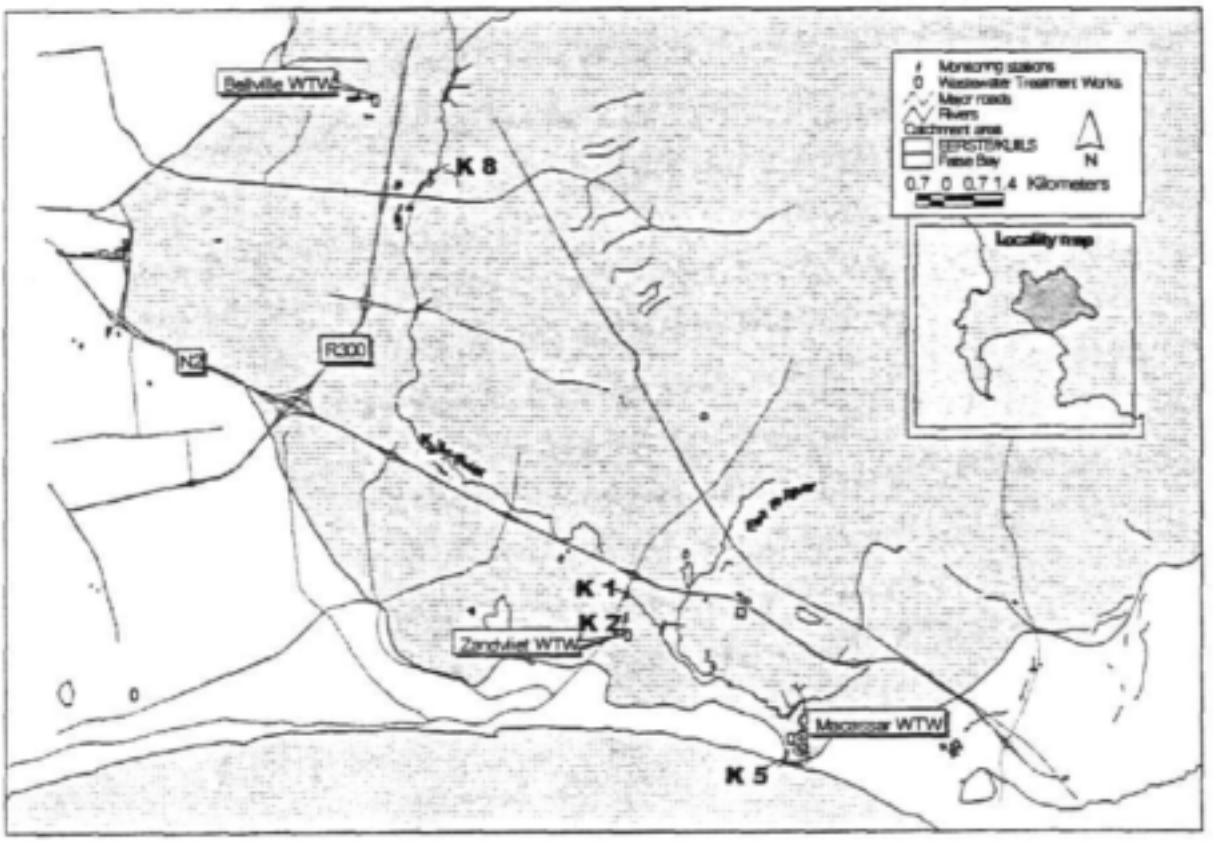


Figure 8.1
Map of selected Western Cape region indicating sampling points used during the study.

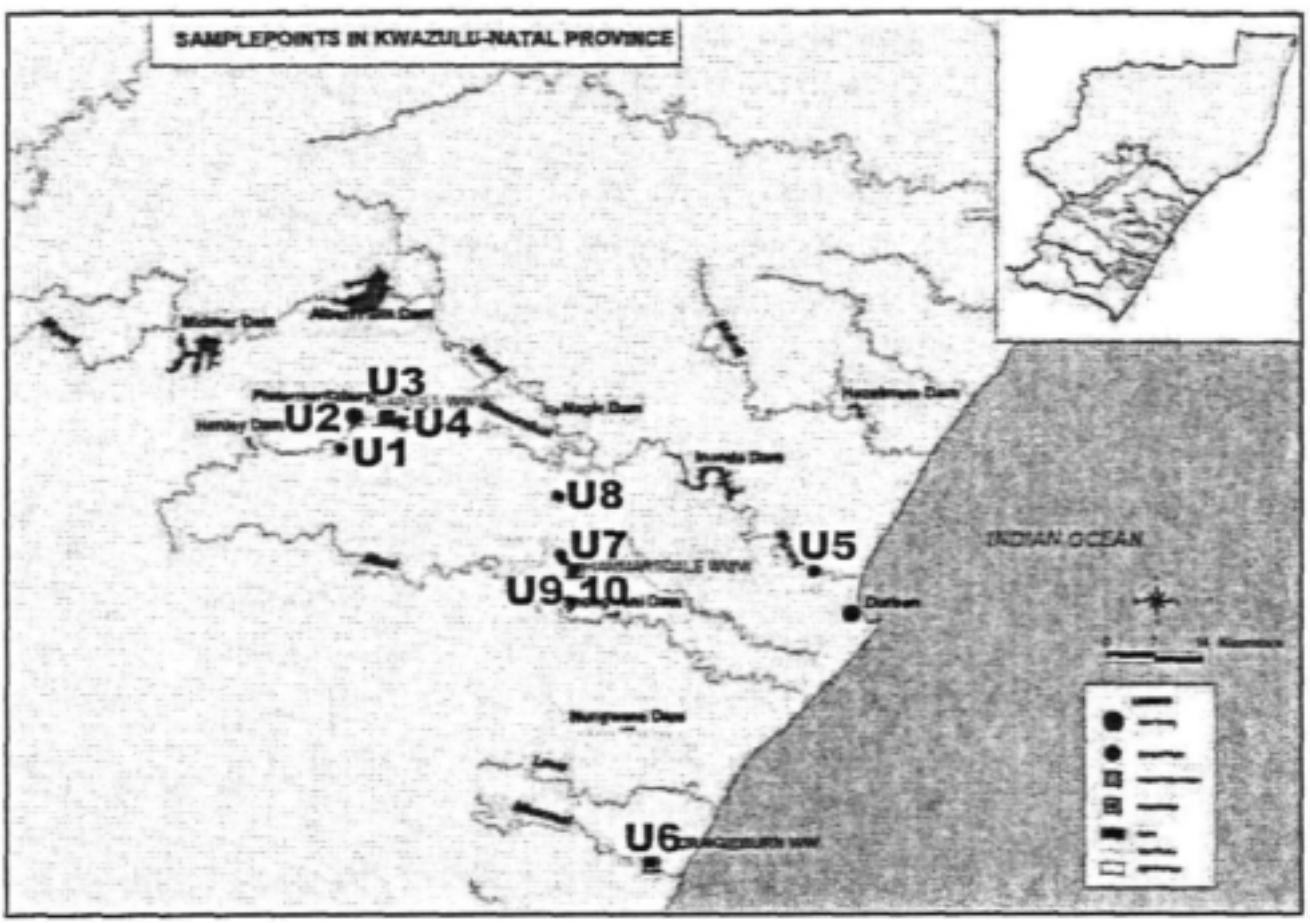


Figure 8.2
Map of selected Kwazulu/Natal region indicating sampling points used during the study.

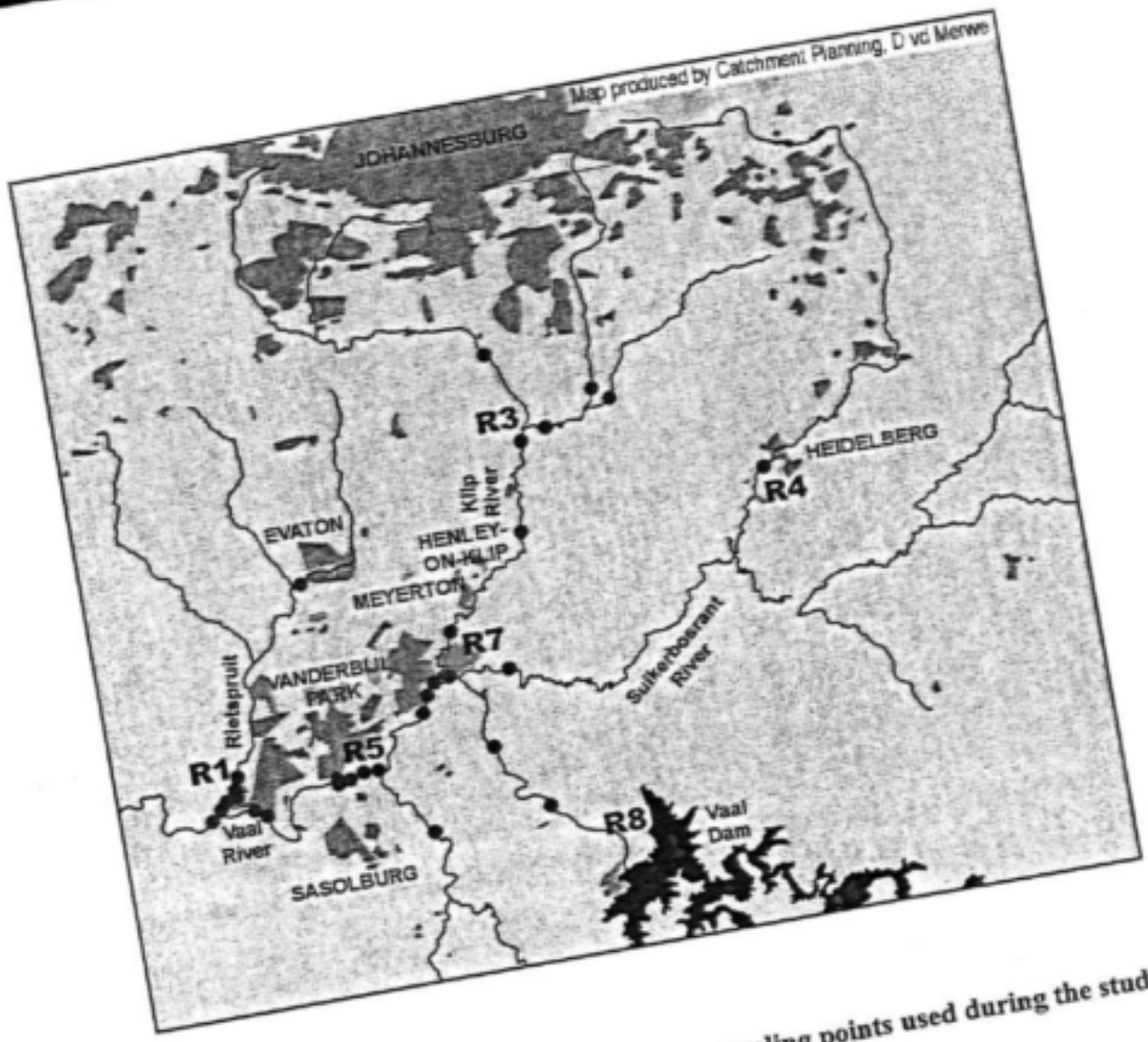


Figure 8.3
Map of selected Gauteng region indicating sampling points used during the study.

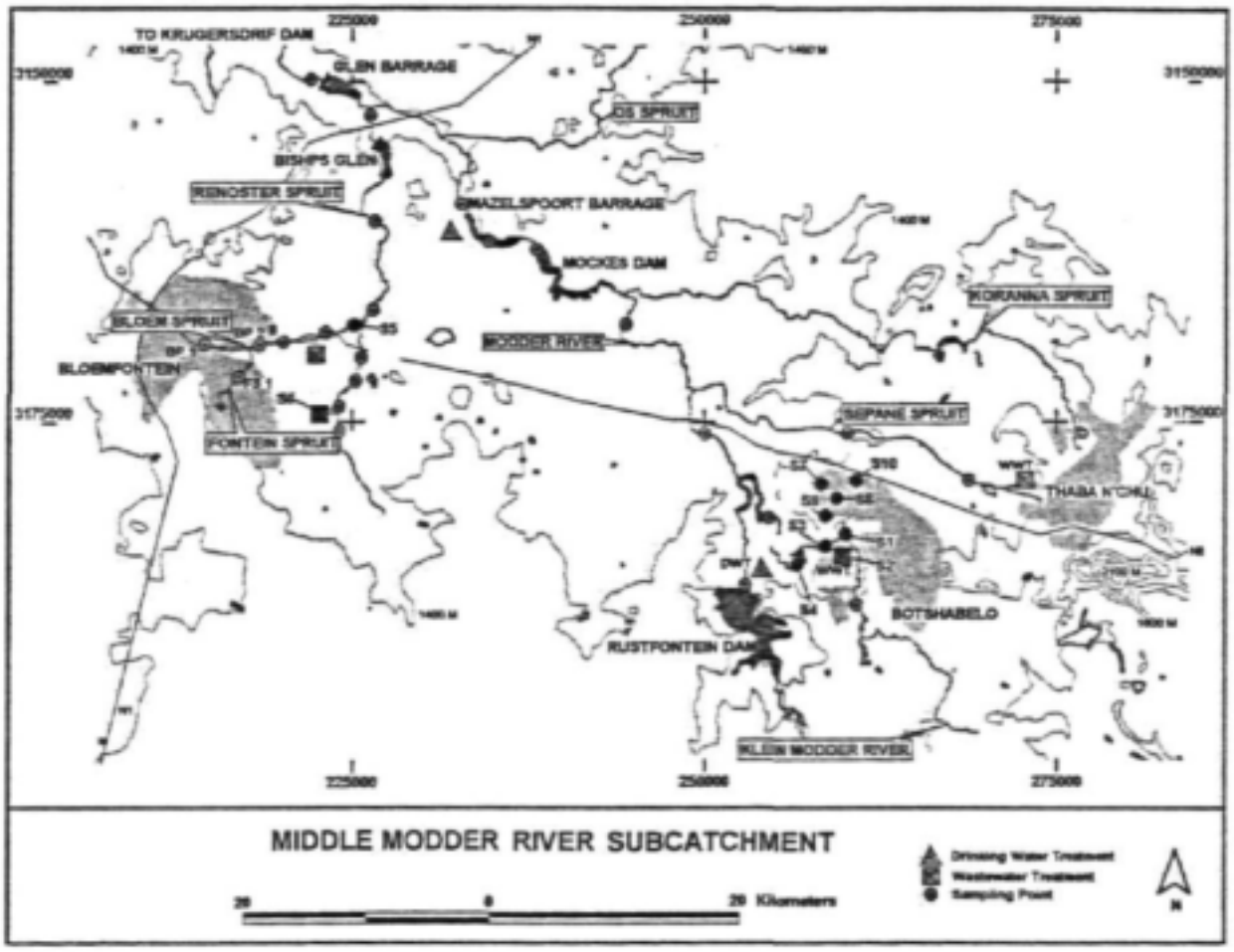


Figure 8.4
Map of selected Free State region indicating sampling points used during the study.

Table 8.3 Sampling results for the Western Cape and Kwazulu-Natal.

Code	Faecal coliforms (cfu/100ml)			*Campylobacter/Arcobacter* (cfu/100ml)		Astroviruses
	Run #1	Run #2	Run #3	Run #1	Run #3	Run #2
K1	5.2×10^{1}	8.6×10^{4}	9.4×10^{4}			
K2	8.8×10^{5}	2.2×10^{2}	3.4×10^{2}			Positive
K3	ND	ND	ND			
K4	ND	ND	ND			
K5	6.1×10^{5}	3.6×10^{3}	4.9×10^{3}			
K7	8.4×10^{4}	3.2×10^{5}	2.5×10^{5}			
K8	5.2×10^{4}	1.3×10^{5}	1.5×10^{5}			
U1	6.5×10^{5}	5.0×10^{0}				
U2	4.0×10^{3}	1.6×10^{2}				
U3	1.0×10^{1}	5×10^{0}				
U4	1.9×10^{7}	5.0×10^{6}		4.5×10^{6} Arco		Positive
U5	2.5×10^{3}	1.4×10^{2}				
U6	ND	ND	ND			
U7	ND	ND	ND			
U8	7×10^{2}	2×10^{2}		5.0×10^{3} Arco		
U9	8.5×10^{7}	1.3×10^{8}			$>10^{4}$ Arco	
U10	1×10^{3}	3.3×10^{2}				

ND = None detected
Arco = *Arcobacter*
Campy = *Campylobacter*
Run # 1 = Late March 2000; Run #2 = July - August 2000; Run #3 = October - November 2000

Table 8.4 Sampling results for Gauteng and the Free State.

Code	Faecal coliforms (cfu/100ml)			*Campylobacter/Arcobacter* (cfu/100ml)		Astroviruses
	Run #1	Run #2	Run #3	Run #1	Run #3	Run #2
R1	3.1×10^4	3.8×10^4	7.0×10^2	1.4×10^2 Arco	10 - 100 Arco	Positive
R2		ND	ND			
R3	9.3×10^4	1.9×10^2	2.9×10^2	1.3×10^2 Arco	2.9×10^2 Arco + Campy	
R4	5×10^0	3.0×10^1	ND		10 - 100 Arco	
R5	3.0×10^3	2.5×10^1	5×10^0			
R6	ND	ND	ND			
R7	7.5×10^3	8.0×10^1	1.3×10^2			
R8	4.5×10^2	ND	ND		$>10^4$ Arco	
R9	1.8×10^1	2.5×10^0	ND			
S1	1.1×10^2	2.0×10^1	5.0×10^0		10 - 100 Arco	
S2	4.5×10^4	1.3×10^3	7.0×10^2			
S3	1.0×10^4	1.2×10^2	3×10^0		100 - 1000 Arco	
S4	ND	ND	1.4×10^1		100 - 1000 Arco	Positive
S5		6.3×10^4	ND			
S6	5.0×10^0	1.3×10^6	2.1×10^4		> 1000 Campy	
S7		ND	ND			
S8		ND	ND			
S9	ND	2.0×10^2	2.5×10^1			
S10	ND	ND	ND			

ND = None detected
Arco = *Arcobacter*
Campy = *Campylobacter*
Run # 1 = Late March 2000; Run #2 = July - August 2000; Run #3 = October - November 2000

CHAPTER 9

CONCLUSIONS AND RECOMMENDATIONS

S N Venter

9.1 PROJECT SUMMARY

The project set out to achieve a number of aims which could summarized by the following two broad objectives:

a) To establish techniques for the detection of selected emerging pathogens in various types of water samples, and

b) To monitor a number of source and treated water samples from different localities in South Africa for the presence of the selected pathogens.

The following pathogens were selected:

Hepatitis E and Astroviruses;

Campylobacter spp and *Helicobacter pylori*; and

The microsporidia

As can be seen from the previous chapters these two objectives were achieved with varying levels of success, depending on the specific pathogen targeted. Although detection techniques were established for all of the selected pathogens apart from Hepatitis E virus, the sensitivity of a number of these techniques were highly unsatisfactory. Efforts to increase either the sensitivity of specificity of the detection techniques were only met with partial success. A summary of major findings of the are presented in the following sections. Detailed discussions of these conclusions are presented in each of the individual chapters.

9.2 VIRUSES

9.2.1 Astroviruses

- In this study a HAstV-specific RT-PCR oligonucleotide probe assay was used for the detection of HAstVs in water samples. This method which combines a molecular assay and cell culture propagation was EM and EIA normally used for the detection of HAstVs in environmental water samples.
- HAstVs were detected in only one sample from each geographical region during the late winter sampling period.
- HAstVs were only detected in surface and waste waters.
- The low prevalence of HAstVs noted in this study corresponds with recent reports of HAstVs in river and dam waters in Gauteng.

9.2.2 Hepatitus E Virus

- The development of a reliable technique for the detection of HEV is still under investigation as the nested-PCR still needs to be optimized for the testing of water samples.
- Seroprevalence studies suggest that HEV is endemic in the swine populations in South Africa.

9.3 BACTERIA

9.3.1 *Campylobacter*

- The enrichment approach was much more efficient than the optimized modified Cape Town Protocol for the isolation of Campylobacters from environmental waters.
- Of the 22 potential *Campylobacter* isolates obtained after enrichment only 3 true *Campylobacter* species were isolated, the rest of the isolates belonged to the genus *Arcobacter*, which forms part of the broader *Campylobacter* group.
- The majority of the isolates were from surface water with only six isolated from sewage. No isolates were obtained from ground and drinking water.
- All isolates from mCCDA were identified as *Arcobacter* according to their 16S rDNA identity, although Bolton enrichment media and mCCDA were supposed to eliminate all non-*Campylobacter* species.

- The primers used to verify all the presumptive *Campylobacter* isolates, were again proven not to be specific to the 9 *Campylobacter* species as claimed.

9.3.2 *Helicobacter pylori*

- A novel seminested PCR assay for the detection of *H. pylori* in environmental water samples has been developed .
- Under the tested parameters, the equivalent to 5×10^2 bacteria, were detectable by the seminested PCR assay.
- The supposed *H. pylori* selective medium was not selective when used for the isolation of *H. pylori* from environmental water samples.
- *H. pylori* was not detected in any of the samples tested but this does not necessarily indicate that they were absent from the tested samples.

9.4 PARASITES

- Inhibition of the PCR method by some of the surface water samples had a negative impact on the sensitivity of the method.
- Staining procedures used for diagnosis of microsporidian spores in clinical specimens are not specific or sensitive enough for environmental water samples.
- Cultivation of microsporidia in cell culture as a means of detection is too labourious and lengthy and is prone to failure due to contamination with specimens from non-sterile sources.

9.5 IMPLICATIONS OF RESULTS

From the results of this study it can be concluded that the emerging pathogens are most likely to be found in surface and waste waters. During this study none of the pathogens were detected in drinking and ground water. Although the results of this study suggest a low incidence of the selected emerging pathogens in various water sources care should be taken when interpreting the

results. The sensitivity and reliability of a number of these techniques were highly unsatisfactory. At present the only method suitable for routine monitoring of water samples would be the one developed for the detection of the human Astroviruses.

This study has shown that a great need still exists for reliable and effective methods for the detection of emerging pathogens in water. It has clearly shown that the methods developed for clinical use are often not able to deal with the complexities of environmental samples. Although progress has been made in the adaptation of a number of detection methods for environmental use, this study has highlighted a number of areas where further research is still required

9.6 RECOMMENDATIONS

- As HAstVs were detected in only one sample from each geographical region the significance of these results is difficult to assess and further studies are required to elucidate the actual risk of infection.
- Molecular techniques for the detection and comparison of Hepatitis E virus in the stool and water samples still need to be developed.
- More work has to be done to refine the use of enrichment media for testing environmental samples for the presence of *Campylobacter*.
- A molecular method for identifying all *Campylobacter* and *Arcobacter* species from environmental waters is needed.
- There is a great need for further research into and the development of an effective isolation method for *H. pylori* based on culturing procedures.
- There is still a need for an effective method for the preparation of environmental samples to be analysed for the presence of microsporidia spores using tissue culture.

Appendix A

Technology transfer, scientific outputs and data availability

Based on the research of this project the following presentations were made at local and international conferences:

S. M. Diergaardt, S. N. Venter, J. Theron, P. A. Gouws and V. S. Brözel Detection of campylobacters from environmental water in South Africa. Campylobacter, Helicobacter and Arcobacter. Summer conference of the Society for Applied Microbiology, Glasgow, Scotland, 11 - 13 July 2000.

S. M. Diergaardt, M. Chalmers, S.N. Venter, J. Theron, P.A. Gouws and V. S. Brözel. Development of a method for the detection and recovery of campylobacters from water. BioY2K combined millenium meeting, Grahamstown, 23 – 28 January 2000.

S. M. Diergaardt, A. Spreeth, S. N. Venter, J. Theron, P. A. Gouws and V. S. Brözel Adaptation of the Cape Town protocol for environmental isolation of Campylobacter. *Campylobacter* 2000. University of the Western Cape. Bellville, South Africa, August 2000.

The original data are available from the following institutions:

Viruses :	Department of Medical Virology, University of Pretoria
Bacteria:	Department of Microbiology and Plant Pathology, University of Pretoria
	Department of Microbiology, University of the Western Cape
Parasites:	Environmentek, CSIR, Pretoria

Other related WRC reports available:

The health impact of waterborne viruses and methods of control in high risk communities

WOK Grabow, MB Taylor, JC Vivier, N Potgieter and MG Gaobepe

Infectious diseases are the most important concern about water quality. Authoritative estimates ascribe about 50 000 deaths per day in the world to waterborne and water-related infectious diseases. In addition to mortality, which affects mainly children in developing communities, waterborne diseases also have far-reaching socio-economic implications. Viruses feature prominently among the wide variety of pathogenic micro-organisms concerned.

Many enteroviruses are cytopathogenic, ie, detectable by cell culture propagation. However, some enteroviruses, notably coxsackie A viruses, are not detectable by cell culture. Detection of these viruses requires inoculation of newborn mice, or recently developed molecular techniques.

Recommendations for the control of waterborne viral diseases in high risk communities are, therefore, largely based on education and training regarding the collection and storage of indoor drinking water supplies. This addresses short-term immediate interventions at minimum cost. The need for supplying safe water closer to homes is also emphasised. This option is more expensive and will take longer to implement. The ultimate goal is to supply running tap water inside households, but this is the most expensive and time consuming option.

Report Number: 743/1/02 **ISBN: 1 86845 859 8**

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