ORIGIN, FATE AND CLINICAL RELEVANCE OF WATER-BORNE PATHOGENS PRESENT IN SURFACE WATERS

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Water Research Commission

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Report to the

Water Research Commission

by

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Report and accompanying CD with maps and colour figures

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EXECUTIVE SUMMARY

BACKGROUND AND MOTIVATION

Access to safe water remains a serious problem worldwide, especially in developing countries. Rapid population growth, urbanization and limited water resources (scarcity) are some of the main factors that have contributed to this problem. Surface water pollution has serious health and economic consequences for communities who rely on such sources for drinking water, irrigation or recreation. The most common source of contamination of surface waters is still human and animal wastes. The introduction of water-borne pathogens by these sources is of particular concern.

At present most of the water industry's efforts are focused on the removal of water-borne pathogens during water treatment. High levels of microbial pollution in source water can, however, lead to breakthrough in the treatment process. With the promulgation of the new Water Act in 1998, and the publication of the World Health Organization's *Drinking-Water Quality Guidelines* in 2004, a strong emphasis has therefore been placed on the protection and management of water quality within catchments. A catchment management approach towards microbial pollution will, however, only be successful if it is based on a clear understanding of the origin, fate, survival and transport of pathogens that have been introduced into the water body.

OBJECTIVES OF THE PROJECT

The scope of this project was to investigate the possible sources, survival and clinical relevance of selected water-borne pathogens in a rural and peri-urban area, and to investigate the environmental factors and social determinants that contribute to the transmission of such diseases. The information provided by this study could form the basis for the development of appropriate catchment management and intervention strategies to reduce the health risk to various water users. In order to achieve the abovementioned objective a number of different aims were set for the study. The intention of these aims was to develop a better understanding of the fate and / or clinical relevance of selected parasites (*Cryptosporidium*) and bacteria (*Salmonella* and *Vibrio cholerae*) in fresh water environments. All of these aims have been met during the reported study. The specific aims for the research were:

- To determine the dominant *Cryptosporidium parvum* genotype involved in human infections in the Pretoria and surrounding areas.
- To investigate the prevalence of *Cryptosporidium* among domesticated animals which may pose a potential contamination risk to water resources.
- To determine the level, source and clinical relevance of *Salmonella* spp isolated from surface waters in a rural area.
- To determine the potential build-up and survival of *Salmonella* spp. in sediments and the possible effect thereof on the microbial quality of the water phase.
- To study the origin, survival and transport of *Vibrio cholerae* strains in a peri-urban catchment.
- To investigate the environmental factors and social determinants that contribute to water-borne outbreaks such as cholera and to present some of the data in a GIS format.

RESEARCH APPROACH

The main focus of this project was to develop a better understanding of the origin, fate and survival of enteric pathogens in fresh water environments. For this purpose the focus was on three pathogens that represented various types of enteric pathogens as well as different perceived behaviour in aquatic environments. The project focused on *Cryptosporidium parvum* a protozoan parasite and *Salmonella enterica* and *Vibrio cholerae* two bacterial pathogens.

Cryptosporidium parvum represents parasitic pathogens that can only grow and multiply within an animal host. These pathogens can only be transmitted by water but cannot multiply in this environment. Oocysts are, however, known to have a thick outer protein layer and they can therefore survive for very long periods in the environment. Due to the problems of separating the parasites from the environmental matrix and therefore obtaining enough biomass for molecular analyses, the present study focused more on the typing of clinical samples to obtain information about the possible sources of environmental contamination and transfer.

Salmonella enteria subsp *enterica* is one of the major food and water-borne bacterial pathogens in both developed and developing countries with approximately 1.4 million cases of non-typhoidal *Salmonella* annually in the USA. As a zoonotic bacterium, it has a wide host range and contamination originates from both domestic and agricultural sources. Although aquatic and terrestrial environments have not been considered to be important reservoirs for this enteric pathogen, recent finding indicated that the bacterium might be well equipped to survive in non-host environments. *Salmonella* therefore serves as a model organism to study the behaviour of enteric pathogens in aquatic and sediment environments. The current project investigated the survival of a *Salmonella* Typhimurium strain under varying environmental conditions. The project also studied the potential link between environmental and clinical isolates in a rural area where the bacterium is endemic.

Vibrio cholerae is widely considered to be an environmental bacterial pathogen. These pathogens are defined as microorganisms that normally spend a substantial part of their lifecycle outside human hosts, but when introduced to humans they cause disease with measurable frequency. These pathogens can only be controlled through an understanding of their environmental ecology, epidemiology and genetic variability. The population dynamics of this pathogen was therefore studied in an inland fresh water catchment to develop an understanding of it's behaviour. Data from the largest cholera outbreak in South Africa was also used to develop an understanding for the environmental and socio-economic factors that may cause a community to be at risk.

SUMMARY OF MAJOR RESULTS AND CONCLUSIONS

Cryptosporidium

Based on the study it seems unlikely that cryptosporidiosis is commonly transferred from animals to humans in the South African context. The high percentage of the human genotype (80%) observed in the cases investigated suggests the importance of person-to-person contact and human sewage contamination of drinking water, recreational water and food sources, as a means of spreading the parasite. This could have been anticipated because of the high population density in the area, as well as poor sanitary conditions in close-by informal settlements.

As noted above, the introduction of oocysts into the environment by livestock may be of lesser importance in the South African context. This was supported by the prevalence data for *Cryptosporidium* oocyst in calves. The prevalence was found to be lower than what have been reported for other countries.. *Cryptosporidium* infection is most prevalent amongst young calves between 1-2 weeks of age. Good management and hygiene practices are however, still required to prevent the spread of *Cryptosporidium* to other animals and the aquatic environment.

Salmonella

The results of the present study have revealed that *Salmonella* was prevalent in environmental and clinical samples examined. This could be due to fecal contaminants from animal and human excreta that may find their way to the river. *Salmonella* isolates were also isolated from raw vegetables (cabbages, tomatoes and onions) obtained from local rural gardens, which used river water for irrigation. Strains isolated from human stools, water, sediments and food were phenotypically and genetically correlated which provide evidence for the epidemiological link between environmental reservoirs and human infection in an endemic area. This confirms that water and food could be important sources of human salmonellosis in rural areas.

Salmonella Typhimurium is the most commonly isolated serotype from clinical cases in the Venda area and a GFP labelled strain was therefore used to investigate the survival and behaviour of *Salmonella* in fresh water sediments. The study indicated that *Salmonella* survived for extended periods of more than 6 months in sediments at temperatures typically associated with fresh water streams and rivers in South Africa. This is of concern as they could easily be released from the sediment into the water phase at high concentrations during rain events or other disturbances of the sediment.

Vibrio cholerae

The *ompW* based PCR identification was found to be ideal for surveillance work and was more reliable than the biochemical identification approach. The identity of *Vibrio cholerae* stains isolated from the Vaal Barage catchment area were confirmed with this PCR technique and thereafter a selection of these strains were typed with AFLP. With the AFLP typing a high level of genetic diversity was seen. It is therefore believed that

the *Vibrio cholerae* population in the Vaal Barrage system is not a product of one or two strains that have adapted to local conditions, nor that it consist of diverse clones that only occupy specific niches in the aquatic system. The population in the Vaal Barrage is rather made up of highly diverse clones that constantly compete, resulting in genetic shifts only perceivable within short time frames and localized regions. Using environmental *Vibrio cholerae* as a model, it is suggested that enterotoxigenic strains may exhibit the same degree of persistence and survival in inland aquatic systems, being able to survive for extended periods and posing as potential future health risk.

For the KwaZulu-Natal outbreak, no statistical correlations could be established between the %CIR and the climatic variables of rainfall, temperatures (minimum, maximum and average) and humidity. There may have been correlations within specific months when cholera was at peak but not when the entire dataset was statistically considered. A number of socio-economic factors could be correlated with high CIR percentages in the DCs. They included traditional households, no sanitation or the use of bucket system, the use of river water for household purposes and the lack of refuse removal. This again stress how the lack of basic services expose communities to the risk of infectious disease and increase their overall vulnerability.

RECOMMENDATIONS FOR FUTURE RESEARCH

A number of issues have not been addressed or resolved during this study and future research projects should address them. These issues include the need to obtain a better understanding of the virulence factors that can help to predict the pathogenic capabilities of pathogens detected in the environment. Only with the ability to distinguish between harmless and pathogenic strains would it be possible to assess the real risk to communities posed by these pathogens present in environmental sources. Understanding of the fate of enteric pathogens in aquatic and terrestrial environments is still limited. The initial survival studies should be extended to address their behaviour within soil and sediments, as well as the effect these environments have on the pathogenicity of the organisms. Enteric pathogens will only be controlled effectively once a thorough understanding of their environmental ecology, epidemiology, virulence, genetic variability and the potential risks posed to exposed populations has been obtained.

RESEARCH OUTPUTS

Based on the research of this project the project team have already published two articles in international peer-reviewed journals, made one presentation at a national conference and five presentation at international gatherings. The details are as follow:

Papers:

Paulsen, I. and Venter S.N. 2003. Genotyping of *Cryptosporidium parvum* isolates obtained from humans in South Africa. *Acta Parasitologica*, **48**, 135 – 137.

Le Roux, W.J., Masoabi, D., de Wet, C.M.E. and Venter, S.N. 2004. Evaluation of a rapid polymerase chain reaction based identification technique for *Vibrio cholerae* isolates. *Water, Science and Technology*, **50 (1)**, 229 – 232.

Presentations:

National:

Said, M.D., Cloete T.E. and Venter, S.N. 2004. Epidemic cholera in KwaZulu-Natal: Population demographics, water and sanitation. Poster presentation at South African Microbiology Society congress, Stellenbosch, 4 – 7 April 2004.

International:

Kgotlagomang, I., Burke, L.M., Brözel, V.S. and Venter, S.N. 2003. *Salmonella enterica* subsp *enterica* survives in, and is readily released from, river sediments. Poster presentation at the International Symposium on Health-Related Water Microbiology, Cape Town, South Africa, 14 – 19 September 2003.

Burke, L.M., Brözel, V.S. and Venter, S.N. 2003. Construction and evaluation of GFP-tagged *Salmonella* isolateds for use in survival and dissemination studies. Poster presentation at the International Symposium on Health-Related Water Microbiology, Cape Town, South Africa, 14 – 19 September 2003.

Said M.D., Venter, S.N. and Cloete, T.E. 2003. The 2000-2002 Cholera epidemic in KwaZulu-Natal: A possible link between population demographics, water and sanitation. Oral presentation at the IWA Specialist Conference: Water: Key to

Sustainable Development in Africa Symposium, Cape Town, South Africa, 14 – 17 September 2003.

Burke, L.M., Brözel, V.S., MacDonald, R and Venter, S.N. 2004. Growth of *Salmonella* in biofilms of a drinking water distribution system. Poster presentation at the 104th ASM General Meeting, New Orleans,USA, 23 – 27 May 2004.

Said M.D., Cloete, T.E. and Venter S.N. 2004. The trend of epidemic cholera in KwaZulu-Natal: 2000 - 2004. Oral presentation at the 5th Waternet / WARFSA symposium, Windhoek, Namibia, 2 – 4 November 2004.

CAPACITY BUILDING

During the study one PhD, three MSc and six BSc (Hon) students were trained. Of these students 6 were black and 6 were female students. The complete list of students is as follow:

University of Venda

MSc: E Musie: Molecular epidemiology of clinical and environmental *Salmonella* isolates in rural Venda communities in the Limpopo Province, South Africa. (Completed)

BSc (Hons): P Ramudigana (Completed)
A L Nemarude (Completed)

University of Pretoria

PhD: M Said: Epidemic cholera in KwaZulu-Natal: The role of the natural and social environment.

MSc: L Burke : Fate of *Salmonella* Typhimurium in biofilms of drinking water distribution systems.
W le Roux: Polulation dynamics of *Vibrio cholerae* in the Vaal Barrage catchment.

BSc (Hons):	I Kotlagomang (Completed)
	L Paulsen (Completed)
	H Said (Completed)
	M Willemse (Completed)

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The original data are available from the following institutions:

Cryptosporidium:	Department of Microbiology and Plant Pathology, University of Pretoria.
Salmonella isolates:	Department of Microbiology, University of Venda for Science and Technology.
Salmonella survival:	Department of Microbiology and Plant Pathology, University of Pretoria.
Vibrio cholerae Identifications:	Scientific Services, Rand Water.
Vibrio cholerae typing:	Department of Microbiology and Plant Pathology, University of Pretoria.
KZN cholera data:	Provincial Department of Health, KwaZulu-Natal
KZN social and analyses:	Department of Microbiology and Plant Pathology, and Department of Geography and Geoinformation, University of Pretoria.

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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)
Mr I Bailey, Water Environment Health Research and Training
Dr S Jooste, Department of Water Affairs and Forestry
Ms J Kadiaka, Department of Health
Prof J Lin, University of KwaZulu-Natal
Ms S Rushworth, KwaZulu-Natal Department of Health
Ms S September, University of Pretoria (Committee Secretary)

Dr MJ van der Linde and Ms R Ehlers from the Department of Statistics, University of Pretoria for their guidance and statistical analyses.

The KwaZulu-Natal Department Health who gave permission for the use of data from their cholera database.

Everyone, who has assisted during the execution of this project, dealt with the financial management and provided support during the preparation of the report.

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LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphisms
BPW	Buffered peptone water
CIR	Cumulative infection rate
DC	District council
GFP	Green fluorescent protein
KZN	KwaZulu-Natal
LB	Luria Bertani medium
MD	Magisterial district
MDR	Multi-drug resistant
MPN	Most probable number
PAGE	Polyacrilamied gel electrophoresis
PBP	Penicillin binding protein
PBS	Physiologically buffered saline
PCR	Polymerase chain reaction
RV	Rappaport-Vassilliadis
RVS	Rappaport-Vassilliadis Soya
SAWS	South African Weather Services
TBE	Tris-borate EDTA buffer
XLD	Xylose Lysine Deoxicolate

INTRODUCTION

CHAPTER 1

OVERVIEW OF PROJECT

S N Venter

1.1 BACKGROUND

Access to safe water remains a serious problem worldwide, especially in developing countries. Rapid population growth, urbanization and limited water resources (scarcity) are some of the main factors that have contributed to this problem. Surface water pollution has serious health and economic consequences for communities who rely on such sources for drinking water, irrigation or recreation. The most common source of contamination of surface waters is still human and animal wastes. The introduction of water-borne pathogens by these sources is of particular concern.

At present most of the water industry's efforts are focused on the removal of water-borne pathogens during water treatment. High levels of microbial pollution in source water can, however, lead to breakthrough in the treatment process. With the promulgation of the new Water Act in 1998, a strong emphasis has therefore been placed on the protection and management of water quality within catchments. A catchment management approach towards microbial pollution will, however, only be successful if it is based on a clear understanding of the origin, fate, survival and transport of pathogens that have been introduced into the water body.

1.2 OBJECTIVES OF THE PROJECT

The scope of this project was to investigate the possible sources, survival and clinical relevance of selected water-borne pathogens in a rural and peri-urban area, and to investigate the environmental factors and social determinants that contribute to the transmission of such diseases. The information provided by this study could form the basis for the development of appropriate catchment management and intervention strategies to reduce the health risk to various water users. In order to achieve the abovementioned objective a number of different aims were set for the study. The intention of these aims was to develop a better understanding of the fate and / or clinical

relevance of selected parasites (*Cryptosporidium*) and bacteria (*Salmonella* and *Vibrio cholerae*) in fresh water environments. The specific aims were:

- To determine the dominant *Cryptosporidium parvum* genotype involved in human infections in the Pretoria and surrounding areas.
- To investigate the prevalence of *Cryptosporidium* among domesticated animals which may pose a potential contamination risk to water resources.
- To determine the level, source and clinical relevance of *Salmonella* spp isolated from surface waters in a rural area.
- To determine the potential build-up and survival of *Salmonella* spp. in sediments and the possible effect thereof on the microbial quality of the water phase.
- To study the origin, survival and transport of *Vibrio cholerae* strains in a peri-urban catchment.
- To investigate the environmental factors and social determinants that contribute to water-borne outbreaks such as cholera and to present some of the data in a GIS format.

1.3 OUTLINE OF THE REPORT

The report has been divided into four sections. The introductory section contains only this chapter and deals with the motivation, objective and aims for this joint project between the Department of Microbiology and Plant Pathology, University of Pretoria, the Department of Microbiology, University of Venda for Science and Technology and Scientific Services, Rand Water.

The second section deals with the possible transmission routes for the infection of humans by *Cryptosporidium*. In Chapter 2 the predominant genotype of *Cryptosporidium parvum* isolates obtained from patients from the larger Pretoria area has been determined. Chapter 3 addresses the issue of the prevalence of *Cryptosporidium* in young calves in order to determine whether these animals may be a major risk factor in the spread of the disease in the human population.

The third section covers the research addressing the routes of transmission and survival of *Salmonella*. In chapter 4 the fate and clinical relevance of *Salmonella* isolates from

aquatic environments are determined by comparing their genetic composition with that of clinical strains from the same area. The antibiotic resistance patterns of these isolated were also determined. Chapter 5 describes the construction of a GFP-labelled strain and it's use during studies to determine the factors that influence *Salmonella*'s survival in fresh water sediments.

In section four some issues pertaining to the fate and spread of *Vibrio cholerae* are addressed. In Chapter 6 a rapid method for the identification of *Vibrio cholerae* isolates obtained from the Vaal Barage area is described. Chapter 7 addresses the occurrence and population dynamics of *Vibrio cholerae* isolated from this inland catchment. Chapter 8 on the other hand, looks at the environmental, health and socio-economic factors that may assist the spread of epidemic cholera. This analysis was based on epidemiological data collected during the 2000 – 2002 cholera outbreak in KwaZulu-Natal. The report is concluded by Chapter 9, which provides a summary of the major findings. Recommendations on possible future research topics to be addressed are also made.

Cryptosoridium

CHAPTER 2

GENOTYPING OF *CRYPTOSPORIDIUM PARVUM* ISOLATES OBTAINED FROM HUMANS IN SOUTH AFRICA

L Paulsen and S N Venter

Abstract

Cryptosporidium parvum is endemic to South Africa. In order to provide direction for prevention strategies, information on the dominant genotype involved in human infections is required. A combination of diethyl ether extraction, sodium chloride floatation and immuno-magnetic separation (IMS) was used for maximum recovery of oocysts from feacal samples. In order to improve the sensitivity of PCR-RFLP analysis of the *Cryptosporidium* oocyst wall protein (COWP) gene, a nested PCR was developed. RFLP analysis was done on a 553 bp segment obtained from the final nested PCR reaction to distinguish between *C. parvum* genotype 1 (human exclusive type) and genotype 2 (broad host range). Overall, genotype 2 was detected in 10 samples, whereas genotype 1 was detected in the remaining 40 samples. The high incidence of genotype 1 parasites (80%) implies that direct and indirect human transmission play a dominant role in the epidemiology of the disease caused by this parasite in the study area.

The research has been published as:

Paulsen, L and Venter S.N. 2003. Genotyping of *Cryptosporidium parvum* isolates obtained from humans in South Africa. *Acta Parasitologica*, **48**, 135 – 137.

CHAPTER 3

PREVALANCE OF CRYPTOSPORIDIUM AMONGST ANIMALS IN THE PRETORIA AREA

H Said, P Solarczyk and S N Venter

3.1 INTRODUCTION

Cryptosporidium parvum, a protozoan parasite, is a significant pathogen of humans and other mammals. Whereas the diarrhea caused by *Cryptosporidium* is self-limited in healthy individuals, AIDS patients and malnourished children generally experience a life-threatening condition. The absence of effective treatment strategies for cryptosporidiosis and a poor understanding of its epidemiology highlights the need to develop preventative measures for those who might be particularly at risk.

Transmission occurs by the fecal-oral route, either directly through contact with infected animals or humans, or indirectly through ingestion of water or food contaminated by fecal oocysts. Two major genotypes of *C. parvum* have been detected in humans: one type occurring exclusively in naturally infected humans (genotype 1) and the other occurring in livestock animals as well as in humans (genotype 2). For a disease such as cryptosporidiosis to be sustained in a community, there constantly needs to be infected hosts and oocysts in the environment for subsequent transmission to susceptible hosts. Rational approaches for controlling the disease depends on a better understanding of both the host reservoirs and the routes of infection. In the 1980s and early 1990s it has been suggested that in humans, cryptosporidiosis is primarily zoonotic, and that transmission is achieved via direct contact with animals or consumption of contaminated water. However, some workers questioned this consensus and suggested that the source of human infection is commonly man. Based on preliminary results obtained in our laboratory, this might also apply to South Africa. A need therefore exists to investigate the prevalence of Cryptosporidium among animals and during this study

the prevalence of *Cryptosporidium* among domesticated animals which may pose a potential contamination risk to water resources, were investigated.

3.2 MATERIALS AND METHODS

Samples were collected from two diary herds in the Pretoria area. On average about 10 calves were born per month on each farm. The age of animals studied ranged between 2 days and 8 weeks. A total of 119 fecal samples were collected from the two farms. In a period of ten months, 73 fecal samples were collected from the first farm while 46 fecal samples were collected from the second farm over a period of seven months. The samples were collected per rectum if possible using disposable gloves. Some of samples were collected from the ground after the animal had recently defecated. In most cases each animal was sampled on two occasions. Details of age, sampling date, date of birth, origin and the identification number of calves, presence/ absence of diarrhea were recorded. Samples were placed in a sample bottle and processed directly or they were preserved in 2.5% (w/v) potassium dichromate solution and were kept refrigerated until processed.

Direct smears were prepared from each fecal sample and stained with the Ziehl-Neelson method. The smear was examined at x100 magnification. The oocyst stained pink, and were observed as thick walled, round structure approximately 4-5 μm in diameter.

All Ziehl-Neelson positive samples were stained using the fluorescent antibody staining method. A drop of sample was placed on a 8-welled Teflon coated glass slide. Slides were air-dried and fixed with acetone for 5 minutes. The slides were again air-dried, spotted with 3μl of Crypto/Giard Cel immunofluorescent reagent (Celllab) and placed in a moisture chamber. The slides were incubated at 37 ^{0}C in the dark for 1 hour. Afterwards the slides were removed and washed with 1x PBS. Finally citifluor was added and a cover slip was placed on the preparation before examining the sample under the fluorescent microscope.

Attempts have been made to initiate a sampling programme for young lambs but after an initial screening of twenty new-borne lambs this part of the study was abandoned

Cryptospordium could not be detected in any of the samples tested. Sampling of young lambs was also found to be logistically and physically very difficult to perform.

Permission was also obtained from the National Zoological Gardens in Pretoria and the Gauteng Veterinary Services to collect samples form a variety of animals in the Zoo. The primary focus of the study was to screen animals indigenous to Southern Africa but a number of animals from other regions of the world were also sampled.

3.3 RESULTS

3.3.1 Prevalence and age distribution

Oocysts were present in the feaces of calves ranging from 2 days to 7 weeks old. Twenty-two (18.4%) of the 119 calves tested were shedding oocysts during at least one sampling. The highest prevalence was observed in animals between 1 and 2 weeks old, with oocysts found in 7 (5.9%) and 9 (7.5%) respectively out of the 22 (18.4%) calves shedding oocysts. The percentage of animals of different ages shedding *Cryptosporidium* oocysts is shown in Figure 3.1.

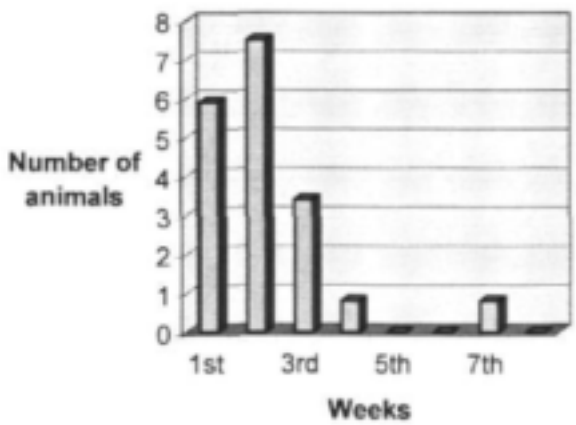

Figure 3.1: Age distribution of calves shedding *Cryptosporidium* oocyst.

3.3.2 Seasonal variations in oocyst shedding

Prevalence of shedding oocysts varied significantly by the season on both of the farms. Of the 119 fecal samples collected *Cryptosporidium* oocysts have been detected as

follows: 1 (0.84%) in May, 1 (0.84) in June, 2 (1.7%) in August, 1 (0.84%) in October, 3 (2.5%) in November, 5 (4.2%) in December, 1 (0.84%) in January, 6 (5%) in February and 2 (1.7%) in March. The highest shedding of oocysts was observed in December and February, with prevalence 4.2% and 5% respectively as it is shown in Figure 3.2.

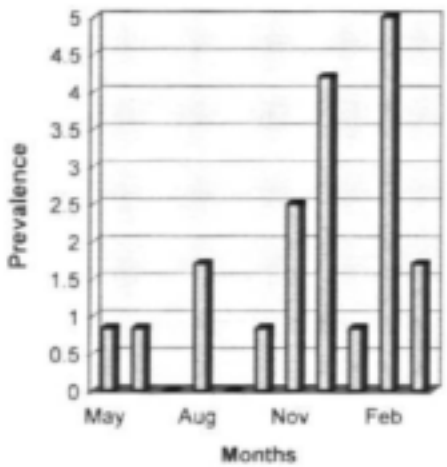

Figure 3.2: Monthly distribution of positive *Cryptosporidium* cases.

3.3.3 Associations with diarrhea in calves

Forty-five (37.8%) out of the 119 fecal samples were diarrheic during the time of sampling. Out of the 22(18.4%) calves shedding *Cryptosporidium* oocyts, 17 (77.2%) were diarrheic but 5 (22.7%) were non-diarrheic.

3.4 DISCUSSION

C.pavum was first found in cattle in 1971 and since then epidemiological studies of *Cryptosporium* infection have been reported from different parts of the world. However the prevalence of *C.parvum* in calves varies from region to region. The figure reported for the United States of America is 22%, it is 6-92% in Europe, 11-15% in Asia, and 25-59% in other areas (Uga *et al.*, 2000). The prevalence rate of 18.4% reported for this study showed that *Cryptosporidium* infections in calves in the Pretoria area are comparable to that found in some of the other countries although higher values are often (Uga *et al.*, 2000).

Of the two farms screened, the second farm had a higher prevalence of *Cryptosporidium* species, with the 26% reported for the study period of seven months. On the other hand a lower prevalence of *Cryptosporidium* species was observed at the first farm, with only 13.7% of the calves shedding oocysts over the study period of ten months. The prevalence of *Cyptosporidium* infections varied with the age of the animals. The highest prevalence was observed in calves 1-2 weeks old, but the rate decreased to 0.84% when the calves were more than one month old. These results confirm those of previous workers who concluded that cryptosporidiosis in calves is generally established during the first two weeks of life (Huetink *et al.*, 2001). The occurrence of high infection rates in this age category is attributed to the poor immunity in the newborn calves and the easiness of contamination through bucket feeding (Mtambo *et al.*, 1997).

The prevalence also varied according to the specific time of the year. The prevalence of *Cryptosporidium* oocysts peaked in late summer during February with 6 (5%) cases. In May only 1 (0.84%) of the calves were shedding oocysts, and in July and September none of the calves were shedding oocysts. Previous researchers reported seasonal variations, which peaked in spring, whereas other researchers reported peaks during winter months or no seasonality at all (Huetink *et al.*, 2001). Explanations offered for peaks in spring and summer were related to a peak in the number of newborn calves and a possible rise in temperature.

Two contradictory results on the relationship between *Cryptosporidium* species infection and occurrence of diarrhoea have been reported previously. The one study indicated a relationship and the other did not find any indication of an association with diarrhea (Uga *et al.*, 2000). Snodgrass *et al.* (1986) and Kaminjolo *et al.* (1993) found no statistically significant association between infections and diarrhoea, although cryptosporidia were detected more frequently in diarrheic than in healthy calves. The results of the present study indicated that, cryptosporidial infection rates were higher in diarrheic than in non-diarrheic calves. From the calves shedding *Cryptosporidium* oocysts, 17 of the calves had diarrhoea at the time of sampling while 5 of the calves had asymptomatic infection. This clinical symptom is however, not necessarily attributed only to cryptosporidiosis, as neither bacterial nor viral infections were assessed. Of the 119 caves sampled 45 (37.8%) had diarrhoea at the time of sampling but only 17 out of the 45 were shedding oocysts. The remaining diarrheic animals without

Cryptosporidium infection at the sampling may reflect the involvement of other infectious agents (e.g. rotavirus, *E.coli*, *Salmonella* or *Giardia*), or environmental and nutritional factors that were not investigated.

Cryptosporidium could not be detected in any of the 77 samples collected from herbivores, carnivores, primates and birds at the National Zoological Gardens in Pretoria.

3.5 CONCLUSIONS

The results of the current study conducted in the two farms demonstrate that the prevalence of *Cytosporidium* oocyst in calves is lower than what have been reported for other countries.. *Cryptosporidium* infection is most prevalent amongst young calves between 1-2 weeks of age. Adequate care must therefore be taken by individuals in the presence of calves to protect themselves from infection and becoming a source of infection. Good management and hygiene practices are also required to prevent the spread of *Cryptosporidium* to other animals and aquatic environments.

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Salmonella

CHAPTER 4

MOLECULAR EPIDEMIOLOGY OF CLINICAL AND ENVIRONMENTAL *SALMONELLA* ISOLATES AND THEIR ANTIBIOGRAMS IN RURAL VENDA COMMUNITIES IN THE LIMPOPO PROVINCE, SOUTH AFRICA.

N Potgieter

4.1 INTRODUCTION

In 1888, the identification of non-typhoidal *Salmonella* as a human pathogen was the result of an outbreak in Germany that affected 58 persons who had consumed raw beef (Gouws, 1999). An organism was isolated from the leftover meat, and was subsequently named *Bacillus enteritidis*, currently known as *Salmonella* Typhimurium (Thong *et al.*, 1995). Today, non-typhoidal *Salmonellae* are known to be a leading cause of food-borne infection and cause a disease called Salmonellosis (Yang and Yu., 2001). It has a worldwide distribution and an estimated annual incidence of 1.3 billion cases and 3 million deaths (Gruner *et al.*, 1994). The strains included in the non-typhoidal *Salmonellae* are *Salmonella* Typhimurium and *Salmonella* Enteritidis (Thong *et al.*, 1995).

Salmonella species are facultative Gram negative, non-sporing, oxidase negative, non-lactose-fermenting bacilli that produce hydrogen sulfide, and belong to the family Enterobacteriaceae. Although members of this genus have pertichous flagella, non-flagellated variants, such as *S.* Pollurum and *S.* Gallinarum, non-motile strains resulting from dysfunctioning flagella do occur (Brenner and McWhoter-Murlin, 1998). The genus *Salmonella* compromises of more than 2 000 serotypes according to a system based on somatic (O) flagella, (H) antigen, known as the Kauffmann-White scheme. Human infections are associated with a limited number of serotypes (Brenner and McWhoter-Murlin, 1994). To be fully pathogenic, salmonellae must possess a variety of attributes called virulence factors. These include the ability to invade cells, a complete lipopolysaccharide coat, the ability to replicate intracellularly, and possibly the elaboration of toxin(s). Non-typhoidal salmonellosis is a worldwide disease of humans and animals. Most non-typhoidal *Salmonellae* enter the body when

contaminated food is ingested. Animals are the main reservoir, and the disease is usually food borne, although it can be spread from person to person. *Salmonella* that cause typhoid fever and other enteric fevers spread mainly from person to person via the fecal-oral route and have no significant animal reservoirs. A symptomatic human carrier ("typhoid Mary's") may spread the disease (Thong *et al.*, 1995).

4.1.1 *Salmonella* species associated with water and sediments

Water and water sources have different qualities which are influenced by natural pollution and provide a habitat for many different microorganisms and is also considered a health risk to humans when it is contaminated with pathogenic organisms (Brock *et al.*, 1986; Thong *et al.*, 1995).

Generaly, microbial populations are more abundant in muddy sediments than in sandy ones depending on the granulometry of particles (Lakshmanaperumalsamy *et al.*, 1986). In sediments, bacteria are present as 'free-living bacteria' and associated with organic or mineral particles. The number of bacterial cells is usually higher where waters are not deep and where there are a large number of organisms. Under these conditions, leaves and other plant and animal residues decay and settle on the bottom before metabolisation. This represents a good nutritional substrate and favours bacterial growth (Cavallo *et al.*, 1999). In aquatic habitats, the most common bacteria are Gram-negative rods. The majority of the isolates belong to the genera *Salmonella*, *Pseudomonas*, *Vibrio*, and *Flavobacterium* (Spring *et al.*, 2000).

Freshwater lakes are important reservoirs of water and some regions of the earth. Nutrient cycles in the environment are largely influenced by metabolic processes localized in the surface layers of sediments. They give rise to many different environmental niches (Spring *et al.*, 2000). They harbour highly complex microbial communities with regard to species composition and metabolic activity. Hendricks and Morrison (1976) found that both multiplication rates and numbers of species of *Salmonella* increased with the density of organic and inorganic nutrients present in water.

4.1.2 *Salmonella* associated with food

Members of the genus *Salmonella* are major cause of food poisoning throughout the world (Tamada *et al.*, 2001). *Salmonella enterica* subsp. *enterica* and serotype Typhimurium (a globally distributed zoonotic serotype that is common in both cattle and poultry) is one of the main causes of human food-borne enteric infection in the world (Tamada *et al.*, 2001). The recent emergence of *S.*Enteritidis as one of the major food-borne pathogens and a cause of acute gastroenteritis worldwide has pointed to an urgent need to effectively monitor the epidemic spread of this pathogen (Olsen *et al.*, 2000). Microbial biofilms are attracting attention of scientists in different areas such as the medical field, aquatic environment, and food processing industries. Microbial biofilms may be detrimental and undesirable in food processing premises. Biofilms by pathogenic bacteria such as *Salmonella* (Humphery *et al.*, 1989) and other enteric pathogens such as *Klebsiella, Campylobacter,* enterohaemorrhagic *E. coli* and *Listeria* have been reported. Such biofilms could be a continuous source of contamination of foods coming in contact with them. Joseph *et al.*, (2001), showed that biofilm cells of *Salmonella* are much more resistant to sanitizers and the efficiency of biofilm formation and the resistance to treatment with sanitizers varied depending on the surfaces. Formation of biofilm by *Salmonella* on various surfaces has implications for the food processing industries. *Salmonella* strains entering the food processing environment either through the meat or through carriers handling meat may survive in the premises by forming biofilms on various surfaces which could be a source of contamination of foods. Joseph *et al.*, (2001) in their study suggested that the cleaning and disinfection protocols in food processing units should therefore consider *Salmonella* biofilms.

During the past 10 years the incidence of food-borne infections caused by *Salmonella spp.* increased dramatically in many countries (Maré *et al.*, 2001). In the United States, *Salmonella* Enteritidis is the most frequently isolated food-borne *Salmonella* bacteria (Rodrigue *et al*, 1990, Schroeter *et al.*, 1994). Outbreaks of *Salmonella* Enteritidis were also reported, with higher incidence in Argentina, Japan, Poland, Coastal Resorts of Southern parts of Spain (Anonymous, 1995). In South Africa the incidence of Salmonellae has escalated since the first poultry outbreak in 1991 (Maré, 1997). Strains of *Salmonella* Enteritidis are probably of more importance regarding the spread of Salmonellosis throughout South Africa (Maré *et al.*, 2001).

4.1.3 Antibiotic susceptibility and resistance of *Salmonella*

Antibiotic resistance is the ability of an organism to withstand the harmful effects of an antibiotic (Forbes *et al.*, 1998). An antibiotic may exert an inhibitory or killing effect on microorganisms (Black, 1999). Antibiotics that inhibit the growth of bacteria are known as bacteriostatics and antibiotics that kill the bacteria are known as bactericidal (Black, 1999). Resistance of bacterial pathogens to many antibacterial agents continues to increase globally and create barriers to treatment of severe and recurrent infections in infected individuals, especially in resource-poor countries because of increased treatment complexity and the need for appropriate treatment (Threlfall and Frost, 1990).

It takes years to develop, test and gain approval for new antibiotic drugs (Molback *et al.*, 1999). So while pharmaceutical companies are slowly developing potent new classes of antibiotics, resistance is developing at a rate faster than the drug companies can develop replacements (Landeras *et al.*, 1996). For example, within the last few years there has been an emergence of bacteria resistant to vancomycin, a last defense drug for some illnesses, including deadly blood infections and pneumonia caused by *Staphylococcus* bacteria and there is evidence that resistant bacteria may have been passed to humans in the meat products from livestock who were fed a similar antibiotic for growth purposes.

Multidrug-resistant *Salmonella enterica* serotype Typhimurium strains have emerged during the last decade as a global health problem because of its association with animal and human diseases. Acquired antimicrobial resistance was found in serotype Typhimurium which were resistant to ampicillin, chloramphenicol, and tetracycline, trimethoprim and sulfonamides (Immerseel, 2004). *S.* Typhimurium DT 104 strains are commonly resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracyclines (Immmerseel, 2004). At present, flouroquinelone antibiotics are the drugs of first choice in the treatment of *Salmonella* infections and recent developments of fluoroquinelone antibiotic resistance are of particular concern because this reduce the efficacy of early empirical treatment (Immmerseel, 2004).

Antibiotics such as cetriazone and chloramphenicol are the drugs of choice for treatment of severe typhoid fever. However, drug sensitivity tests are required because of the continuing appearance of resistance strains. Ampicillins are used as effective alterations

(Caldwell, 1995). Chloramphenicol resistance, MDR *S.* Typhi and now low-level fluroquinolone resistance have emerged as the newer challenges to treatment of typhoid fever (Shrikala and Shalini, 1999). It has been seen that in certain areas especially Northern India *S.* Typhi has lost this acquired resistance and chloramphenicol resistance has reduced from a high of 18% to only 2%. This has been noticed in our Institutes also where chloramphenicol resistance has reduced drastically over the years (Shrikala and Shalini, 1999). It has also been identified that chloramphenicol is still being used to treat typhoid fever in some areas in Indonesia where 96% of strains remain sensitive to chloramphenicol. Chloramphenicol resistance is known in *Salmonella* Typhi since 1972, when plasmids of incompatibility group Inc H, coding for chloramphenicol resistance were found in *S.* Typhi. Multi drug resistance (MDR), against chloramphenicol, ampicillin, co-trimoxazole and tetracycline, has been endemic in the Indian Sub continent and South East Asian countries since 1984 (Parray and Hein, 2002). Though initially, individual plasmids were known to code for resistance to each of these antibiotics, since 1988 a single plasmid was known to code for multidrug resistance. This plasmid belongs to incompatibility group H 11 and is highly transmissible. Low-level fluroquinolone resistance is due to point mutations in the gene coding for DNA gyrase enzyme (Shrikala and Shalini, 1999).

Extended spectrum beta lactamases, coded by transposons and responsible for resistance to third generation cephalosporins have been identified in *S.* Typhi (Cooke and Wain, 2004). Suitable alternatives to treat MDR *S.* Typhi include azitromycin, third generation cephalosporins and carbapenems. Though public health, sanitation and vaccines do have a role to play in control of typhoid fever, it is the antimicrobial therapy which plays a key role in management of typhoid fever. It is likely that *S.* Typhi will continue to acquire genes responsible for antibiotic resistance. The timely detection of these genes and prevention of their spread is the need of the hour to control antibiotic resistance among *S.* Typhi. Cooke and Wain (2004), emphasized on the detection of plasmids encoding for MDR *S.* Typhi and their transmissible nature.

A study carried out by Helms and Molbak (2004) in Denmark showed that a total of 631 patients were hospitalized due to *S.* Typhimurium infection. Of these patterns, 577 had gastroenteritis as their primary diagnosis. Resistance to sulfonamides, tetracycline,

streptomycin, ampicillin, chloramphenicol, kanamycin, quinelones, trimethoprim, gentamycin, and ceftriaxone. In another study by Miriagou et al., (2002) thirteen Salmonella enterica serotype typhimurium and one *Salmonella enterica* serotype Heidelberg were resistant to expanded-spectrum cephalosporins during 2000 to 2001 from infants with gastro enteritis. In all but one serotype of *S.* Typhimurium the resistance was due to the production of a CMY-5-cephalosporinase encoded by a nonconjugative plasmid. The remaining isolate produced an SHV-5-type beta-lactamase (Miriagou et al., 2002).

According to a study carried out by Lee and Puhr (1990), over 80% of *Salmonella* strains from both human and animal sources showed resistance to tetracyclines, sulphonamides, streptomycin, and chloramphenicol. Ampicillin resistance was never found in isolation, and was associated with resistance to at least four antibiotics in 78% of human strains and 83% of animal strains. Resistance patterns were similar among strains from humans and animals: the commonest phenotype comprised resistance to ampicillin, sulphonamides, streptomycin, chloramphenicol, and tetracycline and was found in 76% of human and 73% of animal strains.

The World Health Organization (WHO, 2000) suggest that integrons carrying multiple resistance genes encoding CARB-type beta-lactamase have been acquired and spread in *S. typhimurium* of human and animal origin. These preliminary results strengthen the hypothesis that Salmonella strains may acquire antibiotic resistance by recombination and transfer between them; some strains having all the genetic material required for transfer of resistance genes. Animals are known to be the main reservoir of *Salmonella* and a common source of human contamination, however, ensuring their dissemination and persistence. A link between resistance observed among strains isolated from animals and in human medicine could be established. However a selective pressure also exists in hospital environments that strongly contributes to the increasing resistance of strains isolated in such environments (WHO, 2002).

There is growing concern over the consequences of over-use of antibiotics in livestock operations (Piekering, 2004). Persistent use of antibiotics leads to the development of resistance in bacterial populations (Piekering, 2004). Once a particular type of bacteria has developed resistance to an antibiotic, that antibiotic can no longer be used to combat

the infectious organism. Since 1990s, the frequency of antimicrobial drug resistance in zoonotic *Salmonella* and the number of drugs to which the drugs are resistant have increased, primarily as a consequence of antimicrobial use in food production (Cohen et al., 1986;). The US produces approximately 50 million pounds of antibiotics each year and 40% of that is given to animals, usually as a feed additive to promote growth. More and more evidence shows, however, that infectious bacteria are quickly developing resistance to even the newest, most powerful antibiotics. Researchers have published disturbing reports that antibiotic resistance in *Salmonella* and *Campylobacter*, two human pathogens, is on the rise and evidence is mounting that these resistant bacteria can be passed from chickens and pigs to humans through the food chain. This poses a great health risk to the human population because it makes it easier for humans to become infected with resistant pathogens for which there are few effective treatment options

WHO (2000), indicates that the routine, medically unnecessary use of antibiotics to promote the enhanced growth of livestock is making disease-causing bacteria more resistant to the drugs, which diminishes their power to treat life-threatening diseases in humans. When antibiotics are given again, the resistant bacteria remain. As the resistant strain within the bacterial population increases over time, the drugs become less effective. The more antibiotics we use, the more likely it is that bacteria will become resistant.

The Institute of Medicine, part of the National Academy of Sciences, estimated that the annual cost of treating antibiotic-resistant infections in the U.S. might is high. The increased use of antibiotics in animal production has gone hand-in-hand with the development of industrial-style livestock operations. Thousands of animals are crammed into the unhygienic, crowded quarters of a typical factory operation, and antibiotics are dispensed constantly through the animals' feed. Twenty-five million pounds of antibiotics are fed to American livestock annually. This is about 70% of the total amount of antibiotics produced in the U.S. each year and eight times more than the amount used as human medicine. Scientists do not understand how or why the drugs promote growth. Many of the antibiotics in six of the 17 classes of antibiotics used to promote growth in animals are also used to treat diseases in humans.

According to a study conducted by Holmberg and Osterholm (1984), twenty-one adults and children were identified as having acquired salmonellosis directly or indirectly through contaminated pork. By the time the outbreak was over, 7 had been hospitalized and 1 had died. The outbreak came to light in June of 1983 after five patients were identified as having an unusual strain of *Salmonella* food poisoning. On the same day that this particular strain was identified, the Danish national *Salmonella* surveillance program identified the same strain of *Salmonella* in pork samples collected from a slaughterhouse in late May. Using molecular "fingerprinting" techniques, scientists traced the spread of this new multidrug-resistant strain of *Salmonella* from pigs to humans. Following one lead after another, they sampled nearly 90 herds of swine. In the end, two herds tested positive, one herd in the process of fattening and a second herd that had delivered piglets to and shared machinery with the farm with the first herd. The tracing of this *Salmonella* outbreak through the food chain provided an important opportunity to study how diseases that originate on the farm spread through a community. As might be expected, the largest group of victims consisted of people who ate contaminated pork products. But others were also infected. One of the victims worked at the incriminated slaughterhouse. Two more people probably acquired the disease through exposure to one of the hospitalized victims. A nurse became ill after attending to this patient, while a woman recovering from complications of cancer therapy also fell ill after sharing a room with the same person. The 82-year-old woman later died. So simply changing what we eat is not necessarily enough to protect ourselves from an outbreak of an infectious disease like *Salmonella*.

The problem of antibiotic resistance and its consequences is evident within the β-lactamase class which includes penicillin, penicillin derivatives, cephalosporins, carbapenems, cephamycins, monobactams and monocabams which constitutes 50% of all systematically used antimicrobials (Livemore and Williams, 1996) by virtue of their high efficacy and specificity (Galleni *et al*., 1995), the availability of derivatives and the low toxicity compared to most other broad spectrum antibiotics such as fluoroquinelones or tetracyclines (Livemore and Williams, 1996).

Beta-lactam antibiotics interfere with the final stage of cell wall (peptidoglycan) synthesis by inhibiting bacterial enzymes (transpeptidase, carboxypeptidase, endopeptidase) commonly called penicillin-binding proteins (PBPs), which cross-link

the peptidoglycan polymer (Livemore and Williams, 1990). Peptidoglycan is the most essential component of the bacterial cell wall. It protects the organism from osmotic rupture, determines cell shape and is integral to cell growth and division. It must remain physically continuous during the bacterial cell cyclein order to maintain cell integrity and viability (Jacobs, 1994). This ability is conferred by its net-like structure composed of saccharide chain cross-linked by peptides (Livemore and Williams, 1996).

The efficacy of beta-lactamase antibiotics, specifically against Gram-negetive bacteria, is dependent on accessibility to its target PBPs, the degree of resistance to enzymatic inactivation by beta-lactamases (beta-lactam antibiotic inactivating enzymes), and their ability to inhibit the target PBPs. Altering one or more of these parameters may result in resistance (Bellido *et al.*, 1991). Beta-lactamase strains efficiently catalyse the irreversible hydrolysis of the amide bond of the beta-lactam ring, resulting in biologically inactive products (Matagne *et al.*, 1998). Over 400 beta-lactamases have been documented, varying in their encodement (whether intergron, transposon, plasmid, or chromosomally-mediated), substrate profiles, inhibitin prophiles, molecular mass, isoelectric points, amino acid sequencesand molecular structure (Bauernfeind, 1986; Matagne and Frere, 1995). Factors influencing this mechanism include the quantity of β-lactamase produced, the affinity of the enzyme for the antibiotic, the rate of hydrolyses of the antibiotic and the locatin of the beta-lactamase. Beta-lactamases in Gram-positive organisms are exocellular enzymes excreted into their immediate environment (Nue, 1986). Beta-lactamases are localized in the periplasm in Gram-negetive bacteria where they attempt to maintain the local antibiotic concentration below the bactericidal threshold (Livemore and Williams, 1996).

4.1.4 Objectives of this study

The objectives of this study were to:

- isolate *Salmonella* from environmental samples (sediments and water), food samples and clinical samples (e.g. stools) in the Vhembe region;
- determine the antibiotic sensitivity of human associated *Salmonella* isolates from clinical and environmental samples.

4.2 MATERIALS AND METHODS

4.2.1 Sampling sites and sample collection

This study was carried out in rural communities in the Vhembe region of the Limpopo Province in South Africa. Many of the villages have small vendor stands to make an income and they sell tomatoes, bananas, apples, onions and different types of sweets. Villagers buy apples and tomatoes from these vendors and eat them without washing. Throughout the day various hands touch these foods as peoples decide what to buy and flies /insects are frequently seen to move around on these products in the vendor stands. The rivers are used as a main source of drinking water, washing of clothes, bathing, fishing and livestock watering.

During 2001 and 2003, samples were collected over a wide area in the Vhembe region. Water and sediment samples were collected from the Nzhelele River (site A and site B), Elim bridge River, Tshikuwi River, Shikari River, Ritavi River, Lotanyanda River and from the Sendendza borehole. Stool specimens were obtained from the Elim and Siloam Hospitals. Food samples such as vegetables (onions, cabbages, apples) and frozen chicken portions were collected from various food vendors in the vicinity of the Elim and Siloam hospitals.

During 2004, the study concentrated on the area where the majority of *Salmonella* isolates were obtained from the study conducted during 2002 and 2003. Water and sediments samples were collected from four sampling points (Mbokota 1, 2, 3 and Chavani Bridge) on the Ritavi River running through the Mbokota village. Stool specimens were obtained from the Elim hospital and Chavani clinic serving the Mbokota village. Food samples included tomatoes and apples which were collected from various vendors in the village.

Water and sediment samples were collected aseptically in pre-sterilized 2 l Nalgene containers and transported on ice to the Microbiology laboratory and processed for the isolation of *Salmonella* bacteria within 8 h after collection. Once a week stool specimen were collected from the clinic and hospitals and transported on ice to the laboratory for further isolation and identification studies. Food samples were placed inside separate sterile zip bags with sterile Ringers solution (Oxoid) using sterile gloves.

4.2.2 Isolation of presumptive *Salmonella* spp

The isolation of *Salmonella* spp was carried out using standard culture method as described by Davis *et al*., (2002). One ml of the water, sediments and food samples were added separately to 5 ml of Buffered Peptone Water (BPW) (Oxoid CM509) and incubated at 37°C for 16-20 h. For selective enrichment, Rappaport Vassilliadis Soya (RVS) broth (Oxoid CM 866) was used. The RVS tubes were inoculated with 0.1ml of the pre-enrichment PBW cultures and incubated at 42°C for 48h. Following incubation, 90mm Xylose Lysine Deoxicolate (XLD) agar plates were inoculated with a loopful of the RVS broth and incubated at 37°C for 24 h. Presumptive *Salmonella* colonies from XLD plate were subcultured onto Nutrient agar plates.

4.2.3 Identification of *Salmonella* spp

4.2.3.1 Biochemical tests

Standard tests were performed on presumptive isolates and included Gram staining, hydrogen sulphide production, urea hydrolysis and motility tests.

4.2.3.2 Phenotypic differentiation of *Salmonella* isolates into human and environmental strains

Salmonella enterica, subspecies I strains are usually isolated from humans and warm-blooded animals whereas subspecies II-VI and *Salmonella bongori* are usually isolated from cold-blooded animals and the environment and rarely isolated from humans (Ewing 1986). Biochemical tests are useful for subspecies differentiation of *Salmonella* isolates and were employed to differentiate between *Salmonella subspecies* I (human associated *Salmonella* species) and *Salmonella subspecies* II-IV (cold-blooded animals and environmental associated species) (Ewing, 1986). Biochemical tests used in this study included the gelatin liquefaction and tartrate utilization tests according to the methods described by Ewing (1986). The isolates which were positive for the tartrate utilisation and negative for gelatin liquefaction were identified and grouped as members of subspecies I and those which were negative for tartrate utilisation were identified as representing *Salmonella* subspecies II-VI. Only the human associated *Salmonella* subspecies I isolates were further subjected to PCR for molecular characterisation (Ewing, 1986).

4.2.3.3 Confirmation of *Salmonella* isolates using PCR

The human associated *Salmonella* subspecies I isolates were characterised and typed using PCR-based DNA typing technique according to the methods previously described (Soumet *et al.*, 1999; Pass *et al.*, 2000; Oliveira *et al.*, 2002). Primers used in the study to confirm the isolates as *Salmonella* spp belonging to subspecies I are shown in Table 4.1.

Table 4.1: Primers used for the identification of *Salmonella* subspecies I

Primer Set	Targe t gene	Length basis	Primer sequences	Application product
JHO-For JHO-Rev	*SefA*	20	TCCTCATTCCTATACCTACC AAACGATGAAAAACTGAGCA	188 basepairs
A058 A01	*Sef*A	21 24	GATACTGCTGAACGTAGAAGG GCGTAAATCAGCATCTGCAGTAGC	488 basepairs
Fli15 Tym	*fliC*	22 22	CGGTGTTGCCCAGGTTGGTAAT ACTCTTGCTGGCGGTGCGACTT	559 basepairs

In order to prevent cross contamination, a reagent blank containing reaction mixture but no DNA was included in each set of reaction as a control. Separate rooms were used for DNA isolation, PCR, PCR analysis and PCR product storage. Two sets of pipettes were used to minimize contamination. DNA was extracted from centrifuged bacterial colonies as described by Pass *et al.*, (2000). Briefly: for each isolate, a loop full of overnight pure culture of each presumptive *Salmonella* isolate was inoculated into 100 µl of distilled water then boiled at 100°C for 10 min in order to release bacterial DNA from the bacterial cells. This is known as heat induced bacterial lysis (Pass *et al.*, 2000). The bacterial DNA suspension was centrifuged at 100 rpm for 5 min. Amplification reactions were carried out in the total volume of 50 µl, containing 1.5 U Taq polymerase, 0.6 μmol^{-1} of each primer, 0.2 mM of each dNTPs, 10×PCR buffer (10 $mmol^{-1}$ Tris-HCL). 2.5 Mm $MgCl_2$, 0.1% Tween 20 in nuclease free water and 5 µl of isolated bacterial DNA. Magnesium chloride was used at a concentration of 2.5 mm to optimize the assay. Oligonucleotide primers, Taq polymerase, dNTPs, PCR buffer and $MgCl_2$, were synthesized by Inqaba Biotechnical industries (Pty) Ltd, South Africa. The PCR reaction was centrifuged for 1 min to mix reaction. The PCRs amplifications were carried out using a Gene Amp 9600 Thermocycler (Perkin Elmer

Instruments, Norwalk, CT, USA) with conditions modified from Doran *et al.*, (1996). Briefly: the initial denaturation at 94°C for 1minute was, followed by 35 cycles of denaturation at 94°C for 30 s. Annealing at 58°C for 30 s was followed by extension at 72°C for 30 s, with a final extension at 72°C for 7 minutes. Amplified products (10µl PCR products in 2µl loading buffer) were separated by electrophoresis on a 1.2% agarose gel (Agarose, LE, Analytical Grade) using 1x TBE buffer (89mM Tris, 89mM boric acid, 2mM EDTA) at 80V for 30 min. A 100 bp ladder was used as a DNA molecular weight marker (Soumet *et al.*, 1999; Oliveira *et al.*, 2002).

4.2.4 Antibiotic susceptibility testing of *Salmonella* isolates

Salmonella isolates were sub-cultured onto Nutrient Agar plates for conformation and assessed for antibiotic susceptibility using the Kirby-Bauer method (NCCLS, 2003). Mueller-Hinton broth was used for the enrichment of *Salmonella* isolates and Mueller-Hinton agar was used for antibiotic assays. Aseptic technique using a sterile inoculating needle was used to transfer colonies from the Nutrient Agar plates to the Mueller-Hinton broth. Four colonies of each isolate were transferred to a test tube containing 3 ml of Mueller-Hinton broth. This broth was vortexed for 6 seconds to ensure that a homogenous solution was obtained. The turbidity of the solution was adjusted visually against a 0.5 McFarland turbidity standard in order to obtain an inoculum of 1.5×10^8 cfu/ml (NCCLS, 2003). Within 15 min of adjusting the inoculum against 0.5 McFarland standard, a sterile cotton swab was dipped into the Mueller-Hinton broth and rotated against the walls of the tube to remove any excess fluid. The entire surface of the Mueller Hinton agar plate was then swabbed 3 times rotating the plate approximately 60° without re-emersing the swab into the suspension. Inoculation plates were allowed to stand for 3 to 15 min before applying the antibiotic discs.

Antibiotic discs were commercially obtained from Oxoid (SA) (Table 4.2). A dispenser and a sterile forceps were used in the application of the discs. Gentle pressure was applied to ensure complete contact of each the disc with agar. There was a distance of 24 mm from one disc to the other. Plates were incubated within 15 min after the discs were placed on the agar plates. Plates were incubated at 35°C in an aerobic environment overnight for 16-18 hrs. The diameters of clear zones of inhibition were read using a pair of calipers. Zones of inhibitions by each antibiotic were recorded as

resistant, intermediate, or sensitive according to the National Council for Clinical Laboratory Standards (NCCLS, 2003). Isolates that are beta-lactamase inducers were observed. Beta-lactamase inducers are organisms that produce the enzyme beta-lactamase which is essential for the production of the cell membrane. Beta-lactam antibiotics that inhibit the production of the enzyme beta-lactamase, were used to determine the presence of beta-lactamase inducers. In this case the beta-lactams were cefoxitin, amoxicillin, ceftaxidime and cefotaxime.

Table 4.2: Commercially available antibiotics used, their symbols and the concentrations

Antibiotic agent	Commercial symbol	Concentration
Gentamicin	**CN**	**10µg**
Ampicillin	**AMP**	**10µg**
Ceftazidime	**CAZ**	**30µg**
Cefoxitin	**FOX**	**30µg**
Cefotaxime	**CTX**	**30µg**
Amoxicillin-clavulanic acid	**AMC**	**30µg**
Chloramphenicol	**C**	**30µg**
Ciprofloxacin	**CIP**	**5µg**
Cotrimoxizole	**SXT**	**25µg**
Nalidixic acid	**NA**	**30µg**
Tetracycline	**TE**	**30µg**
Ofloxacine	**OFX**	**5µg**
Meropenem	**MEM**	**10µg**

4.3 RESULTS

4.3.1 Isolation and identification of presumptive *Salmonella* spp from environmental and clinical samples

A total of 637 water and sediment samples, 80 food samples and 180 clinical samples were examined between 2002 and 2004 for the prevalence of *Salmonella* species, of which 514 (57%) tested positive for presumptive *Salmonella* spp using biochemical tests (Table 4.3).

Table 4.3: Presumptive positive *Salmonella* isolates from environmental, clinical and food samples

Source	Sampling Sites	Number of samples tested	Presumptive positive *Salmonella* isolates (percentage %)
Water and sediment	Lotanyanda River	60	56 (93%)
	Ritavi River	34	22 (65%)
	Nzhelele River Site A	29	24 (83%)
	Nzhelele River Site B	50	45 (90%)
	Elim Bridge River	50	40 (80%)
	Shikari River	34	32 (95%)
	Tshikuwi River	30	10 (33%)
	Sendedza Borehole	30	15 (50%)
	Mbokota river site1	80	55 (69%)
	Mbokota river site 2	80	49 (61%)
	Mbokota river site 3	80	20 (25%)
	Chavani river site 4	80	13 (16%)
Food	Food samples	80	36 (45%)
Clinical	Elim hospital	90	53 (59%)
	Siloam Hospital	60	15 (25%)
	Chavani clinic	30	29 (97%)
TOTAL		**897**	**514 (57%)**

4.3.2 Phenotypic differentiation of presumptive *Salmonella* isolates.

All 514 presumptive *Salmonella* isolates were further subjected to phenotypic differentiation using tartrate and gelatin hydrolysis to differentiate between human and environmental associated strains.

Table 4.4: Phenotypic differentiations of presumptive *Salmonella* positive species into human (subspecies I) and environmental (subspecies II-IV) strains

Source	Sampling Sites	Number of isolates tested	Environmental associated strains belonging to subspecies II-VI (percentage %)	Human associated strains belonging to subspecies I (percentage %)
Water and sediment	Lotanyanda River	56	44 (79%)	12 (21 %)
	Ritavi River	22	11 (50%)	11 (50%)
	Nzhelele River Site A	24	23 (96%)	1 (4%)
	Nzhelele River Site B	45	45 (100%)	0 (0%)
	Elim Bridge River	40	40 (100%)	0 (0%)
	Shikari River	32	14 (44%)	18 (56%)
	Tshikuwi River	10	10 (100%)	0 (0%)
	Sendedza Borehole	15	15 (100%)	0 (0%)
	Mbokota river site1	55	43 (78%)	12 (22%)
	Mbokota river site 2	49	41 (84%)	8 (16%)
	Mbokota river site 3	20	15 (75%)	5 (25%)
	Chavani river site 4	13	10 (77%)	3 (23%)
Food	Food samples	36	34 (94%)	2 (6%)
Clinical	Elim hospital	53	18 (34%)	35 (66%)
	Siloam Hospital	15	(0%)	15 (100%)
	Chavani clinic	29	18 (62%)	11 (38%)
TOTAL		**514**	**381 (74%)**	**133 (25%)**

4.3.3 Molecular typing of *Salmonella* isolates by PCR

PCR carried out on the 133 human associated *Salmonella* isolates, indicated that 46 (35%) isolates were *Salmonella* Typhimurium, 87 (65%) isolates were other *Salmonella* strains (Table 4.5).

Table 4.5: Confirmed human associated *Salmonella* isolates

Source	Number of human associated *Salmonella* isolates tested	Number of isolates identified as *S. typhimurium*	Number of other *Salmonella* species
Water and sediment	70	18	52
Food	2	1	1
Clinical	61	27	34
TOTAL	**133**	**46 (35%)**	**87 (65%)**

4.3.4 Antibiogram of human associated *Salmonella* isolates obtained from clinical, food, water and sediment samples

The antibiotic susceptibility of *Salmonella* isolates obtained from clinical, food, water and sediment samples is indicated in Table 4.6. A total of 35 clinical isolates were tested for sensitivity and the isolates were 100% sensitive to gentamicin, ampicillin, ceftazidime, cefoxitin, ofloxacin, cefotaxime, amoxicillin-clavulanic acid, ciprofloxacin, nalidixic acid, ofloxacim and meropenem and 80% of the isolates were sensitive to chloramphenicol, 86% to cotrimoxizole and 83% to tetracycline. However, 20% of the isolates indicated resistance to chloramphenicol, 14 % to cotrimoxizole and 17% to tetracycline. Only 2 food isolates were tested for sensitivity and the isolates were totally (100%) susceptible to all the antibiotics used. A total of 70 isolates from water and sediments were tested for antibiotic susceptibility and the results showed that all isolates (100%) were sensitive to gentamicin, ampicillin, cefoxitin, ofloxacin, cefotaxime, amoxicillin-clavulanic acid, ciprofloxacin, and meropenem. However, only 99% of these isolates were sensitive to tetracycline and chloramphenicol respectively, 98% sensitive to cotrimoxizole, and 70% sensitive to ceftazidime, and nalidixic acid respectively.

Table 4.6: Antibiotic susceptibility of human associated *Salmonella* isolates obtained from clinical specimens, food samples, water and sediment samples

Sources	Human associated *Salmonella* isolates	Antibiotics												
		CN	AMP	CAZ	FOX	CTX	AMC	C	CIP	SXT	NA	TE	OFX	MEM
Clinical	35	35 (100%)	35 (100%)	35 (100%)	35 (100%)	35 (100%)	35 (100%)	28 (80%)	35 (100%)	30 (86%)	35 (100%)	29 (83%)	35 (100%)	35 (100%)
Food	2	2 (100%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)
Water and sediment	70	70 (100%)	70 (100%)	49 (70%)	70 (100%)	70 (100%)	70 (100%)	69 (99%)	70 (100%)	69 (99%)	49 (70%)	69 (98%)	70 (100%)	70 (100%)

4.3.5 Beta-lactamase inducers detected during antibiogram determination

Table 4.7 indicates the presence of *Salmonella* isolates that were beta-lactamase inducers. In total, 7 beta-lactamase inducers were identified from sediment isolates, 2 from water isolates and 1 from clinical isolates. The beta-lactam in this case was cefoxitin.

Table 4.7: The presence of *Salmonella* isolates that were beta-lactamase inducers

Isolate	Number of beta-lactamase inducers	**beta-lactam antibiotics**
Clinical isolates	1	Cefoxitin
Water isolates	2	Cefoxitin
Sediment isolate	7	Cefoxitin

4.3.6 AFLP analysis of human associated *Salmonella* isolates

AFLP analysis were performed on a number of human associated isolates from environmental and clinical samples to establish the link between environmental and clinical samples. The DNA obtained from isolates were digested with *EcoR*I and *Mse*I and the selective amplification was performed with *EcoR*I-G and *Mse*I-T primers. From the dendogram (Figure 4.1) it could be established that there are strong genotypic association between some of the environmental and clinical strains.

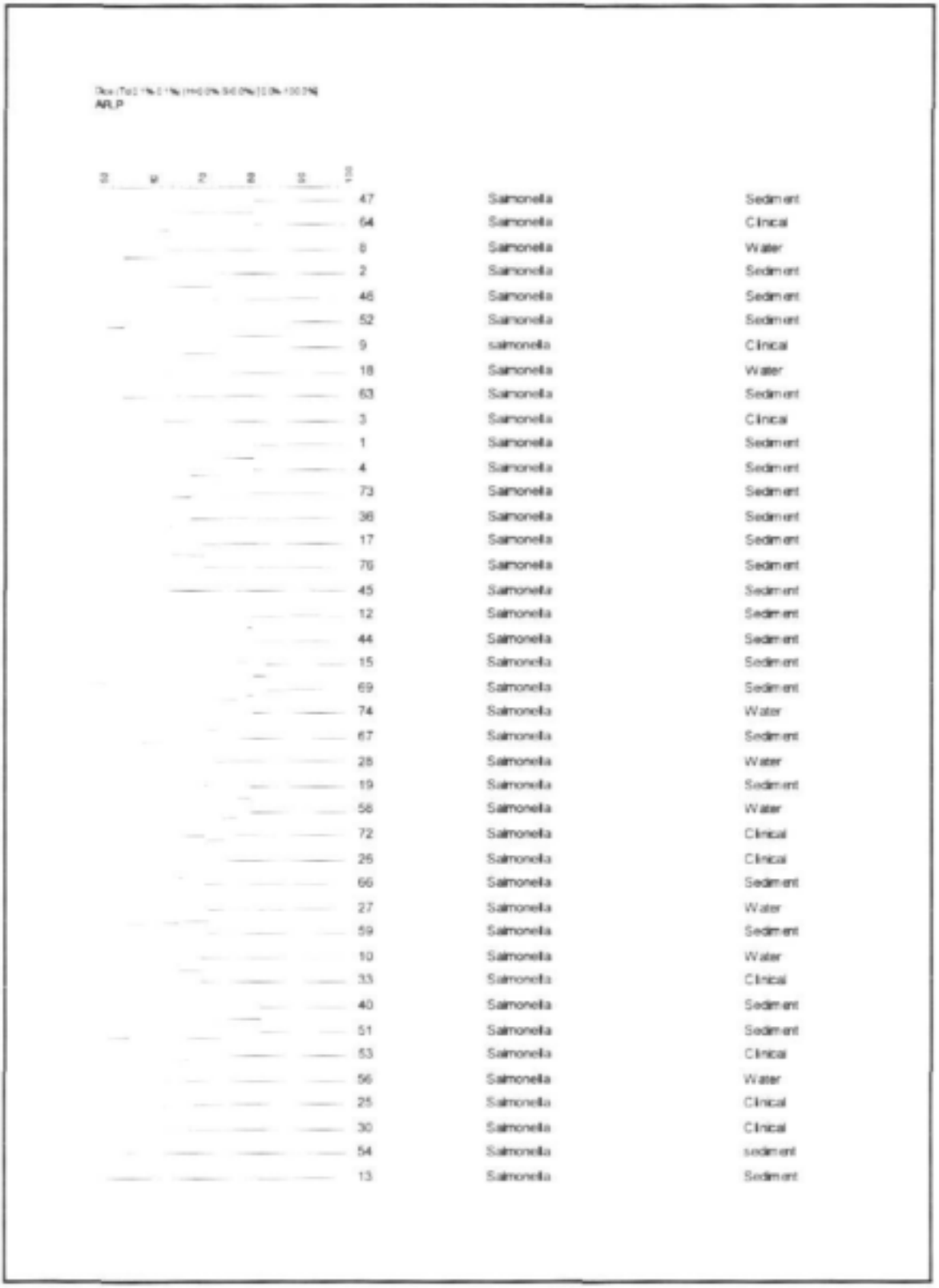

Figure 4.1 Dendogram indicating relationship between human associated *Salmonella* isolates

4.4 DISCUSSION AND CONCLUSION

The availability of clean and safe water in rural areas of South Africa, is still a serious problem. A large proportion of the rural population in South Africa use water from natural sources directly for domestic, daily water needs as well as other non-domestic purposes such as agriculture and recreation (Nevondo and Cloete, 1999). These conditions expose the people to a variety of water related or waterborne diseases (Nevondo and Cloete, 1999). The implications of the presence of *Salmonella enterica* subspecies *enterica* in drinking water sources are grave and of public health significance. *Salmonella enterica* subspecies enterica is a known aetiologic agent of diarrhoea, septicemia. A few of the *Salmonella* serotypes other than *S.* Typhi produce bacteremia. This may lead to the localizaton of the organism in numerous body sites, especially those that are the sites of pre existing diseases. Bronchopneuonia empyma, endocarditis, oestoemylitis, arthritis and meningitis are relatively common (Hampton *et al*., 1998).

The results of the present study have revealed that *Salmonella* bacteria were prevalent in environmental and clinical samples examined. The reason for the presence of *Salmonella* in river water and sediments may be due to the fact that wild and domestic animals seeking water, contaminate the river water through direct defecation. In addition, fecal contaminants from human excreta may also find their ways to the river (Nevondo and Cloete, 1999). Sediments protect enteric bacteria from some of the stresses associated with aquatic environments and provide nutrients that support bacterial growth (Andre and Casper, 2000).

Salmonella isolates were also isolated from raw vegetables (cabbages, tomatoes and onions) obtained from local rural gardens which used river water for irrigation. These findings imply that food such as vegetables or raw products have an equal chance of being contaminated by *Salmonella* bacteria from water and can pose a potential health risk to consumers (Garcia *et al*., 1987; Ait and Hassani, 1999). Therefore, surface decontamination can significantly reduce the level of *Salmonella* on vegetables (Ait and Hassani, 1999). River water irrigation is practised in many countries around the world. However, the problem with using untreated river water for irrigation is that it contains disease causing pathogenic organisms (bacteria, viruses, protozoa and heiminths) which can be a danger to public health when crops are consumed raw (Ait and Hassani, 1999).

This study has shown that human pathogenic *Salmonella* species were isolated from raw vegetables (tomatoes and onions) from local rural gardens which used river water for irrigation. The consumption of salads and raw vegetables irrigated with contaminated river water could lead to *Salmonella* infection (Garcia *et al*., 1987). The water and food link is important as water could also serve as a potential source of food contamination (Garcia *et al*., 1987; Landeras *et al*., 1998). The end users of water and food are humans. Consequently, transmission to humans cannot be underestimated. In humans, infection may manifest as diarrhea and therefore diarrhoeal stools were collected.

Strains isolated from human stools, water, sediments and food were phenotypically and genetically correlated, confirming that water and food could be important possible source of human Salmonellosis in rural areas. The results of this study are in agreement with findings by other researchers who conducted studies on the molecular epidemiology of *Salmonella* (Fantasia *et al*., 1991; Thong *et al*., 1995). Certain molecular types present among the environmental isolates of *Salmonella* Typhi were also found among the human isolates and these provide evidence for the epidemiological link between environmental reservoirs and human infection. A link was also confirmed by Fantasia *et al*., (1991) between food and patients in the serovar Oranienburg outbreak and genetically closely related river isolates were considered to be a result of the outbreak. It should, however, be noted that the presence of *Salmonella* in environmental samples (water and food) is not definitive of the presence of diarrhea causing strains. Most strains of *Salmonella* are classified under the species *S. enterica*, as the number of base differences between *Salmonella* strains is minimal. This species is the only one that contains the strains commonly associated with infections of warm-blooded animals and humans (Ewing, 1986). In this study, phenotypic differentiation (performed to differentiate between human and environmental associated strains) indicated that all clinical isolates and environmental isolates were identified as *Salmonella enterica* subsp. *enterica* or *Salmonella* subspecies I and as *Salmonella* subspecies II-VI. Higher number of human associated *Salmonella* subspecies I was isolated from river sources where there are animal and human activities near the residing place. Most of the river sources are reportedly prone to higher bacterial levels, including *Salmonella*, due to heightened ecological and human activities such as washing, swimming (Nevondo and Cloete, 1999). The presence of human associated and cold-blooded associated environmental *Salmonella* species isolated from various

water sources in this study may confirm that possible sources of contamination of the river water sources include human and animal feaces or introduction of micro-organisms by cold-blooded animals. These multiple sources of contamination are compounded by limited environmental awareness in rural areas (Garcia *et al.*, 1987). Humans and warm blooded animals are considered to be the primary reservoirs of *Salmonella* species and its subsequent shedding can result in environmental contamination of water and vegetables, causing an increased risk of infection for the rural communities (Garcia *et al.*, 1987; Fantasia *et al.*, 1991).

Resistance of bacterial enteric pathogens has shown a progressive increase over the course of time in many areas of the world. Approaches to minimize the development of antimicrobial resistance and optimize therapy include educational interventions, appropriate use of antimicrobial agents, reduction in the use of growth promoters in animal feed and optimization of infection control (Pickering, 2004). Other approaches are measures to prevent spread of resistant organisms, development and use of rapid diagnostic techniques to optimize therapy, use of non absorbable antimicrobial agents, use of non-antibiotic therapeutic and preventive measures including receptor blockers, development and use of enteric vaccines (Pickering, 2004). Multidrug-resistant *Salmonella enterica* serotype Typhimurium strains have emerged during the last decade as a global health problem because of its association with animal and human diseases. According to Pickering (2004), resistant patterns are influenced by geographic location, the year in which isolates were obtained, different classes of antimicrobial agents, the pressure exerted by antimicrobial use, and the source of the isolate.

In this study, 13 antibiotics were tested against human associated *Salmonella* strains obtained from clinical, food, water and sediment samples from 2 separate studies. Antibiotic resistant *Salmonella* strains could not be detected from any of the food isolates, which may be due to limited isolates. Isolation of *Salmonella* from water and sediments indicated that the *Salmonella* isolates were resistant against ceftazidime (35%), chloramphenicol (2%), cotrimoxizole (3%), nalidixic acid (35%) and tetracycline (2%). This may have been as a result of river water polluted through human and animal activities such as recreational activities, washing cloths, and cattle and domestic animals defecating in the vicinity of the water sources.

Since flouroquinelones (ofloxacin, and ciprofloxacin) are the drugs of choice for extra intestinal and serious intestinal complications caused by human *Salmonella*, flouroquinelone resistance has the potential of causing problems with therapy. There is however little evidence of the effect of quinelone-resistant *Salmonella* on human health (Molbak and Beggesen, 1999). Resistance to this antibiotic will reduce the efficicacy of early empherical treatment and may limit therapeutic choice in the management of severely ill patience with culture confirmed infections (Helms *et al*., 2004). In this study, human related clinical, food, water and sediments isolates were found to be susceptible to ofloxacin and ciprofloxacin. It appears that fluoroquinelone resistance is not yet a problem in the rural communities of Vhembe, South Africa. There were no resistance patterns indicated in human associated *Salmonella* from food isolates. However, the results have clearly indicated that Penicillins (ampicllin), the beta-lactams (amoxicilin clavulanic acid), aminoglycosides (gentamycin), carbapenems (meropenem), cefems (cefoxitin and cefotaxime), fluoroquinelones (ofloxacin and ciprofloxacin) are antibiotics in the treatment of *Salmonella* associated symptoms. Beta-lactamase producing *Salmonella* strains have been found to be of importance in epidemiology. In this study 10 Human associates *Salmonella* strains have indicated resistance to currently used beta-lactam antibiotics (cefoxitin). This study has proven that there is an increase in resistance among currently used beta-lactam antibiotics. Antibiotics used against beta-lactamase producing strains should be monitored.

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CHAPTER 5

SURVIVAL OF A GFP-TAGGED *SALMONELLA* ISOLATE IN FRESH WATER SEDIMENTS

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5.1 INTRODUCTION

Salmonella is a major cause of gastroenteritis in humans, characterized by diarrhoea, cramps, vomiting, and fever. The symptoms are usually mild and self-limiting in healthy individuals. However, in the young, the elderly and in individuals with compromised immune systems, dehydration can become severe and life threatening. *Salmonella* are ubiquitous in the environment. Although food has been implicated as the major source of non-typhoidal *Salmonella* infections in the USA (Mead *et al.*, 1999), the role of contaminated water in the transmission of the disease in developing countries is unknown.

Salmonella can persist under hostile environmental conditions in food and composted biosolids. *Salmonella* has been isolated from marine and fresh water sediments where it could act as a contamination reservoir. The accumulation of indicator bacteria and viruses in sediments has been well documented (Pommepuy *et al.*, 1992). This is due to the absorption of the microorganisms to particles suspended in the water, which then sediment out (Davies *et al.*, 1995). Sediments may contain 100-1000 times as many faecal indicator bacteria as the overlying water (Ashbolt *et al.*, 1993; Van Donsel and Geldreich, 1971). *Salmonella* can be widely disseminated in soil and sediment, even in the absence of active fertilization, as a result of water currents, underground springs and rainwater run-off carrying contaminated material (Abdel-Monem and Dowidar, 1990; Chao *et al.*, 1987). Percolation of wastewater through the soil filters out bacteria that become trapped in this environment (Chao *et al.*, 1987). *Salmonella* has been detected frequently in environmental soil samples collected from agricultural and recreational areas (Abdel-Monem and Dowidar, 1990; Thomason *et al.*, 1975a; Thomason *et al.*, 1977; Thomason *et al.*, 1975b). *Salmonella* has been found to survive and multiply in a

soil ecosystem for at least one year (Thomason *et al.*, 1977). The period of pathogen survival in water sources used for recreation, irrigation or as drinking water is of concern as it may have serious health consequences. In order to protect and manage the quality of water sources, a clear understanding of the fate, survival and transport of pathogens such as *Salmonella* is necessary.

The fate and persistence of non-typhoidal *Salmonella* in water environments has not been studied extensively due to the lack of a marked strain distinguishable *in vivo* from the rest of the bacterial community. Technologies have recently been developed which allow the tagging of bacterial strains using Green Fluorescent Protein (GFP). The *gfp* gene encoding green fluorescent protein is obtained from the jellyfish *Aequorea victoria*. GFP fluoresces green upon illumination with blue light and requires only the presence of oxygen during the initial formation of the chromophore. The chromosomal tagging of bacterial strains using GFP allows for the possibility of performing survival studies under mixed culture conditions. GFP is ideally suited to the quantification of tagged organisms in environmental samples as no substrate addition is required and the detection of fluorescence is independent of energy reserves. GFP is a very stable protein (Andersen *et al.*, 1998) and can be detected *in situ* in single cells. As GFP does not interfere with the growth of the host, it is an excellent choice for non-disruptive studies of bacterial systems that require live cells to be studied at the single cell level (Andersen *et al.*, 1998). It has been found that GFP fluorescence is maintained in viable but non-culturable cells, but is lost from dead cells (Lowder *et al.*, 2000). Therefore, when GFP is fused with a growth-regulated promoter, a system like this will show cell growth activity, which is very useful for monitoring bacterial development in environmental samples. In this way, a more realistic indication of the growth potential of *Salmonella* is obtained due to the interaction with competing indigenous microorganisms.

The factors that are considered to be important for the survival and growth of *Salmonella* in sediments are competing microflora, temperature, pH and nutrient availability. *Salmonella* is genetically adapted to persist in nature when conditions are usually sub-optimal. This is the result of the activation of a number of stress response mechanisms, usually triggered during stationary phase (Lin *et al.*, 1995). The factors that influence the stress response are not well understood and may be related to both biotic and abiotic parameters.

In this study clinically relevant *Salmonella* strains were obtained from fresh water sediments and were chromosomally tagged with the GFP marker gene. The isolate was there after used to investigate the factors that might influence the survival of *Salmonella* in fresh water sediments.

5.2 MATERIALS AND METHODS

5.2.1 Isolation and identification of *Salmonella* strains

Sediment samples were taken from rivers in the Gauteng, Limpopo and Free State regions of South Africa. Ten grams of each of the sediments were pre-enriched in 50ml of buffered peptone water (BPW) and incubated for 24 hours at 37°C. Thereafter, 0.1ml of the pre-enrichment sediment was inoculated into 10 ml of Rappaport-Vassiliadis (RV) broth for selective enrichment and incubated for 24 hours at 42°C. Following incubation of the RV enrichment broth, a loop-full from each positive tube of the broth was plated on to XLD (Xylose Lysine Deoxycholate) agar and incubated for 24 hours at 37°C. A bioMérieux API 20E biochemical test strip was inoculated with a presumptive *Salmonella* suspension and incubated for 24 hours at 37°C. After the addition of the prescribed reagents to the cupules, the results were compared with the accompanying identification table. Presumptive *Salmonella* isolates were chosen to undergo serological testing at the ARC-Onderstepoort Veterinary Institute.

5.2.2 Chromosomal tagging

S. ser. Muenchen and *S.* ser. Typhimurium isolates cultured on Luria-Bertani (LB) agar containing 100µg/ml of ampicillin (Amp100) and LB agar containing 100µg/ml of kanamycin (Km100) to confirm susceptibility to these two selective agents. The pUT mini–Tn5 Km transposon (De Lorenzo *et al*, 1990) was used to introduce GFP onto the chromosome of the chosen *Salmonella* strain. *Salmonella* was transformed by tri-parental mating using the *E.coli* strains HB101 (pRK2013) as a helper strain and CC118λpir (pSM1695) as the donor strain (Sternberg *et al*, 1999) and the *Salmonella* isolate as the recipient. A loop-full of each of the donor, helper and recipient strains were mixed together on LB agar plates and incubated for 24 hours at 37°C. After 24 hours the growth obtained was cultured on XLD media containing 100µg/ml of kanamycin and incubated at 37°C for 24 hours. Black colonies were selected and

confirmed as transconjugants by their ability to fluoresce green when exposed to blue light. Confirmed transconjugant colonies were inoculated into 10ml of LB broth and incubated at 37°C. After 24 hours, serial dilutions (10^{-1} – 10^{-6}) were made by transferring 100µl of LB broth to 900µl of Ringer's solution. 100µl of the dilutions were plated out on LB Km100 plates and incubated at 37°C for 24 hours. A dilution plate was selected containing suitably spaced colonies to undergo replica plating. Sterile velvet strips were pressed lightly onto colonies on the LB Km100 plates and transferred to LB Amp100 plates by pressing the fabric on to the replica plates. The LB Amp100 plates were incubated at 37°C for 24 hours. A comparison of the colonies on the replica plates was made. Colonies that did not grow on the LB Amp100 plates were selected from the LB Km100 plates. These colonies were examined for fluorescence with a *Zeiss Axiovert* 200 Inverted Microscope. (Excitation - 490 nm and Emission - 510 nm) and fluorescent isolates were selected.

5.2.3 Stability of the *gfp* gene tagging

Tagged strains and wild-type strains were inoculated separately into 10ml of medium and incubated at 37°C with agitation at 180 rpm. After 24 hours, 10µl of cultured medium was transferred to another 10ml of fresh medium and incubated at 37°C with agitation. This process was repeated for thirty days. The presence and stability of the label was verified by fluorescent microscopy initially, and after constant growth for thirty-days in both nutrient rich (LB broth) and nutrient poor medium ($1/10^{th}$ strength R2A) facilitated by daily transfer into fresh medium.

5.2.4 Evaluation of survival fitness of tagged isolate

The wild-type and tagged strains were inoculated separately into 30ml of LB broth and incubated overnight at 37°C with agitation. One ml of the overnight culture was inoculated into side-arm flasks containing 100ml LB broth. The absorbency readings of the culture were measured with a spectrophotometer set at 600nm from time 0 hours. Measurements were taken every 30 minutes. After each reading, the side-arm flasks were incubated at 37°C with agitation. This was repeated for *S.* ser Typhimurium strains (in duplicate) in side-arm flasks containing a tenth strength R2A medium.

5.2.5 Sediment survival studies

5.2.5.1 Stability of labelled in sediments

The tagged isolate of *S.* Muenchen or *S.* Typhimurium was inoculated into 100ml of LB broth and incubated at 37°C. After 24 hours, the broth was spun down at 4000 rpm for 15 minutes to collect the cells. The broth was discarded and 100ml of 1x PBS was added to each tube and resuspended to wash the cells. The suspension was spun down again at 4000 rpm for 15 minutes. The PBS was discarded and the cells were resuspended in 1ml of 1x PBS and added to 300ml of sterile sediment. The initial concentration of the *Salmonella* isolate was about 10^9 cells per gram of sediment.

Fluorescent microscopy was used to examine sediment during the 30-day period. After 40 days, 1 gram of sediment was added to 25ml of BPW and incubated at 37°C for 24 hours. Hundred µl of the BPW culture was transferred to 10ml of RV broth and incubated at 42°C. After 24 hours, serial dilutions of the RV broth were plated out on XLD plates and incubated at 37°C for 24 hours. Single colonies were examined by fluorescence microscopy.

5.2.5.2 Survival of *Salmonella* in sterile and non-sterile sediments

Using the same spiking procedure as mentioned above in section 5.2.5.1 the survival of *S.* Thyphimurium in sterile and non-sterile sediments was studied at room temperature. For enumeration, 90 ml of a buffered solution containing Zwittergent was used to resuspend 10g of the sediment. This was followed by sonication to assist the release of the bacteria from the sediment. The 90 ml buffered solution contained 0.04g EGTA, 0.12g TRIS, 0.1g peptone and 0.04mg N-dodecyl-N, N dimethyl-3-ammonio-1-propanesulfonate (Zwittergent). Dilutions were prepared in ¼ Ringers solution and 1ml of the dilution were transferred to 9ml of BPW. This procedure was performed in triplicate for each dilution. After incubation at 37°C for 24 hours, 0.1ml of the BPW culture was transferred to 10ml of RV broth and incubated at 42°C. Following incubation of the RV enrichment broth, a loop-full from each of the RV tubes was plated on to XLD (Xylose Lysine Deoxycholate) agar and incubated for 24 hours at 37°C. The MPN table was used to estimate the level of bacteria in each sample by recording the all black colonies as presumptive *Salmonella* isolates. From time to time

some of these colonies were picked and confirmed to be the spiked isolate using fluorescence microscopy to detect the presence of the *Gfp* gene.

5.2.5.3 Survival in sediments under different pH conditions or temperatures

Similar studies were performed to determine the survival of the *S.* Typhimurium strain under variable pH and temperature conditions. For the pH study, instead of resuspending the sediment in sterile river water or PBS, the sediments were suspended in citric acid (pH 5.5), phosphate (pH 7) and Tris-HCl (pH8.5) buffer. The survival in both sterile and non-sterile sediments was determined. For the temperature studies, the spiked sterile and non-sterile sediments were incubated at 8°C, 16°C, 28°C or 37°C respectively. Spiking and enumeration of the *Salmonella* was performed as described in section 5.2.5.2.

5.2.5.4 The effect of nutrient levels on the survival of *Salmonella* in non-sterile sediments.

Five sediment samples were spiked as described in section 5.1.5.1 and were incubated at 37°C. Two samples served at control (sterile and non-sterile) and the other three non-sterile samples were spiked with either 400 µl of a 10% glucose solution, 400 µl of a 5% starch solution or with 100ml of sterile river water on a weekly basis after an initial period of two weeks of incubation. Enumeration of the *Salmonella* was performed as described in section 5.2.5.2.

5.3 RESULTS AND DISCUSSION

5.3.1 Isolation and identification of *Salmonella* strains

Several black colonies could be isolated from the sediment samples. The pre-enrichment in the buffered peptone water allows resuscitation of the injured or stressed bacteria (Edel *et al.*, 1973 and Marinigo *et al.*, 1986). Selective RV enrichment medium and XLD selective media are standard culture methods for the isolation of *Salmonella*. The high prevalence of black colonies isolated from the sediment samples suggested that *Salmonella* is widely abundant in fresh water sediments where it serves as a contamination reservoir. Colonies on the XLD that appeared black due to H_2S production were selected for further characterisation. The API 20E identification table identified all the isolates as *Salmonella* species. Metabolic and biochemical tests are the basis of presumptive identification of microorganisms. Due to the genetic variability arising from mutations and the exchange of plasmids encoding biochemical traits, biochemical tests are confirmed by serotyping in order to identify presumptive *Salmonella* isolates. The isolates were typed as follows:

Isolate 1: *S. enterica* subsp. *enterica* ser. Typhimurium

Isolate 2: *S. enterica* subsp. *enterica* ser. Muenchen

Isolate 3: *S. enterica* subsp. *salamae*

Serotyping involves the agglutination of bacterial surface antigens with *Salmonella*-specific antibodies (Doyle *et al.*, 1997). Isolate 3 belongs to group II of the *Salmonella* species. Serotypes belonging to this group are usually isolated from cold-blooded animals and the environment, but rarely from humans. (Farmer *et al.*, 1984). Isolates 1 and 2 both belong to the enterica subspecies of *Salmonella* (group I). This is the only subspecies of *Salmonella* that are responsible for infections in warm-blooded animals and humans (Popoff *et al.*, 1997). *Salmonella* ser. Muenchen and *Salmonella* ser. Typhimurium were selected for GFP tagging, as they are clinically relevant environmental *Salmonella* isolates. *S.* Muenchen is one of approximately 2400 *Salmonella* serotypes that cause illness in humans. It is however an infrequently isolated serotype, which accounts for 1.6% of human *Salmonella* isolates reported in 1997 to the U.S. Public Health Laboratory Information System (Martin *et al.*, 1995 and CDC, 1998). *S.* Typhimurium was the most common isolate in the United States in 1999, causing 25% of culture confirmed infections according to the CDC *Salmonella* Surveillance 1999 Annual Summary (CDC, 2003)

5.3.2 Chromosomal Tagging

The pUT mini–Tn5 Km transposon (De Lorenzo *et al.*, 1990) was used to introduce GFP into the chromosome of the *Salmonella* strains. The pUT mini–Tn5 Km element has an sfiI cassette containing the kanamycin resistance gene and a single NotI site outside the cassette. (De Lorenzo *et al.*, 1998). The fragment of the pSM1690 plasmid carrying rrnBP1-RBSII-gfpmut3b*-T_0T_2 is flanked by NotI sites and is inserted into the NotI site of the pUT-mini Tn5 Km (Sternberg *et al.*, 1999). The delivery plasmid contains gfpmut3b*, a very bright and stable variant of the green fluorescent protein. A growth-regulated 72bp promoter (rrnBP1) from *E.coli* is inserted in front of the *gfp* gene to drive the expression of the marker gene. The delivery plasmid has a synthetic ribosome binding site, stop codons in all three reading frames and two strong transcriptional terminators (Sternberg *et al.*, 1999).

Prior to tagging, *S.* ser. Typhimurium and *S.* ser Muenchen was sensitive to both ampicillin and kanamycin. The media used to select transconjugants (XLD Km100) counter-selects the helper and donor strains, but selects recipient strains carrying the transposon marker. Once the transposon has integrated onto the chromosome, the recipient should only have resistance to kanamycin. If the suicide delivery plasmid replicates in the recipient and is not lost, the recipient will display resistance to kanamycin and ampicillin.

Following tri-parental mating, two *S.* ser. Typhimurium strains were obtained, and three S. ser Muenchen strains. All strains were sensitive to ampicillin, but resistant to kanamycin. All strains fluoresced green when exposed to blue light (Fig. 5.1).

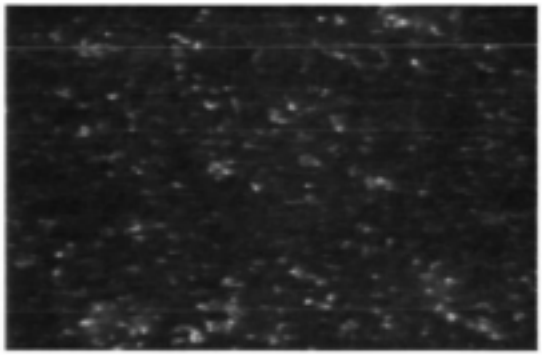

Figure 5.1: Green fluorescence emitted by the recipient cells visualised by fluorescent microscopy. *Colour image on CD.*

5.3.3 Stability of label in sediment

The stability of the GFP labelling was tested by exposing the isolates to a thirty-day growth period under non-selective conditions. The presence of the label was verified by fluorescence microscopy. After 30 days, the GFP was visible in all tagged cells, indicating stable maintenance (Figure 5.2 and 5.3). The GFP fluorescence was visible in cells under nutrient poor conditions (Figure 5.3) indicating that even when cells are subjected to stress, the new gene is still maintained. Nutrient poor conditions reflect the stress induced on microorganisms in aquatic environments. This indicates that the inserted fragment remains stably inherited and does not serve as a burden to the carrier strain.

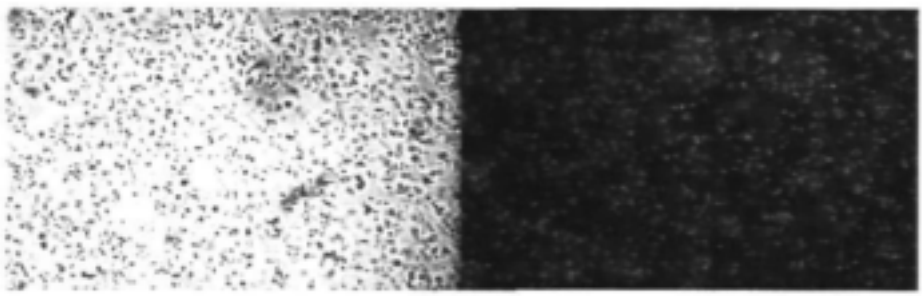

Figure 5.2: Tagged isolate cultured in LB broth viewed with phase contrast (left panel) and fluorescence. Microscopy (right panel) (100X). *Colour image on CD.*

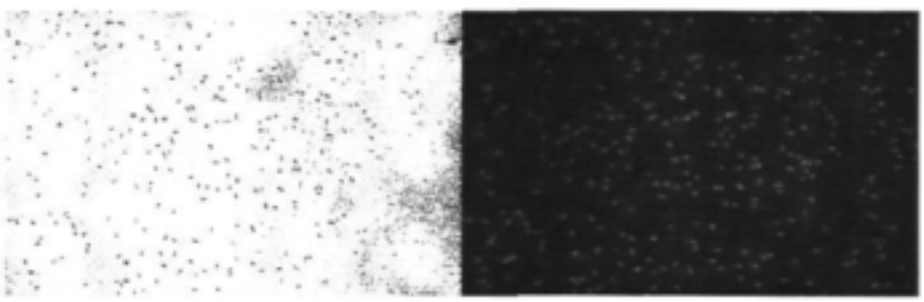

Figure 5.3: Tagged isolate cultured in $1/10^{th}$ strength R2A broth viewed with phase contrast (left panel) and fluorescence microscopy (right panel) (100X). *Colour image on CD.*

5.3.4 Evaluation of the survival fitness of the tagged isolate

Fitness under nutrient-rich and nutrient-poor conditions

A general problem of the mini-Tn5 transposon is that its insertion involves the disruption of a chromosomal sequence. Environmentally important functions can thus

be affected (De Lorenzo *et al.*, 1998). Therefore, all *gfp* strains generated from the transposon insertion must be screened against growth defective lesions.

The labelled strains did not display growth rate deficiencies when compared to the wildtype strains (Figure 5.4, 5.5 and 5.6). GFP continued to fluoresce in cells cultured in nutrient poor medium (Figure 5.6).

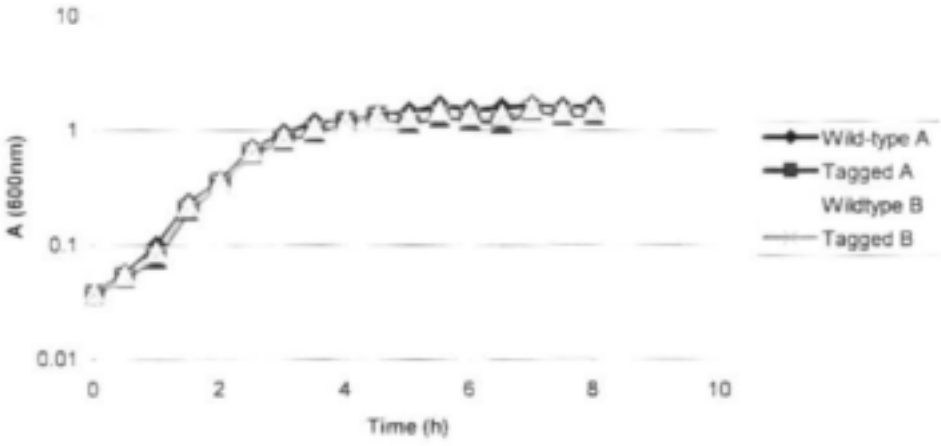

Figure 5.4: Growth of *S.* ser Muenchen wild-type strains vs tagged strains in LB broth.

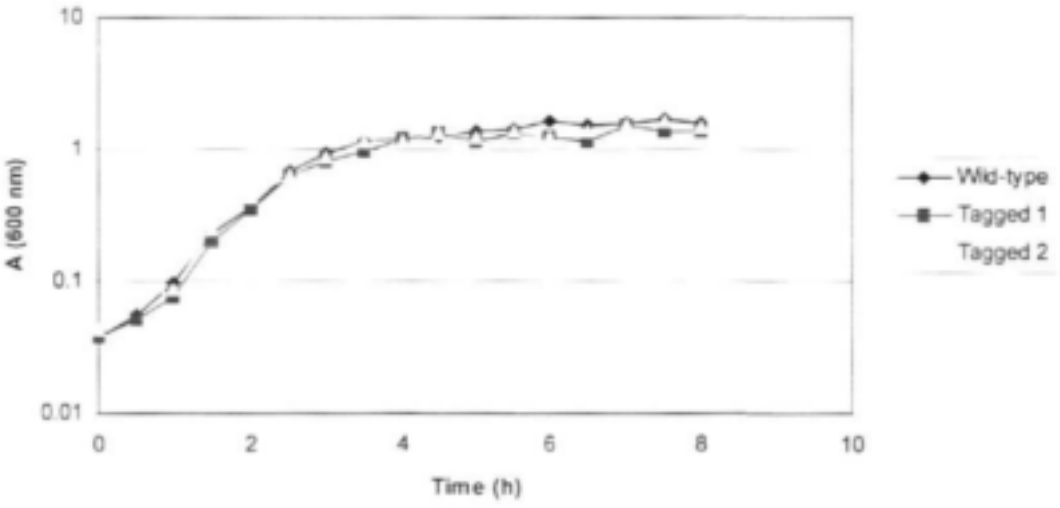

Figure 5.5: Growth of *S.* ser Typhimurium wild-type vs tagged strains in LB broth.

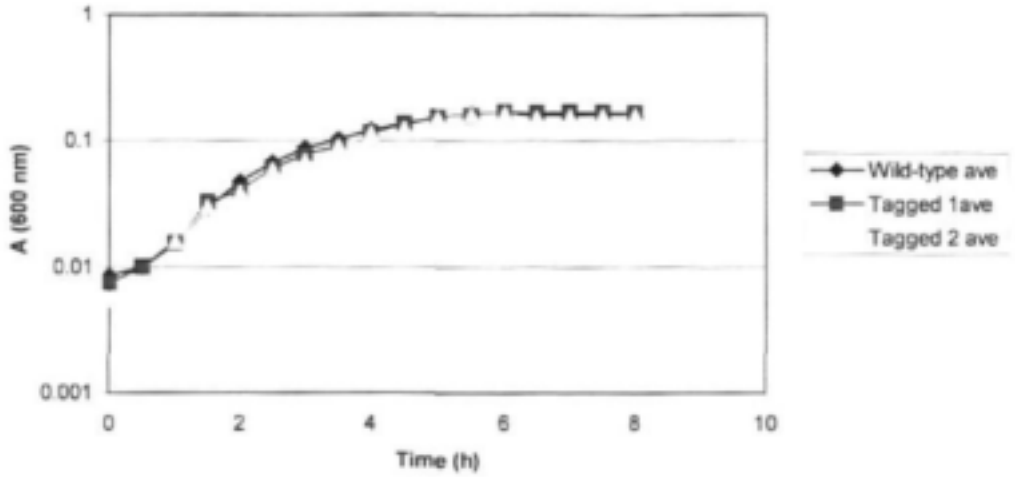

Figure 5.6: Average growth of *S.* ser Typhimurium wildtype vs tagged strains in $^{1}/_{10}$ R2A medium.

The survival studies indicate that the incorporation of the *gfp* gene did not have any detrimental effects on the survival and fitness of the tagged strains. These tagged strains can therefore be used *in vivo* to study the fate and survival of *Salmonella* in water environments.

5.3.5 Sediment survival studies

5.3.5.1 Stability of label in sediments

For the duration of 30 days, the *gfp*-tagged *S.* ser Muenchen strain could be visualised by fluorescence microscopy directly from the sediment. After a further 10-day period, the *gfp* could no longer be detected directly from the sediment, but required enrichment and cultivation on selective media. These colonies continued to exhibit fluorescence. The fate and survival of a tagged *Salmonella* strain in sterile fresh water sediment was investigated. The results indicate that the tagged strain was able to survive in the sediment for a significant time period.

5.3.5.2 Survival of *Salmonella* in sterile and non-sterile sediments

The survival studies has shown that gfp- tagged *S.* ser Muenchen can survive in sterile sediments for longer than 20 weeks. The survival in non-sterile sediments was for a

shorter period but the organisms could still be detected for up to 8 weeks as can be seen in Figure 5.7. A repeat of the experiment produced similar results.

5.3.5.3 Survival in sediments under different pH conditions or temperatures

During the experiments to evaluate the effect of pH, sterile and non-sterile sediment samples were buffered at pH 5.5, 7 and 8.5 and kept at 22°C. The tagged strain survived for about eleven weeks (Figure 5.8) and the selected pH conditions did not show any effect on the bacterium's survival pattern. As observed previously the tagged strain survived slightly longer in sterile sediments than in sediments where the natural microbial population was still present.

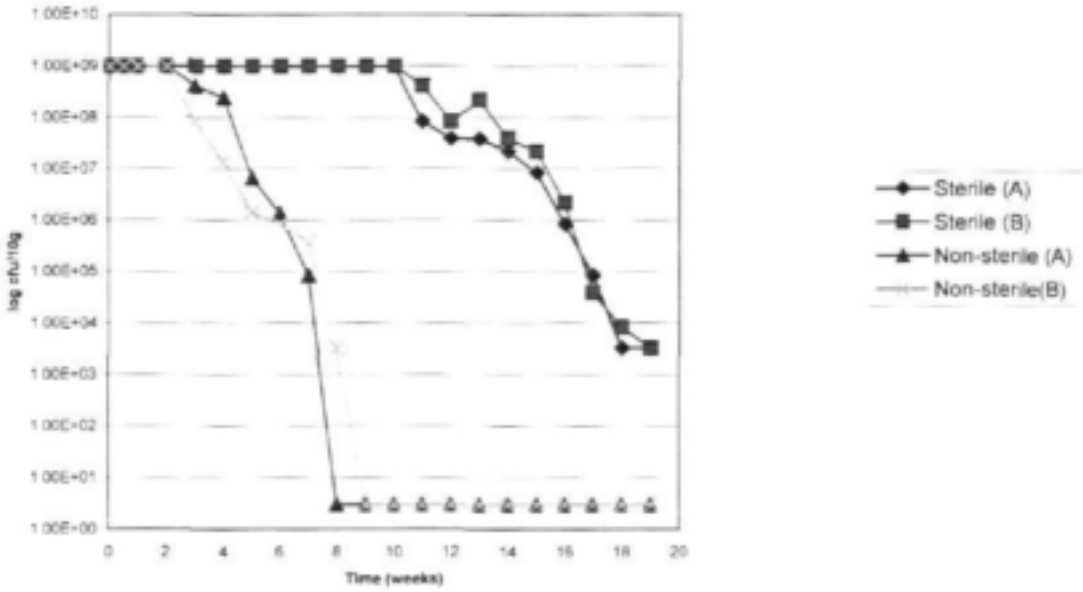

Figure 5.7: Survival of gfp-tagged *Salmonella* in sterile and non-sterile sediments.

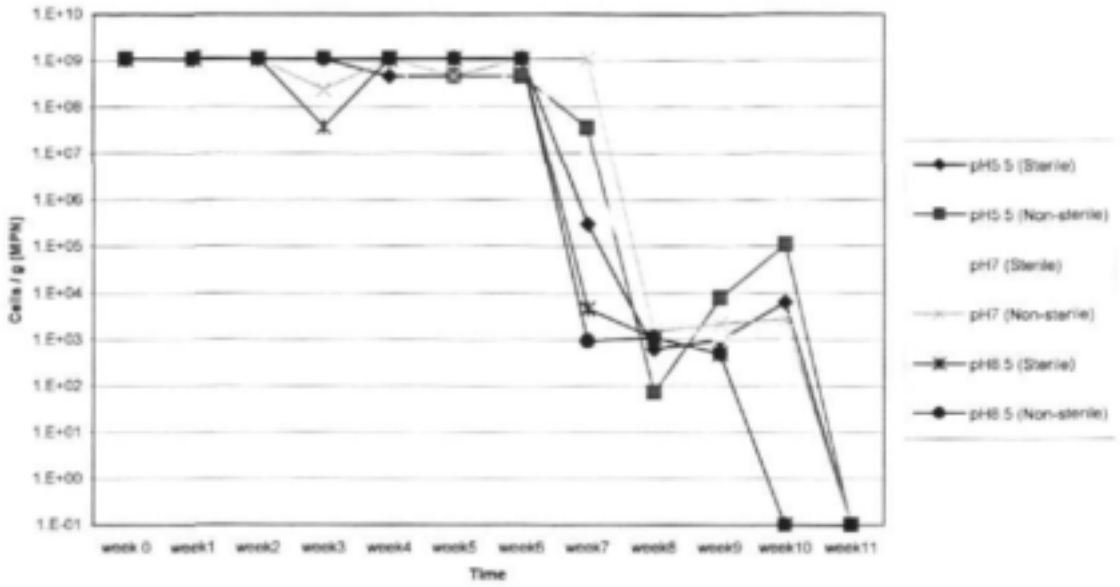

Figure 5.8: The survival of *Salmonella* under different pH conditions.

The effect of temperature on the survival of the bacterium was much more pronounced (Figure 5.9). Bacteria kept at 37°C only survived for a period of 5 to 6 weeks. The bacteria in the non-sterile sediment sample kept at 28°C survived for between 6 to 8 weeks. In contrast the survival in the corresponding sterile sample was for 12 to 13 weeks. The bacteria in the non-sterile sediments kept at 16 and 8°C survived for a period of at least 21 weeks and the bacteria in the sterile samples could still be detected after 23 weeks when the experiment was terminated. This study showed that *Salmonella* could survive for extended periods in sediment at typical environmental temperatures. A repeat of the experiment over a period of 18 weeks produced similar results.

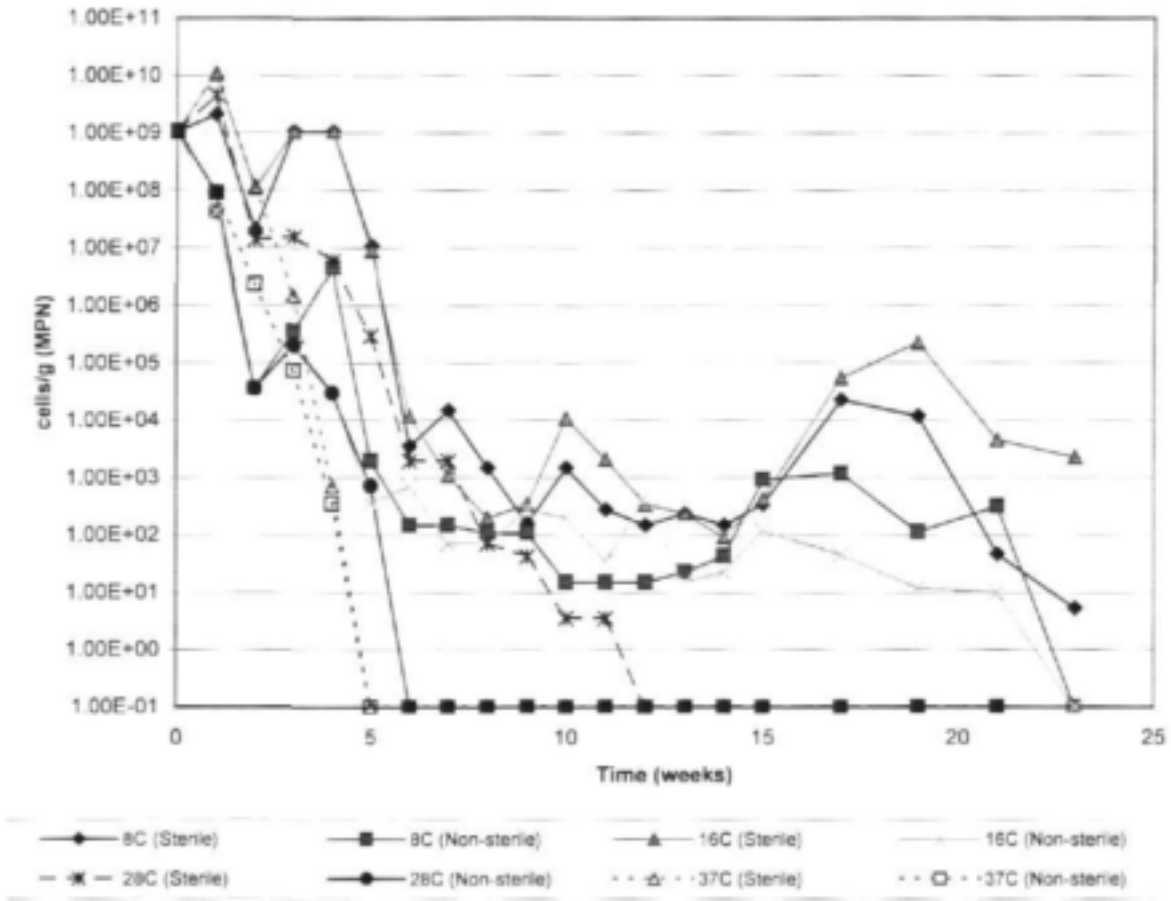

Figure 5.9: The effect of various temperatures and competition on the survival of *Salmonella* in sediments.

5.3.5.4 The effect of nutrient levels on the survival of *Salmonella* in non-sterile sediments.

An experiment was performed to investigate whether the poorer survival of *Salmonella* in non-sterile conditions could be due to an inferior ability of the bacterium to compete for nutrients. During the survival study, samples were supplemented with glucose, starch or sterile river water on a weekly basis after an initial period of two weeks. The results showed (Figure 5.10) that additional nutrients made no significant difference to the survival of the cells. This suggests that the survival of *Salmonella* in sediments is not likely to be dependant on nutrient supply. Other forms of competition should therefore be investigated for their influence on Salmonella's survival in non-sterile sediments. Sidh et al.. support this finding (2001) when they postulated that the rapid

inactivation of vegetative *Salmonella* cells might be the result of the presence of inhibitory or secondary metabolites of other organisms.

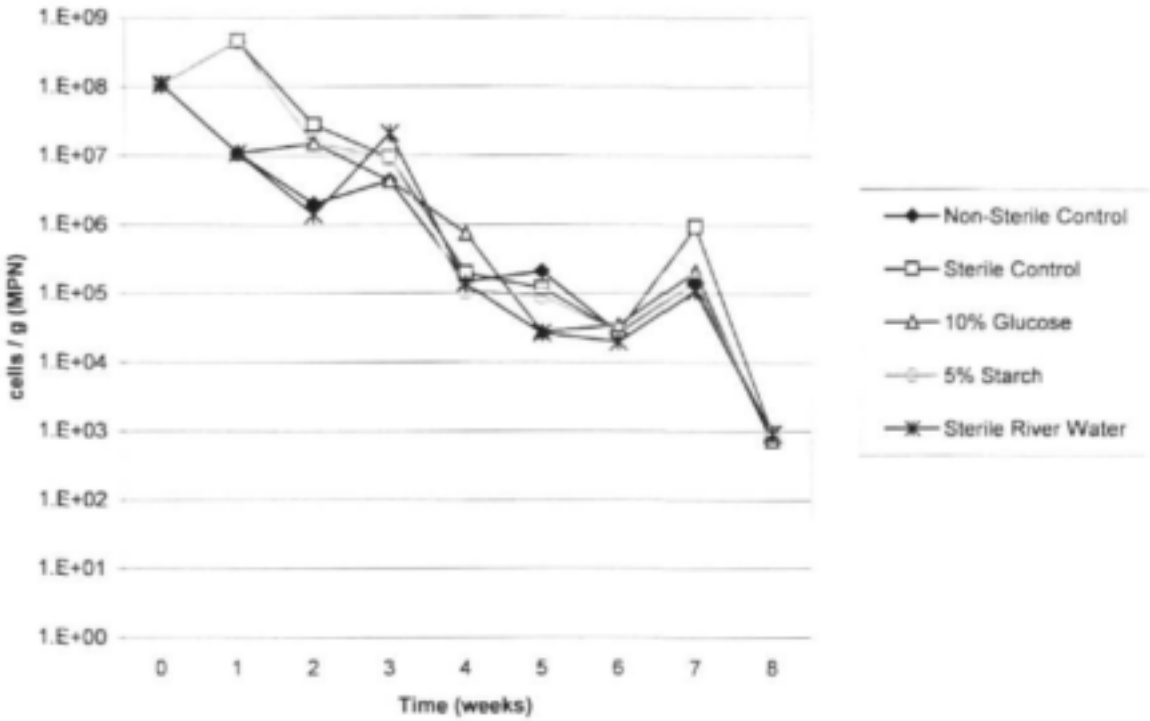

Figure 5.10: The effect of additional nutrients on the survival of *Salmonella* in sediments.

5. 4 CONCLUSIONS

The study showed that *Salmonella enterica enteria* serotype Typhimurium and *Salmonella enterica enteria* serotype Muenchen could be isolated from river sediments in the Venda area. Both isolates could also be successfully tagged on the chromosome with the *gfp* gene. The *gfp* gene was stably maintained during continuous growth for 30 days. The *gfp*-labelled recombinants were not growth rate impaired, in nutrient-rich (LB broth) or nutrient–poor ($1/10^{th}$ R2A broth).

Salmonella Typhimurium is the most commonly isolated serotype from clinical cases in the Venda area and was therefore used to investigate the survival and behaviour of *Salmonella* in fresh water sediments. The study indicated that *Salmonella* survived for extended periods of more than 6 months in sediments at temperatures typically

associated with fresh water streams and rivers in South Africa. This is of concern as they could easily be released from the sediment into the water phase at high concentrations during rain events or other disturbances of the sediment.

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Vibrio cholerae

CHAPTER 6

EVALUATION OF A RAPID POLYMERASE CHAIN REACTION BASED IDENTIFICATION TECHNIQUE FOR *VIBRIO CHOLERAE* ISOLATES OBTAINED FROM THE VAAL BARAGE AREA.

W J le Roux, D Masoabi, C M E de Wet and S N Venter

Abstract

Rapid and accurate identification of waterborne pathogens like *Vibrio cholerae* in drinking water sources is important to enable effective resource management and public health protection. Phenotypic systems currently being used for the identification of *Vibrio cholerae* isolates are time-consuming and the need for the development of suitable molecular techniques that can offer both fast and reliable identification exists. During this study isolates identified as *Vibrio cholerae* by means of two different biochemical test systems (API 20E and VITEK 32) were analysed with the polymerase chain reaction to compare the reliability of the various identification systems. The selected PCR technique amplifies a sequence within the outer membrane protein of *Vibrio cholerae*, a gene specific for *V. cholerae*. It was found that out of 243 isolates biochemically identified as *V. cholerae* with either the API or VITEK system, 21 isolates did not give a positive result with the PCR detection method. Sequencing the 16S rDNA of more than half of these isolates and comparison of the sequences to Internet databases indicated that most of the isolates belonged to the genus *Aeromonas*. The results indicate that the rapid PCR procedure is more accurate than the API or VITEK systems currently being used for the phenotypic identification of *Vibrio cholerae* isolates.

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CHAPTER 7

POPULATION DYNAMICS OF *V. CHOLERAE* IN THE VAAL BARAGE CATCHMENT

W J le Roux and S N Venter

7.1 INTRODUCTION

Cholera remains to be one of the most severe diseases of our time, with seven pandemics being recorded since 1817. The Seventh pandemic is still raging since its start in Asia in 1961 and has lead to major epidemics in developing African countries since being introduced into Africa in 1971 (Lan and Reeves, 2002). South Africa has not escaped, and had two major epidemics since 1980 (Department of Health, South Africa 2004). Due to the large amount of people infected during epidemics the disease has a huge socio-economic impact on regions. This along with the potential for rapid spread calls for effective management strategies to alleviate risk factors.

Vibrio cholerae is indigenous to the aquatic environment, preferring salt water, but can also be readily isolated from fresh water bodies (Halpern *et al.*., 2003). These aquatic systems may serve as possible reservoirs for not only enterotoxigenic *V. cholerae*, which are capable of epidemic spread, but also for non-enterotoxigenic environmental strains. Non-enterotoxigenic strains should also be regarded as clinically important due a variety of secondary pathogenetic mechanisms that enable them to cause disease in humans, though not to the extent of enterotoxin producing strains (Chakraborty *et al.* 2000). Environmental *V. cholerae* strains should also be regarded as a potential health risk in the light of the discovery of the lysogenic filamentous phage CTXϕ, which has been shown to be able to donate enterotoxin genes to non-toxigenic strains. The receptor for CTXϕ invasion (the toxin co-regulated pili) is encoded for by an operon, which forms part of the cholera pathogenicity island. This pathogenicity island has been found to be present in some non-O1 and non-O139 environmental strains suggesting that it can be transferred among *V. cholerae* strains within a population (Karaolis *et al.*, 1998). Not only could environmental *Vibrio cholerae* strains serve as progenitors for the evolution

of epidemic strains, but some strains have also been shown to be genetically closely related to clinical O1 and O139 strains (Thompson *et al.*, 2003). These factors make environmental isolates an ideal model for the study of *Vibrio cholerae* O1 and O139 survival and transport in water systems.

An understanding of *Vibrio cholerae* population structure and cholera molecular epidemiology may give insight into the spread of the disease as well as the possible emergence of novel epidemic strains. After the emergence of the O139 epidemic strain, which served as an example of how novel epidemic strains can arise, many researchers are studying the genetic diversity of both epidemic as well as environmental *V. cholerae* strains. Multilocus enzyme electrophoreses was found to be of more use with environmental strains than with clinical strains obtained from epidemics, due to the fact that epidemic strains mainly belong to the same electrophoretic type (Chen *et al.*, 1991, Beltran *et al.*, 1998). Single-locus sequence typing of the cholera toxin sub-unit B was shown to have low discriminatory ability (Olsvik *et al.*, 1993) whilst multi-locus sequence typing was shown to have higher resolution. Kotetishvili *et al.* (2003) found the technique to compare favourably with pulsed field gel electrophoreses (PFGE). Many researchers used PFGE to characterize *V. cholerae* as the technique exhibits good discrimination between strains (Choudhury *et al.*, 1994, Cameron *et al.*, 1994, Popovic *et al.*, 1995). The one disadvantage is, however, the inability of this technique to type strains that exhibit endonuclease activity (Pichel *et al.*, 2002). At present, the technique to be of value for genetic diversity studies is most likely amplified fragment length polymorphism fingerprinting (AFLP). This technique has been used successfully by various researchers to type both clinical and environmental *V. cholerae* strains (Jiang *et al.*, 2000, Lan and Reeves, 2001, Thompson *et al.*, 2003). AFLP has the advantage that no prior sequence knowledge is required, it is robust and reliable due to the stringent reaction conditions used, and it is sensitive due to the incorporated PCR (Vos *et al.*, 1995). The ability of this technique to discriminate between closely related strains makes it well suited for the typing of epidemic as well as environmental strains.

The aim of this study is determine the genetic diversity of *Vibrio cholerae* in the Vaal Barrage catchment using the AFLP technique. The results obtained could provide insight into the population structure and dynamics of environmental *V. cholerae* with reference to South African conditions.

7.2 MATERIALS AND METHODS

7.2.1 Acquisition and maintenance of cultures

Environmental *Vibrio cholerae* isolates were obtained from Rand Water (Vereeniging, South Africa). The strains were isolated over a two-year period from 15 different sampling sites in the Vaal Barrage Catchment, South Africa (Figure 7.1, Table 7.1). An Enterotoxigenic strain was obtained from the National Collection of Type Cultures, Public Health Laboratory Services, London (strain NCTC 5941). Three other *Vibrio cholerae* O1 clinical strains (isolated from cholera cases in the Kwazulu-Natal and Nelspruit regions) were obtained from the National Health Laboratory Services, Johannesburg, South Africa.

Vibrio cholerae were grown on Nutrient Agar (Biolab, Merck C1) or alternatively on Nutrient Broth No. 2 (Oxoid CM67). Agar plates which were incubated at 37 °C for 24 hours to obtain bacterial growth, were stored at room temperature and were subcultured on a fort nightly basis. Long-term storage of strains (up to three years) was achieved using Microbank™ Cryobeads (Davies Diagnostics).

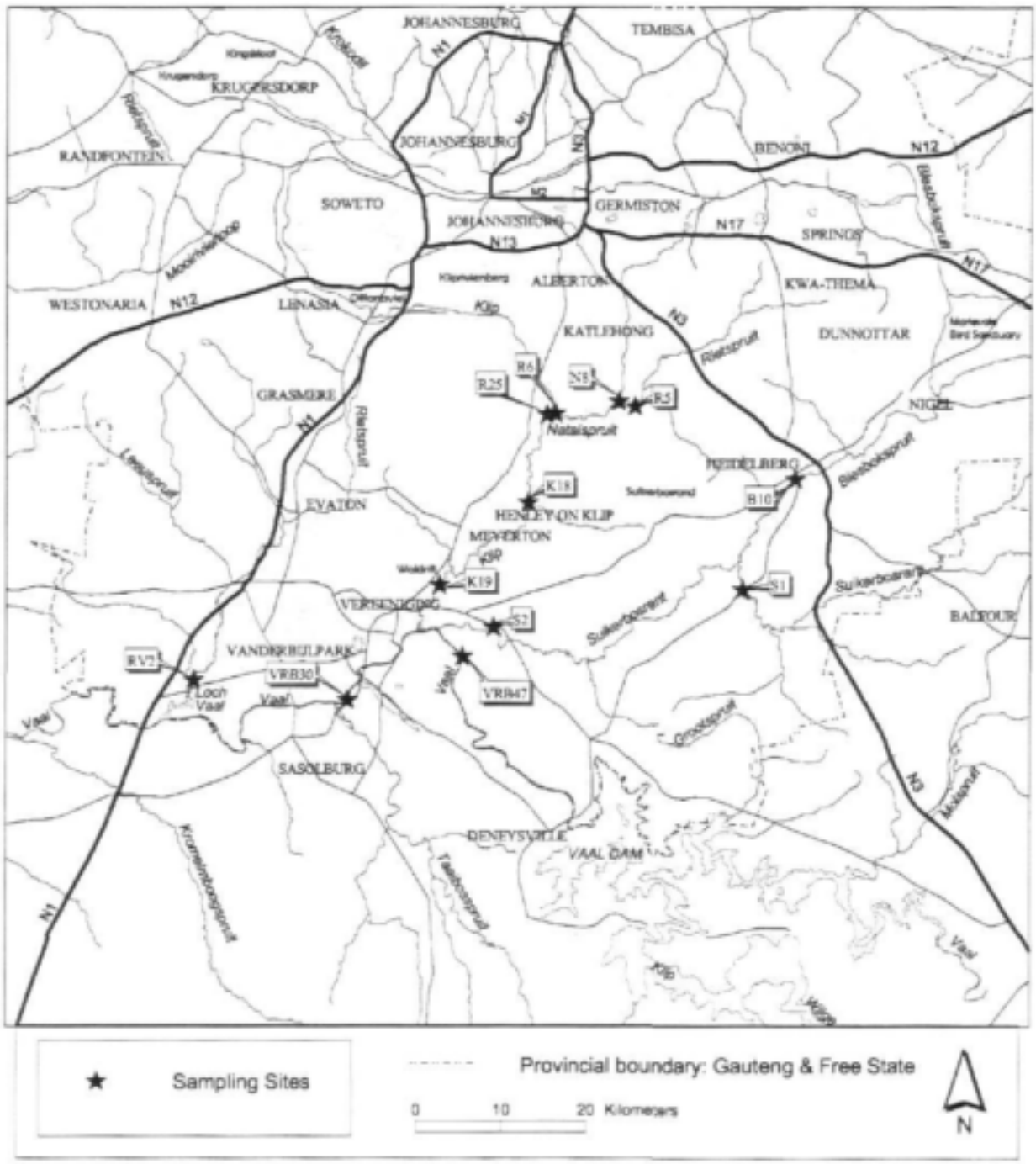

Figure 7.1: Sampling sites in the Vaal Barage Catchment where water was sampled for the presence of *Vibrio cholerae*.

Table 7.1: River sampling sites in the Vaal Barrage Catchment.

Sampling Site	Description
A18 *	Raw water pipeline at Vereeniging
B6 *	Distribution main at Zuikerbosch
B10	Blesbokspruit near Heidelberg
K18	Klip River at Henley-on-klip
K19	Klip River weir at Redan Train Bridge
K25	Klip River
M-Canal *	Canal at Zuikerbosch from Vaal Dam
N8	Natalspruit at Heidelberg road
R5	Rietspruit at Hardman's Farm
R6	Rietspruit weir at Luttig's Farm
RV2	Rietspruit weir at Loch Vaal
S1	Suikerbosrand River near Heidelberg
S2	Suikerbosrand River weir at Three Rivers
VRB30	Vaal River Barrage (Ascott Bridge)
VRB47	Vaal River Barrage (Zb pump station)

* Sites marked with an asterisk are not shown on the accompanying map (Fig 1.)

Table 7.2: Isolates typed with AFLP

SAMPLING SITE	SAMPLING DATE
A18	
53	5-Feb-2001
235	7-May-2001
B10	
1	20-Dec-2000
178	2-Apr-2001
455	26-Nov-2001
515	14-Jan-2002
598	25-Feb-2002
599	25-Feb-2002
655	25-Mar-2002
1029	14-Oct-2002
K18	
46	29-Jan-2001
373	27-Aug-2001
502	7-Jan-2002
602	25-Feb-2002
632	11-Mar-2002
1059	20-Oct-2002
1193	13-Jan-2003

SAMPLING SITE	SAMPLING DATE
1246	10-Feb-2003
1302	10-Mar-2003
K19	
520	14-Jan-2002
521	14-Jan-2002
576	11-Feb-2002
605	25-Feb-2002
K25	
195	9-Apr-2001
226	30-Apr-2001
240	7-May-2001
505	7-Jan-2002
522	14-Jan-2002
523	14-Jan-2002
606	25-Feb-2002
607	25-Feb-2002
1250	10-Feb-2003
1307	10-Mar-2003
1317	10-Mar-2003
1411	29-Apr-2003
M-CANAL	
535	14-Jan-2002
618	25-Feb-2002
619	25-Feb-2002
1265	10-Feb-2003
N8	
3	20-Dec-2000
209	23-Apr-2001
456	26-Nov-2001
501	7-Jan-2002
628	11-Mar-2002
661	25-Mar-2002
1355	24-Mar-2003
1412	29-Apr-2003
R5	
446	12-Nov-2001
506	7-Jan-2002
637	11-Mar-2002
746	13-May-2002
1011	30-Sep-2002
1012	30-Sep-2002
1152	9-Dec-2002
R6	
2	20-Dec-2000
171	26-Mar-2001
241	7-May-2001
269	21-May-2001
391	9-Sep-2001
610	25-Feb-2002
1155	9-Dec-2002

SAMPLING SITE	SAMPLING DATE
1256	10-Feb-2003
1362	31-Mar-2003
1420	29-Apr-2003
RV2	
256	14-May-2001
448	12-Nov-2001
529	14-Jan-2002
612	25-Feb-2002
613	25-Feb-2002
640	11-Mar-2002
1072	30-Oct-2002
S1	
449	12-Nov-2001
615	25-Feb-2002
643	11-Mar-2002
671	25-Mar-2002
672	25-Mar-2002
S2	
11	8-Jan-2001
160	19-Mar-2001
174	26-Mar-2001
244	7-May-2001
510	7-Jan-2002
616	25-Feb-2002
617	25-Feb-2002
644	11-Mar-2002
645	11-Mar-2002
995	23-Sep-2002
1158	9-Dec-2002
VRB30	
1039	14-Oct-2002
1040	14-Oct-2002
1267	12-Feb-2003
1321	12-Mar-2003
1424	30-Apr-2003
1425	30-Apr-2003
VRB47	
13	15-Jan-2001
622	27-Feb-2002
650	13-Mar-2002
1025	2-Oct-2002
1053	14-Oct-2002
1054	14-Oct-2002
1322	12-Mar-2003
165	19-Mar-2001

7.2.2 DNA Extraction

Whole genomic DNA was extracted and prepared using Dneasy Tissue Kit (Qiagen 69504). The manufacturer's protocol was followed, with final elution in 100µL buffer to obtain a high concentration of DNA. The concentration of DNA in the final elute was determined using a spectrophotometer (Nanodrop ND1000, Nanodrop Technologies, Wilmington, USA.)

7.2.3 Restriction Enzyme Digestion of Genomic DNA and Adapter ligation

Restriction enzymes *MseI* [*Tru9*] (Roche 1464817) and *EcoRI* (Roche 1175084) were used to generate fragments from whole genomic DNA. Digestions were performed in 15µL reactions containing 3.0µL 5X Reaction buffer (50mM Tris HAc, 50mM MgAc, 230mM KAc and 25mM DTT), 12 units *EcoRI* and 8 units *MseI* restriction enzymes, and 100 ng template DNA. The digestions were carried out at 37°C for two hours, after which the restriction enzymes were deactivated at 70°C for 10 minutes.

Adapters as described by Lan and Reeves (2002) (Table 7.3) were ligated to the terminal ends of the generated fragments by adding a reaction mix containing the adapters to the restriction enzyme digestion reaction after completion. The adapter ligation reaction mix contained 1X reaction buffer (50mM Tris HAc, 50mM MgAc, 230mM KAc and 25mM DTT), 5 pmol EcoRI and 50 pmol MseI adapters, 0.3 mM ATP and 1 unit T4 DNA ligase (Roche 481220). After addition, the adapters were left to ligate at room temperature for two hours. The final product was stored at -20°C for no more than 24 hours before use as PCR template, extended storage was found to lead to DNA degradation.

Table 7.3: Adapters used in the AFLP ligation reaction

Name	**Sequence**
925 EcoRI	5'-AATTGGTACGCAGTCAGTGAGGTTCATTACCATCC-3'
931 MseI	5'-CCTGATTGCTACAACTGAACGATGAGTCCTGAG-3'

7.2.4 PCR primers

The primers used in this study are shown in Table 7.4. Primers Eco-G was labeled with a fluorescent dye (Cye 5.5) in order to visualize the fragments using an automated system. All primers were synthesized by Inqaba Biotech, South Africa.

Table 7.4: Primer sequences for pre-amplification and selective-amplification

Amplification step	**Primer**	**Sequence**
Pre-amplification	Eco-O	5' – GACTGCGTACCAATTC – 3'
	Mse-O	5' – GATGAGTCCTGAGTAA – 3'
Selective-amplification	Eco-G	5' – GACTGCGTACCAATTCG – 3'
	Mse-T	5' – GATGAGTCCTGAGTAAT – 3'

7.2.5 PCR amplification of fragments

Two PCR amplification steps were carried out, the first was the pre-amplification where all terminally heterogeneous fragments were amplified (fragments generated by *EcoRI* on one end and *MseI* on the other). This was followed by a selective-amplification step where both primers have an additional 3' base, which gives a theoretical 16-fold reduction in the number of fragments amplified. The template for the pre-amplification, adapter ligation reaction as described in Section 7.2.3, was diluted ten fold with nuclease free water (Whitehead Scientific P1193). Each of the pre-amplification reactions contained 2μL of the template (1:10 Dilution of adapter ligation), 0.2μL of each of the dNTP's (Abgene Ab-11.24), 2nM MgCl2 (JMR 801), 1X Reaction Buffer (JMR801), 1μM of each of the primers (Table 11) and 1 unit Taq DNA polymerase (SuperTherm Taq, JMR 801). The reaction was made up to 25μL with nuclease free water (Whitehead scientific P1193), and the PCR carried out in a Perkin-Elmer 2700 Gene-amp (Applied Biosystems). The PCR parameters for pre-amplification were: Initial denaturation at 94°C for 3 minutes, 20 cycles of amplification consisting of denaturation at 94°C for 30 seconds, primer annealing at 56°C for 1 minute and DNA extension 72°C for 1minute. Synthesis was completed at 72°C for 7 minutes. The PCR product was then diluted 50 fold (with nuclease free water) for use as template in the selective-amplification. Each of the selective-amplification reactions contained 5μL of

the template (1:50 Dilution of pre-amplification), 0.2μL of each of the dNTP's (Abgene Ab-11.24), 2nM MgCl2 (JMR 801), 1X Reaction Buffer (JMR801), 1μM of each of the primers (Table 8) and 1 unit Taq DNA polymerase (SuperTherm Taq, JMR 801). The reaction was made up to 20μL with nuclease free water (Whitehead scientific P1193), and the 'touch-down' PCR carried out in a Perkin-Elmer 2700 Gene-amp (Applied Biosystems). The PCR parameters for selective-amplification were: initial denaturation at 94°C for 3 minutes, the first cycle was: denaturation at 94°C for 30 seconds, primer annealing at 65°C for 30 seconds and DNA extension at 72°C for 1 minute. From the first cycle the annealing temperature was lowered with 1°C per cycle until it reached 56°C. The amplification was therefore completed with the following parameters: 23 cycles of amplification consisting of denaturation at 94°C for 30 seconds primer annealing at 56°C for 30 seconds DNA extension at 72°C for 1 minute, and completion of synthesis at 72°C for 7 minutes. To verify that amplification took place the pre-amplification and selective amplification products were electrophoresed on a 1% agarose gel (Hispanagar D-1 LE) and visualized with ethidium bromide staining (10mg/ml). Successful amplification produced smears.

7.2.6 Polyacrylamide gel electrophoresis and visualization

The amplified AFLP reactions were separated using an automated PAGE system (Licor Global IR2 DNA analyzer, Licor Inc. Nebraska, USA). Two μL of the selective-amplification product was mixed with 2 μL loading dye prior to denaturation at 90°C for 10 minutes, thereafter the sample was kept on ice until loaded. A pre-run (10 Watt for 30 minutes) was done to homogenize the gradients within the PAGE gel before the samples were loaded onto the gel. Gel electrophoreses was carried out at 35 Watt for four hours, the temperature being controlled at 55°C. A sizing standard of between 50-700bp (Li-Cor 4200-60[700]) was used as reference. The Automated Li-Cor system generated digitized fingerprints (16 Bit TIFF images) during the gel run, which were analysed with compatible analysis software.

7.2.7 Analysis of fingerprints

Digitized fingerprints were analyzed using GelCompar II software (Applied Maths, Kortrijk, Belgium) following the manufacturers instructions. Images were straightened and normalized by alignment to molecular size standards loaded on each gel. Curve-

based dendograms were generated using Pearson Correlation Coefficient with 1% optimization and 0.14 position tolerance values.

7.3 RESULTS

Restriction enzymes EcoRI and MseI were used to generate DNA fragments from 104 environmental and clinical *Vibrio cholerae* isolates. The AFLP patterns generated with primers listed in Table 7.4 contained 45 to 60 bands per isolate. Diverse banding patterns were observed within environmental isolates, while clinical strains grouped loosely together. Similarity analysis with Pearsons correlation coefficient yielded a number of clusters, with environmental isolates grouping in various clusters, while the four clinical isolates together with two environmental strains were grouped in a separate cluster. The clinical isolates were not of the same origin, two originated from the Nelspruit region, one from the Kwazulu-Natal province, and the last being a *Vibrio cholerae* reference isolate (NCTC 5941). Environmental isolates grouped in clusters showing no or little resemblance to their isolation site and or isolation date (Figure 7.2). Thus there was no correlation between site of isolation and date of isolation with isolates obtained from the various different river systems over a period of three years.

Using a Pearson correlation value of 90% or higher, clusters formed by the environmental isolates were further investigated (Table 7.5). In 6 of the 17 clusters created by using this cut-off value the isolates grouped together were of the same sampling site (or geographically closely related sampling sites). Sampling site N8 and R5 were regarded as being closely related, with the R5 sampling site situated less than 2km upstream of the convergence of the Rietspruit and Natalspruit rivers, which represents site N8. These 6 clusters contained strains isolated on different sampling dates, with the dates of isolation differing with up to seven months. Five of the 17 clusters only consisted of isolates sampled at the same site on the same date. These were disregarded, as they may be duplicates of one strain. This effectively reduced the clusters of interest to 12. Isolates from unrelated sampling sites and different sampling dates made up the remainder of the 12 clusters.

	Isolate no.	Sampling site	Sampling Date

AFLP

	N8	25/03/2002
	K18	10/02/2003
	VRB47	27/02/2002
	VRB47	14/10/2002
	VRB47	14/10/2002
	VRB307	12/03/2003
	M-Canal	10/02/2003
	B10	25/03/2002
	K18	10/03/2003
	R6	29/04/2003
	S2	25/02/2002
	S2	11/03/2002
	S2	26/03/2001
	S1	25/03/2002
	RV2	14/05/2001
	S2	07/01/2002
	S2	25/02/2002
	S2	11/03/2002
	S1	25/03/2002
	R6	25/02/2002
	M-Canal	25/02/2002
	K26	25/02/2002
	N9	14/01/2002
	B10	25/02/2002
	B10	25/02/2002
	VRB477	12/03/2003
	B10	02/04/2001
	VRB30	30/04/2003
	VRB30	30/04/2003
	R6	31/03/2003
	S2	19/03/2001
	M-Canal	25/02/2002
	R6	20/12/2000
	N4	07/01/2002
	R5	07/01/2002
	N8	26/11/2001
	S2	10/03/2003
	R6	26/03/2001
	S1	25/02/2002
	VRB47	02/10/2002
	K18	11/03/2002
	VRB47	13/03/2002
	N8	25/01/2002
	S1	11/03/2002
	RV2	12/11/2001
	B10	26/11/2001
	R6	10/02/2003

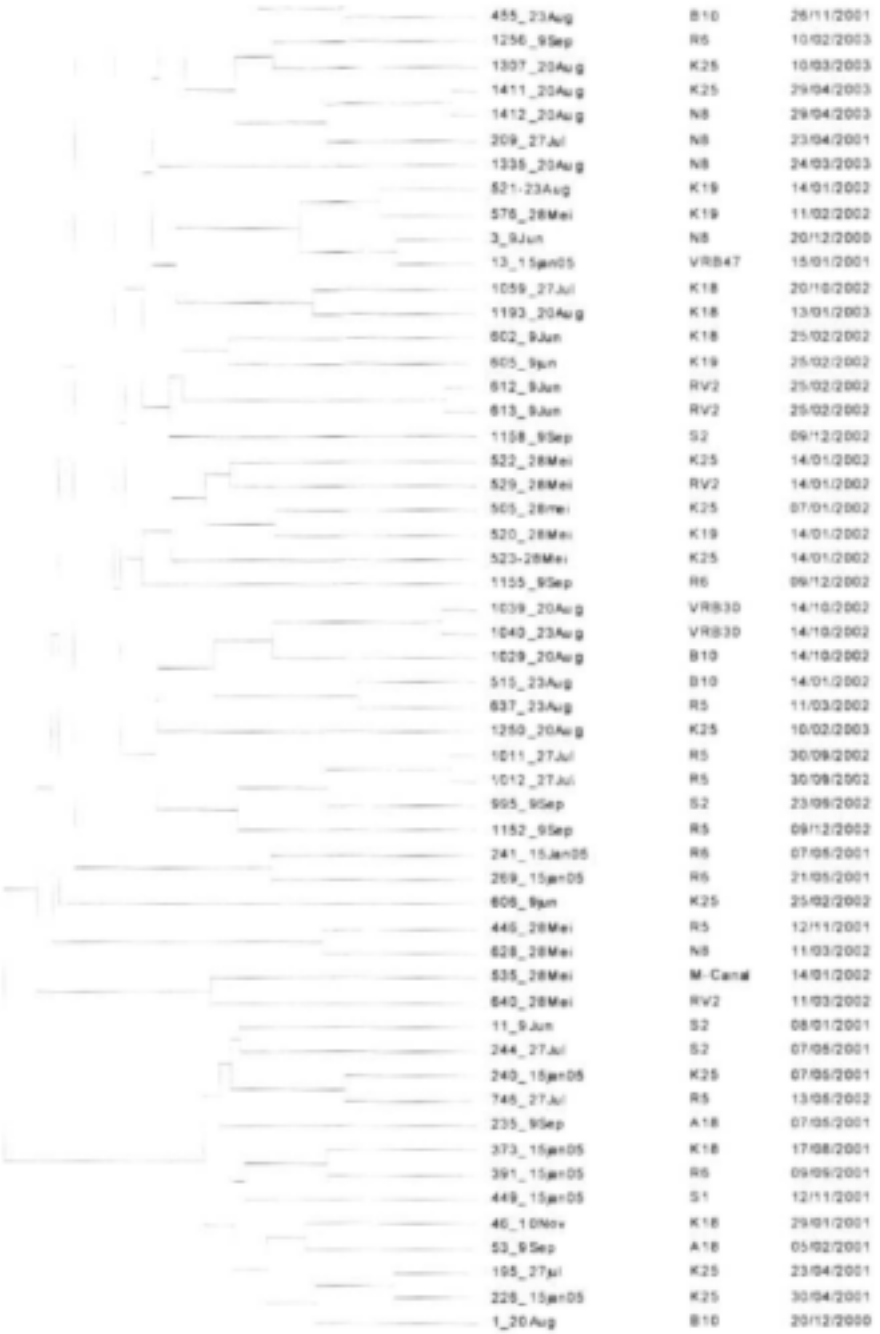

Figure 7.2: Dendogram derived from AFLP patterns of 104 *Vibrio cholerae* strains. Dendogram was constructed using the Pearson coefficient and unweighted pair group method of arithmetic averages.

Table 7.5: Isolates present in clusters showing a Pearson correlation of 90% or higher

Isolate number	Sampling site	Sampling date
Correlation of sampling site		
622	VRB47	27-Feb-2002
1054	VRB47	14-Oct-2002
1053	VRB47	14-Oct-2002
616	S2	25-Feb-2002
645	S2	11-Mar-2002
174	S2	26-Mar-2002
617	S2	25-Feb-2002
644	S2	11-Mar-2002
672	S1	25-Mar-2002
501	N8	7-Jan-2002
506	R5	7-Jan-2002
521	K19	14-Jan-2002
576	K19	11-Feb-2002
195	K25	23-Apr-2001
226	K25	30-Apr-2001
Correlation of sampling site and date - Possible duplicates		
598	B10	25-Feb-2002
599	B10	25-Feb-2002
1424	VRB30	30-Apr-2003
1425	VRB30	30-Apr-2003
612	RV2	25-Feb-2002
613	RV2	25-Feb-2002
1039	VRB30	14-Oct-2002
1040	VRB30	14-Oct-2002
1011	R5	30-Sep-2002
1012	R5	30-Sep-2002
Diverse sampling sites/dates		
256	RV2	14-May-2001
510	S2	7-Jan-2002

Isolate number	Sampling site	Sampling date
610	R6	25-Feb-2002
618	M-Canal	25-Feb-2002
1411	K25	29-Apr-2003
1412	N8	20-Apr-2003
160	S2	19-Mar-2001
619	M-canal	25-Feb-2002
171	R6	26-Mar-2001
615	S1	25-Feb-2002
3	N8	20-Dec-2000
13	VRB47	15-Jan-2001

7.4 DISCUSSION

Molecular techniques are an invaluable tool for determining population structure and dynamics of pathogenic bacteria. Researchers have used a variety of these techniques to study the genetic diversity of *Vibrio cholerae,* including pulsed field gel electrophoreses, restriction fragment length polymorphisms, sequencing of genes, ribotyping and amplified fragment length polymorphism fingerprinting (Cameron *et al.*, 1994, Wachsmuth *et al.*, 1991, Dalsgaard *et al.*, 1995, Olsvik *et al.*, 1993, Kotetishvilli *et al.*, 2003, Jiang *et al.*, 2000, Lan and Reeves 2001). Most of these studies focused on clinical strains, where the epidemic as well as pandemic spread of cholera was the main issue of interest. Only in recent years have researchers turned to studying the genetic diversity of environmental *Vibrio cholerae* isolates (Jiang *et al.*, 2000, Thompson *et al.*, 2003). Amplified fragment length polymorphism DNA fingerprinting has the advantage that it allows the differentiation of highly related bacterial genomes in a highly reproducible fashion. The technique also does not require prior sequence knowledge, and combines the power of RFLP analysis with the flexibility associated with PCR-based technology (Janssen *et al.*, 1996) The combination of these characteristics make AFLP a highly suited tool for determining genetic diversity within environmental *Vibrio cholerae* populations. This study focused on closely-related environmental *V. cholerae* strains and a primer pair with high discriminatory ability were sought. The discriminatory power of a typing technique can be measured by the Simpson index of

diversity (D). Lan and Reeves (2001) showed that the combination of primers Eco-G and Mse-T could obtain a D-value of 0.837 when discriminating closely related seventh pandemic strains. The decision was therefore made to use this primer pair in this study as they showed the highest D-value within the Eco/Mse group.

Vibrio cholerae is an autochthonous inhabitant in the Vaal Barrage Aquatic system, with environmental strains being isolated on a regular basis. Factors that have been shown to influence the survival of *V. cholerae* in inland water systems include water temperature, water pH, salinity and association with water fauna (Jiang *et al.*, 2000, Huq *et al.*, 1983, Huq *et al.*, 1984, Halpern *et al.*, 2003). The conditions in the Vaal Barrage system is favourable for *Vibrio cholerae* propagation and survival with water temperatures ranging between 17°C and 23°C, pH values averaging around 8.0, and Na present in concentrations ranging between 5 and 300 mg/ml (as measured from Jan 2001 to Oct 2003). These favourable conditions have lead to the establishment of diverse *V. cholerae* populations in the river system. The clusters generated using AFLP patterns of the environmental isolates, contained isolates from different isolation sites and sampling dates. This indicates that *V. cholerae* strains with similar genotypes are spread throughout the aquatic system and are able to survive for extended periods. It was seen that the population is genetically diverse. Many of the clusters were only distantly related to other *Vibrio cholerae* clusters, indicating how well established and diversified the *V. cholerae* population is. This diversity is not only observed when looking at the entire aquatic system, but also when focusing on specific sampling sites. Isolates from the same sampling site rarely grouped closely together. Where clusters do contain highly related isolates from the same sampling site the sampling dates do not differ by more than a few months. The similarity is possibly lost due to small genome rearrangements over time. The clinical isolates used in this study grouped together, even though originating from different sources. The clinical strains originating from Nelspruit and Kwazulu-Natal showing an extremely high level of similarity suggesting that they were derived from the same strain. The inclusion of an environmental isolate in the same cluster as the previously mentioned clinical isolates suggests that there could be a high degree of relatedness between some of the enterotoxin producing strains and environmental non-O1, non-O139 strains.

With the high level of genetic diversity seen in this study the *Vibrio cholerae* population in the Vaal Barrage system is probably not a product of one or two strains that have adapted to local conditions, nor does it consist of diverse clones that only occupy specific niches in the aquatic system. The population in the Vaal Barrage is rather made up of highly diverse clones that constantly compete, resulting in genetic shifts only perceivable within short time frames and localized regions. This constant adaptation with the associated genetic rearrangements gives an indication of the genetic flexibility of environmental *Vibrio cholerae* strains, and may portray the potential of environmental populations to serve as reservoirs for future epidemic strains. Using environmental *Vibrio cholerae* as a model, it can be suggested that enterotoxigenic strains may exhibit the same degree of persistence and survival in inland aquatic systems, being able to survive for extended periods and posing as potential future health risk.

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CHAPTER 8

EPIDEMIC CHOLERA IN KWAZULU-NATAL: THE ROLE OF THE NATURAL AND SOCIAL ENVIRONMENT

M D Said, S N Venter, T E Cloete S Rushworth and I Booysen

8.1 INTRODUCTION

Cholera, since first documented, has been around the world in seven pandemics, the latest (seventh), which is still in progress, originated in Sulawesi, Indonesia in 1961 (Barua, D. 1992). This seventh pandemic first crossed into Africa in 1970 in West Africa, which had not experienced the disease for more than 100 years (WHO, 1991; Barua, D. 1992). Cholera outbreaks made their mark in South Africa in the 1970s, when several gold mines reported outbreaks of cholera (Isaäcson *et al* 1974). In October 1980, cholera was reported in Kangwane (Mpumalanga) resulting in 2,748 cases with 36 deaths (Kustner *et al* 1981). Following this, a cholera outbreak was reported in Lebowa (Mpumalanga) in November 1981 (Sinclair *et al* 1982). At about the same period, there was a cholera outbreak in Transkei (Eastern Cape) between October 1982 and September 1983 (Transkeian Dept of Health, 1982; Tshibangu and Stawski, 1984). In 1998, there was yet again a cholera outbreak in Mpumalanga (Athan *et al* 1998; Keddy & Koornhof 1998). The initial cases were all from the eastern Transvaal (Mpumalanga) close to the Mozambique border. Later on, the epidemic spread into Natal as well as into Gauteng. The epidemic declined and was mostly confined to Natal province (Kustner *et al* 1981). From 1986 up to mid 2000, there were random reports of cholera cases, with KwaZulu-Natal (KZN) reporting 456 cases, accounting for 87% of the national total of 526 (Dept of Health, 2000).

South Africa experienced a new wave of the disease when cholera swept the eastern coast of South Africa in August 2000. Cholera had a firm grip on the rural parts of North-eastern KZN, as it developed into the most serious epidemic yet experienced in KZN with cases still continuing to be reported well in the year 2004. KZN is a province of over nine million residents, with more than half of the population who live in crowded, rural and underdeveloped conditions that favour the spread of the disease

(Laskow 2001). As cholera struck KZN, it became obvious that most who had fallen ill lived in rural areas without access to tap water or sanitation (Reeves and Boshielo, 2001). The first cases of cholera in the epidemic of 2000 were reported in the Empangeni-Eshowe area of KZN. Despite prompt medical intervention and health education and media campaigns, cholera continued to spread to most corners of the district in a matter of weeks. By March 2004, the official statistics of cholera cases in KZN, since the August 2000 outbreak, stood at 143 814 cases (Dept-KZN Health, 2000). The death toll being 484 which translates to a percentage fatality rate of 0.33%; the lowest compared to the previous epidemics recorded in South African (Kustner *et al* 1981; Küstner and du Plessis, G. 1991).

Cholera is a highly contagious disease caused by *Vibrio cholerae*. Infection normally starts with the most common route of transmission, i.e. oral ingestion of food or water contaminated with *V. cholerae* (Reidl and Klose, 2002). Thus, hygiene, clean water, and sanitary food handling are considered very important in cholera prevention (St. Louis *et al* 1990; Barua, 1992; Rabbani & Greenough, 1999). Nonetheless, the role of other factors such as climate, geographic location, socio-economic status and the environment are being increasingly appreciated in the dynamics of the disease (Climate Change 2001; Pascual *et al* 2002) as also shown in Figure 8.1. The incubation period ranges from several hours to 5 days (Sánchez & Taylor 1997). The majority of infected patients develop only mild or no illness, although the bacterium is present in their faeces for 7-14 days. In most cases, such sub-clinical illness resolve in 4-6 days (Sánchez & Taylor 1997). Clinical manifestations range from asymptomatic carrier state; mild, watery diarrhoea to an acute diarrhoea characteristic of cholera (Keen & Bujalski, 1992). Fortunately, most cases of cholera can be treated adequately by giving a solution of oral rehydration salts as prescribed in the WHO/UNICEF standard formulation (Nalin *et al* 1968; Duggan *et al* 2004).

The hypothesis is put forward that climatic conditions play a significant role in the spread of cholera in KwaZulu-Natal. In addition, socio-economic variables like sanitation, clean water supply, population density, health service delivery etc., contribute to the vulnerability of communities to the risk of cholera.

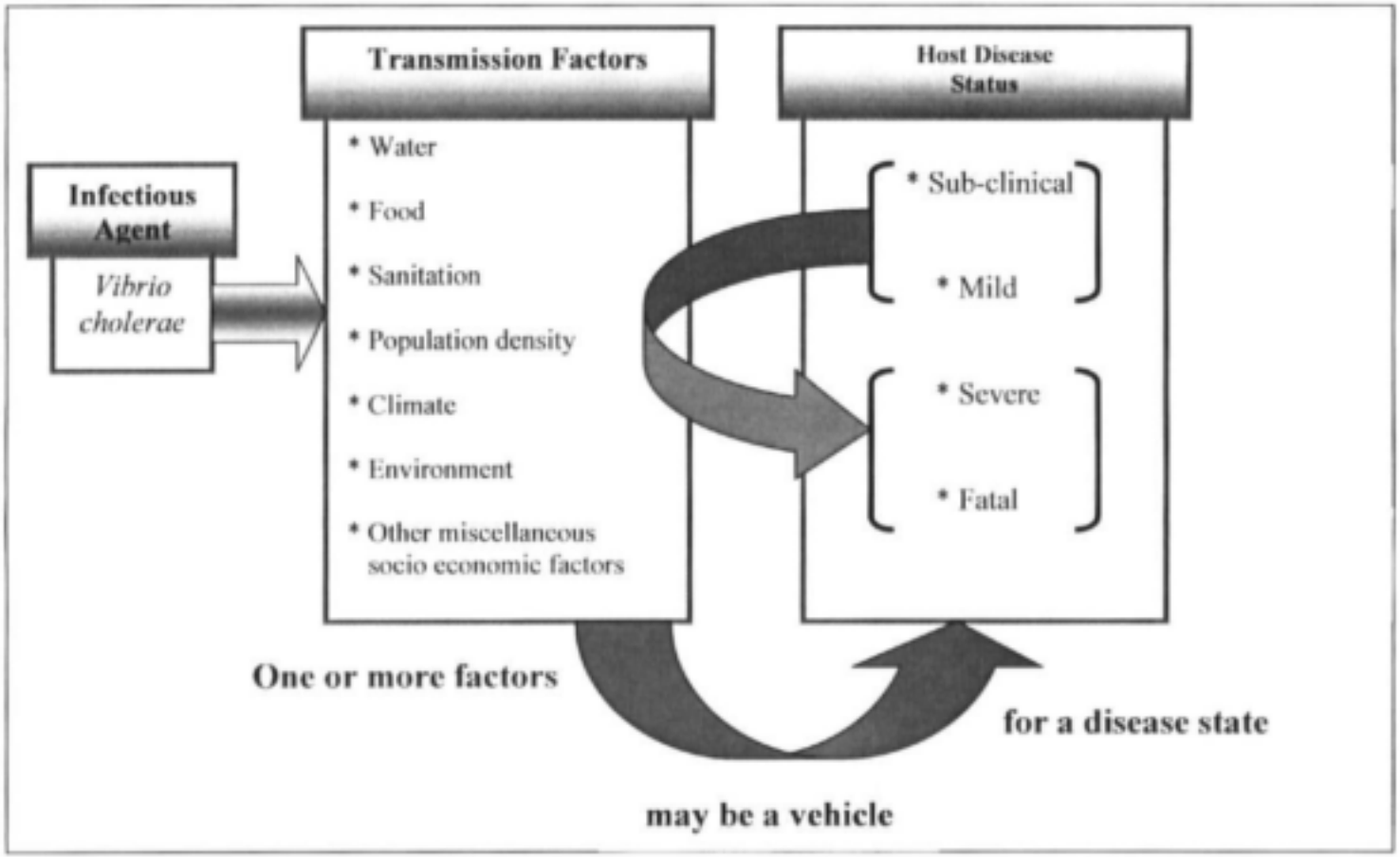

Figure 8.1: The possible relationships between the cholera pathogen, transmission factors and the human host.

8.2 OBJECTIVES

8.2.1 Specific objectives

To evaluate the dynamics of the 2000-2004 cholera epidemic in KZN, with respect to the natural environment, i.e. temperature, rainfall and humidity and the socio-economic status of the communities in that region.

8.2.2 General objectives

a. To investigate how factors such as sanitation, water supply, population density; and other socio-economic factors expose communities to the risk of cholera.

b. To develop GIS maps that will spatially demonstrate how the important risk factors relate to one another in the spread of the disease.

8.3 METHODOLOGY

The surveillance of morbidity and mortality statistics of the 2000-2004 cholera epidemic, was put in place by the GIS Unit of the KZN provincial Department of Health (DOH) in Pietermaritzburg. This exercise led to the creation of the KZN Cholera Dataset, which formed the central basis of this study. In addition to the cholera data, there were four other major groups of parameters that were considered as well. These included demographic, socio-economic, geographical and climatic parameters. For the collective analyses of all the data types involved, an attribute dataset was created which lists all the places in KZN and their corresponding cholera cases, demographic profiles, basic services (water, sanitation etc.), other socio-economic data and climatic profiles. The attribute dataset types and categories (where applicable) of data type are explained below.

8.3.1 The KZN cholera dataset

The cholera data set describes the cholera situation from August 2000 onwards with an inventory of all the cholera patients that were registered in the health institution linked to the dataset collecting system of KZN.

8.3.1.1 Climatic data

The climatic data specific to the areas affected by cholera in KZN was requested from the South African Weather Service (SAWS). The climatic data used in the study covered the same period in time as the cholera dataset i.e. from August 2000 to February 2004. The climatic variables included in the attribute dataset were: maximum, minimum and average temperatures, rainfall and humidity.

8.3.1.2 Demographic and socio-economic data

All the data types included in this category were sought from the Census 1996 data obtained from Statistics South Africa (STATSSA, 1998). It should be noted that the study used the 1996 census information for all its demographic and socio-economic data. The reason being that when this study commenced, the 2001 census data was not yet available. Later on, as the Census 2001 became available, it was realised that some of the KZN magisterial demarcations had changed. Thus, to conserve the consistency of the cholera dataset, which had used the same Census 1996 demarcations for communities/townships/village during the data collection exercise (Personal communications), it was likewise decided that all the demographic and socio-economic data used for this study, should be from the same source. Demographic indicators included in the study were age and gender. While water supply, sanitation services, refuse collection, electricity, types of dwellings, income and types of health services represented the socio-economic indicators. Place names and the area in sq km, their P codes (place codes), magisterial districts, district councils, their elevation above sea level, and the type of water resources (perennial rivers, dams, pans, wetlands and canals) were the geographical parameters considered.

8.3.2 Data assessment and processing

8.3.2.1 General approach

The attribute dataset was exploited using Microsoft Excel 2000 to study the general disease trend. The case distributions were considered in absolute numbers as well as by %CIR (Percentage Cumulative Infection Rate) which is defined as the risk of individuals in the population getting the disease during a specified period (Beaglehole *et al* 1993). The %CIR represents the incidence rate per 100 of the population, and is calculated as:

$$CI = \frac{\text{Number of people who get a disease during a specified period}}{\text{Number of people free of the disease in the population at risk}} \times 100$$

8.3.2.2 Statistical approach

Derived values were acquired through statistical data processing using SAS® (version 8.2) obtainable under licence from SAS Campus Drive, Cary, NC 27513. The derived values formed the basis of all the statistical correlations.

8.3.2.3 Spatial approach

The outputs from both spreadsheet and statistical processing supported the production of GIS maps using ArcView 3.2 GIS Software to give a spatial picture of the different aspects of the epidemic in space and time.

8.4 RESULTS

A multitude of analyses were used to get an insight on the modus operandi of the 2000-2004 cholera epidemic from a descriptive, statistical and spatial point of view.

8.4.1 The general trend of the cholera epidemic in KZN

Analyses from spreadsheet exploration of the database were presented as tables and graphs. The spread of cholera on a monthly basis from August 2000 to February 2004 was represented (Figure 8.2), which also showed the distribution of the disease from a gender perspective. Figures 8.3 and 8.4 continued to highlight the epidemic in the confines of District Councils (DCs) clearly showing the most affected DCs within KZN. Figure 8.5 revealed the most reported age groups within the various DCs. At the Magisterial District (MD) level, Figure 8.6 shows the most affected MDs as well as the DCs each belongs to. The monthly patterns of the climatic variables versus the spread of cholera throughout the epidemic period are highlighted (Figures 8.7 – 8.9).

8.4.2 Statistical correlations

Partial correlations were performed using the derived values of all the variables included in the attribute dataset i.e. demographic, socio-economic and climatic variables; against %CIR cholera ($p<0.05$) (Table 8.1).

Table 8.1: Variables that a positive correlation with cholera (%CIR)

% CIR → Variable↓	Correlation	P-value ($p<0.05$)
Traditional house	0.27255	0.0978
Homeless	0.38525	0.0169
Piped water	0.3691	0.0267
Borehole	0.33617	0.045
River water	0.50754	0.0016
Bucket toilet	0.30015	0.0634
Unspecified sanitation	0.41089	0.0094
No refuse service	0.39147	0.0125
Income: R6,000–R16,000/month	0.29336	0.078

8.4.3 Spatial GIS mapping

The variables statistically associated with cholera formed the basis of the type of geo-spatial representation in the form of GIS maps. While in the mapping process, high cholera areas became automatically demarcated and were observed spatially. GIS maps 1-4 (Appendix-A on CD) portray the distribution of cholera between Aug'00 to Dec'03 as well as the most affected areas.

8.4.4 Data verification

Of the 158,896 entries captured in the Cholera Dataset, only 136,262 (85.8%) were included in the attribute database after verification. This disadvantaged the outcome more bearing in mind that as it were, the cholera cases represented in the cholera dataset are just a fraction of the real case scenario.

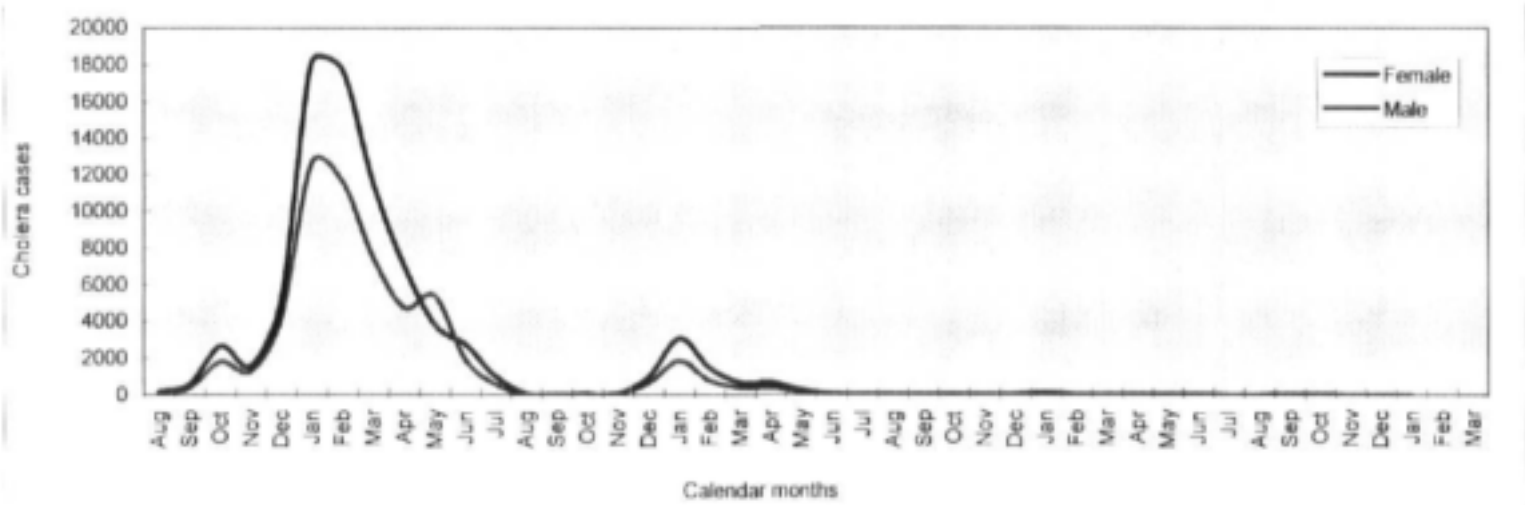

Figure 8.2: The KZN annual trends of the cholera epidemic between Aug 00 to Feb 04.

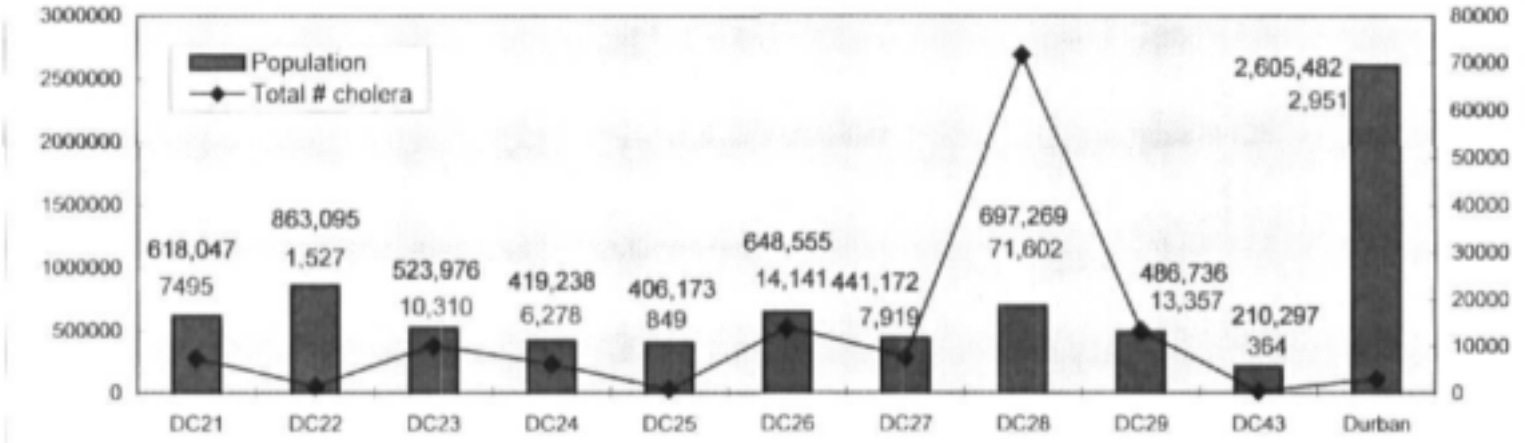

Figure 8.3: The distribution of cholera among the KZN District Councils during the 2000-2004 epidemic.

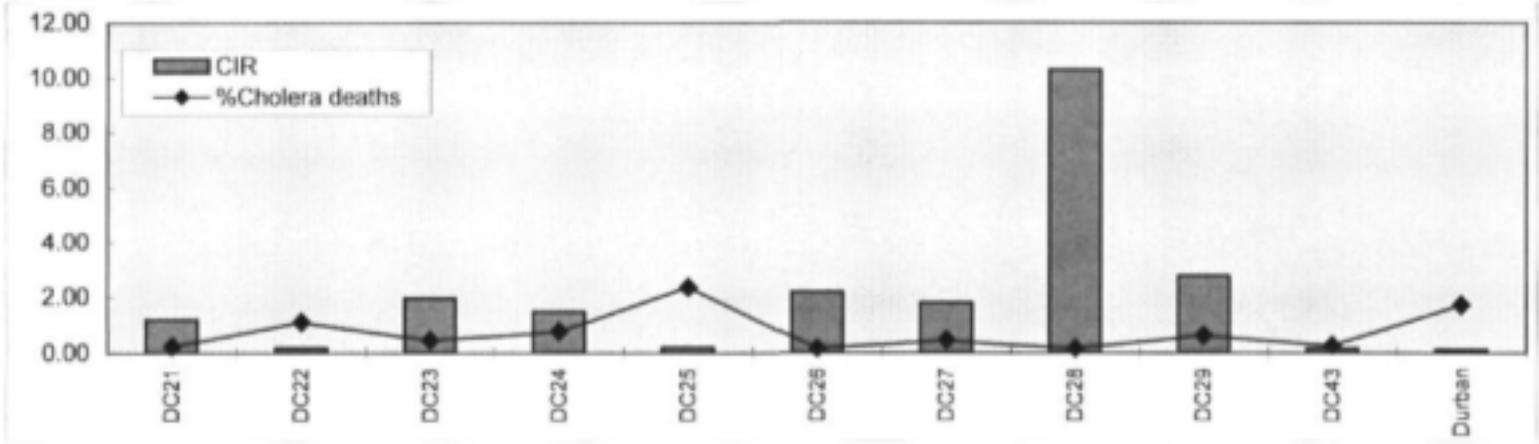

Figure 8.4: A comparison of the % CIR of cholera and cholera related deaths among the KZN DCs during the 2000-2004 epidemic.

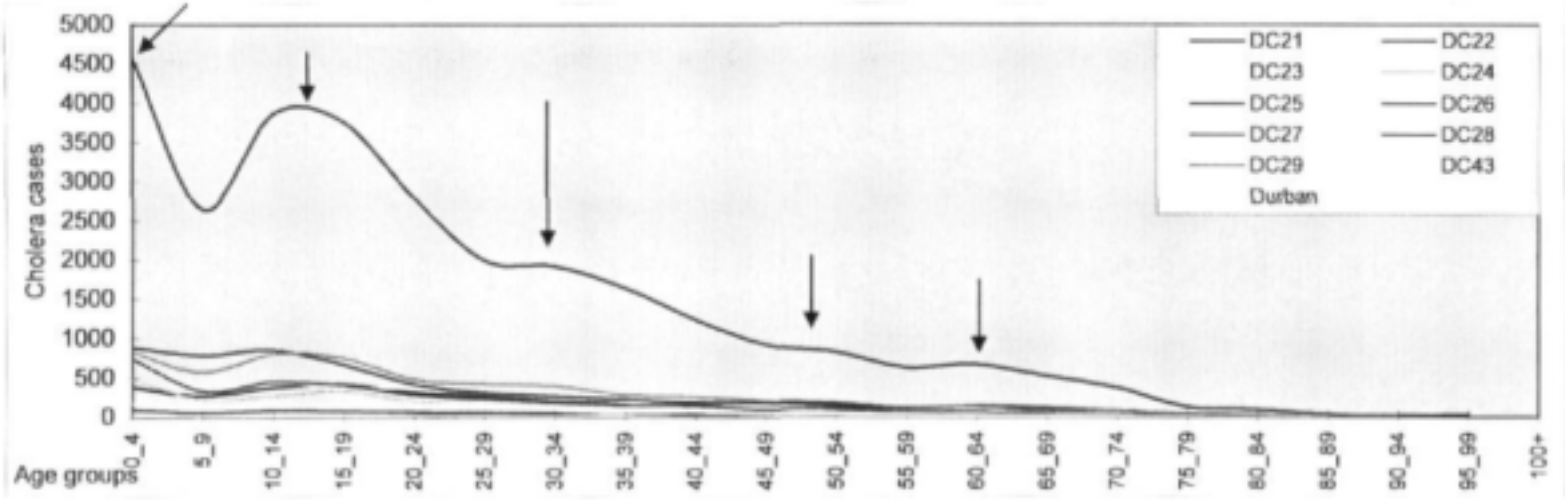

Figure 8.5: Distribution of cholera among the different age groups in the KZN District Councils.

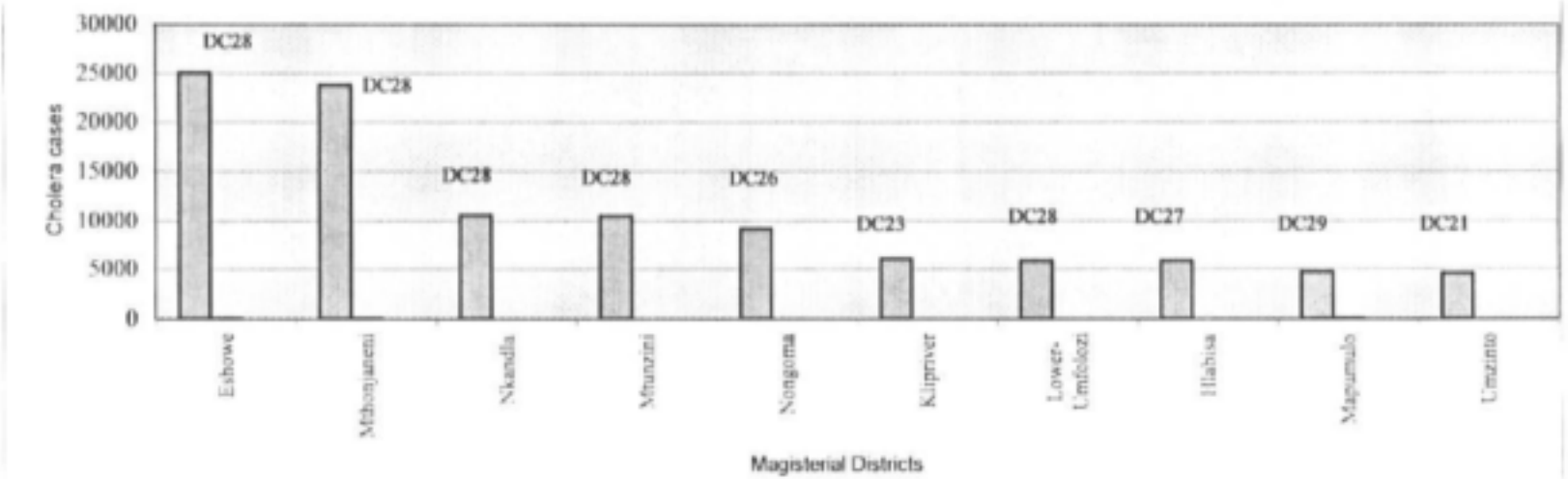

Figure 8.6: Magisterial Districts that reporting the highest cholera cases during the epidemic period Aug00-Feb04.

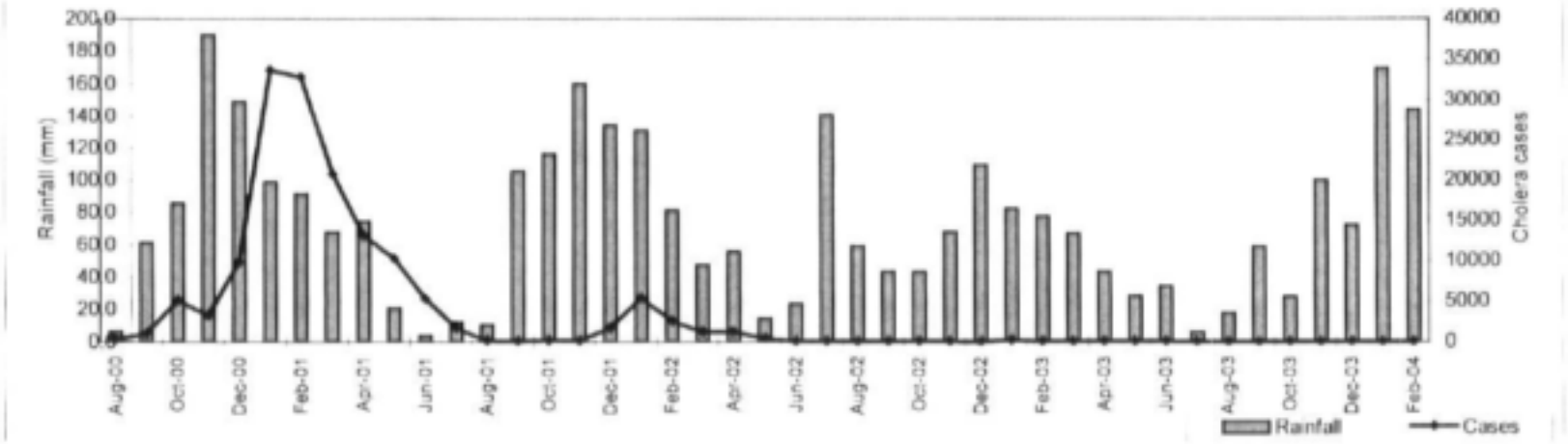

Figure 8.7: Variations in monthly rainfall (mm) patterns and the number of monthly cholera cases during the epidemic.

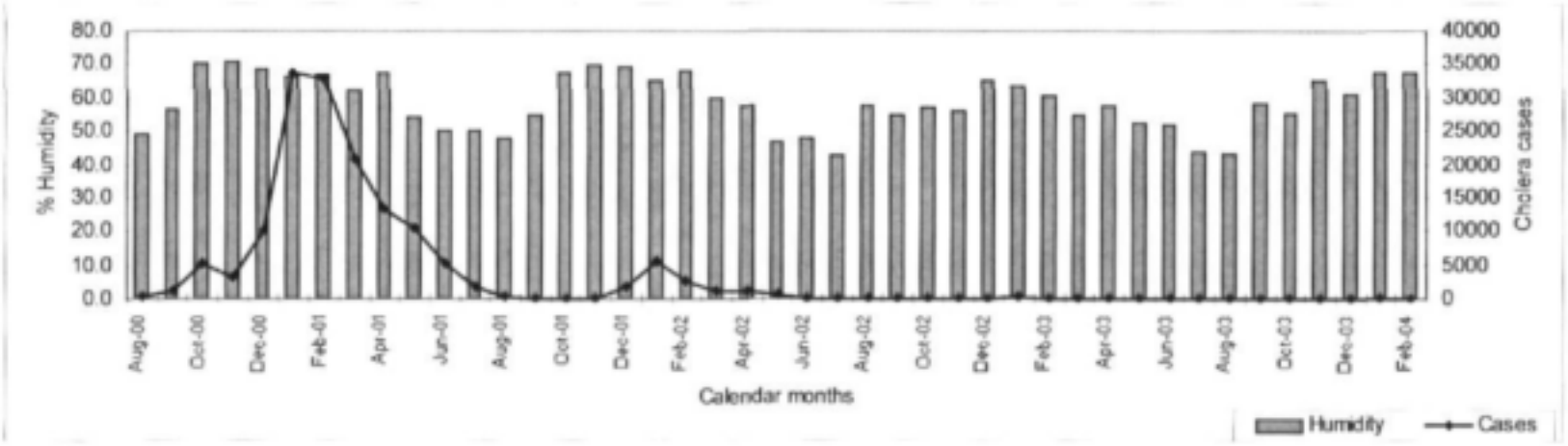

Figure 8.8: Variations in monthly humidity (%) and the number of monthly cholera cases during the epidemic.

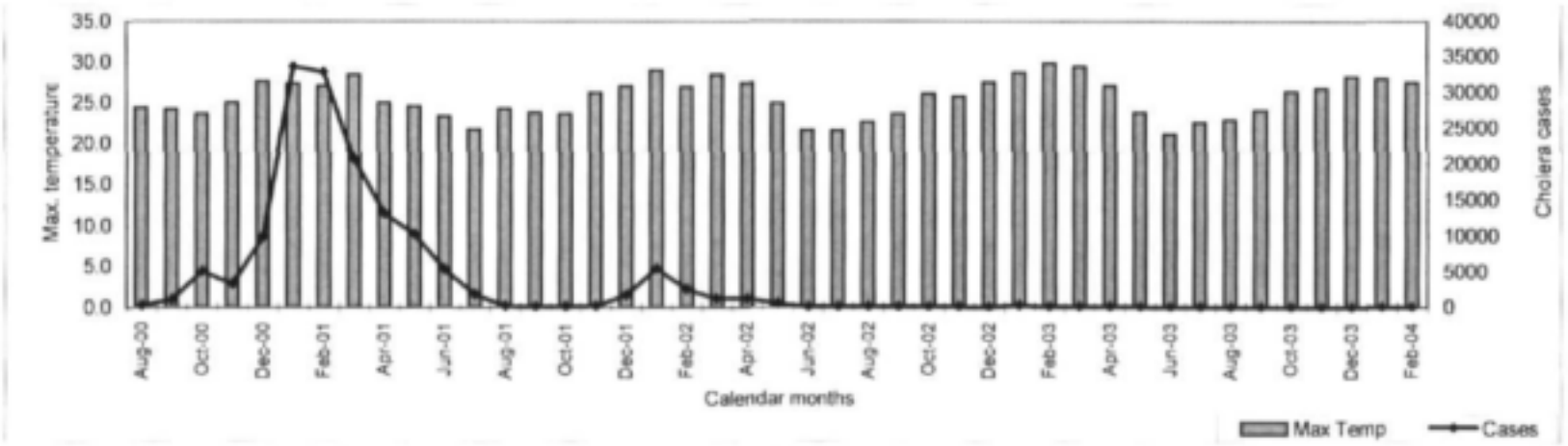

Figure 8.9: Variations in monthly maximum temperatures (^{0}C) and the number of monthly cholera cases during the epidemic.

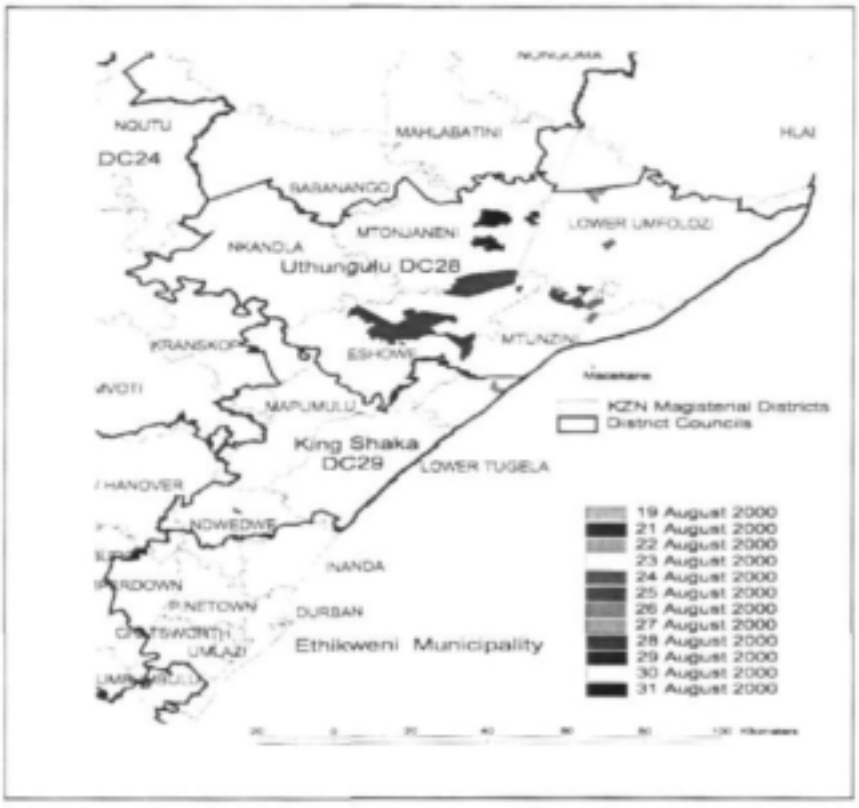

Figure 8.10: The initial cases reported in the month of August 2000 in KZN.

8.5 DISCUSSION

Cholera had an upward trend from November 2000 and experienced its major peak between Dec'00 to Feb'01. As intervention measures took control of the epidemic, cholera numbers waned to a minimum in July 2001. Another upward trend in cholera cases was repeated between December 2001 and February 2002 where the cholera epidemic experienced a minor peak. Both the major and minor cholera peaks showed male cholera cases being outnumbered by those of females by a ratio of approximately 1:1.5 respectively (Fig 8.2). In generally all the age groups were represented in the cholera dataset i.e. from infants of less than a year to the elderly above 100 years. Albeit, results indicate that the age groups 0-4, 10-14 and 15-19 featured more in the overall epidemic picture. The trend in age related cholera cases was more pronounced in DC28 (Uthungulu).

It is apparent from the results that DC28 was the most affected area, boasting a 52.2% of the cholera cases from of KZN. The %CIR of DC28 was also the highest in relation to the other DCs (Fig 8.3). Evidently, as the cholera epidemic started in August 2000, all the cases reported in that month were exclusively confined to DC28; which was the

disease focal point (Fig 8.10). A similar pattern is seen at the MD level whereby among the 10 most affected MDs, 50% belong to DC28 (refer Fig 8.6), and three MDs (MD26, MD27 & MD29) of the remaining five were neighbours of DC28. Furthermore, an interesting feature of the 10 most affected MDs is that eight of them have a coastal border with the Indian Ocean.

Spatial representation of the most affected areas, reveal them to be mostly rural, made up of 20-60% traditional houses. The areas also have relatively high population densities of 17-99 persons/sq km with 0.01-0.12% of their population classified as homeless (Maps 5-7 Appendix-A on CD). The most affected MDs, individually accommodated 2-3% of the provincial population (Map 8 Appendix-A on CD), of whom, up to 85% had no form of income (Maps 8-9 Appendix-A on CD); implying a high level of poverty in the area. This state of affairs dictated the type of basic services the population could afford in that only 15-47% had piped water in their dwellings, 6-14% depended on boreholes while the majority 23-55% sourced their water from rivers, streams and springs (Maps 10-12 Appendix-A on CD). The sanitation coverage was such that 9-53% households had pit latrines while 1-6 % had no specified form of sanitation; which is a point of concern. In an epidemic situation, people with no specified sanitation will probably include disease carriers who may continuously seed the aquatic and terrestrial environmental with vibrios. Interestingly, the areas where the initial cholera cases were reported still had 3% of households using the bucket toilet system (Maps 13-15 Appendix-A on CD), which can expose households to unsanitary conditions if not properly serviced by the local authorities. Similarly, a positive correlation (r=.30 p 0.06) was also seen between the %CIR and use of the bucket toilet system. Overall, 12-41% of the households in these MDs had no form of rubbish disposal system; supported by the positive correlation (r=0.39 p 0.01) between %CIR and lack of refuse services; implying a significant level of indiscriminate pollution which may be an environmental factor in the transmission of cholera (Map 16 Appendix-A on CD). Though a shortage of clean water and adequate sanitation still carry the banner of blame for the epidemic, HIV has also been implicated as one of the underlying causes of the persistence of cholera in KwaZulu-Natal, when one takes into account that the province has the highest HIV infection rate in the country (UN-IRIN, 2001). Nonetheless, it is quite apparent that shortfalls in socio economic services contribute to the vulnerability of communities to cholera.

Figures 8.7 – 8.9 attempts to explore the relationships between cholera and the climatic variables of rainfall, humidity and maximum temperature that were experienced in KZN during the epidemic period. Of the three climatic variables, the rainfall graph revealed an interesting trend whereby, both the major and the minor cholera peaks were experienced just after the peak rainfall months. Spatially, the Eshowe/Mtunzini areas that recorded the most cases during the peak of the epidemic, had relatively more rainfall in Dec'00 (239-299 mm) and Feb'01 (106-212 mm) (Maps 17-19 Appendix-A on CD). As the Dec'00 to Feb'01 rainfall patterns were spatially superimposed with cholera cases of the time, most of the cholera cases seen are clearly confined within the boundaries of DC28. The relative humidity maps clearly shows the confinement of the cholera cases between Dec'00 – Feb'01 in areas where humidity ranged from 73-83% along the coastal MDs and 53-68% in the more interior ones (Maps 20-22 Appendix-A on CD). Overall, cholera started in the coastal MDs with relatively more rainfall and higher humidity and only later spread to the interior, where there was less rain and lower humidity. No statistical correlations could be established between the %CIR and the climatic variables of rainfall, temperatures (minimum, maximum and average) and humidity. There may have been correlations within specific months when cholera was at a peak but not when the entire dataset was statistically considered.

In KZN it was easy for cholera to spread from DC28 to the adjacent DCs and subsequently spread further because the status of the basic service delivery was either at the same level as that of DC28 or most of the time even worse off as demonstrated spatially (Maps 5-16 Appendix-A on CD). In addition to the socio economic disadvantages of the area, spatially, there appear to have been a concurrence between climate and cholera incidence (%CIR) in the most affected areas. The initial cases and the most number of cases coincided with the same areas where there was relatively more rain, high humidity and high maximum temperatures (Maps 17-25 Appendix-A on CD).

8.6 CONCLUSIONS

District Council 28 showed the highest cholera infection rate in KZN, wherein all the variables that came up as statistically significant during the cholera epidemic were those

that have been previously implicated in the spread of cholera in South Africa. In general, use of river water, inadequate sanitation and lack of refuse services came out with relatively stronger correlations; once more stressing the importance of basic services in creating vulnerable communities. The role (if any) of the local climatic variables in KZN in the transmission of cholera were not statistically supported.

There is a high likelihood that cholera has become endemic in KZN considering the number of years that this epidemic has taken thus far. The epidemic may eventually wane completely but unless the socio economic situation improves, people in areas with low delivery of basic services may still have to opt for untreated water sources and alternative sanitation measures irrespective of the consequences. In the meantime, individuals with healthy immune systems will continue to be challenged as they only have limited immunity. The immuno-compromised individuals will however always be at a high risk.

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PROJECT SUMARRY

CHAPTER 9

CONCLUSIONS AND RECOMMENDATIONS

S N Venter

9.1 APPROACH

Water is an important vehicle for the transmission of pathogens and is of particular concern because it can place an entire community at risk. The main focus of this project was to develop a better understanding of the origin, fate and survival of enteric pathogens in fresh water environments. For this purpose the studies have focused on three pathogens to represent various types of enteric pathogens as well as different perceived behaviour in aquatic environments. The project focused on *Cryptosporidium parvum* a protozoan parasite and *Salmonella enterica* and *Vibrio cholerae* two bacterial pathogens.

Cryptosporidium parvum represents to parasitic pathogens that can only grow and multiply within an animal host. These pathogens can only be transmitted by water but cannot multiply in this environment. Oocysts are, however, known to have a thick outer protein layer and they can therefore survive for very long periods in the environment. Due to the problems of separating the parasites from the environmental matrix and therefore obtaining enough biomass for molecular analyses, the present study focused more on the typing of clinical samples to obtain information about the possible sources of environmental contamination and transfer.

Salmonella enteria subsp *enterica* is one of the major food and water-borne bacterial pathogens in both developed and developing countries with approximately 1.4 million cases of non-typhoidal *Salmonella* annually in the USA. As a zoonotic bacterium, it has a wide host range and contamination originates from both domestic and agricultural sources. Although aquatic and terrestrial environments have not been considered to be important reservoirs for this enteric pathogen, recent finding indicated that the bacterium might be well equipped to survive in non-host environments. *Salmonella* therefore serves as a model organism to study the behaviour of enteric pathogens in

aquatic and sediment environments. The current project investigated the survival of a *Salmonella* Typhimurium strain under varying environmental conditions. The project also studied the potential link between environmental and clinical isolates in a rural area where the bacterium is endemic.

Vibrio cholerae is widely considered to be an environmental bacterial pathogen. These pathogens are defined as microorganisms that normally spend a substantial part of their lifecycle outside human hosts, but when introduced to humans they cause disease with measurable frequency. These pathogens can only be controlled through an understanding of their environmental ecology, epidemiology and genetic variability. The population dynamics of this pathogen was therefore studied in an inland fresh water catchment to developed an understanding of it's behaviour. Data from the largest cholera outbreak in South Africa was also used to develop an understanding for the environmental and socio-economic factors that may cause a community to be at risk.

9.2 *CRYPTOSPORIDIUM*

Based on the study it seems unlikely that cryptosporidiosis is commonly transferred from animals to humans in the South African context. The high percentage of the human genotype (80%) observed in the cases investigated suggests the importance of person-to-person contact and human sewage contamination of drinking water, recreational water and food sources, as a means of spreading the parasite. This could have been anticipated because of the high population density in the area, as well as poor sanitary conditions in close-by informal settlements.

As noted above, the introduction of oocysts into the environment by livestock may be of lesser importance in the South African context. This was supported by the prevalence data for *Cytosporidium* oocyst in calves. The prevalence was found to be lower than what have been reported for other countries. *Cryptosporidium* infection is most prevalent amongst young calves between 1-2 weeks of age. Good management and hygiene practices are however, still required to prevent the spread of *Cryptosporidium* to other animals and the aquatic environment.

9.3 *SALMONELLA*

The results of the present study have revealed that *Salmonella* was prevalent in environmental and clinical samples examined. This could be due to fecal contaminants from animal and human excreta that may find their way to the river. *Salmonella* isolates were also isolated from raw vegetables (cabbages, tomatoes and onions) obtained from local rural gardens which used river water for irrigation. Strains isolated from human stools, water, sediments and food were phenotypically and genetically correlated which provide evidence for the epidemiological link between environmental reservoirs and human infection in an endemic area. This confirms that water and food could be important sources of human salmonellosis in rural areas.

Salmonella Typhimurium is the most commonly isolated serotype from clinical cases in the Venda area and a GFP labelled strain was therefore used to investigate the survival and behaviour of *Salmonella* in fresh water sediments. The study indicated that *Salmonella* survived for extended periods of more than 6 months in sediments at temperatures typically associated with fresh water streams and rivers in South Africa. This is of concern as they could easily be released from the sediment into the water phase at high concentrations during rain events or other disturbances of the sediment.

9.4 *VIBRIO CHOLERAE*

The *ompW* based PCR identification was found to be ideal for surveillance work and was more reliable than the biochemical identification approach. The identity of *Vibrio cholerae* stains isolated from the Vaal Barage catchment area were confirmed with this PCR technique and thereafter a selection of these strains were typed with AFLP. With the AFLP typing a high level of genetic diversity was seen. It is therefore believed that the *Vibrio cholerae* population in the Vaal Barrage system is not a product of one or two strains that have adapted to local conditions, nor that it consist of diverse clones that only occupy specific niches in the aquatic system. The population in the Vaal Barrage is rather made up of highly diverse clones that constantly compete, resulting in genetic shifts only perceivable within short time frames and localized regions. Using environmental *Vibrio cholerae* as a model, the study suggests that enterotoxigenic strains may exhibit the same degree of persistence and survival in inland aquatic systems, being able to survive for extended periods and posing as potential future health risk.

For the KwaZulu-Natal outbreak, no statistical correlations could be established between the %CIR and the climatic variables of rainfall, temperatures (minimum, maximum and average) and humidity. There may have been correlations within specific months when cholera was at peak but not when the entire dataset was statistically considered. A number of socio-economic factors could be correlated with high CIR percentages in the DCs. They included traditional households, no sanitation or the use of bucket system, the use of river water for household purposes and the lack of refuse removal. This again stress how the lack of basic services expose communities to the risk of infectious disease and increase their overall vulnerability. Other factors or combinations of factors not addressed in this study e.g. transport systems and HIV infections may also have played a role in the spread of cholera and could be investigated should such data become available.

9.5 RECOMMENDATIONS

A number of issues have not been addressed or resolved during this study and future research projects should address them. These issues include the need to obtain a better understanding of the virulence factors that can help to predict the pathogenic capabilities of pathogens detected in the environment. Only with the ability to distinguish between harmless and pathogenic strains would it be possible to assess the real risk to communities posed by these pathogens present in environmental sources. Understanding of the fate of enteric pathogens in aquatic and terrestrial environments is still limited. The initial survival studies should be extended to address their behaviour within soil and sediments, as well as the effect these environments have on the pathogenicity of the organisms. Enteric pathogens will only be controlled effectively once a thorough understanding of their environmental ecology, epidemiology, virulence, genetic variability and the potential risks posed to exposed populations has been obtained.

APPENDIX

APPENDIX A: LIST OF MAPS:

Map 1: Incidence of cholera in KZN in 2000.

Map 2: Incidence of cholera in KZN in 2001.

Map 3: Incidence of cholera in KZN in 2002.

Map 4: Incidence of cholera in KZN in 2003.

Map 5: Distribution of traditional dwellings in KZN.

Map 6: The population density of the MDs in KZN.

Map 7: Distribution of homeless persons in KZN.

Map 8: The proportion of people per MD in relation to the KZN population.

Map 9: Percentage households in KZN with no income.

Map 10: Percentage households in KZN with piped water in their dwellings.

Map 11: Percentage households in KZN using dam and river water.

Map 12: Percentage households in KZN using bore-holes, rainwater, tanks, and wells.

Map 13: Percentage households in KZN with pit latrines.

Map 14: Percentage households in KZN with the bucket toilet system.

Map 15: Percentage households in KZN with unspecified sanitation.

Map 16: Percentage households with no refuse removal services.

Map 17: Superimposition of Dec'00 cholera cases with Dec'00 rainfall in KZN.

Map 18: Superimposition of Jan'01 cholera cases with Jan'01 rainfall in KZN.

Map 19: Superimposition of Feb'01 cholera cases with Feb'01 rainfall in KZN.

Map 20: Superimposition of Dec'00 cholera cases with Dec'00 humidity in KZN.

Map 21: Superimposition of Jan'01 cholera cases with Jan'01 humidity in KZN.

Map 22: Superimposition of Feb'01 cholera cases with Feb'01 humidity in KZN.

Map 23: Superimposition of Dec'00 cholera cases with Dec'00 maximum temperature in KZN.

Map 24: Superimposition of Jan'01 cholera cases with Jan'01 maximum temperature in KZN.

Map 25: Superimposition of Feb'01 cholera cases with Feb'01 maximum temperature in KZN.

Please Note: Electronic copy of maps on accompanying CD

Other related WRC reports available:

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Diseases caused by protozoan parasites have gained attention in the last decade, especially due to its prevalence among children, the elderly and immuno-compromised patients. There is a need to detect and quantify these emerging pathogens in order to control and minimise the risk to human health.

Aims are to:

- Establish techniques for the detection and quantification in source and treated water of the following pathogens:
  - *Campylobacter* spp. and *Heliobacter pylori*
  - *Cyclospora cayetanensis*
  - Hepatitis E and astro-virus;
- Compare environmental isolates with clinical isolates (of *Campylobacter* spp.) to establish the importance of water in the transmission of these pathogens;
- Establish known techniques in the laboratories of participating organisations;
- Monitor various source and treated waters for the presence of the above-mentioned emerging pathogens; and
- Formulate management strategies should the pathogens be found to pose a possible health risk.

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