THE PREVALENCE OF *Vibrio cholerae* AND OTHER *Vibrio* spp. IN SURFACE WATER OF RURAL COMMUNITIES IN THE LIMPOPO PROVINCE OF SOUTH AFRICA

by

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A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS OF THE MASTERS DEGREE IN MICROBIOLOGY (MSc MBY)

to the

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April 2016

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DEDICATION

- I dedicate this study to the almighty God for sustaining me, giving me guidance and direction throughout my journey.
- I dedicate this study to my wonderful parents; Mr Mbuelo Hampson and Mrs Shonisani Constance Masindi for all the love, encouragement, patience and support.
- I also dedicate this study to Prof N Potgieter and Dr AN Traore who have been great supervisors and mentors to me.

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ACKNOWLEDGEMENTS

- I thank my grandmothers Ellen Netshiumoni and Mpfunzeni Mamphiswana and my grandfathers Solomon Netshiumoni and Jackson Mutavhatsindi who have constantly been on my side
- I thank my siblings: Elisa, Mark, Kevin, Jedidiah, Rudzani, Vhutali and Tshimangadzo Masindi for all the encouragement and moral support they have given me
- I thank Mr Thomas and Mrs Ruth Mathoho for lending help whenever needed and being pillars of strength
- To my auntie, Anna Mutavhatsindi, thank you for all the advice and guidance
- I thank Shammah, Adonai, Hadasah Mathoho for being there.
- To all the members of the Water research team, I appreciate and cherish the advice and technical support you gave me through my journey.
- I thank Mr Kabue JP for all the advice and mentorship.

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DECLARATION

I, Wontonda Masindi (Student no: 11595357), declare that this dissertation for the award of Masters degree in Microbiology (MSc MBY) at the university of Venda has not previously been submitted for a degree at this or any other institution and that all the references materials contained herein have been duly acknowledged.

..... 16/08/2016

Signature Date

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LIST OF ABBREVIATIONS

%	-	percentage
ºC	-	degree Celsius
<	-	greater than
>	-	less than
µl	-	microlitre
µS/cm	-	micro Siemens per centimeter
µm	-	micrometer
ace	-	accessory cholera enterotoxin
API	-	Analytical Profile Index
APW	-	Alkaline Peptone Water
bp	-	base pair
Camp	-	Cyclic adenosine monophosphate
cep	-	core-encoded pilin
cm^{-1}	-	per centimeter
CPS	-	Capsule Polysaccharide
ct	-	cholera toxin
ctxAB	-	Subunit A and B of cholera toxin
DFA-DVC	-	Direct fluorescent antibody – Direct viable count
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxynucleotide Triphosphate
DWAF	-	Department of Water Affairs and Forestry
EDTA	-	Ethylene Diamine Tetraacetic Acid
Flae	-	Flagellin
g	-	gram
GuSCN	-	Guanidium thiocyanate
h	-	hour
hlyA	-	alpha hemolysin gene

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i.e	-	that is
kb	-	kilobyte
LT	-	heat-labile toxin
mg/L	-	milligram per Litre
min	-	minute
mL	-	millilitre
mm	-	millimeter
NaCl	-	sodium chloride
NAG-ST	-	heat stable enterotoxin
nm	-	nanometer
orfU	-	gene encoding unknown function
pH	-	potential of hydrogen
PCR	-	Polymerase Chain Reaction
rfb	-	O- antigen biosynthesis
rpm	-	revolutions per minute
rtx	-	repeats in toxin
s	-	second
SA	-	South Africa
Sec	-	seconds
sodB	-	superoxide dismutase
spp	-	species
ST	-	heat-stable toxin
stx	-	shiga-like toxin
TAE	-	Tris acetate-ethylenediamine tetraacetate
TCBS	-	Thiosulfate-Citrate-Bile-Sucrose
tcpA	-	toxin regulated pilli
tdh	-	thermo-stable direct hemolysin
TDS	-	Total Dissolved Solids

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tl	-	thermolabile hemolysin
TMPD	-	Tetramethyl-p-phenylenediamine
toxR	-	central regulatory protein
trh	-	hemolysin-related hemolysin
UV	-	Ultra violet
VBNC	-	Viable but non culturable
Vcm^{-1}	-	Volume per centimeter
WHO	-	World Health Organisation
WRC	-	Water Research Commission

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ABSTRACT

Introduction: *Vibrio cholerae* is a waterborne pathogen that has caused several outbreaks in developing parts of the world due to a lack of access to clean water.

Objectives: The aim of the study was to investigate the prevalence of *Vibrio cholerae* and other *Vibrio* species in surface waters used by rural communities in the Limpopo province of South Africa.

Materials and method: A total of 120 water samples were collected over a period of three months from 13 rivers in the rural communities of the Vhembe district. The study area encompassed 3 of the 4 Vhembe municipalities namely Thulamela, Mutale, Makhado municipalities. The water samples were collected from three points on each river (upstream, middle stream and downstream). Physico-chemical parameters (pH, Temperature, Total dissolved solids (TDS), electric conductivity (EC)) were measured for each sample. Isolation of *Vibrio* spp. was done using membrane filtration technique, followed by pre-enrichment of the bacteria in Alkaline Peptone Water after which the bacteria was cultured onto Thiosulfate Citrate Bile Salts (TCBS) selective agar. Presumptive *Vibrio* isolates where identified using a series of biochemical identification techniques which included: Gram staining, Oxidase test and API 20 E Identification strips. DNA was extracted from all samples which were identified as *Vibrio* spp by the API 20 E strips. Two optimized multiplex conventional PCR assays were used to identify the pathogenic strain of the presumptive *Vibrio* isolates. The first PCR assay targeted 16S rRNA *(Vibrio* species*), ctxA* (cholera toxin) and *sodB* (Multiplex 1) genes and the second PCR assay targeted 16S rRNA *V. cholerae* O1 *rfb* and *V.p flae* (Multiplex 2) genes.

Results and discussion: *Vibrio cholerae* non O1 was the only prevalent strain identified over the study period. Most physico-chemical parameters of the rivers (pH, Temperature, TDS and electric conductivity were within the (DWAF 1998), (WRC 1998) and (WHO 2011) standard guideline limits.

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Conclusion: The results showed that only the non O1 serogroups *Vibrio cholerae* was prevalent in rivers in the Vhembe district of the Limpopo province of South Africa. Although this strain is not toxigenic, several studies have reported that this strain is responsible for several diarrhea outbreaks.

Keywords: *V. cholerae,* Rivers, Rural communities, Diarrhea, multiplex conventional PCR, *Vibrio* species.

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Chapter 1

GENERAL INTRODUCTION

1.1 STUDY RATIONALE

In developing countries, rural areas generally lack safe and proper water distribution systems. People living in these areas rely on natural water sources such as river, streams, unprotected fountains and boreholes for their daily household activities (Nevondo and Cloete, 1991). The use of unsafe drinking water may lead to transmission of waterborne pathogens such as *Vibrio cholerae, Aeromonas spp* and etc (Gleick *et al.*, 2002).

Vibrio cholerae which causes cholera is recognized as one of the most important diarrhea causing agents in the developing world. In 1994, cholera was reported as a re-emerging major disease, with a global increase in its incidence: cases were notified from 94 countries—this was highest number of countries ever reported in one year (World Health Organization (WHO), 1995). Between 2000 and 2003, South Africa suffered a serious cholera outbreak which affected a total of six provinces including Limpopo and approximately 128 408 cases and 391 deaths were reported during this outbreak (Mudzanani *et al.*, 2003). In 2008, a total of 26 497 cases and 1 518 deaths due to cholera were reported in Zimbabwe. This outbreak spread to neighbouring countries like South Africa and Botswana. In South Africa the outbreak was reported in some rural parts of Vhembe district (WHO, 2008). Between 2010 and 2012 Haiti experienced a major outbreak of cholera, a total of 534,647 cases, 287,656 hospitalizations, and 7,091 deaths were reported (Centers for Disease Control and Prevention, 2012). Studies on cholera outbreaks in Zimbabwe indicated

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that the principal cause may be the lack of access to safe drinking water, improper sanitation and poor hygienic conditions (Thornycroft, 2008).

Vibrio cholerae have been subdivided into O1, O139 and non O1 serogroups. *Vibrio cholerae* belonging to O1 serogroup is the most pathogenic strain responsible for various epidemics worldwide and have become pandemic in some parts of Asia (Nair *et al.*, 1994; Bhattacharya *et al.*, 1993). *V. cholerae* O139 serogroup was discovered late 1992 and found to be a causative agent of cholera outbreaks occurring in Madras, India on the year (Cholera Working Group, 1993). The non O1 and non O139 *V. cholerae* serogroups are generally not known to cause epidemics, However the Centers for Disease Control and Prevention (CDC) reported that *Vibrio cholerae* non O1 has become one of the most commonly reported group of *Vibrio* bacteria. Nearly 40 cases of non-O1 *Vibrio cholerae* were reported each year since 2000 (CDC, 2012). Recent studies have also shown that human diarrheal episodes in many countries, such as India, are due to *V. cholerae* non-O1 (Dalsgaard *et al.*, 1995). Other studies of diarrheal cases done in Thailand showed that the prevalence of *V. cholerae* non-O1 was comparable to that of *V. cholerae* O1 (Dalsgaard *et al.*, 1999).

Other known pathogenic *Vibrios* spp includes *V.parahaemolyticus*, *V.mimicus, V. Vulnificus* and *V. fluvialis*. These bacteria are known to cause various diseases ranging from severe gastroenteritis; some infect open wounds and cause septicaemia (Hlady *et al.*, 1993).

Therefore this study is aimed at assessing the prevalence of *V. cholerae* and other *Vibrio* species in surface water (rivers) in rural communities within the Vhembe district of South Africa.

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1.2 SIGNIFICANCE OF THE STUDY

Developing countries like South Africa still suffer from scarcity of proper water supply especially in the rural parts such as the Vhembe district (Nevondo and Cloete, 1991). During 2008, the Vhembe district of the Limpopo had a huge outbreak of cholera due to cross border contamination linked to the outbreak in Zimbabwe. Several people were affected by gastroenteritis and some died as a result (WHO, 2008).

People living in rural communities of the Vhembe district rely primarily on surface waters including rivers, streams, fountain and dams for drinking and other domestic purposes which are unimproved water sources that may lead to transmission of waterborne pathogens such as *V cholerae*. Although no outbreak in the Vhembe district has occurred since 2008, the environmental conditions can change again to become favourable for another cholera outbreak. Due to this, the microbiological assessment of surface water is important in order to stop the potential route of transmission.

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1.3 OBJECTIVES OF THE STUDY

1.3.1 General objective

- To determine the prevalence of pathogenic *Vibrio cholerae* and other *Vibrio* species in surface water (different rivers) within the rural areas of the Vhembe District located in the Venda region of South Africa.

1.3.2 Specific objectives

- To isolate *Vibrio* spp. from drinking water using the membrane filtration technique;
- To identify isolated *Vibrio* spp. using traditional culture methods and biochemical identification techniques;
- To investigate the pathogenecity of the identified *Vibrio* spp. using an optimized multiplex PCR detection methods.

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Chapter 2

LITERATURE REVIEW

2.1 GENERAL INTRODUCTION

Vibrio cholerae belongs to the family Vibrionaceae in the genus *Vibrio*, it is a Gram-negative, Oxidase-positive, non-spore-forming curved rod, about 1.4–2.6mm long, facultative anaerobe capable of respiratory and fermentative metabolism; it exists as free-living organism or bound to a variety of substrates, including suspended mineral particulates, plants and the exoskeleton of zooplankton (Fraga *et al.*, 2007; Jores *et al.*, 2003; Hervio-Heath *et al.*, 2002; Baumann *et al.*, 1984). Almost 200 serotypes of *V. cholerae* have been identified; however, Cholera epidemics have mostly been associated with the toxigenic serogroups *V. cholerae* O1 and also O139 (Kaper *et al.*, 1995).

Vibrio cholerae O1 serogroups are those that agglutinate to "O" antiserum on the basis of biochemical tests (as shown in Figure 2.1). They have been subdivided into Ogawa and Inaba serotypes as well as El Tor and classical biotypes. Strains that are identified as *V. cholerae* but that do not agglutinate with "O" antiserum are generally termed non-O1 *V. cholerae*. Studies have showed that non-O1 strains are only occasionally isolated from cases of diarrhoea (Ramamurthy *et al.*, 1993). Recent studies are showing an increment in the occurrence of non O1 serogroups isolated from clinical samples of diarrhea patients. *V. cholerae* non-O1 has also been isolated from extra intestinal infections, from wounds, and from the ear, sputum, urine, and cerebrospinal fluids (Morris and Black, 1985).

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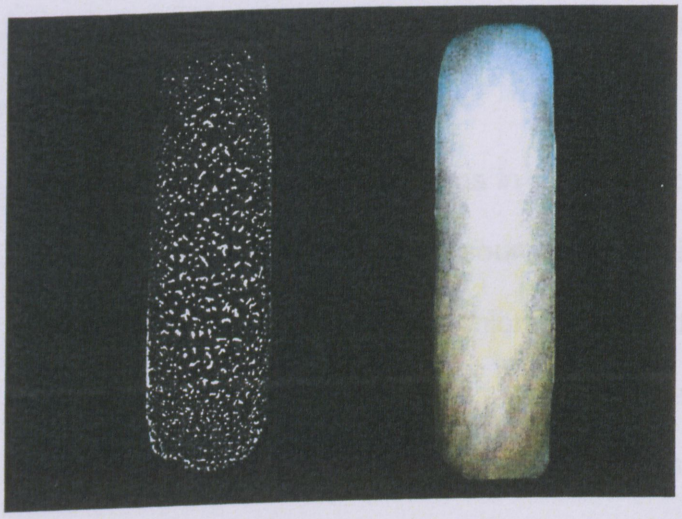

Figure 2.1: Agglutination test ; *V. cholerae* O1 serogroup shows agglutination to "O" antiserum (left) *V. cholerae* non O1 serogroup does not show agglutination (right).(**Taken from** *Centers* for Disease Control and Prevention **Laboratory Methods lab manual)**

Vibrio cholerae are generally abundant in marine environments such as river, sea, lake and dam (Thompson *et al.*, 2004). In previous studies, *Vibrio cholerae* has been isolated from surface water (Fraga *et al.*, 2007) as well as in brackish water (Colwell *et al.*, 2004). A study conducted by Percival (2004) showed that *Vibrio cholerae* can survive for contradictory periods of time in marine environment. Table 2.1 is indicative of the period in which *Vibrio cholerae* can survive.

Table 2.1: Survival period of *Vibrio cholerae* in different marine environments

Type of water	Days	Temperature	Reference
Polluted River water	26 – 39	22 – 28^{0}C	Patel *et al.*, 2004
Well water	1 – 20	20 – 30^{0}C	Pandit *et al.*, 1967
Dechlorinated water	31	4^0 C	Feachem *et al.*, 1981

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Patel et al (2004) showed that *Vibrio cholerae* could survive for up to 39 days in polluted river water at 22 – 28^{0}C.

Due to the lack of portable water systems in rural developing areas, rivers are one of the most important and frequently used sources of drinking water. This implies that if proper hygienic steps such as boiling, chlorination (adding bleach) are not followed prior to consumption of these untreated water sources, outbreaks of waterborne diseases may occur. Another study has shown that infection by *Vibrio cholerae* is commonly of environmental origin (Morris, 1990). Therefore there is a potential health risk; resulting in contraction of waterborne diseases such as cholera (WHO, 2005).

Vibrio cholerae is a major health concern in the developing world such as Africa where there is inadequate water quality. WHO reported a total of 95 560 cases in Africa, accounting for 94% of the global total of officially notified cholera cases (WHO, 2005). In 2003, South Africa suffered a major cholera outbreak which affected six of the nine provinces; 128408 cases and 391 deaths were reported (Mudzanani *et al.*, 2003).

Other *Vibrio* species such as *V. parahaemolyticus*, *V. mimicus, V. Vulnificus* and *V. fluvialis* may be associated with human infections (WHO, 2003). However *V. cholerae* still remains the most important species within the genus as it is a causative agent of cholera which continues to cause many epidemics and several pandemics resulting in millions of deaths worldwide (Kaper *et al.,* 1995; Albert *et al.,* 1994; Blake, 1994).

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2.2 PHYSICO-CHEMICAL PARAMETERS AS DRIVING FACTORS *OF Vibrio* SPECIES OCCURRENCE

Vibrio species are ubiquitous in warm oceans. Changes that occur in the density and diversity of the *Vibrio* community may be correlated with fluctuations taking place in the physico-chemical parameters such as temperature and pH (Thompson *et al.*, 2004). As a consequence of these external factors, the abundance of the *Vibrio* population, exhibits a complex seasonal distribution which is driven by fluctuations in physiochemical parameters such as temperature and pH. A study by (Thompson *et al.,* 2003) reported that *Vibrio* species were abundant in warm water temperatures (>15^{0}C). According to the Water Research Commission (WRC, 1998) standard guideline limit, water temperature should be below 25 ^{0}C.

pH of water can be used to define the measure of hydrogen ions concentration in water. It ranges from 0 to 14. The pH of 7 is considered neutral, pH<7 as acidic while pH>7 is basic. According to (DWAF, 1998) water quality standards, pH should not exceed 6.0 to 9.0. Studies have shown *Vibrios* to be basophilic meaning, they tolerate alkaline environments that kill most intestinal commensals, but are sensitive to acidic pH<6 (Finkelstein *et al.*, 1992). Other physico-chemical parameters such as electric conductivity and TDS have been reported to be important especially when evaluating water quality. Electrical conductivity (EC) is used to measure the ability of water to conduct electricity and may be used to indicate the salinity of the water samples Department of Water Affairs and Forestry standard guideline entails that electric conductivity should not exceed 700µS/cm (DWAF, 1998). EC does not have a direct impact on human health; however water with high EC value may be corrosive to metal (Rahman *et al.,* 2015)

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Total Dissolved Salts (TDS) are a measure of the amount of various inorganic salts that are dissolved in the water, this include carbonate, chloride, nitrate, magnesium, calcium and other ions (DWAF, 1996). Total Dissolved Solids (TDS) not to be >1000mg/L (DWAF, 1998).

In a study done by (Nevondo and Cloete, 1999), it was indicated that surface water may be polluted in the upstream (point situated in the opposite direction from which a stream or river flows) and even downstream (point situated in the direction in which the river flows) due to human activities (such as washing of clothes/cars, bathing, and disposal of waste materials as well as open defecation) and/or animal activities.

The pollution that occurs on the upstream of the river is likely to spread towards the middle stream and the downstream due to the flow of water. Open defecation by human and animal excretion in drinking water sources such as rivers are of major concern since they contribute to the faecal oral route on the transmission of bacteria such as *Vibrio cholerae, E. coli, Aeromonas* spp and others.

2.3 EPIDEMIOLOGY OF CHOLERA WORLDWIDE AND IN SOUTH AFRICA

The epidemiology of cholera has always been of concern because of its ability to rapidly spread up to a global scale by means of pandemics (Table 2.2) (Hunter, 1997). In the 19th century, cholera spread from Ganges delta, which was suspected to be the source, to a world wide scale and then, the epidemic receded in South Asia.

There have been six cholera pandemics which affected Africa, Europe and the Americas, claiming millions of lives (WHO, 2008). In 1961, Asia experienced the

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beginning of the seventh pandemic (Lan and Reeves, 2002). The pandemic reached South Africa in 1991, the same year marked the beginning of the eighth cholera pandemic reported to be the first ever pandemic caused by a non O1 *cholerae* strain. The pandemic occurred in India following its spread in other Asian countries including Bangladesh, China, Nepal, Thailand and Malaysia (Hunter, 1997).

V. cholerae non-O1 strains exist in abundance in the aquatic environment. However, recent studies have concluded that considerable incidence of human diarrheal episodes in many countries, including India, is due to *V. cholerae* non-O1 and non-O139 (Dalsgaard *et al.*, 1995). Studies of diarrheal cases in Thailand between 1993 and 1995 revealed that the prevalence of non-O1 *V. cholerae* was comparable to that of O1 *V. cholerae* O1 (Dalsgaard *et al.*,1999).

The seventh cholera pandemic that occurred in 1991 was believed to have spread to South Africa. The country has long been considered to be at risk of cholera due to scarcity of safe water supply as early as 1971 (Igbinosa and Okoh, 2008). The first cases were recorded in 1973 (Mugero and Hoque, 2001) in mines located around the Limpopo province. In August 2000, an outbreak of cholera was reported in a rural area of Ndabayake in the Kwazulu Natal (KZN) province. Thereafter, the epidemic spread to five other districts of KZN and also other provinces in the country, namely Limpopo, Mpumalanga, Eastern Cape, Gauteng and North West (Hoque and Worku, 2005).

In July 2001, 106 389 people were infected and 229 died, however epidemics subsequent to 2001 declined in magnitude and duration. Between August 2001 and December 2002, a total of 18 224 cases of cholera and 122 deaths were reported to the National Department of Health (NDoH, 2001). By September 2003, a total of 3 795 cases and 40 deaths were reported to the NDoH (Mudzanani *et al.*, 2003).

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However, in December 2008, 26 497 cases and 1 518 deaths were reported in Zimbabwe while in the same year 1 279 cumulative cases and 12 deaths were reported in South Africa with the majority of the cases (1 194) found in the Limpopo province neighbouring Zimbabwe.

Table 2.2: Summary of the 8 pandemics of cholera (adopted from (Hunter, 1997))

Pandemic	Organism	Origin	Duration	Affected regions
First	O1 Classical	Bangladesh	1817-1823	India, SE Asia, Middle East, East Africa
Second	O1 Classical	Bangladesh	1826-1851	India, SE Asia, Middle East, Africa, Europe, Americas
Third	O1 Classical	Bangladesh	1852-1859	India, SE Asia, Middle East, Africa, Europe, Americas
Fourth	O1 Classical	Bangladesh	1863-1879	India, SE Asia, Middle East, Africa, Europe, Americas
Fifth	O1 Classical	Bangladesh	1881-1896	India, SE Asia, Middle East, Africa, Europe, Americas
Sixth	O1 Classical	Bangladesh	1899-1923	India, SE Asia, Middle East, Africa, Europe, Americas
Seventh	O1 El Tor	Indonesia	1961-	India, SE Asia, Middle East, Africa, Europe, Americas
Eighth	O139	India	1991-	India, SE Asia

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2.4 THE PATHOGENESIS AND VIRULENCE FACTORS OF *Vibrio* SPECIES

Pathogenesis of *Vibrio cholerae*

Infection by *V. cholerae* is caused by ingestion of contaminated water or food. After the passage of the contaminated material through the acid barrier of the stomach, the bacteria begins to colonize the epithelium of the small intestine by means of the toxin-coregulated pili (Taylor *et al.*, 1987) and possibly other colonization factors such as the different haemagglutinins, accessory colonization factor, and core-encoded pilus, all of which seem to play a role. cholera enterotoxin (CT) produced by the adherent *Vibrios* is secreted across the bacterial outer membrane into the extracellular environment and disrupts ion (Na^- Cl^+) transport by intestinal epithelial cells.

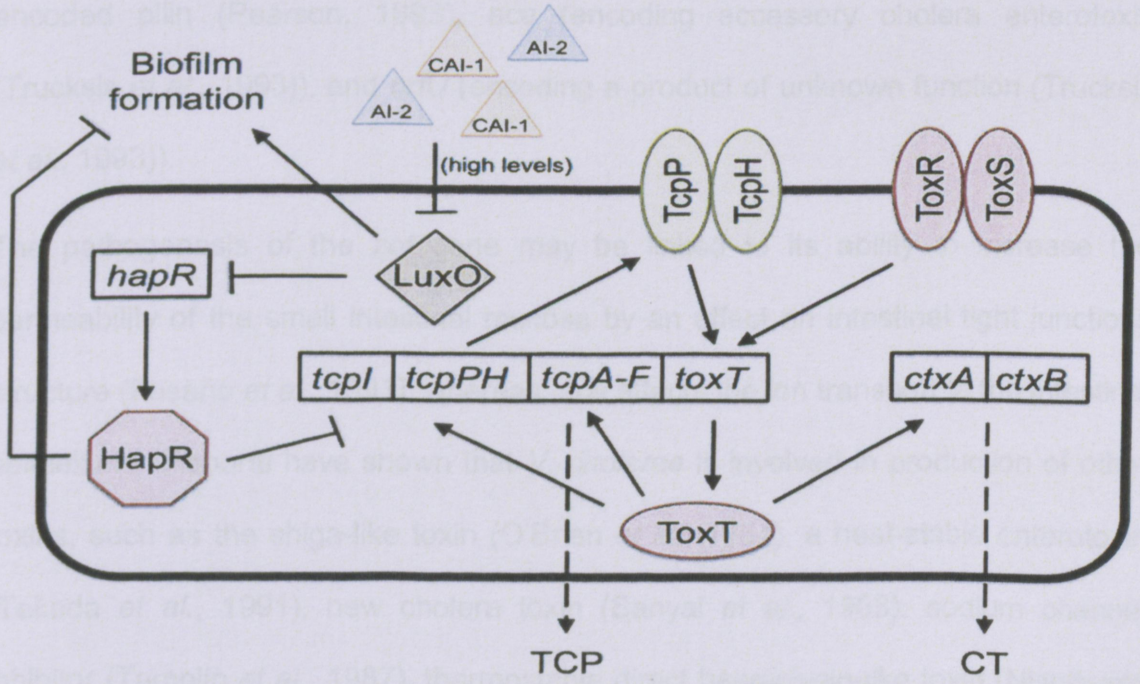

Figure 2.2: Different genes of *Vibrio cholerae* (who adapted it from images by Dr. Jun Zhu (Harvard Medical School) and Dr. Douglas Higgins (Princeton University)) (*accessed April 2016*).

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The subsequent loss of water and electrolytes leads to the severe diarrhoea which is characteristic of cholera. The cholera toxin structure has different subunits, namely: A subunit and 5 smaller identical B subunits (Finkelstein and Lospalluto, 1969). The A subunit acts intracellularly and possesses a specific enzymatic function; it raises the cellular level of Camp and thereby resulting in change of the net absorptive tendency of the small intestine to one of net secretion. The B subunit is known to serve in the binding of toxin to the eukaryotic cell receptor, ganglioside GM1. Neuraminidase is an enzyme which enhances the binding of CT to epithelial cells.

Studies have indicated that virulence cassette (dynamic 4.5-kb core region) is present in the toxigenic *V. cholerae* O1. The Virulence cassette comprises of at least six genes, which include *ctxAB* (encoding the A and B subunits of CT), *zot* (encoding zonula occludens toxin (Fasano *et al.*, 1991)), *cep* (encoding core-encoded pilin (Pearson, 1993), *ace* (encoding accessory cholera enterotoxin (Trucksis *et al.*, 1993)), and *orfU* (encoding a product of unknown function (Trucksis *et al.*, 1993)).

The pathogenesis of the *zot* gene may be linked to its ability to increase the permeability of the small intestinal mucosa by an effect on intestinal tight junctions structure (Fasano *et al.*, 1991), whereas, *ace* affects the ion transport in the intestinal epithelium. Reports have shown that *V. cholerae* is involved in production of other toxins, such as the shiga-like toxin (O'Brien *et al.*, 1984), a heat-stable enterotoxin (Takeda *et al.*, 1991), new cholera toxin (Sanyal *et al.*, 1983), sodium channel inhibitor (Tamplin *et al.*, 1987), thermostable direct haemolysin-like toxin (Nishibuchi *et al.*, 1992), and a cell-rounding cytotoxic enterotoxin known as the non-membrane-damaging cytotoxin (Saha and Nair, 1997; Saha *et al.*, 1996). One of the most important distinctions between the toxigenic *V. cholerae* O1 and non O1 serogroups

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is the presence of a virulence cassette on the O1 serogroup, which is absent in the non O1 serogroup (Trucksis *et al.*, 1993).

There is evidence for horizontal transfer of "O" antigen among *V. cholerae* serogroups. This horizontal gene transfer between O1 and non-O1 strains is a phenomenon suspected to cause evolution leading to non-toxigenic strain acquiring toxigenecity, thus the emergence of *V. cholerae* O139 as a new serogroup was evident of probable of evolution (Bik *et al.*, 1995). Other studies have shown that some of the *Vibrio cholerae* non O1 produce heat stable toxin NAG-ST (coded by the *stn/sto* gene), hemolysin (hlyA) and the pore-forming toxin RtxA which may be contributory to the pathogenesis of this species (Bidinost *et al.*, 2004). Recently, there has been an increase in occurrence of the non-O1 serogroup as well as outbreaks of diarrhoea associated with *V. cholerae* serogroups; O10 and O12 (Rudra *et al.*, 1996; Dalsgaard *et al.*, 1995).

This has brought more attention to the *V. cholerae* serogroups; non-O1 (Karaolis *et al.*, 1995).

Pathogenesis of other *Vibrio* species

V. parahaemolyticus causes gastroenteritis and has been associated with virulent factor encoding the thermo-stable direct hemolysin (*tdh*) and the thermo-stable direct hemolysin-related hemolysin (*trh*) genes (Igbinosa EO and Okoh A. 2010). These virulent factors have also been associated with *V. mimicus* (Igbinosa EO and Okoh A, 2010). Gastroenteritis caused by *V. mimicus* is usually linked to consumption of contaminated seafood. *V. mimicus* can also cause cholera like diarrhea when carrying genes that encode cholera toxin (MacEachern D *et al.*, 2010).

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Virulence factors of *V. fluvialis* include; enterotoxin-like substances, lipase, proteases, cytotoxins and hemolysin. Gastroenteritis caused by this species is similar to that caused by *V. cholerae*, except for the frequent occurrence of blood in stools. *V. fluvialis* has also been associated with wound infection leading to primary septicemia in immunocompromised individuals (Igbinosa EO and Okoh A. 2010; Tall *et al.*, 2003).

The primary virulence factor for *V. vulnificus* is the extracellular Capsule Polysaccharide (CPS), which allows bacteria survival in the human host. *V. vulnificus* bacteria can cause infections resulting in fulminant primary septicaemia (often with necrotizing cutaneous lesions) associated with a high mortality rate reaching up to 61% (Wallace *et al.*, 1999; Rippey, 1994; Weber *et al.*, 1994)

2.5 ISOLATION AND IDENTIFICATION OF *Vibrio* SPECIES

Vibrio requires sodium chloride (NaCl) or has its growth being stimulated by its addition. All members of the genus *Vibrio* except *V. metschnikovii* and *V. gazo genes* are oxidase positive and reduce nitrates to nitrites (CDC, 2016). Identification of *V. cholerae* O1 requires only serologic confirmation of the presence of O1 serotype antigens, however, a more complete characterization of the organism may be required and may include various biochemical tests and the determination of other characteristics.

The various methods as listed in Table 2.3 include:

- Traditional methods
- Molecular methods
- Immunological methods

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Table 2.3: Summary of methods used in isolation and detection of *V. cholerae* (CDC, 2016)

Methods	Tests
Isolation and identification of V. cholerae using traditional methods	Conventional bacteriological culture method for V. cholerae
Molecular methods for detection and identification of V. cholerae	Direct PCR, Multiplex PCR & Colony blot hybridization with labeled DNA or RNA hybridization probes
Direct detection and enumeration of V. cholerae using immunological methods	Direct fluorescent antibody-direct viable count (DFA-DVC), Indirect fluorescent antibody (IFA) method

2.5.1 Traditional culture method

Traditional culture based techniques for isolation of *Vibrio cholerae* involve pre-enrichment of samples using selective enrichment broth namely alkaline peptone water (APW) along with nutrient enriched modified alkaline peptone water such as egg- APW and blood-APW (Percival *et al*, 2004). Another selective enrichment media used is TTP media which has been reported to have a greater isolation to *Vibrio cholera* as compared to APW media, the limitation of TTP is that it requires a longer incubation period than APW media (Kay *et al.*, 1994).

After enrichment in APW, the samples are plated onto Thiosulfate Citrate Bile Salt (TCBS) selective agar, which contains 1% NaCl, and has an alkaline pH of 8.6. These factors help suppress the growth of unwanted bacteria such Gram positive bacteria of the family enterobactericae under genus Pseudomonas. On TCBS agar *Vibrio* colonies appear yellow, flat and 2-3 mm in diameter.

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Vibrio can be well isolated and identified using culture methods; however there are shortcomings which are linked to the viable but non-culturable (VBNC) state of *Vibrio.* Previous research has revealed the existence of a dormant state in which *V. cholerae* can enter in response to unfavourable conditions such as extreme temperature, salinity and nutrient deprivation (Oliver, 2005). This stage is known as the viable but non culturable (VBNC). The bacteria in this state fail to grow in routine bacteriological media on which they would normally grow and form colonies; however they remain metabolically active and capable of culturability (Olivier, 2005; Vora *et al.*, 2005). Other shortcomings may be that culture methods are unable to show distinction between toxigenic and non-toxigenic *Vibrio cholerae,* are time consuming and labour- intensive (Park *et al.*, 2013).

2.5.2 Biochemical tests: Oxidase and API 20E tests

Biochemical confirmation tests such as Oxidase test, and API 20 E, can be used in identifying presumptive *Vibrio* isolates

Oxidase test

Oxidase strips or discs impregnated with tetramethyl-p-phenylenediamine (TMPD) are used in this test. A fresh colony grown in a non-carbohydrate containing media (eg. nutrient agar) is placed on the oxidase strip or disc and colour change is observed. The colour change is present in bacteria that produce cytochrome C oxidase such as most *Vibrio* species with the exception of *V. metschnikovii* and *V. gazo* (Huq *et al.*, 2006).

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API 20E test

The API 20 E is a fast identification system which combines different conventional tests. This test allows identification of Gram negative enterobacteriaceae and non-enterobacteriaceae. API 20 E test is comprised of 20 small reaction tubes containing dehydrated substrates. The test is done using fresh microbial culture of presumptive isolate (Huq *et al.*, 2006).

2.5.3 Molecular techniques used in identification of *Vibrio spp*

In contrast to traditional culture methods, molecular technique such as Polymerase Chain Reaction (PCR) have several advantages; they are sensitive, rapid, very selective and less tedious (Le Roux and Van Blerk, 2011). PCR is a technique in which DNA is amplified using the enzyme DNA polymerase. There are three steps in PCR namely: Denaturation were DNA template is heated to 94° C breaking down the weak hydrogen bonds that hold DNA strands together, allowing the strands to separate creating single stranded DNA. This is followed by the annealing step at temperature between 50-70° C (60° C for *V. cholerae*) which allows the primers to bind (anneal) to the complementary sequence of the single stranded DNA template. Extension is the final step, it occurs at temperature of 72° C, which is the optimal temperature for DNA polymerase. The general function of DNA polymerase is to extend the primers, adding nucleotides onto the primer in a sequential manner, using the target DNA as a template (Kramer and Coen, 2001). PCR can be performed in uniplex PCR targeting one gene or multiplex targeting multiple genes. PCR can be performed in combination with gel electrophoresis, probe hybridization or real time fluorescence for detection (Liu *et al.*, 2008).

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2.5.4 Immunological methods used in detection of *Vibrio species*

Traditional culture methods are not effective in detecting *V. cholerae* which have entered the Viable but non culturable (VBNC) state. Direct Fluoresence Antibody-Direct Viable Count (DFA-DVC) Immunological technique has the ability to distinguish between viable cells from VBNC cells. It is a two-step method. Step1 involves incubation of samples in yeast extract media (supplemented with nalidixic acid) after which viable cells become enlarged and elongated (Kogure *et al.*, 1979).

Following that step, smears prepared from this suspension on a glass slide are stained with fluorescently labelled monoclonal antibody raised against the "A" factor of *V. cholerae* O1 Lipopolysaccharide, which reacts with both Ogawa and Inaba (Hassan and Bernstein, 1994).

2.6 SUMMARY OF LITERATURE REVIEW

Vibrio cholerae are gram negative, Oxidase-positive, facultative anaerobic, rod shaped bacteria (Hervio-Heath *et al.,* 2002). They have been described as important causes of mortality worldwide. They remain a major health concern in rural areas of developing countries as opposed to developed countries. This may be due to the scarcity of water and safe water distribution systems in rural areas which often leads to most rural residences relying on surface water for drinking and other domestic purposes (Bhagwan *et al.*, 2008). Consequently surface water is a natural habitat for waterborne pathogens such as *Vibrio cholerae* and *E. coli*. Previously outbreaks of cholera where exclusively linked to serogroups O1 and O139 but studies have shown outbreaks linked to non O1 therefore indicating the importance of *V. cholerae* non O1/139 in causing potential health risk. Other pathogenic *Vibrio* species known

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are: *V. parahaemolyticus*, *V. mimicus, V. Vulnificus* and *V. fluvialis* (WHO, 2003). However *V. cholerae* still remains the most important species within the genus as it is a causative agent of cholera which continues to cause many epidemics and several pandemics resulting in millions of deaths worldwide (Kaper *et al.,* 1995; Albert *et al.,* 1994; Blake, 1994).

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Chapter 3

MATERIALS AND METHODS

3.1 STUDY SITE

The Vhembe District is located in the northern part of the Limpopo province It shares borders with Zimbabwe and Botswana in the north-west and Mozambique in the south-east through the Kruger National Park. Vhembe district is approximately 25 597km and is one of the five districts found in the Limpopo province with a population of about 1 472 615 with a total number of 821 communities. There are 4 Local Municipalities within the Vhembe district municipality, namely: Musina, Mutale, Makhado and Thulamela. Figure 3.1 shows a map of Vhembe district showing the water sources and municipalities.

Rivers in the rural areas of Limpopo province within the Vhembe district were used as study sites for this project. Some of the rivers include the ones highlighted by a red triangle in Figure 3.1 which are; Dzindi, Mutshundudi, Mutale & Sambandou. Other rivers that were sampled included, the Luvuvhu (Albasini, Karringmelkspruit, Lutanandwa, Piesanghoek, Springfield & Tshino), Madadzhe, Mbwedi and Tshinane rivers. These rivers are shared by both human and animals with people collecting water from the river while animals (such as cattle) were seen walking in the river; other people were washing clothes and some even bathing in these rivers.

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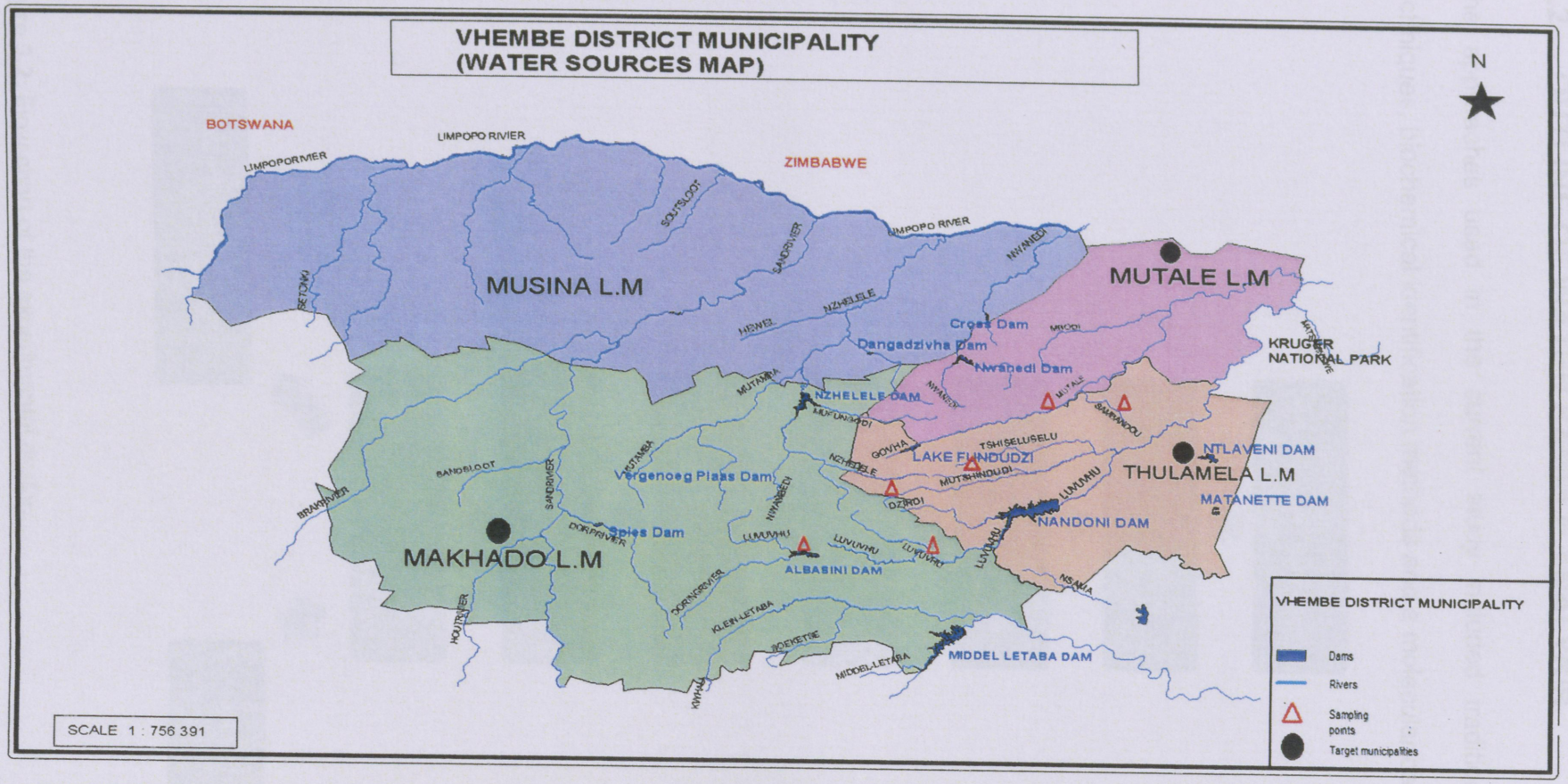
The Vhembe District Map



DRAFT IDP REVIEW FOR 2014/15 MUTALE LOCAL MUNICIPALITY

Figure 3.1: Map of the Vhembe district. The red triangle on the map represent some of the rivers sampled, the black dot represent the three municipalities in which the rivers were situated

3.2 OUTLINE OF WATER SAMPLE ANALYSIS

The approaches used in the current study included traditional culture based techniques, biochemical identification methods and a molecular based technique.

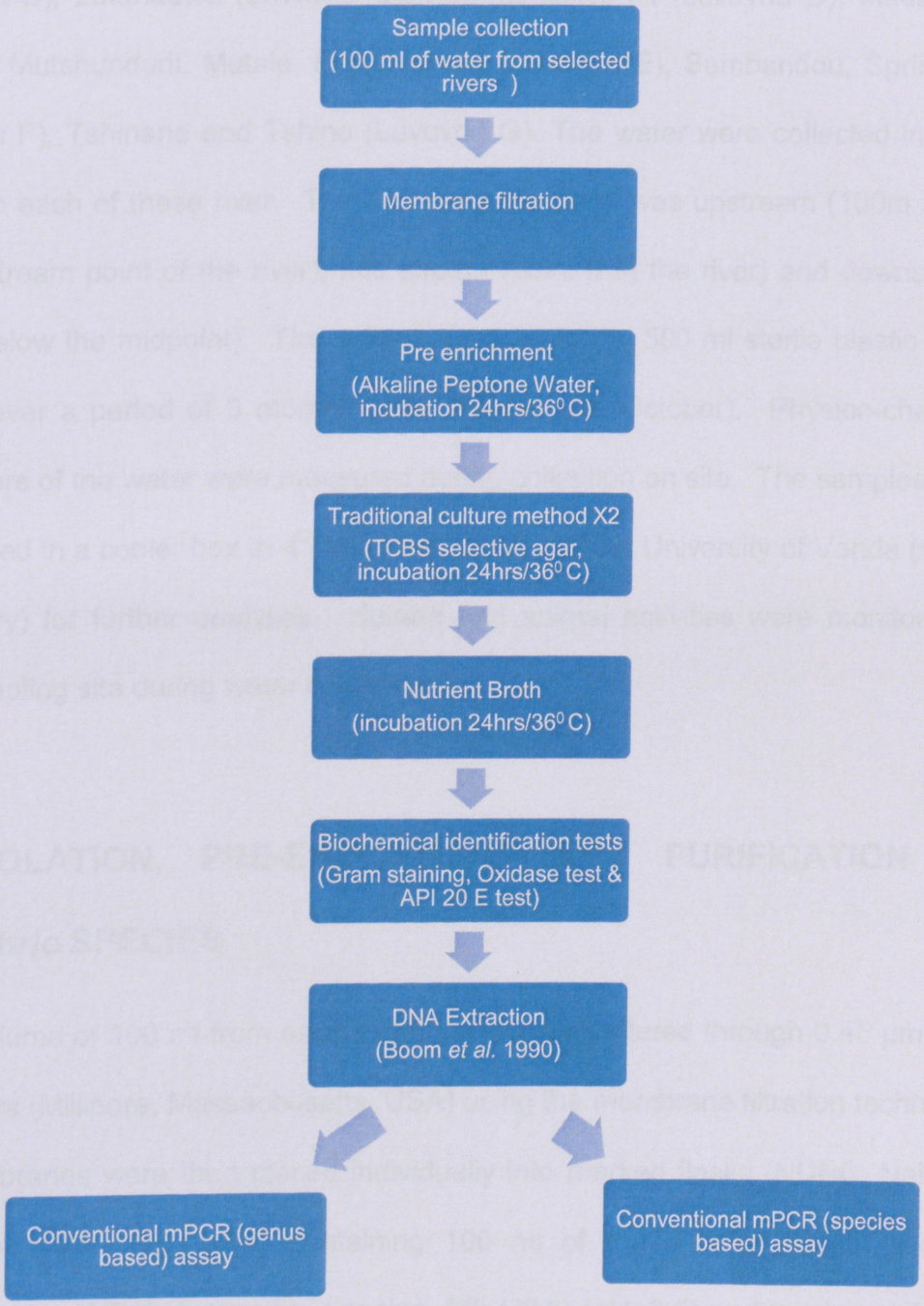


Figure 3.2: Flow chart of the experimental design

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3.3 SAMPLE COLLECTION

A total of 120 water samples were collected in 2015 (once-off sample collection) from 6 rivers and 7 sites of the Luvuvhu River, namely: Albasini (Luvuvhu A), Dzindi (Luvuvhu B), Lutanadwa (Luvuvhu C), Karringmelkspruit (Luvuvhu D), Madadzhe, Mbwedi, Mutshundudi, Mutale, Piesanghoek (Luvuvhu E), Sambandou, Springfield (Luvuvhu F), Tshinane and Tshino (Luvuvhu G). The water were collected in three points on each of these river. The first sampling point was upstream (100m above the midstream point of the river), mid stream (point 0 in the river) and downstream (100m below the midpoint). The water was collected in 500 ml sterile plastic water bottles, over a period of 3 months (July, August and October). Physico-chemical parameters of the water were measured during collection on site. The samples were then placed in a cooler box at 4^0C and transported to the University of Venda (Water Laboratory) for further analyses. Human and animal activities were monitored at each sampling site during water collection.

3.4 ISOLATION, PRE-ENRICHMENT AND PURIFICATION OF *Vibrio* SPECIES

A total volume of 100 ml from each water sample was filtered through 0.45 µm pore sized filters (Millipore, Massachusetts, USA) using the membrane filtration technique. The membranes were then placed individually into marked flasks (NUNC, Nalgene Nunc, Rochester, NY; USA) containing 100 ml of the enriching broth alkaline peptone water (APW; Acumedia, Lansing, MI; USA) (pH=8.5) and incubated for 18 hrs at 36°C (*Esco*, Esco Technologies Inc, Horsham, PA; USA). Hundred (100 µl) of the broth was taken from the top aerobic surface layer in each flask using a sterile

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pipette (Gilson Inc, Middleton, WI; USA) and placed onto 90 mm plates (Plastpro scientific, Edenville, Gauteng, SA) containing TCBS selective agar (Acumedia, Lansing, MI; USA). TCBS selective media has high pH (8.5-9.5) which suppresses growth of intestinal flora other than *Vibrio* spp (Koneman and Winn, 2006). The bile salts inside the media inhibit growth of Gram-positive microorganisms while the NaCl which is incorporated into the medium provides the optimum growth and metabolic activity needed for the halophilic *Vibrio* spp (Koneman and Winn, 2006). On TCBS agar, *Vibrio* colonies appear yellow, flat and 2-3 mm in diameter. The plates were incubated at 36°C for 18 – 24 hrs. Representative sample of yellow colonies were purified through sub-culturing the presumptive *Vibrio* isolates twice using the streaking plate method: first on TCBS media and then on Nutrient broth (Acumedia, Lansing, MI; USA). Both times the plates were incubated at 36°C for 18 – 24 hrs. Pure colonies were then tested using the Gram staining technique (Lasec, Johannesburg, SA) and all Gram-negative presumptive isolates were streaked onto Nutrient agar plates for further confirmational biochemical identification tests.

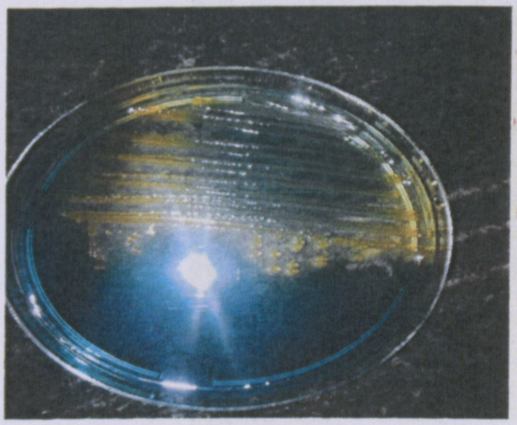

Figure 3.3: *Vibrio* colonies grown TCBS selective media (Masindi, 2015)

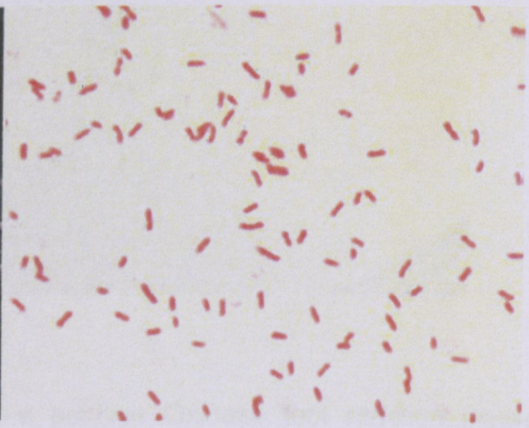

Figure 3.4: Gram negative, rod shaped bacteria viewed microscopically

(Picture taken from http://www.nlm.nih.gov/cgi/mesh/2013/MB_cgi?mode=&index=14126)

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3.5 BIOCHEMICAL IDENTIFICATION TESTS

3.5.1 Oxidase Test

The Oxidase test was performed using commercially available oxidase strips (Mast Diagnostics, Liverpool, UK) according to the manufacture's protocol. A pure colony was isolated from 24 h Nutrient agar plate and placed onto an oxidase impregnated strip using a sterile swab. After 10 seconds, the colour was monitored for any change. The oxidase positive isolates turned purple in colour (Figure 3.5) and were therefore considered as potential *Vibrio* isolates. Colourless colonies were considered as oxidase negative and identified as non-enteropathogens and were not analysed further.

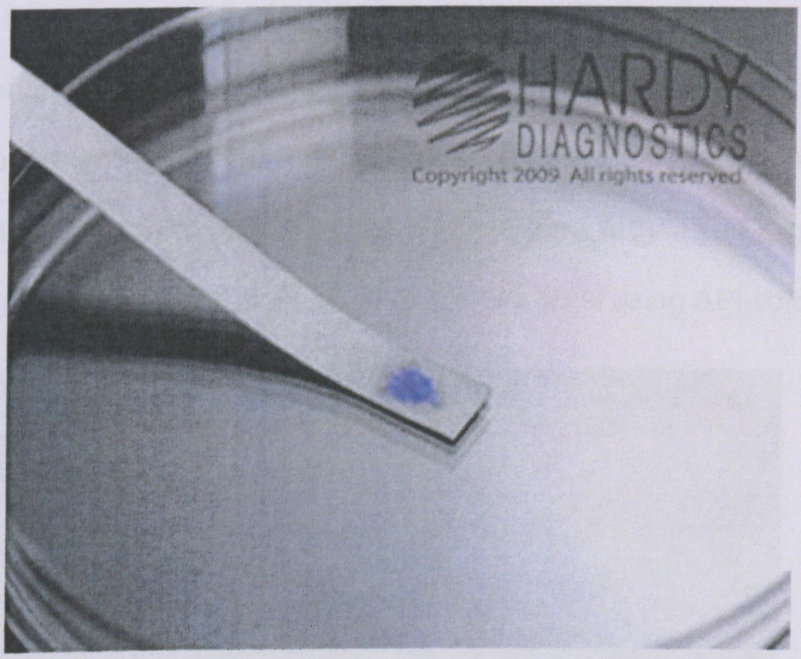

Figure 3.5: Display of a positive Oxidase test reaction (Picture taken from http://www.nlm.nih.gov/cgi/mesh/2013/MB_cgi?mode=&index=14126)

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3.5.2 Analytical Profile Index (API) 20 E Test

The API 20 E test (Bio-Merieux, Marcy l'Etoile; France) was performed according to the manufacturer's protocol. An incubation box (a tray and a lid) was used for each purified isolate and 5ml of distilled water was added onto the tray to create a humidified atmosphere before placing the API 20E test strip onto the tray following which 1 ml of bacterial suspension was prepared by adding 1 colony of the purified presumptive isolates into 1ml of distilled water and mixing. The bacterial suspension was then carefully pipetted onto the API 20 E test strip and incubated at 37°C for 24hrs (*Esco*, Esco Technologies Inc, Horsham, PA; USA). The results were recorded. Microtubules which required addition of further reagents namely (TDA - Tryptophan deamination, IND – Indole fermentation, VP – Actetoin fermentation, GLU – Glucose fermentation; Bio-Merieux, Marcy l'Etoile; France), were treated accordingly and their results were recorded after the addition of reagents. All results were read by referring to the reading table supplied by the manufacture and recorded to the total number of 7 digits code (Figure 3.6). The organisms were identified by interpreting the 7 digits code to species level using API software.

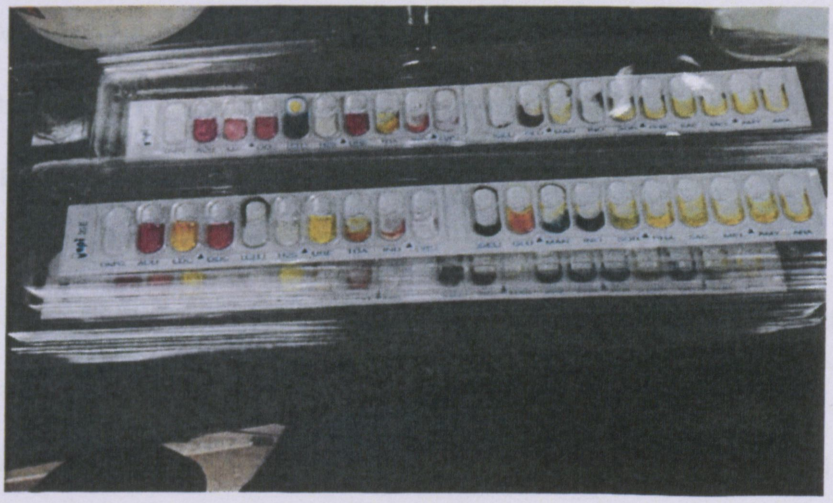

Figure 3.6: API 20 E reading after incubation (Masindi, 2015)

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3.6 DNA EXTRACTION PROCEDURE FOR THE ISOLATION OF BACTERIAL DNA

An adapted version of the protocol described by Boom et al (1990) was used. This method entails the centrifugation (13,300 rpm, 60 sec; (Spectra 24D centrifuge, Labnet International, Inc; Edison, NJ; USA) of 2 ml of enrichment broth. The resulting bacterial pellet was resuspended in 700 µl of lysis buffer, followed by incubation at 70^{0}C for 10 min. A volume of 250 µl 100% (v/v) ethanol (Rochelle chemicals and Lab equipment cc, SA) was added to the mixture and further incubated at 56^{0}C for 10 min. After incubation, 50 µl of celite solution (Sigma-Aldrich, Germany) was added followed by incubation at room temperature for 10 min. A clean, sterile spin column (Sigma-Aldrich, Germany) was placed in a sterile 2 ml microfuge tube. 500µl lysis buffer was loaded followed by centrifugation at 13300 rpm (Spectra 24D centrifuge, Labnet International, Inc; Edison, NJ; USA) for 30 sec (repeatedly until buffer and celite were separated). This process was done repeatedly until all of the lysis mixture was loaded into the column. The column was then washed twice by adding 400µl of the wash buffer, followed by centrifugation at 13,300 rpm, for 30 sec (Spectra 24D centrifuge). Two more washing steps were done using 400µl of 70% ethanol solution, following centrifugation at 13,300 rpm, for 2 min (Spectra 24D centrifuge). To ensure complete elimination of the alcohol (ethanol), the column was then transferred into a clean 1.5 ml microfuge tube (Eppendorf, Hamburg; Germany), followed by the addition of 100µl elution buffer (AE buffer; Qiagen, Hilden; Germany) and incubation for 2min at 56^{0}C. To elude the DNA from celite the solution was centrifuged at 13300 rpm, for 2 min (Spectra 24D centrifuge), after which the column was discarded. The AE buffer containing the DNA was collected in the 1.5 ml microfuge tube and stored at -80^0 C until further use.

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3.7 CONVENTIONAL MULTIPLEX POLYMERASE CHAIN REACTION (M-PCR) ASSAY

Two multiplex conventional PCR assays were optimised for the detection of toxigenic and non-toxigenic *V. cholerae* (O1 serotypes). The first multiplex conventional PCR assay was genus specific and targeted the *ctxA* (cholera toxin), 16 S rRNA and *sodB* (superoxide dismutase gene). The second assay was species specific targeting *V. cholerae* O1 (*Vibrio cholerae* O1 serogroup), *V. p flae* (*V. parahaemolyticus* species) and 16 S rRNA) genes. The PCR assays were multiplexed with the *gfp* assay, the latter serving to detect the *E. coli*-GFP gene added as a positive process internal control. The primers for the two conventional PCR were obtained from integrated DNA Technologies (Coralville, Iowa; USA) as listed in Table 3.1.

Conventional Multiplex PCR was performed using a Biorad thermal cycler (Biorad, Carlifornia; USA) in a total reaction volume of 20µl. For both the multiplex conventional PCR, each reaction consisted of 10 µl 2xQiagen m-PCR master mix (consisting of HotStartTaq DNA Polymerase, m-PCR buffer with 2 mM $MgCl_2$ and dNTP Mix); 1µl 5x Qiagen Q-solution; 2 µl of primer mix,1-5µl extracted DNA and PCR grade water. (Applied Biosystems, Foster City, California; USA). Reaction conditions for m-PCR1 were as follows:15 min enzyme activation step at 95^0C followed by 35 cycles of denaturation at 92^0C for 40 sec, annealing was at 57^0C for 60 sec and extension at 72^0C for 90 s followed by the final step of elongation at 72^0C for 7 min.

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Table 3.1: Primers used in the conventional multiplex PCR detection assays.

Primers	Sequence(5'-3')	Conc (µM)	Amplicon size(bp)	References
ctxA-F	TCCGGAGCATAGAGCTTGGA	0.3	308	Lipp *et al* 2003
ctxA-R	TCGATGATCTTGGAGCATTCC	0.3		
16S rRNA-F	CGCTTTATTGTTCGATGCGTTA	0.3	663	Tarr *et al* 2007
16S rRNA-R	ACTCGGTTATCGTCAGTTTGG	0.3		
V.pflae-F	CCTGTCCTTTTACCAGACAACCA	0.05	897	Tarr *et al* 2007
V.pflae-R	GGTCTCTCTTTTCGTTGGGATCT	0.05		
O1rfb-F	CCAGATTGTAAAGCAGGATGGA	0.3	192	Lipp *et al* 2003
O1rfb-R	GGTCATCTGTAAGTACAAC	0.9		
V.cSodB-F	CATACCAACGCCCTTATCCATT	0.1	248	Tarr *et al* 2007
V.cSodB-R	GCATGACTGGCATCCCAAAAT	0.1		
16S rRNA-F	CGCTTTATTGTTCGATGCGTTA	0.3	663	Tarr *et al* 2007
16S rRNA-R	ACTCGGTTATCGTCAGTTTGG	0.3		

For m-PCR 2, the thermal cycling profile was as follows:15 min enzyme activation step at 95^0C; 35 cycles of denaturation at 94^0C for 45 sec, annealing was at 55^0C for 45 sec and extension at 72^0C for 60 sec followed by the final step of elongation at 72^0C for 5 min. Positive, negative and no-template controls were used in every experiment.

After running the PCR using the Biorad thermal cycler (Qiagen, Germany),DNA was analyzed using a 2.5% (w/v) agarose (Whitehead scientific (Pty) Ltd) gel in TAE buffer (40nmmol^{-1}tris-acetate; 2mmol^{-1} EDTA, pH 8.3) with 0.5µgml^{-1} of the intercalating agent ethidium bromide (Whitehead scientific (Pty) Ltd, Johannesburg

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SA). The electrophoresis was conducted at $8Vcm^{-1}$ for one to two hours (Qiagen, Germany). After the gel had run after the required time, it was visualized under UV light (Gene Genius Bio-Imaging System, Syngene, Cambridge, UK). The relative size of DNA fragments was estimated by standard samples on each gel using either a 1Kb or 100bp markers (Fermentas; Waltham, Massachusetts, USA).

3.8 DATA ANALYSIS

The data obtained were entered into Microsoft Excel Spread Sheets for simple statistical analyses such as minimum, maximum and geometric mean. The results thereafter were used for comparisons to determine the prevalence of pathogenic *Vibrio cholerae* and other *Vibrio* species in rivers from the study area.

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Chapter 4

RESULTS AND DISCUSSION

4.1 PHYSICO- CHEMICAL PARAMETERS OF RIVERS

All physico-chemical parameters (pH, Temperature, TDS and Electrical Conductivity) were measured using a portable multi parameter (multi-meter MM40, Crison, Johannesburg, South Africa). The results are indicated in the tables 4.1 to 4.4.

The ideal environment for *Vibrio* species is alkaline pH above 6 (Finkelstein and Lospalluto, 1969) and temperature of 25^0C (Huq *et al.*, 2005).

4.1.1 pH of the rivers

The pH of water is known as the measure of hydrogen ions concentration in water. It ranges from 0 to 14. The pH of 7 is considered neutral, values which are lower than the neutral pH are referred to as acidic and those greater than pH-7 are referred to as basic. According to the DWAF (1998) guideline, standard pH of water should be between 6.0 to 9.0. The WHO (2011) guideline standard for pH of water is 6.5 to 8.5. In a study by (Finkelstein and Lospalluto, 1969) on the *Vibrio cholerae*, it was indicated that *Vibrios* flourish in much more alkaline environment however are sensitive to low pH and die rapidly in pH below 6.

In Table 4.1, it can be seen that pH of the river samples with low pH was within the standard guideline limits, whereas river samples with alkaline pH such as Luvuvhu A, C, D, E, F & G, occasionally had pH values beyond the DWAF (1998) and WHO (2011) guideline limits. The high alkaline pH values observed from the river water samples may be due to activities such as bathing and laundry washing which were observed in some of these rivers during sample collection. Similar pH values were

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observed in a study done by (Obi *et al.*, 2004) on Rivers within the Vhembe district. Water with alkaline pH above 8.5 may taste soapy or bitter (DWAF, 1998).

Table 4.1: Geometric mean values of pH of the rivers

Rivers	Upstream	Middle stream	Downstream
Albasini (Luvuvhu A)	8.24	9.16	9.15
Dzindi (Luvuvhu B)	7.21	7.53	7.45
Karringmelkspruit (Luvuvhu C)	8.11	9.1	9.04
Lutanandwa (Luvuvhu D)	8.81	8.92	9.14
Piesanghoek (Luvuvhu E)	7.42	7.91	9.86
Springfield (Luvuvhu F)	9.1	8.77	8.32
Tshino (Luvuvhu G)	9.05	9.09	9.15
Madadzhe	8.3	8.1	8.79
Mbwedi	6.56	6.57	6.57
Mutshundudi	7.27	7.48	6.93
Mutale	6.53	6.66	6.63
Sambandou	7.43	7.45	7.79
Tshinane	7.25	7.08	7.39

pH: 6.5-8.5; WHO standard guideline (2011) pH: 6.0-9.0 DWAF (1998)

4.1.2 Temperature of the rivers

According to the (WRC, 1998) standard guidelines, safe water temperatures should not exceed 25^{0}C. It can be shown in Table 4.2 that none of the river waters exceeded the (WRC, 1998) standard temperature limit. These waters had values varying between 17.17-24.77, well within the 15 and 30^{0}C limit which *Vibrio* can grow. The optimum temperature for growth of *V. cholerae* in the natural environment has been reported to be 25 ^{0}C (Huq *et al.*, 2005). In Bangladesh a study carried out on the occurrence of cholera showed that cholera occurred when temperatures where exceeding 18^{0}C (Huq *et al.*, 2005). In the current study the water temperature was between 17.17 and 24.77^{0}C which is ideal for the growth of *Vibrio* species

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Table 4.2: Geometric mean values of temperature (^{0}C) of the rivers

Rivers	Upstream	Middle stream	Downstream
Albasini (Luvuvhu A)	20.45	20.51	21.81
Dzindi (Luvuvhu B)	22.66	23.70	22.15
Karringmelkspruit (Luvuvhu C)	17.40	17.17	17.99
Lutanandwa (Luvuvhu D)	17.45	17.47	17.19
Piesanghoek (Luvuvhu E)	18.20	20.24	19.70
Springfield (Luvuvhu F)	20.45	20.51	21.81
Tshino (Luvuvhu G)	20.55	21.79	21.49
Madadzhe	18.48	18.95	18.49
Mbwedi	24.77	20.68	21.30
Mutale	21.74	21.92	20.67
Mutshundudi	23.42	23.25	22.41
Sambandou	24.64	24.09	24.57
Tshinane	20.07	19.36	20.30

Temperature ≤25^{0}C; (WRC (1998)) standard guideline,

4.1.3 TDS of the rivers

TDS are a measure of the amount of various inorganic salts that are dissolved in the water, this include carbonate, chloride, nitrate, magnesium, calcium and other ions (DWAF, 1996). According to the (World Health Organisation, 2011) standard guideline TDS should not be greater than 1000 mg/L. TDS of water which is greater than the recommended standard limit of >1000mg/L is considered to be unpalatable in taste and therefore is not fit for human consumption. As shown in Table 4.3 none of the river sites had TDS values above the (WHO, 2011) prescribed limit.. The overall TDS values ranged from 64.97 to 318.64 mg/L. A study by (Mohammed *et al.*, 2013) in rivers within rural Shadrah town (India) had TDS values of 406 - 694 mg/L. This shows that the water quality of both countries is were good for human consumption

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Table 4.3: Geometric mean values of TDS (mg/L) of the rivers

Rivers	Upstream	Middle stream	Downstream
Albasini (Luvuvhu A)	120.14	123.99	126.96
Dzindi (Luvuvhu B)	113.19	112.97	114.71
Karringmelkspruit (Luvuvhu C)	318.64	314.00	310.21
Lutanandwa (Luvuvhu D)	64.97	66.63	68.93
Piesanghoek (Luvuvhu E)	67.73	73.63	69.90
Springfield (Luvuvhu F)	102.26	100.60	97.97
Tshino (Luvuvhu G)	82.15	78.25	77.23
Madadzhe	219.50	180.65	204.61
Mbwedi	65.21	67.42	66.13
Mutale	76.50	79.94	82.69
Mutshundudi	103.61	100.23	103.83
Sambandou	78.25	74.31	67.78
Tshinane	111.23	114.49	125.38

TDS<1000 mg/L; WHO (2011) & DWAF (1998)

4.1.4 Electrical Conductivity of rivers

The safe water standard guideline set by DWAF for Electric Conductivity (EC) is <700 µS/cm. Electrical conductivity measures the ability of water to conduct electricity. The presence of EC gives an indication of presence of dissolved ions in water. Electric Conductivity values exceeding the standard guideline can be indicative of poor water quality and as a result may lead to water borne diseases (Mohammed *et al.*, 2013). Most of the river water did not exceed that standard limit (Table 4.4). WHO (2011) standard guideline limit for EC is 400 µS/cm. The Karringmelkspruit River point had an EC value of 486.25 µS/cm, which was beyond the (WHO (2011)) standard limit. In a study conducted in Pakistan on rivers within rural areas EC values were found to vary between 401 and 429µS/cm which is similar to the current study (Mohammed *et al.*, 2013)

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Table 4.4: Geometric mean values of EC (µS/cm) of the rivers

Rivers	Upstream	Middle stream	Downstream
Albasini (Luvuvhu A)	186.20	196.40	198.71
Dzindi (Luvuvhu B)	231.25	228.58	219.33
Karringmelkspruit (Luvuvhu C)	484.00	489.56	485.21
Lutanandwa (Luvuvhu D)	99.60	101.30	103.40
Piesanghoek (Luvuvhu E)	101.74	112.69	112.30
Springfield (Luvuvhu F)	163.20	169.10	151.74
Tshino (Luvuvhu G)	77.34	121.34	120.77
Madadzhe	328.44	268.14	410.26
Mbwedi	131.41	128.85	128.56
Mutale	140.10	142.99	139.22
Mutshundudi	177.06	170.74	175.17
Sambandou	151.78	152.33	141.26
Tshinane	211.46	233.91	242.62

Electrical conductivity<400µS/cm WHO (2011), DWAF (1998); Electrical conductivity <700 µS/cm

4.2 ISOLATION AND IDENTIFICATION OF *Vibrio* SPECIES

Presumptive *Vibrio* isolates were identified using Gram staining and Oxidase test. (113/120) 94% appeared as gram negative rod shaped bacteria. Further analysis was done on the Gram negative isolates using oxidase test method, wherein (109/120) 91% of the presumptive *Vibrio* isolates were oxidase positive. API 20 E method was then used to identify the Gram negative and Oxidase positive presumptive *Vibrio* isolates which was (109/120) 91% of the isolates. API 20 E biochemical identification technique is shown in Tables 4.5 and 4.6. Of all the 120 river water samples collected, API 20 E identification showed that 83% (100/120) samples had *Vibrio* species. *Vibrio cholerae* was prevalent in 71% (71/100) of the identified samples; thus encompassing all of the rivers. Other *Vibrios* species 29% (29/100) identified included: *V. parahaemolyticus, V. mimicus, V. fluvialis* and *V. vulnificus*. Conventional mPCR assay showed that 57% (57/100) of the presumptive

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isolates were *Vibrio cholerae* and 14% (14/100) were identified as other *Vibrio* species. A report by (Baron *et al.,* 2007) showed that 85.5% (662/774) of presumptive *Vibrio cholerae* isolates were correctly identified using API 20E system and mPCR assay. The primers used in multiplex PCR assays were more specific for *Vibrio cholerae* species and also *V. parahaemolyticus* species thus did not target other *Vibrio* species.

The API 20E test is a traditional biochemical identification method, which detects 20 biochemical reactions for the identification of *Enterobacteriaceae* such as *Vibrio* species (Koneman et al., 1997). Results obtained in the present study shows that m-PCR is more sensitive as compared to API 20 E for detection of *Vibrio* isolates. A study by (O'Hara *et al.*, 2003) on the evaluation of six commercial methods (including API 20 E) used to detect *Vibrio* species in clinical samples, found that many isolates are inaccurately identified using these commercial methods, with accuracy rates ranging from 63.9% to 80.9% (O'Hara *et al.*, 2003). PCR is highly sensitive and specific to the presence of DNA even in minute amounts, making it much more reliable for detection than simple biochemical tests (Chiu and Ou, 1996). However a limitation when using PCR is that the method cannot readily distinguish between viable and non-viable organisms as both contain DNA (Ntema and Barnard, 2013).

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Table 4.5: Biochemical and Molecular identification of *Vibrio* species from different sites of the Luvuvhu River.

Luvuvhu sampling point	Sampling Points on the Rivers	API 20 E Biochemical analysis of *Vibrio* species					mPCR analysis of *Vibrio*spp
		V. Cholerae	*V. Parahaemolyticus*	*V. vulnificus*	*V. mimicus*	*V.fluvialis*	
Albasini (Luvuvhu A)	Upstream	+			+	+	*V.cholerae*
	Midstream		+	+		+	*Vibrio spp*
	Downstream	+					*V.cholerae*
Dzindi (Luvuvhu B)	Upstream	+				+	*V.cholerae*
	Midstream	+				+	*V.cholerae*
	Downstream	+	+	+	+		*V.cholerae*
Karringmelkspruit (Luvuvhu C)	Upstream	+			+		*V.cholerae*
	Midstream	+	+				*V.cholerae*
	Downstream	+		+	+	+	*V.cholerae*
Lutanandwa (Luvuvhu D)	Upstream	+				+	*V.cholerae*
	Midstream	+			+		*V.cholerae*
	Downstream	+			+		*V.cholerae*
Piesanghoek (Luvuvhu E)	Upstream	+					*V.cholerae*
	Midstream	+					*V.cholerae*
	Downstream			+		+	*Vibrio spp*
Springfield (Luvuvhu F)	Upstream	+	+	+	+	+	*V.cholerae*
	Midstream	+					*V.cholerae*
	Downstream			+		+	*V.cholerae*

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Table 4.6: Biochemical and molecular identification of *Vibrio* species from different Rivers

Rivers	Sampling points of the Rivers	API 20 E Biochemical analysis of *Vibrio* spp					mPCR analysis of *Vibrio* spp
		V. Cholerae	*V. Parahaemolyticus*	*V. vulnificus*	*V. mimicus*	*V. fluvialis*	
Madadzhe	Upstream	+					*V. cholerae*
	Midstream	+		+	+	+	*V. cholerae*
	Downstream	+		+			*V. cholerae*
Mbwedi	Upstream	+		+	+		*V. cholerae*
	Midstream	+	+			+	*V. cholerae*
	Downstream	+		+	+	+	*V. cholerae*
Mutale	Upstream	+	+	+	+	+	*V. cholerae*
	Midstream	+	+	+	+	+	*V. cholerae*
	Downstream	+	+	+	+		*V. cholerae*
Mutshundudi	Upstream	+				+	*V. cholerae*
	Midstream	+			+		*V. cholerae*
	Downstream	+			+	+	*V. cholerae*
Sambandou	Upstream	+			+	+	*V. cholerae*
	Midstream	+	+	+	+	+	*V. cholerae*
	Downstream	+	+	+	+	+	*V. cholerae*
Tshinane	Upstream	+	+		+		*V. cholerae*
	Midstream	+			+		*V. cholerae*
	Downstream	+	+	+	+		*V. cholerae*
Tshino (Luvuvhu G)	Upstream	+					*V. cholerae*
	Midstream	+					*V. cholerae*
	Downstream				+		*Vibrio spp*

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4.3 CONVENTIONAL MULTIPLEX PCR ANALYSIS

Figures 4.1 and 4.2 show the conventional multiplex PCR products of DNA extracted using the (Boom *et al.*, 1990) method. The m-PCR assays were performed in a Biorad Mycycler ™ thermal cycler under optimised conditions (Ntema and Barnard, 2013). The amplified PCR products were ran using gel electrophoresis as described on material and methods (Chapter 3). The products were then visualized under UV light (Gene Genius Bio-imaging System, Syngene, Cambridge, UK).

Two optimized multiplex PCR assays targeting a total of six genes where ran. The first mPCR was genus based, targeting 16 S rRNA, *V. c* sodB, *V.p*flaE genes. The second mPCR was species specific and targeted 16 S rRNA, *ctxA*, *V. cholerae* O1 rfb genes. The PCR results show that *Vibrio cholerae* was prevalent in all the rivers

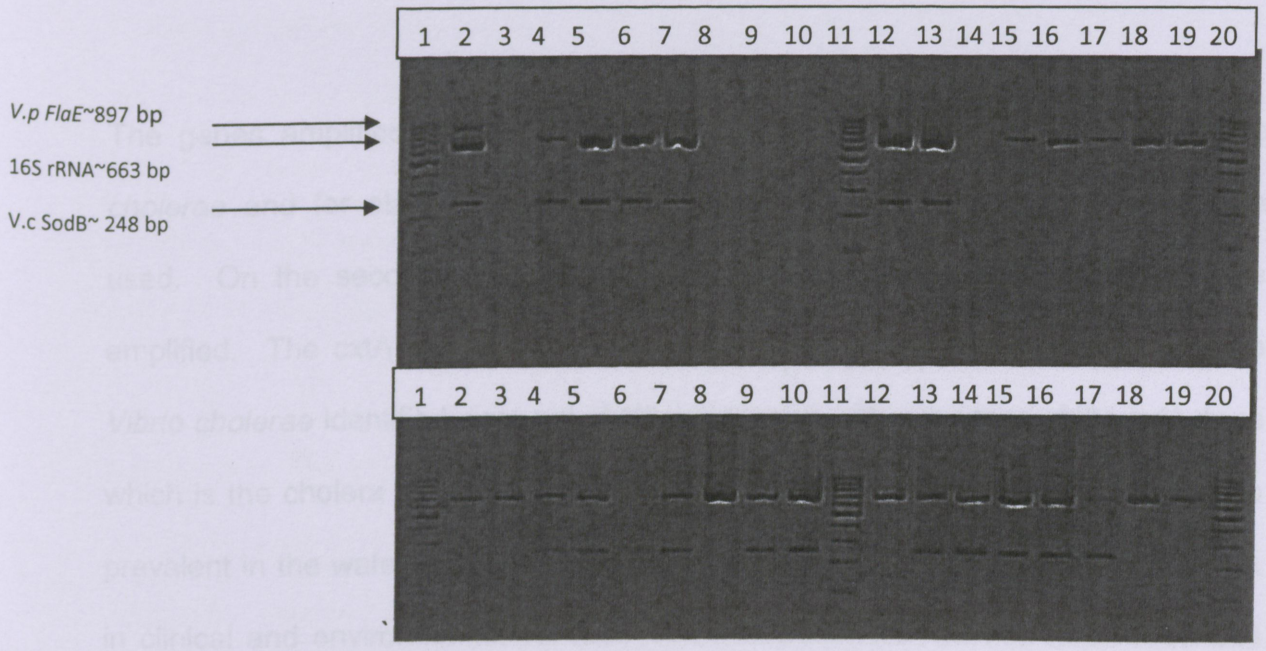

Figure 4.1: Agarose gel showing results of PCR product obtained, targeting *Vibrio* species. 100 bp ladder (lane 1, 11 & 20) multiplex PCR (*V. p Flae*, 16S rRNA, *sodB*) lane 2, the negative control on (lane 3), Samples (lane 4 to 19)

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Figure 4.2: Agarose gel showing results of PCR product obtained, targeting *V. cholerae* O1 serogroup. 100 bp ladder (lane 1,11 & 20), 16S rRNA, ctxA, *V. cholerae* O1 genes for positive control (lane 2), negative control (lane 3), Samples (lane 4 to 19)

The genes amplified on the first m-PCR where 16S rRNA and sodB for *Vibrio cholerae* and for other *Vibrio* species, *V parahaemlyticus, V.pflaE* primers were used. On the second mPCR which was species specific, only 16S rRNA was amplified. The cxtA, *V.c* O1 rfb genes were not expressed. This shows that the *Vibrio cholerae* identified were not of O1 serogroups. The absence of the cxtA gene which is the cholera toxin producing gene clearly indicates that the *V. cholerae* spp prevalent in the water is non-toxigenic. In previous studies the absence of the ctxA in clinical and environmental samples *Vibrio cholerae* non-O1 has been described (Blackstone *et al.*, 2003; Fraga *et al.*, 2007; Singh *et al.*, 2001).

In this study we found that there was a high prevalence of *V. cholerae* non O1, thus 57% (57/100). (Ntema and Barnard, 2013) also reported a high prevalence thus 71%

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(483/676) of *Vibrio cholerae* non O1 in South Africa River water. *Vibrio cholerae* non O1 is non-toxigenic as indicated previously, however they are pathogenic. A recent study showed that a number of non-O1 *V. cholerae* were characterized in clinical strains that were isolated from hospitalized patients with diarrhea (Chatterjee *et al.*, 2009). The detection of *Vibrio cholerae* non O1 serogroups in local rivers within rural areas is of a public health concern as the bacteria are likely to cause diarrhea which can cause mortality and morbidity. In the present study, it was observed that most rural residents relied on local rivers as a primary water supply due to the scarcity of water. River water may be used for drinking purposes without any form of treatment prior to use (Nevondo and Cloete, 1999). This creates a potential transmission route for waterborne bacteria such as pathogenic *Vibrio cholerae*.

Studies have shown that there has been considerable incidence of human diarrheal episodes in many countries due to *V. cholerae* non-O1 (Dalsgaard *et al.*, 1995). A study done in Kolkata (India) also shows increase in diarrhea incidences caused by *Vibrio cholerae* non O1 where patients were hospitalized after suffering from diarrhea. Diarrheal cases in Thailand between 1993 and 1995 had prevalence of non-O1 *V. cholerae* which was comparable to that of O1 *V. cholerae* (Dalsgaard *et al.*, 1999).

Additionally, (Bik *et al.*, 1995) reported that there was evidence of horizontal gene transfer between *Vibrio cholerae* O1 and non-O1 strains which may lead to newly acquired virulence for non O1 strains. It has also been reported that *V. cholerae* non-O1 strains encode some putative virulence factors such as NAG-ST enterotoxin, proteases and hemolysins (Bidinost *et al.*, 2004) which have not been investigated in the present study.

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Therefore showing that non toxigenic *Vibrio cholerae* may pose a major health risk (Ntema and Barnard, 2013). The high prevalence of *Vibrio cholerae* non O1 found in this study is a serious health concern as it has been revealed in previous studies that the bacteria has major health risk implications especially in sensitive population groups (e.g. immune compromised individuals) (Ntema and Barnard, 2013).

4.4 THE OCCURRENCE OF *Vibrio cholerae* IN RIVERS

Water was collected from three points of the river, namely the upstream point, mid stream point and downstream point. In Table 4.7, it is shown that *Vibrio cholerae* non O1 was prevalent in all the river sites. The highest prevalence was observed on up and down stream points. *Vibrio cholerae* non O1 was also prevalent in the mid stream point. A study done by (Nevondo and Cloete, 1999) on water microbial quality showed that upstream may be the source of pollution in a river, therefore the stream may have high prevalence of bacteria; this may be caused by various factors such as human activities and animal activities.

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Table 4.7: The distribution of *Vibrio cholerae* in the different River sites

Rivers sites	Percentage
Upstream	25 (34.25%)
Middle stream	23 (31.5%)
Downstream	25 (34.25)

Table 4.8 and 4.9 show human activities such as car washing, clothes washing, waste disposal and animal activities such as grazing and excretion which were observed at the rivers. Most of these activities occurred on the upstream as well as the downstream points. Activities that were observed in the midstream included bathing, swimming and car washing. Since *Vibrio cholerae* can be transmitted through the faecal oral route, human and animal activities such as defecation may be responsible for the high prevalence of the *Vibrio* spp in the rivers. In rivers such as Tshino (Luvuvhu G) and Sambandou, people were seen collecting drinking water which they consume without any further purification due to their religious belief which commands that the water is used as it is without purification (common knowledge in the region).The consumption of the water may be a contributing factor to potential health risk for the individuals.

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Table 4.8: Activities observed in the rivers during water sample collection

Sample point	Human and animal activities
Albasini (Luvuvhu A)	
Upstream	Faecal contamination by wild animals
Midstream	People bathing
Downstream	Cattles faecal contamination
Dzindi (Luvuvhu B)	
Upstream	Cattles faecal contamination, subsistence farming
Midstream	disposal of garbage
Downstream	Disposed diapers
Karringmelkspruit (Luvuvhu C)	
Upstream	Cattle and wild animal faecal matter
Midstream	
Downstream	Disposed diapers
Lutanandwa (Luvuvhu D)	
Upstream	Animals faecal contamination
Midstream	
Downstream	Disposed diapers
Madazhe	
Upstream	Sewage discharge
Midstream	Sewage discharge
Downstream	Cattle faecal contamination
Mbwedi	
Upstream	Cattles faecal contamination
Midstream	bathing and car washing
Downstream	Washing laundry
Mutale	
Upstream	Cattles drinking water
Midstream	Car and laundry washing, swimming
Downstream	Farm activity
Mutshundudi	
Upstream	Cattle faecal contamination
Midstream	People bathing and laundry washing
Downstream	Laundry washing
Piesanghoek (Luvuvhu E)	
Upstream	Cattles farming and contamination
Midstream	
Downstream	Wild animals faecal contamination

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Table 4.9: Activities observed in the rivers during sample collection

Sample point	Human and animal activities
Sambandou	
Upstream	Cattles fecal contamination, collection of drinking water
Midstream	
Downstream	people bathing
Springfield (Luvuvhu F)	
Upstream	Cattles and wild animal fecal pollutants
Midstream	
Downstream	Farming activities
Tshinane	
Upstream	Cattles fecal contamination
Midstream	People bathing and swimming
Downstream	Car and laundary washing
Tshino (Luvuvhu G)	
Upstream	Cattle fecal contamination, collection of drinking water
Midstream	garbage disposal
Downstream	Car and laundary washing

4.5 LIMITATION OF THE STUDY

The limitation of the present study may be the conventional PCR assay that was used, which has a low accuracy compared to real-time PCR (Paiva-Cavalcanti *et al.*, 2010). The primers used may also be considered as a limitation to the study as they did not target a broad range of genes which made it hard to confirm the presence of other *Vibrio* species detected by API 20 E biochemical confirmation test

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Chapter 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

The aim of this study was to determine the prevalence of *Vibrio cholerae* and other *Vibrio* species. In order to achieve this aim, the study had three objectives.

Objective 1 was to isolate *Vibrio cholerae* and other *Vibrio* species from river water using membrane filtration technique. This objective was achieved by the culturing technique and isolation of presumptive isolates after enrichment.

Objective 2 was to identify *Vibrio cholerae* and other *Vibrio* species using traditional culture methods and biochemical identification techniques, the objective was achieved and results are shown in Table 4.2 and 4.3.

Objective 3 was to investigate the pathogenecity of *Vibrio* species, this objective was achieved by using the PCR method and results can be seen in Figures 4.1 & 4.2.Hence all the study objectives were achieved.

In conclusion, this study has shown that *Vibrio cholerae* non O1 is prevalent in Rivers within rural areas of the Vhembe district. The mPCR methods were found to be suitable for this type of studies.

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5.2 RECOMMENDATION

Even though *Vibrio cholerae* non O1 is non toxigenic, studies have shown that it can cause gastroenteritis, septicaemia and extra-intestinal infections (Fraga *et al.*, 2007) Therefore the present study suggests the importance of public intervention which involves educating people about basic water purifying methods such as boiling and chlorination, in order to prevent the transmission of the pathogenic waterborne bacteria *Vibrio cholerae*.



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