

Genotypic characterization of virulent *Vibrio* species and their endemic bacteriophages

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Microbiology)* at the North-West University

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DECLARATION

I, Maje Mpho Defney, declare that the dissertation entitled “**Genotypic characterization of virulent *Vibrio* species and their endemic bacteriophages**”, is my own work, unless where acknowledged. It is submitted for the degree of Master of Science in Biology (Molecular Microbiology) at the Mafikeng Campus of the North-West University and it has not been submitted for any other degree or examination in any other university for obtaining a qualification. All materials used and quoted herein have been duly acknowledged.

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DEDICATION

I dedicate this project to my beloved Mother (The late Mrs. M.E Maje), to my family, my partner and friends.

To God Be the Glory

ACKNOWLEDGEMENTS

Firstly, I would like to thank God for His protection and guidance throughout this study. I could have not made it without Him.

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ABSTRACT

Vibrio are gram negative curved bacteria commonly found in aquatic environments. Some species are pathogenic to humans as well as to aquatic animals and have been associated with serious public health complications in their hosts. Water quality is presently a great issue of public health concern globally as well as in South Africa. Water-borne diseases still pose significant threats to public health and a number of outbreaks have been associated with pathogenic *Vibrio* species. The aim of this study was to determine the virulence profiles of pathogenic *Vibrio* species and assess the bio-control potential of *Vibrio* species-specific endemic bacteriophages against environmental isolates. A total of 136 water samples were randomly collected from areas around North West Province, South Africa and 57 presumptive *Vibrio* isolates were obtained using the Thiosulfate bile salt sucrose agar. Typical yellow and green colonies were selected, purified and subjected to both preliminary and confirmatory tests which are specific to *Vibrio* species. All the isolates were Gram negative rods while only 38% of the isolates were oxidase positive. All the presumptive isolates were further subjected to PCR in order to amplify bacterial 16S rRNA gene fragments and all isolates were positive for this gene. Of the 57 presumptive isolates a relatively small proportion of 22 (38.60%) isolates were confirmed as *Vibrio* species based on the presence of *vhh* (45.45%), *flaE* (22.72%), *sodB* (13.64%), *sodB* (1) (9.09%) and *hsp* (9.09%) gene fragments. Furthermore, isolates were subjected to a panel of 11 antimicrobial agents in order to determine their antibiotic resistant profiles using the CLSI guidelines. Results revealed that isolates displayed high levels of resistance to Cephalothin (95.45%), Ampicillin (77.27%) and Streptomycin

(40.91%). On the contrary, small proportions (4.55% to 27.27%) of the isolates were resistant to Chloramphenicol, Ciprofloxacin, Tetracycline, Gentamycin, Kanamycin, Nalidixic acid, Trimethoprim-Sulfamethoxazole and Trimethoprim. All the 22 confirmed *Vibrio* isolates were screened for the presence of virulence genes. Out of 6 virulence genes targeted, only 3 were positively amplified. *Vibrio parahaemolyticus* possessed *tdh* (18.18%) and *trh* (18.18%) virulence genes while *Vibrio cholerae* and *Vibrio mimicus* harboured (13.64%) and (4.55%) of the *zot* gene fragment respectively. The presence of these genes might indicate the public health implication these isolates may pose on humans. The biofilm formation potentials of 22 *Vibrio* species was determined using microtiter plate assay at different temperatures. The *Vibrio* species displayed potentials to form biofilm at different temperatures. Although the isolates were not able to form strong biofilms at 37 °C, small proportions (4.55% and 27.27%) of the isolates produced strong biofilms at 25 °C and 35 °C respectively. These findings indicate that the isolates of this study may be capable of colonizing a wide range of ecological niches and persist when associated with human infections. A further objective of the study was to assess the virulence potentials of *Vibrio* species-specific bacteriophages as biocontrol agents against antibiotic resistant and virulent environmental *Vibrio* isolates obtained in this study. Phage virulence assays showed that the phages isolated in this study presented lytic potentials. Two of the employed bacteriophages Phage A and Phage B were able to inhibit bacterial growth of more than one host strain. These findings indicate that phages isolated in this study have presented potential characteristics of being used as effective agents for biological control of environmental *Vibrio* bacterial host strains that also

possess multidrug resistant determinants. These findings are of great epidemiological significance implications associated with *Vibrio* infections.

Keywords: *Vibrio* species, Antimicrobial agents, Antimicrobial resistance, PCR, Thiosulfate bile salt sucrose agar, Biofilm formation, Bacteriophages, Water-borne diseases

TABLE OF CONTENTS

Contents

DECLARATION	i
DEDICATION	iii
ACKNOWLEDGEMENTS	iv
ABSTRACT	v
TABLE OF CONTENTS	viii
LIST OF TABLES	xi
LIST OF FIGURES	xii
LIST OF APPENDICES	xiv
LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS	xv
DEFINITION OF CONCEPTS	xvii
CHAPTER 1	1
INTRODUCTION AND PROBLEM STATEMENT	1
1.1 GENERAL INTRODUCTION	1
1.2 PROBLEM STATEMENT	4
1.3 AIM AND OBJECTIVES OF THE STUDY	7
1.3.1 Aim of the study	7
1.3.2 Objectives of the study	7
CHAPTER 2	9
LITERATURE REVIEW	9
2.1 <i>VIBRIO SPECIES</i>	9
2.1.1 BACKGROUND	9
2.2 WATER CONTAMINATION	10
2.1.2 DISEASES CAUSED BY <i>VIBRIO SPECIES</i> AND CLINICAL PRESENTATION	11
2.1.3 TRANSMISSION OF <i>VIBRIO SPECIES</i>	14
2.1.4 LABORATORY DIAGNOSIS AND DETECTION	14
2.1.5 PREVENTION, TREATMENT AND CONTROL OF <i>VIBRIO</i> INFECTIONS	16
2.2 ANTIBIOTIC RESISTANCE IN <i>VIBRIO SPECIES</i>	17

2.3 VIRULENCE FACTORS ASSOCIATED WITH <i>VIBRIO</i> SPECIES.....	18
2.4 BIOFILM FORMATION	20
2.5 BACTERIOPHAGES AS POTENTIAL BIO-CONTROL AGENTS AGAINST	24
<i>VIBRIO</i> SPECIES.....	24
CHAPTER 3	29
MATERIALS AND METHODS	29
3.1 MATERIALS AND METHODS	29
3.1.1 Area of the study.....	29
3.1.2 Ethical Clearance.....	29
3.1.3 Collection of samples and sample sites	29
3.2 ANALYSIS OF WATER SAMPLES AND BACTERIAL ISOLATION.....	31
3.2.1. Processing of water samples and Isolation of <i>Vibrio</i> species	31
3.3 BIOCHEMICAL IDENTIFICATION OF <i>VIBRIO</i> SPECIES	31
3.3.1 Cellular morphology.....	31
3.3.2 Oxidase test.....	32
3.3.3 Triple Sugar Iron agar test.....	33
3.3.4 Simmons Citrate Agar	34
3.3.5 Salt Tolerance Test.....	34
3.3.6 Motility Test	34
3.4 BACTERIAL STORAGE.....	35
3.5 MOLECULAR CHARACTERISATION OF <i>VIBRIO</i> SPECIES	35
3.5.1 DNA Extraction.....	35
3.5.2 Determination of DNA quality and quantity	35
3.6 PCR AMPLIFICATION OF 16S rRNA GENE OF <i>VIBRIO</i> SPECIES.....	36
3.7 SEQUENCE ANALYSIS OF PCR AMPLICONS	40
3.8 ANTIMICROBIAL SUSCEPTIBILITY TEST.....	40
3.8.1 Antimicrobial susceptibility test (AST)	40
3.8.2 Plasmid DNA extraction	41
3.8.3 Detection of <i>Vibrio</i> virulence genes.....	44
3.8.4 Biofilm formation assay	46
3.9 BACTERIOPHAGE ASSAY.....	47
3.9.1 Isolation of <i>Vibrio</i> species infecting phages using the double layer assay	47
3.9.2 Purification and enumeration of phages in stocks	48

3.9.3 Phage propagation	48
3.9.4 Characterisation of bacteriophages	49
3.10 STATISTICAL ANALYSIS	50
3.11 DATA CAPTURING	51
CHAPTER 4	53
RESULTS	53
4.1 DETECTION OF <i>VIBRIO</i> ISOLATES FROM WATER SAMPLES	53
4.2 DISTRIBUTION OF <i>VIBRIO</i> ISOLATES IN WATER SAMPLES	54
4.3 IDENTIFICATION OF ISOLATES BASED ON PRELIMINARY AND CONFIRMATORY BIOCHEMICAL TESTS.	54
4.4 GENOMIC DNA EXTRACTION AND GEL ELECTROPHORESIS	57
4.5 THE 16S rRNA GENE PCR ANALYSIS	57
4.6 IDENTIFICATION OF <i>VIBRIO</i> SPECIES USING SPECIES SPECIFIC PCR AMPLIFICATION	58
4.7 ANTIBIOTIC SUSCEPTIBILITY PROFILES OF ISOLATES	62
4.8 CLUSTER ANALYSIS OF <i>VIBRIO</i> ISOLATES BASED ON BACTERIA GROWTH INHIBITION ZONE DIAMETER DATA	64
4.9 PLASMID PROFILES	67
4.10 PROPORTIONS OF <i>VIBRIO</i> ISOLATES POSITIVE FOR THE DIFFERENT VIRULENCE GENES	67
4.11 BIOFILM FORMATION CAPACITY OF <i>VIBRIO</i> SPECIES AT DIFFERENT TEMPERATURES	69
4.12 CHARACTERIZATION OF <i>VIBRIO</i> SPECIFIC BACTERIOPHAGES	71
4.12.1 Transmission electron microscopy	71
4.12.2 Bacteriophage virulent assay	72
4.12.3 Stability of phage at different conditions	74
CHAPTER 5	78
DISCUSSION	78
5. GENERAL DISCUSSION	78
CHAPTER 6	88
CONCLUSION AND RECOMMENDATIONS	88
6. CONCLUSION AND RECOMMENDATIONS	88
REFERENCES	90
APPENDICES	114

LIST OF TABLES

TABLE 3. 1: AREAS FROM WHICH WATER SAMPLES WERE COLLECTED	30
TABLE 3. 2: OLIGONUCLEOTIDE PRIMERS USED FOR AMPLIFICATION OF PCR.....	39
TABLE 3.3: ANTIBIOTICS USED IN THE STUDY	43
TABLE 3.4: OLIGONUCLEOTIDE PRIMERS USED FOR THE AMPLIFICATION OF VIRULENCE GENES.....	45
TABLE 4. 1: DISTRIBUTION OF POTENTIAL <i>VIBRIO</i> ISOLATES IN THE DIFFERENT TYPES OF WATER SAMPLES ANALYSED IN THE STUDY	54
TABLE 4. 2: PROPORTION OF ISOLATES IDENTIFIED USING VARIOUS BIOCHEMICAL TESTS.....	56
TABLE 4.3: THE DISTRIBUTION PATTERNS OF DIFFERENT <i>VIBRIO</i> SPECIES ISOLATED FROM THE DIFFERENT SAMPLING SITES AND TYPES OF SAMPLES.....	60
TABLE 4. 4: THE PERCENTAGE REPRESENTATION OF <i>VIBRIO</i> ISOLATED FROM DIFFERENT SPECIES WITHIN THE VARIOUS CLUSTERS	65
TABLE 4. 5: VIRULENCE POTENTIALS OF BACTERIOPHAGES AGAINST ENVIRONMENTAL RESISTANT BACTERIA <i>VIBRIO</i> ISOLATES. THE ACRONYM PHAGE A AND PHAGE B INDICATES BACTERIOPHAGES ISOLATED USING A <i>VIBRIO</i> ISOLATE AS THE HOST BACTERIA STRAIN. ISOLATES WITH THE SUPERSCRIPTS “*” WERE SUSCEPTIBLE TO PHAGE A WHILE THOSE WITH THE SUPERSCRIPTS “*+” WERE SUSCEPTIBLE TO PHAGE B. ISOLATES WITH THE SUPERSCRIPTS “**” WERE SUSCEPTIBLE TO BOTH PHAGE A AND PHAGE B.	73

LIST OF FIGURES

FIGURE 2. 1: SCHEMATIC STRUCTURE OF THE PRIMARY SITE OF INFECTION OF DIFFERENT PATHOGENIC <i>VIBRIO</i> AFFECTING HUMANS (PLAZA <i>ET AL.</i> , 2017).....	13
FIGURE 2. 2: SCHEMATIC STRUCTURE OF BIOFILMS AND THE ROLE THEY PLAY AS ENVIRONMENTAL RESEVOIRS OF HYGIENICALLY RELEVANT MICROORGANISMS AS WELL AS SOURCE OF CONTAMINATION AND INFECTION IN DRINKING WATER SYSTEMS (WINGERDER, 2011)	23
FIGURE 2. 3: DIAGRAMMATICAL STRUCTURE OF THE PHAGE (ADAPTED FROM THE SCIENTIST.COM).....	25
FIGURE 4. 1: DIFFERENT COLOUR OF COLONIES OBSERVED ON TCBS AGAR	53
FIGURE 4. 2: AGAROSE GEL (1% W/V) IMAGE DEPICTING GENOMIC DNA EXTRACTED FROM PRESUMPTIVE <i>VIBRIO</i> ISOLATES. LANE 1-57= DNA EXTRACTED FROM PRESUMPTIVE <i>VIBRIO</i> ISOLATES.	57
FIGURE 4.3: AGAROSE [2% W/V] GEL IMAGE SHOWING 16S rRNA GENE FRAGMENTS AMPLIFIED FROM ALL PRESUMPTIVE <i>VIBRIO</i> ISOLATES. LANE M= DNA MARKER (O'GENERULER 1 KILO BASE PAIRS DNA LADDER), LANE 1-57= 16S rRNA GENE FRAGMENTS OF PRESUMPTIVE <i>VIBRIO</i> ISOLATES FROM WATER SAMPLES.....	58
FIGURE 4. 4: AGAROSE [2% W/V] GEL IMAGE SHOWING AMPLIFICATION OF <i>FLA</i> E, <i>HSP</i> , <i>SOD</i> B (1) AND <i>SOD</i> B GENE FRAGMENTS. LANE M= DNA MARKER (100 BASE PAIRS DNA LADDER), LANES 1-5= <i>FLA</i> E GENE FRAGMENTS, LANES 6-7= <i>HSP</i> GENE FRAGMENTS, LANES 8-10= <i>SOD</i> B (1) GENE FRAGMENTS AND LANES 11-12= <i>SOD</i> B GENE FRAGMENTS.....	61
FIGURE 4. 5: AGAROSE [2% W/V] GEL IMAGE SHOWING AMPLIFICATION OF <i>VHH</i> GENE FRAGMENTS. LANE M= DNA MARKER (100 BASE PAIRS DNA LADDER), LANES 1-12= <i>VHH</i> GENE FRAGMENTS.	61
FIGURE 4. 6: DISTRIBUTION OF <i>VIBRIO</i> ISOLATED FROM WATER SAMPLES BASED ON SPECIES SPECIFIC PCR ANALYSIS.....	62
FIGURE 4. 8: RESISTANCE PATTERN OF ISOLATES OBTAINED FROM WATER SAMPLES.....	63

FIGURE 4. 9: DENDROGRAM SHOWING THE RELATIONSHIP BETWEEN ISOLATES FROM WATER SAMPLES BASED ON ANTIMICROBIAL INHIBITION ZONE DIAMETER DATA.	66
FIGURE 4. 10: AGAROSE (2% W/V) GEL IMAGE DEPICTING PLASMID PROFILES OF TESTED <i>VIBRIO</i> SPECIES. LANE M= DNA MARKER (1 KILO BASE DNA LADDER), LANES 1-8= PLASMID DNA FRAGMENTS AMPLIFIED FROM <i>VIBRIO</i> ISOLATES.....	67
FIGURE 4. 11: AGAROSE (2% W/V) GEL IMAGE DEPICTING THE VIRULENCE GENE FRAGMENTS OF <i>ZOT</i> , <i>TRH</i> AND <i>TDH</i> GENE. LANE M= DNA MARKER (100 BASE PAIRS DNA LADDER), LANES 1-4= <i>TRH</i> GENE FRAGMENTS, LANES 5- 8= <i>ZOT</i> GENE FRAGMENTS AND LANES 9-12= <i>TDH</i> GENE FRAGMENTS.	68
FIGURE 4. 12: BIOFILM FORMATION POTENTIALS OF <i>VIBRIO</i> SPECIES AT DIFFERENT TEMPERATURES.....	70
FIGURE 4. 13: BIOFILM FORMATION POTENTIALS OF THE 22 <i>VIBRIO</i> ISOLATES AT 37°C.	70
FIGURE 4. 14: BIOFILM FORMATION POTENTIALS OF THE 22 <i>VIBRIO</i> ISOLATES AT 35°C.	70
FIGURE 4. 15: BIOFILM FORMATION POTENTIALS OF THE 22 <i>VIBRIO</i> ISOLATES AT 25°C.	71
FIGURE 4.16: TRANSMISSION ELECTRON MICROGRAPHS OF <i>VIBRIO</i> SPECIFIC BACTERIOPHAGES ISOLATED FROM SEWAGE WATER.	72
FIGURE 4. 17 STABILITY OF PHAGE A AT DIFFERENT PH LEVELS.....	74
FIGURE 4. 18 STABILITY OF PHAGE B AT DIFFERENT PH LEVELS.....	75
FIGURE 4. 19 STABILITY OF PHAGE A AT DIFFERENT TEMPERATURES	75
FIGURE 4. 20 STABILITY OF PHAGE B AT DIFFERENT TEMPERATURES	76

LIST OF APPENDICES

APPENDIX 1. A: DETAILS OF ALL THE MATERIALS; CULTURE MEDIA, CHEMICALS ENZYMES AND REAGENTS USED IN THIS STUDY.....	114
APPENDIX 1. B: ANTIBIOGRAM DATA OF ALL ISOLATES DERIVED FROM THE INHIBITION ZONE DATA.....	125
APPENDIX 1. C: INTERPRETATION OF BIOFILM FORMATION RESULTS.....	128
APPENDIX 1. D: VIRULENT ASSAY DETAILED RESULTS.....	129

LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

The following abbreviations, acronyms and symbols have been used in this dissertation following the style recommended by American Society for Microbiology for Journals

AST	: Antimicrobial susceptibility test
Bp	: Base Pair
Blast	: Basic Alignment Search Tool
°C	: Degree Celsius
DNA	: Deoxyribonucleic Acid
CLSI	: Clinical Laboratory Standard Institute
<i>et al.</i>	: Et alia
EB	: Ethidium Bromide
EDTA	: Ethylenediamine-tetraacetic acid
g	: gram
h	: hour/ Hours
min	: minute/minutes
mL	: Millilitre
NaCl	: Sodium Chloride
NCBI	: National Centre for Biotechnology Information
PCR	: Polymerase Chain Reaction
rRNA	: Ribosomal Ribonucleotide Acid
TAE	: Tris acetic acid EDTA buffer

TBE	: Tris-borate EDTA
<i>tdh</i>	: Thermostable Direct Hemolysin
<i>trh</i>	: Thermostable Direct Hemolysin-Related
TCBS	: Thiosulfate Citrate Bile Salt Sucrose
TSA	: Tryptone Soy Agar
TSI	: Triple Sugar Iron Agar
μL	: Microlitre
WHO	: World Health Organization

DEFINITION OF CONCEPTS

Antibiotics: These are the compounds that inhibit the growth or kill the microorganisms.

Antibiotic resistance: The ability of a given bacteria to survive the exposure to a defined concentration of an antimicrobial agent.

Antimicrobial: Agents that are capable of destroying or inhibiting the growth of microorganisms and especially pathogenic microorganisms.

Bacteriophages: are bacterial viruses that only infect and replicate within their specific hosts.

Biofilm: A material enclosing colonies of microorganisms attached to a surface.

Cluster analysis: A comparative analysis of typing data collected for variety of bacterial isolates in order to group them based on the similarity of their data.

Enterobacteriaceae: Are gram-negative, rod shaped bacilli, non-spore forming and facultative anaerobes bacteria.

Genotyping: The determination of the detailed genetic make-up, or genotype, of an individual, usually in respect to particular genes or sets of genes.

Plasmid: Is a circular DNA molecule that can replicate independently from the chromosome and promote lateral transfer among different species of bacteria through the conjugation process.

Plaques: are clear zones formed in a lawn of bacterial cells due to lysis by phage.

Primer: Is a strand of short nucleic acid sequences (generally about 10 base pairs) that serves as a starting point for DNA synthesis. It is required for DNA replication because the enzymes that catalyze this process, DNA polymerases, can only add new nucleotides to an existing strand of DNA.

Strain: An isolate or group of isolates that can be distinguished from isolates of the same genus or other genus of the same species either by phenotypic characteristics and/or genotypic characteristics.

Species: Collection of bacterial cells which share an overall similar pattern of traits in contrast other bacteria whose pattern differ significantly.

Virulence: The ability to cause disease.

CHAPTER 1
INTRODUCTION AND PROBLEM STATEMENT

CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

1.1 GENERAL INTRODUCTION

Vibrio species are catalase and oxidase positive, Gram-negative rod-shaped bacteria that are also capable of fermenting a variety of carbohydrates (Austin, 2010; Ceccarelli *et al.*, 2013; Yu *et al.*, 2013; Zhang and Orth, 2013; Velazquez-Roman *et al.*, 2014; Wu *et al.*, 2014). These organisms are also non-spore forming, and motile through the possession of polar flagella. In addition, these species are usually sensitive to *Vibriostatic* agents and this serves as a very important characteristic that is used to distinguish these organisms from other closely related species. Furthermore, most *Vibrio* species require sodium chloride for optimum growth. *Vibrio* species are also mesophilic and chemoorganotrophic hence, they can grow on thiosulfate citrate bile salt sucrose agar (Austin. 2010; Ceccarelli *et al.*, 2013; Yu *et al.*, 2013; Zhang and Orth, 2013; Velazquez-Roman *et al.*, 2014; Wu *et al.*, 2014).

Vibrio species belong to the newly classified family *Vibrionaceae* that also comprise organisms belonging to the genera *Aeromonas*, *Campylobacter* and *Helicobacter* (Thompson *et al.*, 2014). Even though the genus *Vibrio* is made up of approximately 63 species (Thompson *et al.*, 2014), *Vibrio cholerae* and *Vibrio parahaemolyticus* that are responsible for cholera and gastroenteritis respectively are mostly associated with human infections (Nair *et al.*, 2007, Kirschner *et al.*, 2011). Despite the fact that other *Vibrio*

species such as *Vibrio alginolyticus*, *Vibrio harveyi*, *Vibrio anguillarum*, *Vibrio mimicus*, *Vibrio metschnikovii*, and *Vibrio fluvialis* have been detected particularly in marine environments (Gennari *et al.*, 2012), they are not pathogenic to humans and potentially cause infections in fish (Gennari *et al.*, 2012). Given that some studies have indicated that these non-pathogenic *Vibrio* species have the potential of developing into emerging pathogenic strains that are capable of causing mild to severe diseases in humans (Ashton *et al.*, 2008), there is need for constant evaluation of their presence as well as their pathogenicity.

Freshwater bodies serve as the key water resources in rural areas and water from these unprotected sources are used for cooking, irrigation and other agricultural processes as well as drinking in greater parts of communities that do not have access to clean and safe drinking water (Osunla and Okoh, 2017). Furthermore, rapid population growth, urbanization and developments along the banks of unprotected water bodies such as rivers significantly contributes towards pollution (Ashton *et al.*, 2008). It is therefore reported that continuous microbial contamination of waterbodies in both developed and most importantly developing countries is a significant cause of a large proportion of water-associated disease epidemics (Guerrant *et al.*, 2003; Cabral, 2010; Chigor *et al.*, 2013; Lutz *et al.*, 2014). In addition, a number of *Vibrio* species that are found in freshwater bodies have been reported to be transmitted to marine environments through rainfall run-offs (Cavallo and Stabili, 2002).

Infections caused by *Vibrio* species result from the consumption of contaminated water as well as undercooked seafood products (CDC, 2016). In addition, person-to-person transmission has also been documented (Baker-Austin *et al.*, 2018). The pathogenicity of virulent *Vibrio* species is largely associated with their potential to produce certain enzymes that are toxic to humans (Austin *et al.*, 1997). However, a number of studies have indicated that overcrowding and poor sanitation are the most important factors that facilitate the persistence of *Vibrio cholerae* in environmental settings (Huq *et al.*, 2012) and this explains the need to constantly monitor their presence. Moreover, the strict implementation of strategies to reduce cross-contamination may significantly limit the occurrence of human infections.

Vibrio species continues to be an important public health pathogen in developing and middle-income countries where infectious diseases and poverty are very prevalent, and they cause complications that range from septicemia, diarrhea, gastroenteritis to the more complicated sepsis and necrotizing fasciitis (CDC, 2016). In order to treat these infections, doxycycline is recommended as the preferred drug for elderly individuals while azithromycin is administered to pregnant women and children (CDC, 2016). Given that antimicrobial resistance is currently a very important issue of public health concern, it is important to have full knowledge of the antimicrobial resistance profiles of *Vibrio* species in a given geographical location or patient before the administration of antimicrobial agents (CDC, 2016). This will greatly reduce human suffering.

Antibiotics and other antimicrobial agents have been used since their discovery for treating both old and emerging infections that are caused by bacteria species, thus facilitating the control and management of infections in humans and animals (Saga *et al.*, 2005; Aminov, 2011). However, the ever-increasing resistance displayed by a number of bacteria strains against commonly used and recommended antimicrobial agents present a severe challenge therapy and may account for a large proportion of therapeutic failures resulting in high morbidity and mortality within clinical settings (Carlet *et al.*, 2011; Finley *et al.*, 2013). This has renewed interests in the search for novel and alternative therapeutic or bio-control agents against clinically relevant bacteria strains. Recently, bacteriophages have been regarded as possible and promising antimicrobial agents that possess antibacterial properties against different pathogens (Shin *et al.*, 2012). Despite the safety concerns associated with the use of bacteriophages, these viral agents have produced significant results in both Laboratory and field experiments highlighting their importance as novel therapeutic agents.

1.2 PROBLEM STATEMENT

Recently, there has been an increase in the occurrence of infections caused by *Vibrio* species worldwide (Scallan *et al.*, 2011). This suggests the need for rapid and reliable detection methods that will ultimately assist in efficient control strategies that will limit the transmission of *Vibrio* species to humans (Han *et al.*, 2011). Pathogenic *Vibrio* species possess a variety of virulence factors that are responsible for pathogenicity (Kalatzis *et al.*, 2018). Generally, human infections caused by *Vibrio* species are classified into two distinct groups viz *Vibrio cholerae* infections and non-*cholera Vibrio* infections (Dalsgaard

et al., 1996; Dalsgaard, 1998; Noriega-Orozco *et al.*, 2007; Ottaviani *et al.*, 2009). The non-cholerae infections range from self-limiting gastroenteritis to severe life-threatening septicaemia. On the other hand, *Vibrio cholerae* infections can be fatal if not properly managed (Sung *et al.*, 2001; Thompson *et al.*, 2004; Chrisolite *et al.*, 2008). In addition, *Vibriosis* has been reported to be a major cause of both production and financial loss in aquatic farms worldwide (Sung *et al.*, 2001; Thompson *et al.*, 2004; Chrisolite *et al.*, 2008).

Vibrio infections are usually associated with the consumption of contaminated sea food and water thereby highlighting the health risks associated with these pathogens. South Africa is located in a semi-arid region and receives very less rainfall and this results in shortage of water especially potable water. In addition, increased industrialization and frequent establishment of informal settlements also affects the quality of water especially in rural areas. Due to scarcity of potable water most individuals revert to water from other unprotected sources such as boreholes, rivers and dams for daily activities. These unprotected sources of water can easily be contaminated with faecal matter from human and animal sources. Although majority of *Vibrio* species occur naturally in sea food, *Vibrio cholera* is primarily found in water or food products that have been contaminated with faeces. This highlights the need for proper risk control strategies.

There is reliable evidence that *Vibrio* species are highly zoonotic and some genetic fingerprinting data have revealed a close similarity between isolates from clinical samples of infected patients and those from animals. Despite this, there is, however, paucity of

information on the occurrence, virulence potentials and molecular epidemiology of *Vibrios* in the aquatic systems in the North-West Province of South Africa. This therefore indicates the need for accurate and rapid identification of human pathogenic *Vibrio* species in water samples from a public health point of view. An overview of the virulence as well as antibiotic resistance profiles of the isolates will be of great epidemiological importance. Data generated will be crucial for effective diagnosis and treatment of diseases in the given area.

The continuous use of antibiotics in aquaculture and in clinical setups has encouraged the emergence of antibiotic resistant *Vibrios*. Thus, safer control and treatment options are increasingly gaining grounds (da Costa *et al.*, 2013). One of these is the use of specific bacteriophages to lyse pathogenic bacteria. Consequently, vibro-specific phages have the potential to lyse pathogenic *Vibrios* and can be used as reliable biocontrol and therapeutic agents. This study was therefore designed to determine the plasmid profiles, biofilm formation potentials, genotypic fingerprints and also to isolate and determine the virulent capabilities of *Vibrio* specific bacteriophages from water samples against environmental antibiotic resistant isolates isolated from water. Data obtained in this study may be very useful for purposes of source tracking, especially in situations of outbreaks. Therefore, the importance of this study cannot be over-emphasised.

1.3 AIM AND OBJECTIVES OF THE STUDY

1.3.1 Aim of the study

The main aim of this study was to determine the virulence profiles of *Vibrio* species and bio-control potentials of *Vibrio* specific endemic bacteriophages from water samples.

1.3.2 Objectives of the study

The specific objectives of the study were:

- To isolate and determine the presumptive identities of potential *Vibrio* isolates using conventional microbiological methods and *Vibrio* specific PCR analysis
- To determine the antibiotic resistance and virulence gene profiles of *Vibrio* isolates
- To isolate plasmids from ampicillin resistant *Vibrio* species
- To determine the biofilm forming potentials of *Vibrio* isolates at different temperatures
- To isolate and determine the morphologies of *Vibrio* specific bacteriophages using electron microscopy
- To determine and evaluate the lytic capabilities of *Vibrio* specific bacteriophages against multi-drug resistant *Vibrio* isolates using the virulence micro-plate assays.

CHAPTER 2
LITERATURE REVIEW

CHAPTER 2

LITERATURE REVIEW

2.1 *VIBRIO SPECIES*

2.1.1 BACKGROUND

As indicated in Bergey's Manual of Determinative Bacteriology (1994) and Bergey's Manual of Systematic Bacteriology, *Vibrios* (*Vibrionaceae* strains) are classified under the Gammaproteobacteria, and these bacteria are Gram negative, typically motile rods, that are mesophilic, chemoorganotrophic, undertake a facultative fermentative metabolism, and are found in aquatic environments such as estuaries, marine coastal waters and sediments and aquaculture and may also be associated with eukaryotes (Madigan *et al.*, 2004; Thompson *et al.*, 2004; Barbieri *et al.*, 1999).

In the late 1880s the Dutch microbiologist Martinus Beijerinck discovered the first non-pathogenic *Vibrio* species, *Vibrio fischeri*, *Vibrio splendidus*, and *Photobacterium phosphoreum* that were isolated from the aquatic environments (Thompson *et al.*, 2004). Later on, several species that include *Vibrio cholerae*, *Vibrio fischeri*, *Vibrio haliotocoli*, *Vibrio metschnikovii*, *Vibrio harveyi*, *Vibrio parahaemolyticus* and *Vibrio alginolyticus* were reported to cause food-borne infections in humans usually associated with the consumption of undercooked seafood (Thompson *et al.*, 2004). Currently, *Vibrio* species constitute a variety of

strains some of which are human pathogens and are responsible for gastrointestinal and wound infections (Dalmasso *et al.*, 2009). Most of these species are transmitted to humans through the ingestion of contaminated fishes and shellfish (Dalmasso *et al.*, 2009).

The growth of almost all *Vibrio* species is stimulated by the presence of sodium which is an absolute growth requirement for most strains (Cabral, 2010). *Vibrio* species require saline in order to grow, except for *Vibrio cholerae* and *Vibrio mimicus* that are non-halophilic (Paydar, 2013). In addition, these organisms are sensitive to acidic pH and therefore are tolerant to alkaline pH environments. *Vibrio* species produce catalase and as a result form visible bubbles during catalase test. Moreover, they are able to ferment sugars without producing gas and hydrogen sulphide and their failure to produce gas distinguishes them from *Aeromonads* (Madigan *et al.*, 2004). *Vibrio* species appear as two morphologically distinct coloured colonies on thiosulfate citrate bile salts agar (TCBS), in which case sucrose fermenters form yellow colonies while non-sucrose fermenters form green colonies (Kaysner *et al.*, 1992). Moreover, *Pseudomonas*, Enterobacteriaceae and Gram-positive bacteria are not able to grow on TCBS (Harwood *et al.*, 2004).

2.2 WATER CONTAMINATION

Vibrio species which are mostly aquatic are either free-living or exist in close association with zooplanktons or phytoplanktons (Chakraborty *et al.*, 2000). Waterborne diseases still pose a major public health risk to humans worldwide

despite the recent advances in water quality and wastewater treatments (Zhou and Smith, 2002). Unlike other enteric pathogens that are found in aquatic environments as a result of the uncontrolled discharge of wastewater, *Vibrio* species on the other hand are not principally introduced into the aquatic ecosystems through faecal pollution (Osunla and Okoh, 2017). Consequently, when communities rely on untreated environmental water sources for various uses such as bathing, cooking and drinking, contamination may accidentally occur through the ingestion of other contaminated food products (Colwell *et al.*, 1990).

2.1.2 DISEASES CAUSED BY *VIBRIO* SPECIES AND CLINICAL PRESENTATION

Infections caused by *Vibrio* species remain a major public health challenge in middle-income countries such as Africa and Asia, and as a result present a severe risk to the health of individuals in the population who have weak immune response (Mackintosh and Colvin, 2003). In addition, *Vibrio* species are considered as one of the most important pathogens associated with foodborne and waterborne diseases and they have been frequently associated with many food poisoning outbreaks (Paydar, 2013). Moreover, there are some *Vibrio* strains that are pathogenic and can cause *Vibriosis*, which is a serious infectious disease in both cultured and wild finfish and shellfish (Li *et al.*, 1999). *Vibrio* species account for an estimate of more than 2.2 million deaths per year and about 1.4 million of these deaths occur among children (Ramírez-Castillo *et al.*, 2015). A large proportion of

the deaths are as a result of diarrhoea, gastrointestinal diseases and systematic illnesses that are now known to be a global burden the medical profession (Ramírez-Castillo *et al.*, 2015).

The *Vibrio* genus has at least twelve known species that are pathogenic to humans (Oliver, 2015; Plaza *et al.*, 2017). These species comprise *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio damsela*, *Vibrio fluvialis*, *Vibrio vulnificus*, *Vibrio mimicus*, *Vibrio cincinnatiensis*, *Vibrio furnisii* and *Vibrio metschnikovii* among others (Oliver, 2015; Plaza *et al.*, 2017). In addition, these pathogens are able to cause a variety of clinical complications that include wound infections, gastroenteritis and septicemia (Oliver, 2015). Moreover, in developing countries where there is lack of appropriate sanitary facilities, as well as improperly managed water purification and poor water supply facilities coupled with poor socioeconomic conditions there has large proportions of cases associated with bloody diarrhoea, and septicemia especially in immunocompromised individuals as well as necrotizing fasciitis have been implicated with *Vibrio* species (Igbinosa and Okoh, 2010). In addition, natural tragedies such as tsunami and floods have been reported to play a significant role in the spread of these outbreaks (Qadri *et al.*, 2005).

The clinical indications of disease with these *Vibrios* in humans are different, for example, *Vibrio harveyi* causes vasculitis, gastro-enteritis, and eye injuries; *Vibrio anguillarum* causes dormancy, red spots on the ventral and sidelong territories,

ulcers, and bleeding; also, *Vibrio alginolyticus* causes septicaemia, exophthalmia, corneal mistiness, ascites, dormancy and melanosis (Yu *et al.*, 2013) (Figure 2.1).

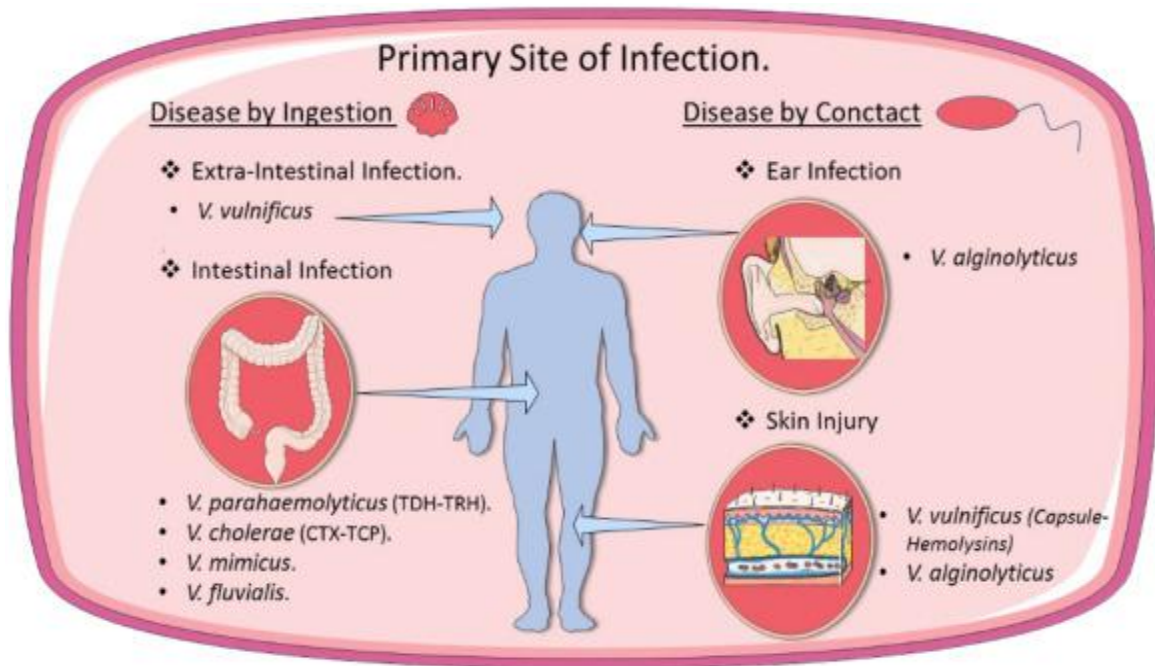


Figure 2. 1: Schematic structure of the primary site of infection of different pathogenic *Vibrio* affecting humans (Plaza *et al.*, 2017)

There are a variety of pathogenic *Vibrios* species infecting humans and some of them such as *Vibrio cholerae* and *Vibrio parahaemolyticus* are well characterized based on their principal virulence factors (Plaza *et al.*, 2017). In addition, some species such as *Vibrio mimicus* and *Vibrio alginolyticus* are considered emerging pathogens due to their recent involvement in disease (Plaza *et al.*, 2017).

2.1.3 TRANSMISSION OF *VIBRIO* SPECIES

The transmission of *Vibrio* species to humans may occur through a variety of sources and these may include through wound infections, the consumption of undercooked contaminated food products especially seafood; finfish, molluscs and crustaceans (Paola et al., 2000; Austin, 2010). In addition to these, the consumption of untreated contaminated water that has come in contact with sewage either directly or indirectly is considered a very important source of human contamination (De Paola et al., 2000; Austin, 2010).

2.1.4 LABORATORY DIAGNOSIS AND DETECTION

Although conventional culture-, biochemical-based methods have frequently been used in the detection of foodborne pathogens, these methods require a number of sequential procedures (Yamazaki *et al.*, 2008a) and may not be very sensitive. PCR is one of the molecular biological DNA-based diagnostic methods that has been considered very reliable for rapid and precise identification of bacteria species including *Vibrio* species. In addition, PCR complements the lack of precision from standard microbiological culture-based methods (Jones *et al.*, 2012).

Although some species of *Vibrio* may require growth factors and/or vitamins, but generally *Vibrios* are fairly easy to isolate from both clinical and environmental samples (Thompson *et al.*, 2004). Depending on the strain under investigation, *Vibrios* grow optimally at temperatures between 15 and 30°C. Although there are

a number of culture media that can be used to grow *Vibrios*, thiosulfate citrate bile salts agar (TCBS; Oxoid) is an ideal growth medium and has proven to be highly selective for the isolation of *Vibrios* (Thompson *et al.*, 2004). Despite the fact that some non-*Vibrio* bacteria species such as *Staphylococcus*, *Flavobacterium*, *Pseudoalteromonas*, and *Shewanella* are capable of exhibiting slight growth on TCBS. *Vibrio cholerae* produce distinct yellow colonies resulting from their ability to metabolise sucrose while other strains within the genus that cannot metabolise sucrose form green colonies (Thompson *et al.*, 2004).

PCR has been used for the detection of pathogens from various sources (Ateba and Mbewe, 2011) and the 16S rRNA gene sequencing has high discriminatory power for molecular identification of pathogens (Song, 2005). This is due to the fact that this gene is highly conserved and has variable regions which contain signatures that are unique for each bacterium (Song, 2005; Ateba and Mbewe, 2011). It is against this background that 16S rRNA gene sequencing has been considered a gold standard technique for the identification of bacterial species.

A variety of housekeeping genes that include *rpoB*, *recA*, and *rpoA* have been used in the identification of *Vibrio* species (Nhung *et al.*, 2007a, 2007b). Despite the fact that it is difficult to distinguish *Aeromonads* from *Vibrio* species using 16S rRNA gene analysis (Tarr *et al.*, 2007), reports have shown that the *rpoB* gene requires further DNA sequencing to confirm the identities of isolates (Mollet *et al.*, 1997). It is against this background that the *dnaJ* gene which has been reported

to show low sequence similarities across related genus is now considered a reliable target for the identification of *Vibrio* species (Nhung *et al.*, 2007a, 2007b).

2.1.5 PREVENTION, TREATMENT AND CONTROL OF *VIBRIO* INFECTIONS

The treatment of infections caused *Vibrio* species is administered according to the causative pathogen (Baker-Austin *et al.*, 2018). For *Vibrio cholerae* infections rehydration therapy is recommended and debridement of infected tissues is considered appropriate for management of disease. However, for *Vibrio vulnificus*-associated infections, antibiotic therapy is recommended to address the severe cholera and systemic clinical implications that are associated with the disease (Baker-Austin *et al.*, 2018). In addition, several antibiotics such as Norfloxacin, Polymyxin B, Amoxicillin, Ampicillin, Chloramphenicol, Gentamicin, Kanamycin, Nalidixic acid, Vancomycin, Quinolone, Streptomycin, Spectinomycin, Erythromycin, Fluoroquinolone, Furazolidone Neomycin, sulfamethoxazole–trimethoprim, Cotrimoxazole, Ciprofloxacin, Doxycycline, Sulphonamides, Tetracycline and Trimethoprim have been used in treating various *Vibrio* infections (CDC; Lima, 2001; Kitaoka *et al.*, 2011; Lagana *et al.*, 2011). Moreover, previous studies have established that Rifampicin, Kanamycin, Polymyxin B, Streptomycin and Tetracycline were active against *Vibrio* species (Igbinosa and Odjadjare, 2016). However, studies have shown that both environmental and clinical *Vibrio* strains harbor antibiotic resistance genes (Cabello *et al.*, 2013; Lutchumanan *et al.*, 2015; Shrestha *et al.*, 2015) and these attributes may affect the management of disease in susceptible individuals.

2.2 ANTIBIOTIC RESISTANCE IN *VIBRIO* SPECIES

The phenomenon of antibiotic resistance among various *Vibrio* species is well-known and has been widely documented globally (Saga and Yamaguchi, 2009). Antimicrobial resistance is a serious global health concern and consequently immediate solutions are needed to limit the spread of resistant determinants and also reduce human suffering (da Costa *et al.*, 2013). Antimicrobial agents are elements that selectively inhibit the growth of bacteria cells while causing minimal or no effect the host cell (Willey *et al.*, 2014). However, the mechanism of action of these agents vary significantly and these may range from their effect on the permeability of the cell wall to the drug, effect on the nucleus, cytoplasm (Willey *et al.*, 2014). It must be mentioned that the mode of action may also depend on the group to which antibiotic belongs (Willey *et al.*, 2014).

Amongst marine bacteria, *Vibrios* are among the most important with respect to the production of antibiotics (Long and Azam, 2001). Antimicrobial resistance is typically defined as the ability of a bacterial cell to survive exposure to a distinct concentration of an antimicrobial agents (Tenover, 1995; Acar and Rostel, 2001). Antimicrobials such as antibiotics have long been used to control the unwanted effects of microbial growth (Kümmere, 2009). Organisms of the same species “inherit” the resistance during the process of cell division (vertical resistance transfer). In other micro-organisms such as *Pseudomonas aeruginosa* primary resistance against penicillin G is naturally present while secondary resistance only

develops during contact of micro-organisms with the antibiotic. In this regard, conjugation (horizontal resistance transfer) has been responsible for the transfer of extra-chromosomal genetic material between different bacterial species. These resistant determinants can later be transmitted to humans especially if proper hygiene practices are not implemented (Kümmere, 2009).

Micro-organisms have the ability to move easily between ecosystems, from the soil and water to animals and later to humans and vice versa. This therefore implies that, antimicrobial resistance genes acquired by organisms in one ecosystem can easily be transferred to organisms in different environments that have a common ecological link. It is against this background that it is important to constantly determine the correlation between the use of antimicrobial agents and the development of antimicrobial resistant bacteria in a given ecosystem or environments (Patterson *et al.*, 2007).

2.3 VIRULENCE FACTORS ASSOCIATED WITH *VIBRIO* SPECIES

A number of virulence factors produced by human pathogenic *Vibrios* cause mild to fatal diseases (Eyisi and Iroegbu, 2013). Generally, the ability of *Vibrio cholerae* to cause disease depends on the production of toxin-coregulated pilus (TCP) and cholera toxin (CT). The CT is coded by the *ctxB* and *ctxA* genes present on the integrated prophage CTX ϕ and it is implicated in diarrhoea with dehydration and electrolyte loss (Waldor and Mekalanos, 1996). By contrast, the TCP coded by *tcpA* helps *Vibrio cholerae* to colonize the epithelium of small intestine (Waldor and

Mekalanos, 1996) and the expression of these genes is regulated by the *toxR* regulon in response to external stimuli (Bina *et al.*, 2003). In addition, the thermostable direct hemolysin-related gene (*trh*) and the thermostable direct hemolysin (*tdh*) are the main contributors to *Vibrio parahaemolyticus* pathogenicity (Terzi *et al.*, 2009; Terzi Gulel and Martinez-Urtaza, 2016) due to its biological impacts including its enterotoxic and cytotoxic effects (Hiyoshi *et al.*, 2010). In addition, *Vibrio vulnificus* virulence is encoded by virulence-related gene (*vcg*). Variations in the degree of virulence which is connected to the origin of the bacterial strain have been established in two main genotypes, with clinical isolates demonstrating higher virulence than environmental strains.

Conversely, *Vibrio fluvialis* is shown to produce numerous potent toxins such as the heat stable enterotoxin even though their functions in pathogenesis are not fully established (Chakraborty *et al.*, 2006). Like in *Vibrio cholera*, the *toxR* is involved in the resistance to bile by *Vibrio fluvialis* which is the first phase in the establishment of disease in their hosts. Additionally, heme utilization protein gene (*hupO*), *Vibrio fluvialis* protease gene (*vfp*) and extracellular haemolysin gene (*vfh*) are other virulence factors (Liang *et al.*, 2013) that are associated with *Vibrio* species. Despite the fact that pathogenic *Vibrios* have been isolated from effluent of waste water treatment plants in South Africa (Igbinosa *et al.*, 2009; Igbinosa *et al.*, 2011; Okoh *et al.*, 2015), the relationship of their occurrence in treated water was not determined. In the present study an analysis of these isolates in both raw and treated water presents an overview of the public health implication of

pathogenic *Vibrios* on humans in the area and data generated may be of great epidemiological importance.

2.4 BIOFILM FORMATION

Biofilms are generally defined as communities of microorganisms attached to a surface (Percival, 2017.). The formation of bacterial biofilms occurs when unicellular organisms combine to form a community that is adhered to a solid surface and enclosed in an exopolysaccharide (EPS) matrix (Mah and O'toole, 2001). The EPS components serves provides both structural and protective functions to the bacteria (Guinta, 2010). In addition, biofilms can be made up of single or a variety of bacterial species (Hall-Stoodley and Stoodley, 2005). Properties expressed by biofilm-forming cells are distinct from those of planktonic cells, one of which is increased resistance to antimicrobial agents (Mah and O'toole, 2001).

Organisms compete for space due to different conditions, for example, fluid flow, nutrient limitation, desiccation, UV radiation, toxic chemical gradients and temperature fluxes (Hall-Stoodley and Stoodley, 2005). Therefore, biofilm formation represents a facile microbial survival strategy in which microorganisms, including pathogens, exist in a dynamic equilibrium where cell clusters form, mature and detach to disseminate to new surfaces (Hall-Stoodley and Stoodley, 2005). Thus, the fundamental key component to ensure environmental survival and transmission is the ability to form biofilms (Yildiz and Visick, 2009). Resistance

of biofilms to antibiotics has been on an increase compared to that which has normally been observed with planktonic cells (Mah and O'toole, 2001). The adaptation of *Vibrio* species to changing environmental parameters in aquatic ecosystems, as well as in their respective hosts, is important to their survival and colonization that may result to establishment of diseases (Yildiz and Visick, 2009).

In the microbial, the production of biofilms during growth is the preferred lifestyle since it enhances growth and survival by providing access to nutrients and protection from predators and antimicrobial compounds (Yildiz and Visick, 2009). An increase in contaminations resulting from a decrease in quality of water, and a reduction in efficacy of heat exchangers are common problems associated with biofilms in water and sewage treatment facilities (Guinta, 2010).

Biofilms are produced by several bacterial pathogens found in the marine environments. Despite the progress that is being made in documenting both molecular and genetic mechanisms to provide an overview of how bacteria species produce biofilms, as well as its role in disease transmission, so far, reports indicate that extensive work has been done on *Vibrio cholerae*, which is the causative agent of cholera (Huq *et al.*, 2008). Biofilm formation in *Vibrio* species in recent years have focused on colony morphology as an indicator of biofilm formation (Yildiz and Visick, 2009). Furthermore, a number of key proteins, including those involved in the biosynthesis of flagella, pili and polysaccharide, as well as their regulators that

control their expression, have been identified to significantly contribute to biofilm formation potentials (Yildiz and Visick, 2009).

Nosocomial infections and chronic illnesses are often as a result of biofilms. In most cases, antibiotics are not able to resolve biofilm-related infections and persistently reoccur. A major example of biofilm-driven infections is chronic respiratory illnesses that are associated with cystic fibrosis (CF) patients (Guinta, 2010). To ensure the survival of pathogens during harvest, transport, production, and distribution, biofilms “offer” the attachment, protection, and resistance essential qualities (Guinta, 2010).

Generally, treating biofilm-related infections with the use of antibiotics do not result in successful cures. Consequently, common outcomes are those of persistent infections which as a result cause significant discomfort and illness (Guinta, 2010). In regards to transmission and persistence of human disease it has been suggested that biofilms play a crucial role. In addition, for human pathogenic bacteria, to allow bacteria from the host immune system to withstand killing doses of antibiotics, biofilms offer protection to the bacteria (Huq *et al.*, 2008). Resistance of biofilms to many antibiotics such as ampicillin, tetracycline, penicillin, erythromycin and chloramphenicol have been document (Guinta, 2010). Laboratory microcosm experiments involving cells of *Vibrio cholerae* revealed that these cells protect themselves through the expression of exopolymer barriers

which are formed as a result of these cells forming biofilms on both biotic and abiotic surfaces (Huq *et al.*, 2008).

It is only recently that the clinical relevance of biofilm forming bacteria especially those that dominate in environmental processes have gained significant attention (Wingender and Flemming, 2011). This is due to the fact that the pathogen can accidentally attach to biofilms even when they are present at very low detection limits in water and as a result can act as their environmental reservoirs thus serve as a potential source of water contamination (Wingender and Flemming, 2011).

Figure 2.2 illustrates the role of biofilms in water contamination.

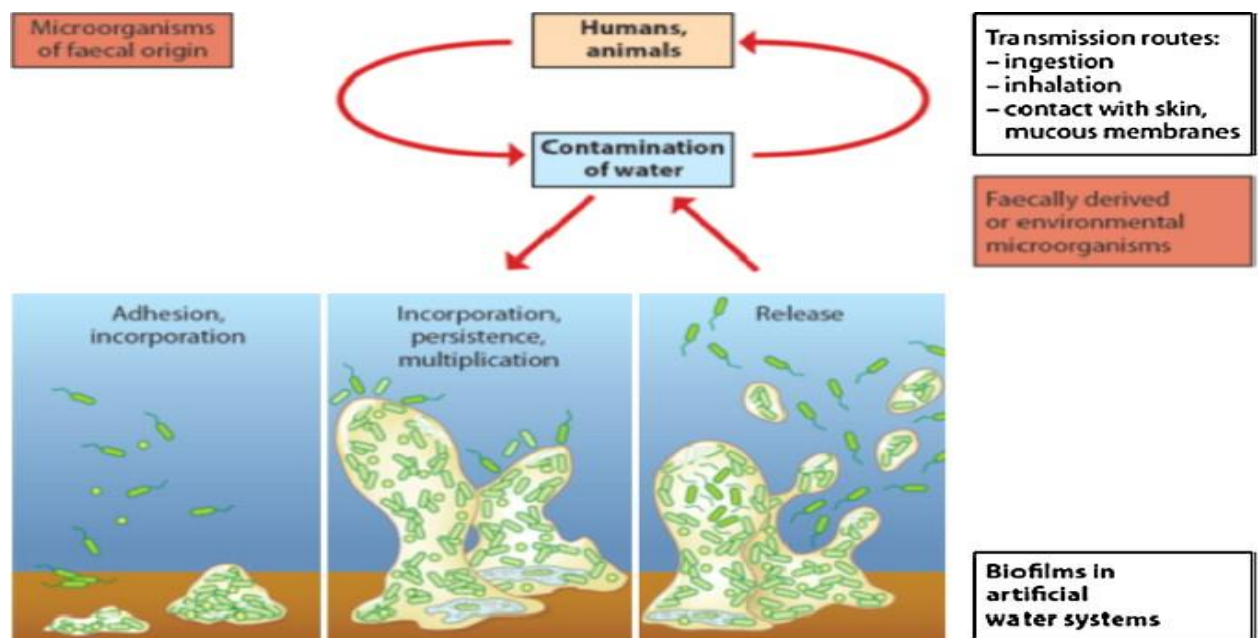


Figure 2. 2: Schematic structure of biofilms and the role they play as environmental resevoirs of hygienically relevant microorganisms as well as source of contamination and infection in drinking water systems (Wingerder, 2011)

2.5 BACTERIOPHAGES AS POTENTIAL BIO-CONTROL AGENTS AGAINST *VIBRIO* SPECIES

2.5.1 Classification of phages

Bacteriophages are viruses that infect bacteria. In addition, phages are specific to bacterial their bacteria host cells that they infect (Santiago-Rodriguez, 2013). Bacteriophages encompass a nucleic acid that is present in a protein coat (capsid), which could be additionally be surrounded by a lipid layer (Nicklin, Graeme-Cook *et al.*, 1999). Moreover, tailed bacteriophages have a tail that may either be non-contractile (phage λ) or contractile (T4 phage). Furthermore, they can also have extra structures such as basal plate, collar, spikes and tail fibres that facilitate the attachment of the phage to the bacterium in order to inject their nucleic acid into the host cell.

The International Committee on Taxonomy of Viruses (ICTV) generally classifies bacteriophages according to morphology and nucleic acid (Murray *et al.*, 1981). Moreover, as compared to bacteria species bacteriophages are more stable in the environment since they are highly resistant to the water disinfection processes (Simkova and Cervenka, 1981; Santiago-Rodriguez, 2010). Additionally, due to their ability to infect and kill bacteria cells as is the case with lytic phages they are considered to be useful in the prevention and treatment of bacterial diseases (Goyal *et al.*, 1987). However, due to the rapid development and dissemination of resistant bacteria might present a challenge to this technology (Goyal *et al.*, 1987).

However, some studies have revealed that the combination of different phage stocks in a single experiment have improved the host range as well as virulence capabilities against resistant strains when compared to individual phages.

Bacteriophages are able to exist with their host in two ways, either through lysogenic in where they insert themselves into the bacterial genome and thus cause modification or via lytic in which they replicate inside the host and release more new phages that will infect more bacterial and consequently destroying host bacteria (Harper *et al.*, 2014). The fact that they can be used as models or surrogates for human viruses in basic genetic research as well as water quality assessment has turned out to be one of many other major benefits of bacteriophages (Grabow *et al.*, 2006). The structure of the phage can be further illustrated in a diagrammatical representation on the Figure 1.3.

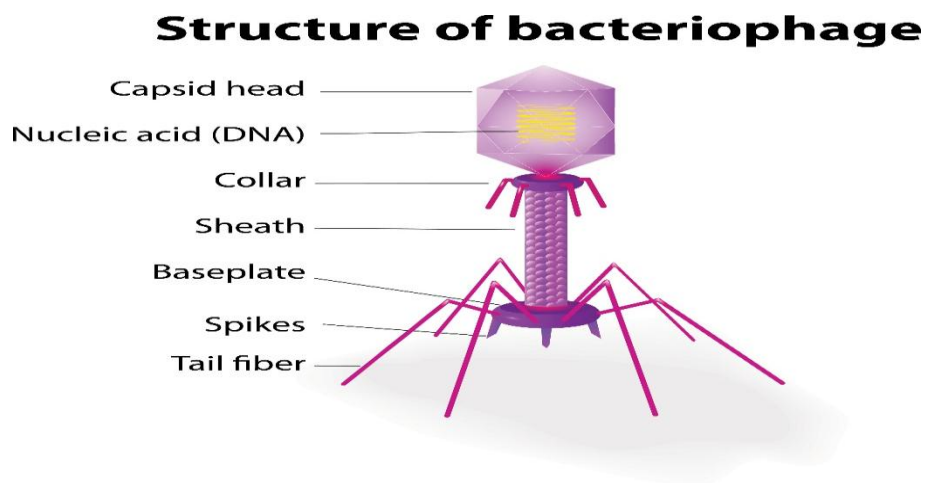


Figure 2. 3: Diagrammatical structure of the phage (Adapted from the scientist.com)

2.5.2 Phage therapy

Phage therapy is regarded as the use of bacteriophages to destroy or reduce bacteria which are resistant to antibiotics (Harper *et al.*, 2014). In addition, lysogenic phages are the most favoured phage type that has been used for therapeutic processes (Harper *et al.*, 2014). Phage therapy and phage bio-control are receiving significant attention as a result of the increasing occurrence of bacterial antibiotic resistance to the majority of available antibiotics (Tanji *et al.*, 2004). Bacteriophages have numerous advantages over the conventional antimicrobial agents since they target specific hosts, are self-limiting and self-replicating, have low inherent toxicity to their hosts and they are able to prolong their shelf life in food products (Sillankorva *et al.*, 2012).

Bacteriophages are fairly easy to isolate since they are usually found in the same habitat as their bacterial hosts (Sillankorva *et al.*, 2012) and this makes the approach of phage therapy and phage bio-control very cheap (Atterbury *et al.*, 2003). Phages can either be used as mono-phage preparations (which involves only one type of phage) or as cocktails (where a combination of different phages is used) (Tanji *et al.*, 2004). The application of phage cocktails decreases the ability of bacteria developing resistance against single phages and thus increases the host range (Tanji *et al.*, 2004). Furthermore, ability of bacteriophages to inhibit the growth of multi-drug resistant bacteria has renewed interests on the use of phages as bio-control agents in recent years worldwide (Shin *et al.*, 2012; Osayande, 2014). Consequently, this has increased the focus and more research

on the application of bacteriophages as potential biocontrol agents against resistant bacteria (Osayande, 2014). This therefore implies the importance of laboratory investigations designed to assess the virulence potentials of endemic phages in destroying virulent and antibiotic resistant bacteria strains such as those belonging to the genus *Vibrio* cannot be over-emphasised.

CHAPTER 3
MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS AND METHODS

3.1.1 Area of the study

The research study was conducted at the Molecular Microbiology Laboratory in the Department of Microbiology, North-West University-Mafikeng Campus, South Africa.

3.1.2 Ethical Clearance

This study was approved by the Research Ethics Committee of the North West University, South Africa and ethical clearance number (NWU- 00725-18-A9) was issued prior to the collection of samples.

3.1.3 Collection of samples and sample sites

A total of 136 water samples were collected from taps, boreholes and dams in randomly selected areas and communities in the North-West Province using sterile 500 mL Duran Schott bottles. The samples were properly labelled and transported on ice to the Molecular Microbiology Laboratory in the Department of Microbiology for analysis. Upon arrival in the laboratory, water samples were immediately analysed for the presence of *Vibrio* species using selective media. The numbers of samples collected from the different areas are shown in Table 3.1.

Table 3. 1: Areas from which water samples were collected

Sampling Area	Sample site and No. of Samples		
	Tap water	Borehole water	Dam water
Dryharts	4		
Choseng	4	4	
Pudimoe	4	4	
Ntswanahatshe	4	4	
Vryburg	4		
Pella	4	4	2
Silverkraans	4		
Swartruggens	4	4	
Coligny	4	4	2
Ventersdorp	4		
Lichtenburg	4		
Marico Bosveld			2
Moorriver	4		
Potchefstroom	4		
Phola	4	6	
Ramosadi	4	6	
Motlhabeng	4	6	
Dibate	4	6	
Seweding	4	6	
Lonely park	2	2	
Total	74	56	6

3.2 ANALYSIS OF WATER SAMPLES AND BACTERIAL ISOLATION

3.2.1. Processing of water samples and Isolation of *Vibrio* species

Upon arrival in the laboratory, water samples were analysed immediately using standard methods (APHA, 1998). An aliquot of 100 mL from each water sample was filtered through a 0.45 µm filter paper (Whatman®Glass Microfiber GS Filter paper) on a water pump machine (model, Sartorius 16824). Sterile forceps were used to remove the membrane filters from the machine and the filter papers were placed in the centre of Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar (Biolab, South Africa) plates. The water pump machine was sterilized with alcohol and flamed after each sample was analysed. The inoculated TCBS plates were incubated aerobically at 37 °C for 24 hours. After incubation green and yellow coloured colonies were indicative *Vibrio* species in the samples. The presumptive *Vibrio* colonies were purified by sub-culturing on TCBS agar plates and plates were incubated aerobically at 37 °C for 24 hours. Presumptive pure isolates were subjected to several biochemical identification tests that are specific for *Vibrio* species.

3.3 BIOCHEMICAL IDENTIFICATION OF *VIBRIO* SPECIES

Presumptive *Vibrio* isolates were identified using the following criteria:

3.3.1 Cellular morphology

The cellular morphologies of pure yellow and green colonies on the TCBS agar was determined through Gram staining using a standard technique (Cruikshank *et al.*, 1975). Isolates belonging to the family *Vibrionaceae* are Gram negative comma-shaped rods,

hence all isolates that satisfied this criterion were subjected to preliminary biochemical identification tests.

3.3.2 Oxidase test

The oxidase test is used to detect the production of intracellular oxidase enzyme by bacterial species. Cytochromes are iron-containing proteins present in all aerobic organisms thus, exhibiting oxidase activity (Winn and Koneman, 2006). This test facilitates the differentiation of members of the family *Vibrionaceae* which are oxidase positive from those belonging to the family *Enterobacteriaceae* which are oxidase negative. To perform the test, oxidase strips (Microbact™ Oxidase Detection Strips) were used according to the manufacturer's instructions. A single colony was transferred to an Oxidase Detection Strip using a disposable sterile plastic loop. The colony was spread on the strip and observed for colour change within 5 to 10 seconds. A deep blue or violet colour was recorded as a positive reaction. An oxidase test is used to identify the presence of cytochrome c oxidase in bacteria and the cytochrome c oxidase catalyses the transportation of electrons between electron donors and redox dye. The oxidase test is based on the principle that tetramethyl-p-phenylenediamine is oxidised by bacterial cytochrome in the presence of atmospheric oxygen to form purple coloured compound. When cytochrome c oxidase oxidizes the reagent tetramethyl-p-phenylenediamine it is reduced to iodophenol which results to the production of a purple colour and therefore interpreted as an oxidase positive result. On the contrary, no colour change is interpreted as negative result for the oxidase test. Bacteria belonging to the family *Vibrionaceae* are oxidase-positive and all the results obtained were recorded.

3.3.3 Triple Sugar Iron agar test

The Triple Sugar Iron agar test was performed using the Triple Sugar Iron (TSI) agar obtained from Merck, South Africa on all the isolates. TSI agar is used to differentiate *Vibrionaceae* based on their ability to ferment three sugars glucose, lactose and sucrose that are present at concentrations of 0.1%, 1.0% and 1.0% respectively with or without the production of gas and hydrogen sulphide (Prescott *et al.*, 2002). Triple Sugar Iron agar medium is a differential medium used to differentiate Gram negative enteric pathogens based on their physiological ability to metabolise carbohydrates (Forbes and Weissfeld, 1998). The degradation of sugars is detected with the help of a pH indicator phenol that changes its colour from red to yellow in the presence of acids after degradation of the sugars. In addition, some species are capable of reducing thiosulphate to hydrogen sulphide, which reacts with an iron salt to produce a black iron sulphide precipitate in the centre of colonies. In performing the test, the media was prepared and aliquots of 5 mL were poured into sterile McCartney bottles. The media was sterilized and bottles kept in slanting positions in order to obtain a slant and butt after the medium solidifies. All isolates were aseptically stab-inoculated into the butt of the medium using a sterile loop and later streaked on the slant. The inoculated bottles were loosely closed and incubated at 37°C for 24 hours. After incubation, the isolates were evaluated for the ability to ferment the sugars present with or without the production of acid, gas and hydrogen sulphide (H₂S). Results were recorded and analysed as previously recommended (Forbes and Weissfeld, 1998).

3.3.4 Simmons Citrate Agar

All the 57 presumptive isolates were subjected to the Citrate utilization test using the Biolab Simmons Citrate Agar (SCA) obtained from Merck, South Africa. In the absence of fermentable carbohydrates, some enteric pathogens are capable of utilising sodium citrate as the sole source of carbon (Macfaddin, 2000; Prescott, 2002) and this ingredient is included in the Simons Citrate agar (Macfaddin, 2000). In performing the test, isolates from a pure culture were streaked stab-inoculated into the butt of Simmons citrate agar (Fluka, Biochemika) using a sterile loop and later streaked on the slant. The inoculated cultures were incubated at 37°C for 24 h. After incubation a colour change from green to blue was recorded as a positive reaction and vice versa (Brenner, 1984).

3.3.5 Salt Tolerance Test

According to the procedure adopted by West (1984), different concentrations (0%, 3%, 6% and 10%) of NaCl were prepared using peptone water. Each isolate was inoculated into the different concentrations of salt contained in 15 mL Centrifuge tubes and tubes were incubated aerobically at 37 °C for 24 hours. After incubation, tubes were observed for visible bacteria growth by assessing its turbidity with reference to the control samples. Evidence of growth indicated tolerance of potential *Vibrio* species to a specific concentration of salt (West, 1984).

3.3.6 Motility Test

The Sulfide Indole Motility (SIM) medium was prepared in sterile 15 mL centrifuge tubes and presumptive colonies were stab-inoculated to about one-half the length of the medium using a straight sterilized needle (Cheesbrough, 2006). The caps of the tubes

were loosely closed and tubes were incubated at 37 °C for 18-24 hours. The identification of visible growth away from the line of inoculation denotes motility of the presumptive isolate.

3.4 BACTERIAL STORAGE

In order to conserve the isolates for future use, 1 ml of 20 % (v/v) glycerol was aliquoted into 1.5 µL sterile eppendorf tubes. A single pure colony for each isolate was transferred into the tubes using sterile disposable plastic loops. The contents of the tubes were homogenised by vortexing and stored at -80 °C.

3.5 MOLECULAR CHARACTERISATION OF *VIBRIO* SPECIES

3.5.1 DNA Extraction

Genomic DNA was extracted from all presumptive *Vibrio* isolates using the Cetyltrimethyl ammonium bromide (CTAB) method as described earlier by Doyle and Doyle (1999). A ChemiDoc Imaging System (Bio-RAD ChemiDoc™ MP Imaging System, UK) was used to capture the image using Gene Snap (version 6.00.22) software to determine the presence of DNA. The DNA samples were stored at -20°C for future use.

3.5.2 Determination of DNA quality and quantity

The quality and purity of genomic DNA extracted from the isolates was determined using a spectrophotometer (version UV Visible spectrophotometer model S-22, Boeco, Germany) at wavelengths of 260 nm and 280 nm as previously described (Sambrook *et al.*, 1989).

3.6 PCR AMPLIFICATION OF 16S rRNA GENE OF *VIBRIO* SPECIES

The 16S rRNA gene is a conserved universal gene found in all bacteria and has a variable portion which possesses a signature unique to each bacterium (Song, 2005). Bacterial 16S rRNA gene fragments were amplified as an internal control for all presumptive isolates. Bacteria 16S rRNA gene fragments were amplified using universal oligonucleotide primers 27F and 1492R whose sequences appear in Table 3.2 to amplify a 1420 bp gene fragment (Korzeniewska and Harnisz, 2013). Amplifications were performed using DNA thermal cycler (model- Bio-Rad C1000 Touch™ Thermal Cycler, Johannesburg, South Africa). The reactions were prepared in standard 25 µL volumes that constituted 12.5 µL of 2X DreamTag Green Master Mix (0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP, 4mM MgCl₂ and loading buffer), 11 µL nuclease free distilled water, 0.5 µL mixture (0.25 µL of each forward and reverse primer) of the forward and reverse primers and 1 µL of template DNA. All the PCR reagents used in this study were Fermentas, USA products supplied by Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa. The cycling conditions used for amplification of the 16S rRNA gene fragments are shown in Table 3.2. The PCR amplicons were resolved by electrophoresis on a 2% (w/v) agarose gel. A ChemiDoc Imaging System (Bio-RAD ChemiDoc™ MP Imaging System, UK) was used to capture the image using Gene Snap (version 6.00.22) software. A 1 kilo base pairs molecular weight DNA marker was included in the gel and used to determine the relative size of amplicons.

3.7 GENUS SPECIFIC PCR

3.7.1 Confirmation of identities of potential *Vibrio* isolates through PCR amplification of *ompW*, *rfbO1*, *flaE*, *sodB* (1), *hsp*, *sodB* and *vhh* gene fragments

The species-specific primers were used in the identification of all the presumptive *Vibrio* isolates. In order to avoid any bias, in the present study different genes that are specific to different *Vibrio* species were used to provide an overview of the species composition among the presumptive isolates. The *flaE* gene was used as a target for *Vibrio parahaemolyticus*, *hsp* for *Vibrio vulnificus*, *sodB* (1) for *Vibrio cholera*, *sodB* for *Vibrio mimicus*, *vhh* for *Vibrio harveyi*, *OmpW* for *Vibrio cholerae* and *rfbO1* for *Vibrio cholera*-Serogroup O1 (Menezes *et al.*, 2014; Raissy *et al.*, 2015). The primer sequences, target genes and amplicon sizes of the different *Vibrio* specific PCR amplicons are listed in Table 3.2. Amplifications were performed using DNA thermal cycler (model- Bio-Rad C1000 Touch™ Thermal Cycler, Johannesburg South Africa). The reactions were prepared as standard 25 µL volumes that comprised 12.5 µL of 2X DreamTag Green Master Mix (0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP, 4mM MgCl₂ and loading buffer), 11 µL nuclease free distilled water, 0.5 µL mixture of the forward and reverse primers and 1 µL of template DNA. All the PCR reagents used in this study were Fermentas USA products supplied by Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa. The cycling conditions for the amplification *Vibrio* species-specific genes are listed in Table 3.2. The amplicons were resolved by electrophoresis on a 2% (w/v) agarose gel at 90 V for 50 mins. A ChemiDoc Imaging System (Bio-RAD ChemiDoc™ MP Imaging System, UK) was used to capture the images using Gene Snap (version 6.00.22)

software. A100 bp DNA molecular weight marker was included in the gel and used to determine the relative sizes of amplicons. The amplicon sizes obtained for the genes was verified by running an *in vitro* PCR using the In-Silico PCR amplification tool (<http://www.insilico.ehu.es/PCR/>).

Table 3. 2: Oligonucleotide primers used for amplification of PCR.

Primers	Sequence (5'-3')	Targeted gene	Amplicon size (bp)	PCR conditions and cycles	Reference
27F	AGAGTTTGATCATGGCTCAG	16S rRNA	1420	94°C, 3 min. 25 times (94°C, 1 min; 55°C, 1 min) 72°C, 2 min	(Korzeniewska et al., 2010)
1492R	GGTACCTTGTTACGACTT				
<i>Vibrio cholera</i>	CACCAAGAAGGTGACTTTATT GTG	ompW	304	94°C, 10 min. 30 times (94°C, 1 min; 59°C, 1 min; 72°C, 2 min) 72°C, 10 min	(Goel et al., 2007)
	GGTTTGTCTGAATTAGCTTCACC				
Cholera-Serogroup O1	TCTATGTGCTGCGATTGGTG	rfbO1	638		
	CCCCGAAAACCTAATGTGAG				
<i>Vibrio parahaemolyticus</i>	GCAGCTGATCAAAACGTT GAGT	flaE	897		
	ATTATCGATCGTGCCACTCAC				
<i>Vibrio cholera</i>	AAGACCTCAACTGGCGGTA	sodB (1)	248	95°C, 10 min. 35 times (92°C, 40 s; 57°C, 1 min; 72°C, 1.5 min)	(Tarr et al., 2007)
	GAAGTGTTAGTGATCGCCAGA GT				
<i>Vibrio vulnificus</i>	GTCTTAAAGCGGTTGCTGC	Hsp	410		
	CGCTTCAAGTGCTGGTAGAAG				
<i>Vibrio mimicus</i>	CATTCGGTTCTTTCGCTGAT	sodB	121		
	GAAGTGTTAGTGATTGCTAGA GAT				
<i>Vibrio harveyi</i>	CTTCACGCTTGATGGCTACTG	Vhh	235	95°C, 10 min. 30 times (95°C, 1 min; 50°C, 1 min; 72°C, 1 min)	(Maiti et al., 2009)
	GTCACCCAATGCTACGACCT				

3.7 SEQUENCE ANALYSIS OF PCR AMPLICONS

The 16S rRNA PCR amplicons were subjected to sequence analysis using the Illumina sequencer at the Department of Microbiology Potchefstroom Campus of the North West University, South Africa. Sequences were subjected to a BLAST Search using the tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) in order to confirm the identities of the isolates.

3.8 ANTIMICROBIAL SUSCEPTIBILITY TEST

3.8.1 Antimicrobial susceptibility test (AST)

An *in vitro* disc diffusion test was used to determine the antimicrobial resistance patterns of confirmed *Vibrio* isolates using a standard protocol (CLSI, 2010). *Vibrio* isolates were screened against a panel of eleven (11) different antimicrobial agents obtained from Mast Diagnostics, South Africa (Table 3.3). In performing the test, a pure colony from each isolate was transferred into an eppendorf containing 0.8 % (w/v) sterile physiological saline solution and vortexed. The optical density of bacteria suspension was adjusted to 0.5 McFarland standard suspension and aliquots of 100 µL inoculum from each suspension (10^6 cells) was spread-plated onto Mueller-Hinton agar. Each antimicrobial disc impregnated with therapeutic concentrations of a specific drug was placed at equitable distances on the inoculated plates. The different antimicrobial agents obtained from Mast Diagnostics, UK as well as their concentrations were as follows: ampicillin (AMP), 10µg; cephalothin (CEF), 30µg; chloramphenicol (CHL), 30µg; ciprofloxacin (CIP), 5µg; tetracycline (TET), 30µg; gentamicin (GEN), 10µg; kanamycin (KAN), 30µg; nalidixic acid (NAL), 30µg; sulfafurazole (SUL), 300µg; streptomycin (STR), 10µg; and

trimethoprim (TMP), 5µg. The plates were incubated aerobically at 37 °C for 24 hours. After incubation, plates were observed for visible clear zones and bacteria growth inhibition diameter data of zones around the discs were measured in millimetres. Values of the bacteria growth inhibition diameter data were used to classify isolates as susceptible, intermediate resistant, or resistant to a particular drug (Bauer *et al.*, 1966; CLSI, 2010).

3.8.2 Plasmid DNA extraction

Plasmid DNA was extracted from all presumptive isolates using the Zyppy™ Plasmid Miniprep Kit (Biotechnical Industries (Pty) Ltd Sunnyside, Pretoria, South Africa) following the manufacturer's instructions. Bacterial culture from glycerol stock culture was revived on TSA media and plates were incubated at 37 °C for 24 hours. A single pure colony of the isolate was streaked on Luria-Bertiani (LB) medium supplemented with ampicillin (100 µL) and plates were incubated at 37 °C for 24 hours. A single colony from ampicillin resistant isolates was inoculated into 15 mL of pre-autoclaved Luria-Bertiani (LB) broth supplemented with 100 µL ampicillin contained in 15 mL Falcon conical centrifuge tubes. The tubes were vortexed and the suspension was incubated at 37 °C for 24 hours while shaking at 160 rpm. After incubation, 600 µL of bacterial culture grown in LB broth was transferred to a 1.5 µL microcentrifuge tube and used for isolation of plasmids. An aliquot of 100 µL of 7X lysis buffer was added to the tube and mixed by inverting the tube 4-6 times. The tubes were incubated at room temperature for 2 minutes. Aliquots of 350 µL of cold Neutralization buffer was added and the contents of the tubes were mixed

thoroughly by inverting 4-6 times. The tubes were centrifuged at $\geq 11,000 \times g$ for 4 minutes. The Zymo-SpinTM IIN column was placed in a collection tube and supernatant was transferred into the Zymo-SpinTM IIN column. The Zymo-SpinTM IIN column was centrifuged at $\geq 11,000 \times g$ for 15 seconds. An aliquot of 200 μL of the Endo-wash buffer was added to the column and centrifuged at $\geq 11,000 \times g$ for 1 minute. After centrifugation 400 μL of the ZyppyTM wash buffer was added to the centre of the column and centrifuged at $\geq 11,000 \times g$ for 1 minute. The column was transferred into a clean 1.5 mL microcentrifuge tube and 30 μL of ZyppyTM elution buffer was added directly to the column matrix. The column was incubated at room temperature for one minute. The microcentrifuge tube was centrifuged at $\geq 11,000 \times g$ for 30 seconds to elute the plasmid DNA. In order to assess for the presence of plasmid DNA, 5 μL of the extract was resolved by electrophoresis on a 2% (w/v) agarose gel in 1X TAE buffer. A ChemiDoc Imaging System (Bio-RAD ChemiDocTM MP Imaging System, UK) was used to capture the images using Gene Snap (version 6.00.22) software. In order to determine the sizes of plasmids a 1kb DNA molecular weight ladder was included in the gel. The plasmid DNA was stored at -20°C .

Table 3.3: Antibiotics used in the study

Group	Antibiotic	Abbr.	Disc conc.(µg)	Reference values		
				Sensitive	Inhibition	Resistant
PENICILINS AND β-LACTAM/β-LACTOMASE INHIBITORS COMBINATIONS						
	Ampicillin	AP	10	≥17	14-16	≤13
CEPHALOSPRINS						
	Cephalothin	KF	30	≥18	14-17	≤13
QUINOLONES						
	Ciprofloxacin	CIP	5	≥21	16-20	≤15
PHENICOLS						
	Chloramphenicol	CHL	30	≥18	13-17	≤12
FOLATE PATHWAY INHIBITORS						
	Trimethoprin2.5 Sulfamethoxazole 23.75	TS	25	≥16	11-15	≤10
TETRACYCLINES						
	Tetracycline	T	30	≥15	12-14	≤11
AMINOGLYCOSIDES						
	Streptomycin	S	10	≥15	12-14	≤11
	Kanamycin	K	30	≥18	14-17	≤13
	Gentamicin	GM	10	≥15	13-14	≤12
QUINOLONES						
	Nalidixic	NA	30	≥19	14-18	≤13
SULPHONAMIDES						
	Trimethoprim	TM	5	≥16	11-15	≤10

3.8.3 Detection of *Vibrio* virulence genes

The presence of gene coding for virulence determinants in *Vibrio* species was determined through amplification of the following gene *ctxAB*, *zot*, *tdh*, *trh*, *flrA*, and *vpsR* sequences (Terzi *et al.*, 2009; Gennari *et al.*, 2012; Menezes *et al.*, 2014). The oligonucleotide primer sequences, target genes and amplicon sizes of the different genes are listed in Table 3.4. Amplifications were performed using DNA thermal cycler (model- Bio-Rad C1000 Touch™ Thermal Cycler, Johannesburg South Africa). The reactions were prepared as standard 25 µL volumes that comprised 12.5 µL of 2X DreamTag Green Master Mix (0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP, 4mM MgCl₂ and loading buffer), 11 µL nuclease free distilled water, 0.5 µL mixture of the forward and reverse primers and 1 µL of template DNA. All the PCR reagents used in this study were Fermentas USA products supplied by Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa. The cycling conditions for the different target genes are shown in Table 3.4. The amplicons were resolved by electrophoresis on a 2% (w/v) agarose gel at 90 V for 50 mins. A ChemiDoc Imaging System (Bio-RAD ChemiDoc™ MP Imaging System, UK) was used to capture the image using Gene Snap (version 6.00.22) software and a 100 bp DNA molecular weight marker was included in the gel in order to determine the relative sizes of amplicons.

Table 3.4: Oligonucleotide primers used for the amplification of virulence genes.

Primers	Sequence (5'-3')	Amplicon size (bp)	PCR conditions and cycles	Reference
<i>Tdh</i>	GTAAAGGTCTCTGACTTTTGGAC	269	94°C, 3 min. 30 times (94°C, 1 min; 58°C, 1 min; 72°C, 1 min) 72°C, 5 min	(Terzi et al., 2009)
	TGGAATAGAACCTTCATCTTCACC			
<i>Trh</i>	TTGGCTTCGATATTTTCAGTATCT	500	94°C, 10 min. 30 times (94°C, 1 min., 59°C, 1 min., 72°C, 2 min) 72°C, 10 min	(Menezes et al., 2014)
	CATAACAAACATATGCCCATTTC			
<i>ctxAB</i>	GCCGGGTTGTGGGAATGCTCCAAG	536	95°C, 5 min. 35 times (95°C, 45 s; 72°C, 45-180 s) 72°C, 15 min	(Gennari et al., 2012)
	GCCATACTAATTGCGGCAATCGCATG			
<i>Zot</i>	TCGCTTAACGATGGCGCGTTTT	947		
	AACCCCGTTTCACTTCTACCCA			
<i>flrA</i>	GAGGCAACAGCACCATCAAA	503		
	CATAACAAACATATGCCCATTTC			
<i>vpsR</i>	GGTGAGTAGCCATAAGCAAG	911		
	CGCATCTATATCAGGGACAA			

3.8.4 Biofilm formation assay

The potential of isolates to form biofilm was determined using the microtitre plate method (O'Toole *et al.*, 1999). Bacteria was grown in nutrient broth at 37°C for 24 hours. Overnight bacterial cultures were diluted 1:100 using sterilized nutrient broth. Two hundred micro litre of the diluted culture was dispensed into the wells of the microtitre plate and plates were incubated at 37°C for 24 hours. In each experiment, a well that contained sterilized uninoculated nutrient broth served as a negative control. After incubation, the cultures in the wells were discarded and the wells were washed twice with phosphate buffer saline. After washing, 200 µL of 1% (w/v) crystal violet dye was added to each well and the micro titre plate was incubated at room temperature for 1 hour. After incubation the dye was discarded and the wells were washed five times with phosphate buffer saline. Wells were allowed to air dry at room temperature and 200 µL of 95% (v/v) ethanol was added to each well of the micro titre plate. The plates were incubated at room temperature for 5 minutes. After incubation, the ethanol was transferred from the wells of the micro titre plate to wells of new micro titre plates and the OD at 630 nm was recorded. The same protocol was repeated but at temperatures of 35°C and 25°C respectively.

The following criteria was used to determine and classify isolates based on their biofilm producing potentials:

Optical density interpretation	Biofilm formation potential
$OD_s < OD_c$	no biofilm formation
$OD_c < OD_s < 2OD_c$	weak biofilm formation
$2OD_c < OD_s < 4OD_c$	moderate biofilm formation
$4OD_c < OD_s$	strong biofilm formation

OD_c = OD of negative control; OD_s = OD of test isolate (sample)

3.9 BACTERIOPHAGE ASSAY

3.9.1 Isolation of *Vibrio* species infecting phages using the double layer assay

Vibrio reference strains and confirmed environmental isolates were used to specifically isolate bacteriophages from sewage water samples (Ceyssens, 2009). To isolate phages from sewage water samples, 10 mL of water was suspended in 10 mL lambda diluent [10 mM Tris HCl (pH 7.5), 8 mM $MgSO_4 \cdot 7H_2O$], vortexed thoroughly and were kept at room temperature for 60 minutes. Samples were later centrifuged at 5250 g for 10 minutes using a Hermle Ultra Centrifuge (model Z300) to sediment bacteria cells. Aliquots of 1.8 mL from each supernatant was extracted, centrifuged at 11,000 g for 10 minutes and filtered through a 0.22 μ m pore syringe filter (GVS Filter Technology, USA). Bacteriophages in the filtrates were enriched using Tryptic Soy broth (TSB) by incubating at 37 °C for 60 minutes. The enriched samples were later used for detection of phages based on the double layer agar technique (Niu *et al.*, 2012). In order to isolate bacteriophages, ten-fold serial dilutions of the phage lysates were prepared using Lambda diluent. Overnight cultures of *Vibrio* species host bacteria cells were also

prepared using TSB containing 10 Mmol/L MgSO₄. Aliquots (100 µL) of each diluted phage lysate was added to 100 µL of the log phase bacteria cultures and incubated at room temperature for 10 minutes to facilitate attachment. An aliquot of 3 mL of 0.6 % (w/v) agarose was added into each tube containing the diluted phage lysate and the bacteria host culture. The contents were mixed by swirling and poured on solidified Modified Nutrient (MNA) agar plates. The plates were kept at room temperature to solidify and incubated aerobically at 37°C for 24 hours. After incubation plates were observed for the presence of plaques.

3.9.2 Purification and enumeration of phages in stocks

Phages were purified 3 times by single-plaque isolation and stock filtrates were prepared using suitable host strains as described previously (Niu *et al.*, 2012). Titres of isolated phages in stock filtrates was also determined using the soft layer overlay technique (Sambrook and Russell, 2001).

3.9.3 Phage propagation

Plaques were picked from plates using sterile pipette tips and inoculated in 1.5 mL of Difco phage broth (DPB) and incubated overnight at 4°C for phages to diffuse out of the agarose. The broth was centrifuged at 10000 *xg* for 2 mins. One milliliter of filtrate and 1 ml of overnight broth culture of each *Vibrio* strain were added to a 50 mL Falcon tube containing 5 mL of DPB and incubated at 37°C in a shaking water bath for 6-7 hours. The phage lysates were centrifuged at 9000 *xg* for 15 mins to remove bacterial debris, and

the supernatant was filtered through 0.2 µm pore-size super acrodisc syringe filter (Ribot *et al.*, 2006).

3.9.4 Characterisation of bacteriophages

3.9.4.1 Electron microscopy

Phages were purified using PEG (polyethylene glycol) according to a standard procedure (Sambrook and Russell, 2001). Phage suspensions were centrifuged at 25 000 *xg* for 60 minutes and washed twice in ammonium acetate (0.1 mol l⁻¹, pH 7.0). Sediments were subjected to transmission electron microscopy at the Electron Microscopy Unit, Potchefstroom Campus in order to determine the morphology of the phages.

3.9.4.2 Phage microplate virulence assay and determination of host range

Host specificity and lytic capabilities of phages was assessed using a microplate phage virulence assay. For this, 180 µL of the overnight cultures was mixed with 20 µL of the filtered phage lysates in 96-well microplates and incubated at 37°C for 5 hours in order to estimate the multiplicity of infection (MOI). TSB was used as a blank while the overnight cultures were used as the negative control. After incubation, wells were examined visually for turbidity due to bacterial growth. In addition, a spectrophotometer was used to read the results and compare the OD readings with results from visual analysis of plates. For each isolate-phage combination, the highest dilution of phage that results in complete lysis (no discernible turbidity) of bacteria was recorded. Ten-fold serial dilutions of bacteria host cultures were performed and aliquots of 50 µL from these dilutions were spread-plated on duplicate TSA plates. Plates were incubated at 37°C for 24 hours.

Colony forming units were counted and recorded. The mean Colony forming units per isolates was also computed and this represent the initial number of bacteria cells added during the phage virulence assay (Ribot *et al.*, 2006).

3.9.4.3 Determination of Multiplicity of Infection (MOI) for phages

The MOI for each phage-host assay was calculated by dividing the initial number of phages in the well with the greatest-dilution by the initial number of bacteria added, as determined from plate counts of serially diluted bacterial cultures. Isolates were then defined as sensitive ($\text{MOI} < 100$) or resistant ($\text{MOI} \geq 100$; complete lysis (no bacterial growth occur) to the phages tested. Sensitivity to the phages were further categorized as follows: extremely susceptible ($\text{MOI} < 0.01$), highly susceptible ($0.01 \leq \text{MOI} < 1$), moderately susceptible ($1 \leq \text{MOI} < 10$), and minimally susceptible ($10 \leq \text{MOI} < 100$) (Ribot *et al.*, 2006).

3.9.4.4 Stability of phage at different conditions

The stability of the phages to heat and pH was assessed in this study. The potential of the phages to resistance different heat treatments at 37 °C, 45 °C, 55 °C and 60°C as well as different pH values (0, 4.2, 6.3, 7, 8 and 10) was determined as previously described (Carlson, 2005).

3.10 STATISTICAL ANALYSIS

Statistical analysis of antibiotic resistance data was performed using the Minitab Release software (version 13.31). Correlations between antibiotic resistant isolates from the various sources were determined using the Pearson's product of moment and scored as

significant if $P \leq 0.05$. Furthermore, cluster analysis of antibiotic susceptibility data for isolates from the different stations was determined using Wards algorithm and Euclidean distances on Statistica version 12 (Statsoft, US). The fingerprints were compared and analysed with the TotalLab Phoretix 1D Pro software (UK). The presence, absence and intensity of band data were obtained, exported to Microsoft Excel (Microsoft Office, 2013) and used to generate a data matrix. Unweighted pair group method with arithmetic mean (UPGMA) and complete linkage algorithms was used to analyse the percentage similarity and matrix data. Relationships between the various profiles and/or lanes were expressed as dendrogram.

3.11 DATA CAPTURING

Data was stored in Ms Excel[®] worksheet and also as hard copy in a book. Data was presented in form of Tables and graphs.

CHAPTER 4

RESULTS

CHAPTER 4

RESULTS

4.1 DETECTION OF *VIBRIO* ISOLATES FROM WATER SAMPLES

A total of 136 water samples were collected from areas around the North West province and analysed for characters of *Vibrio* species using Thiosulfate Citrate Bile Salt Sucrose agar (TCBS). A total of 57 non-repetitive presumptive *Vibrio* isolates that presented with different macroscopic colonial morphologies were selected and subjected to both preliminary and confirmatory biochemical identification tests. Based on the morphological appearance of colonies on TCBS agar a large proportion (68%) of the isolates were yellow in colour while 32% appeared as green coloured colonies (Figure 4.1).

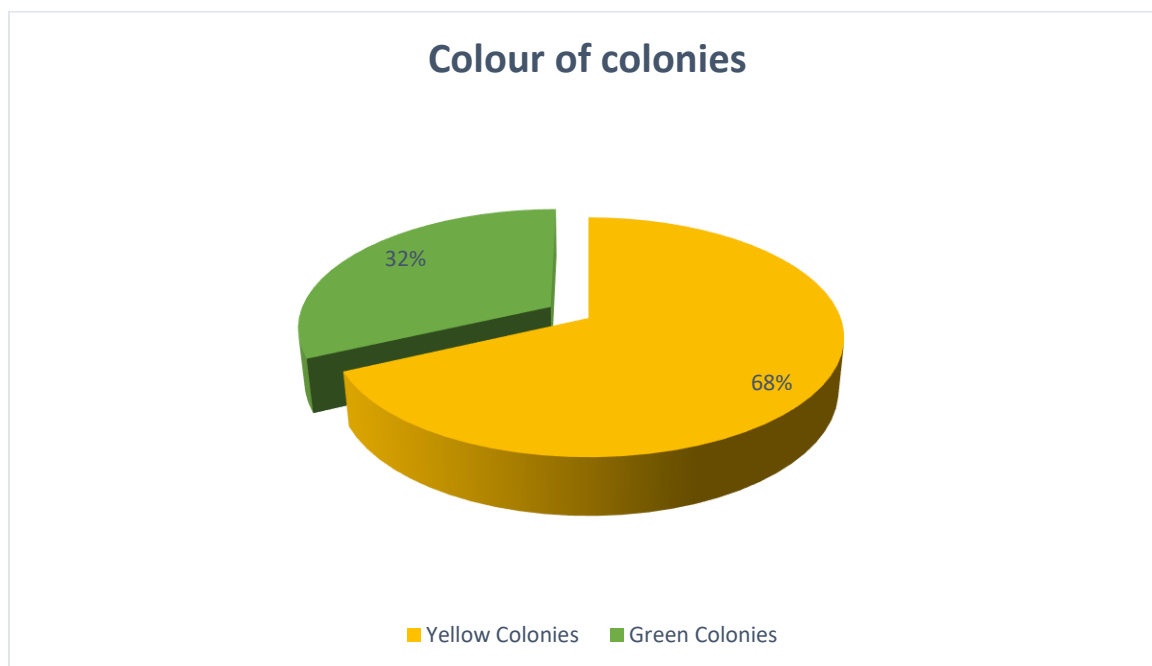


Figure 4. 1: Different colour of colonies observed on TCBS agar

4.2 DISTRIBUTION OF *VIBRIO* ISOLATES IN WATER SAMPLES

Using the TCBS agar, presumptive isolates were identified as potential *Vibrio* species. Based on the colour of the colonies, 18 (32%) were identified as green colonies and these comprised 4 (7.02%) from Tap water and 14 (24.56%) were from borehole water samples. On the contrary a much larger proportion 39 (68%) comprising 18 (31.58%) from borehole water, 16 (28.07%) from tap water and 5 (8.77%) from dam water appeared as yellow coloured colonies. The proportion of isolates with yellow and green coloured morphologies from the different types of samples are summarised in Table 4.1.

Table 4. 1: Distribution of potential *Vibrio* isolates in the different types of water samples analysed in the study

Site of samples	Yellow colonies	Green colonies	Total
Tap water	16	4	20
Borehole water	18	14	32
Dam water	5	0	5
Total	39	18	57

4.3 IDENTIFICATION OF ISOLATES BASED ON PRELIMINARY AND CONFIRMATORY BIOCHEMICAL TESTS.

A total of 57 isolates were successfully isolated from water samples and subjected to a number of preliminary identification tests as indicated in Section 3.3 of the Materials and Methods. A detailed summary of the results obtained are shown in Table 4.2. As indicated in Table 4.2, all the isolates were Gram negative rod-shaped bacteria that fermented the sugars in the TSI medium and produced hydrogen sulphide gas. In addition, all these isolates were not able to utilized sodium citrate as the sole source of carbon. All the isolates grew at 0% (w/v) NaCl solution. A similar growth pattern was observed when

isolates were inoculated in 3% (w/v) NaCl solution. However, all but one of the isolates tolerated the very high salt contents of 6% and 10% NaCl thus satisfying the presumptive identification criteria for *Vibrio* species. However, in order to avoid bias all the 57 isolates were included for further molecular identification tests.

Table 4. 2: Proportion of isolates identified using various Biochemical tests

					TESTS														
Sampling Site	No. of Samples	Colonies	Gram-Stain (-)	TSI							Salt tolerance				SIM				
				Glu (+)	Suc (+)	Lact (+)	Gas (-)	H ₂ S (-)	Citrate (-)	Oxidase (-/+)	0%	3%	6%	10%					
Taung	NT=17	27	27	27	27	27	27	27	27	NN= 20 NP= 7	G= 27	G= 27	G=26 NG=1	G=26 NG=1	M=24 NM=3				
Vryburg	NT=3	3	3	3	3	3	3	3	3	NN=3	G=3	G=3	G=3	G=3	M=3				
Mafikeng	NT=11	15	15	15	15	15	15	15	15	NN=8 NP=7	G= 15	G=1 5	G=15	G=15	M=15				
Ventersdorp	NT=1	1	1	1	1	1	1	1	1	NP=1	G= 1	G=1	G=1	G=1	M=1				
Coligny	NT=4	4	4	4	4		4	4	4	NN=1 NP=3	G= 4	G=4	G=4	G=4	M=4				
Rustenburg	NT=7	7	7	7	7	7	7	7	7	NN=3 NP=4	G= 7	G=7	G=7	G=7	M=6 NM=1				

Glu= glucose, **Suc**= sucrose, **Lac**= lactose, **H₂S**= hydrogen sulphide, **NN**=Negative for the test; **NP**=Positive for the test,

NT= number tested, **G**= growth, **NG**= no growth, **M**= motile, **NM**= nonmotile

4.4 GENOMIC DNA EXTRACTION AND GEL ELECTROPHORESIS

Genomic DNA was extracted from all presumptive isolates (57) and the presence of DNA was confirmed by electrophoresis on a 1% (w/v) agarose gel as depicted in Figure 4.2. DNA was of good quality with no fragmentation.

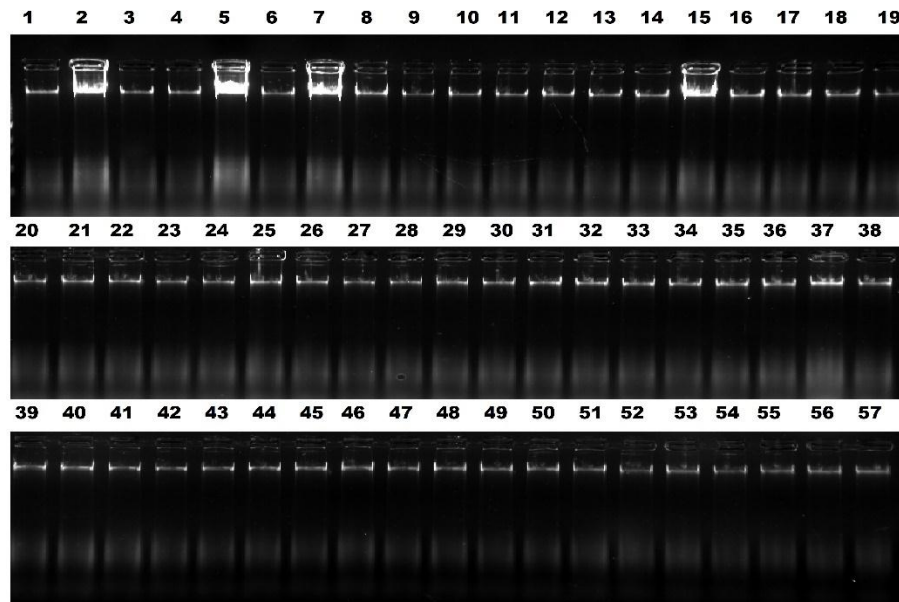


Figure 4. 2: Agarose gel (1% w/v) image depicting genomic DNA extracted from presumptive *Vibrio* isolates. Lane 1-57= DNA extracted from presumptive *Vibrio* isolates.

4.5 THE 16S rRNA GENE PCR ANALYSIS

Bacterial 16S rRNA gene fragments were amplified from all the 57 presumptive isolates. Amplification was performed as described in Section 3.8 (Materials and Methods) and the 16S rRNA gene fragment was successfully amplified in all (100%) 57 isolates. An agarose gel image of the amplified 16S rRNA gene is depicted in Figure 4.3. The amplified amplicons with the expected size (1420 bp) were obtained. All the isolates were subjected to the genus specific PCR identification assay specific for *Vibrio* species.

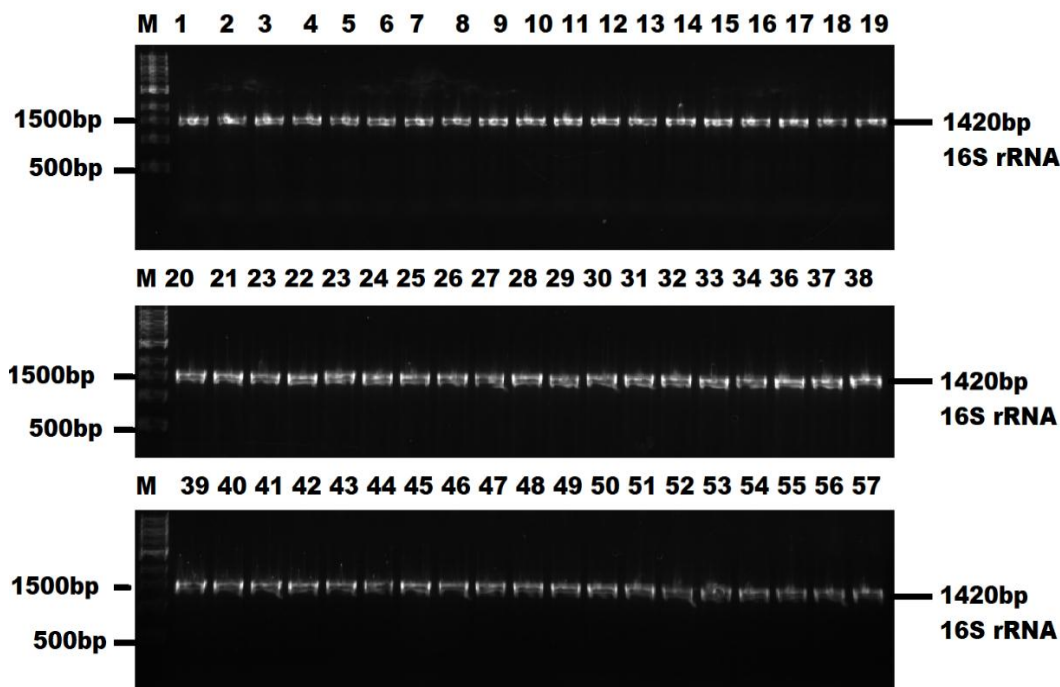


Figure 4.3: Agarose [2% w/v] gel image showing 16S rRNA gene fragments amplified from all presumptive *Vibrio* isolates. Lane M= DNA marker (O'GeneRuler 1 kilo base pairs DNA Ladder), Lane 1-57= 16S rRNA gene fragments of presumptive *Vibrio* isolates from water samples.

4.6 IDENTIFICATION OF *VIBRIO* SPECIES USING SPECIES SPECIFIC PCR AMPLIFICATION

A total of 57 presumptive isolates were subjected to *Vibrio* species specific PCR and only 22 isolates were positively identified. A detailed summary of the number of *Vibrio* species and the species composition that was detected in the different samples are outlined in Table 4. 3. Out of 22 isolates obtained, a large proportion 10 (45.45%) were detected as *Vibrio harveyi* and these comprised 1, 2 and 4 from borehole water in Pudimoe, Choseng

and Ntswanahatshe respectively. In addition, 1 and 2 *Vibrio harveyi* isolates were also obtained from tap water in Ntswanahatshe and Vryburg respectively. In addition, 5 (22.73%) isolates were identified as *Vibrio parahymolyticus* and these comprised 2 isolates each from borehole water from Lonely park and Ramosadi respectively as well as 1 isolate from tap water in Lonely park. On the contrary, only 3 (13.64%) isolates were positively identified as *Vibrio cholerae* and these comprised 2 isolates from dam water and 1 from tap water collected in Pella. Only 2 isolates each of *Vibrio mimicus* and *Vibrio vulnificus* were detected in the study (Table 4.3). *Vibrio mimicus* isolates comprised 1 (9.09%) isolate each from borehole water in Seweding and dam water in Coligny respectively while *Vibrio vulnificus* isolates were isolates from dam water collected in Marico Bosveld as well as tap water in Ventersdorp. These results indicate that *Vibrio* species were dominant in borehole water samples that do not normally undergo treatment and purification before consumption. Moreover, the detection of *Vibrio* species in tap water that has undergone treatment was a cause concern. It is therefore suggested that these water sources may serve as a potential route for the transmission of *Vibrio* species to consumers and this amplifies the need to implement strategies that will limit cross contamination. In general, 5 of the 7 genes were successfully amplified and agarose images of the amplicons of representative isolates are shown in Figures 4.4 and 4.5. The expected amplicon sizes for the *flaE* (897 bp), *sodB* (1) (248 bp), *hsp* (410 bp) and the *sodB* (121 bp) genes were obtained. However, for the *vhh* gene an amplicon size of 235 bp was obtained. Figure 4.6 provides a detailed overview of the distribution of *Vibrio* species isolated from water samples based on species specific PCR analysis.

Table 4.3: The distribution patterns of different *Vibrio* species isolated from the different sampling sites and types of samples

Sampling area	Source/ sample site	<i>Vibrio vulnificus</i>	<i>Vibrio parahaemolyticus</i>	<i>Vibrio mimicus</i>	<i>Vibrio harveyi</i>	<i>Vibrio cholerae</i>
Pudimoe	Borehole (1)	0	0	0	1	0
Choseng	Borehole (1)	0	0	0	2	0
	Tap (1)					
Lonely	Tap (1)	0	3	0	0	0
	Borehole (2)					
Seweding	Borehole (1)	0	0	1	0	0
Ramosadi	Borehole (2)	0	2	0	0	0
Ntswanahatshe	Borehole (4)	0	0	0	5	0
	Tap (1)					
Vryburg	Tap (2)	0	0	0	2	0
Marico Bosveld	Dam (1)	1	0	0	0	0
Pella	Dam (2)	0	0	0	0	3
	Tap (1)					
Coligny	Dam (1)	0	0	1	0	0
Ventersdorp	Tap (1)	1	0	0	0	0
Total	22	2	5	2	10	3

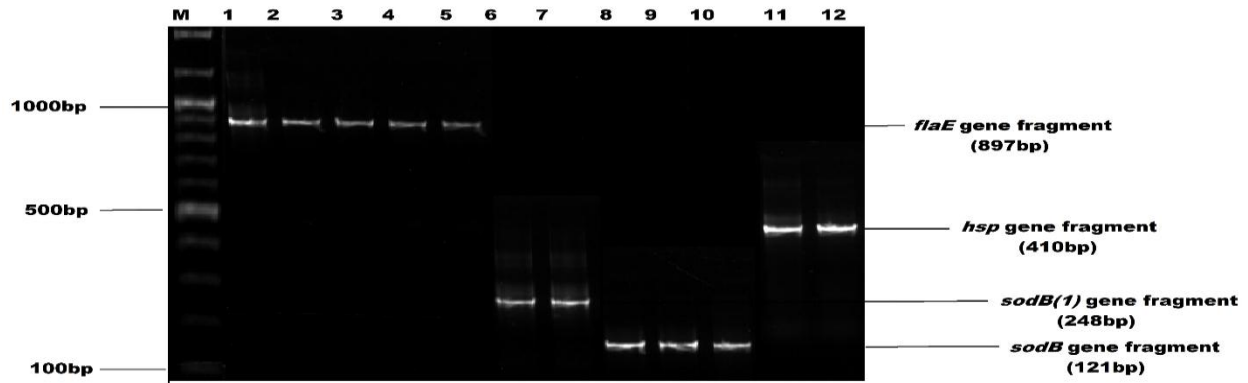


Figure 4. 4: Agarose [2% w/v] gel image showing amplification of *flaE*, *hsp*, *sodB* (1) and *sodB* gene fragments. Lane M= DNA marker (100 base pairs DNA Ladder), Lanes 1-5= *flaE* gene fragments, lanes 6-7= *hsp* gene fragments, lanes 8-10= *sodB* (1) gene fragments and lanes 11-12= *sodB* gene fragments.

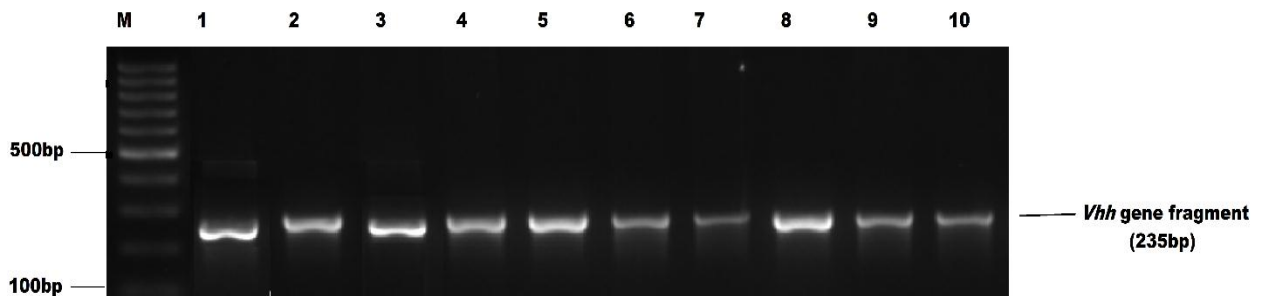


Figure 4. 5: Agarose [2% w/v] gel image showing amplification of *vhh* gene fragments. Lane M= DNA marker (100 base pairs DNA Ladder), Lanes 1-12= *vhh* gene fragments.

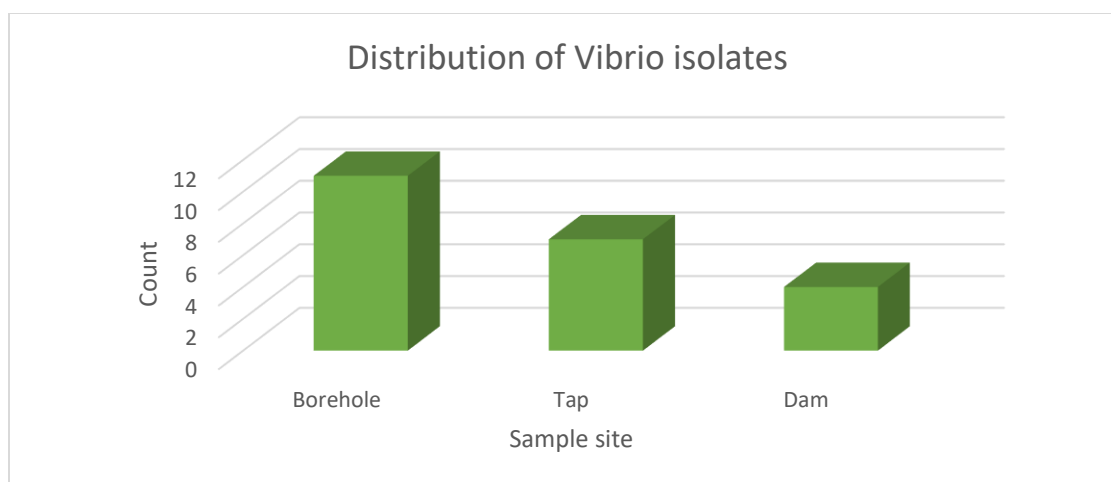


Figure 4. 6: Distribution of *Vibrio* isolated from water samples based on species specific PCR analysis.

4.7 ANTIBIOTIC SUSCEPTIBILITY PROFILES OF ISOLATES

The antibiotic susceptibility profiles of twenty-two isolates that were positively identified as *Vibrio* species based on specific PCR analysis was determined using the disc diffusion test against a panel of 11 antimicrobial agents as previously described (Bauer *et al.*, 1966; CLSI, 2010). The proportion of isolates that were resistant to the different antibiotics was represented as percentages and is indicated in Figure 4.7. A detailed overview of the susceptibility profiles of the isolates indicating individual bacteria growth inhibition zone diameter data is presented in Appendix 1B and 2B. As indicated in Figure 4.7, large proportions of the isolates were resistant to Cephalothin (95.45%), Ampicillin (77.27%) and Streptomycin (40.91%). Despite this, small proportions of the isolates were resistant to Trimethoprim (13.64%), Trimethoprim-sulfamethoxazole (4.55%), Chloramphenicol (9.1%) and Gentamycin (13.64%), Kanamycin (13.64%) and Ciprofloxacin (4.55%). All isolates that were isolated from borehole water were resistant to Cephalothin and this

was a cause for concern since this drug is not used regularly in the treatment of human infections. Moreover, water from boreholes are consumed by inhabitants of the sampled rural communities without any form of treatment and may thus serve as a potential source for the transmission of antibiotic resistant determinants or resistant bacteria to humans.

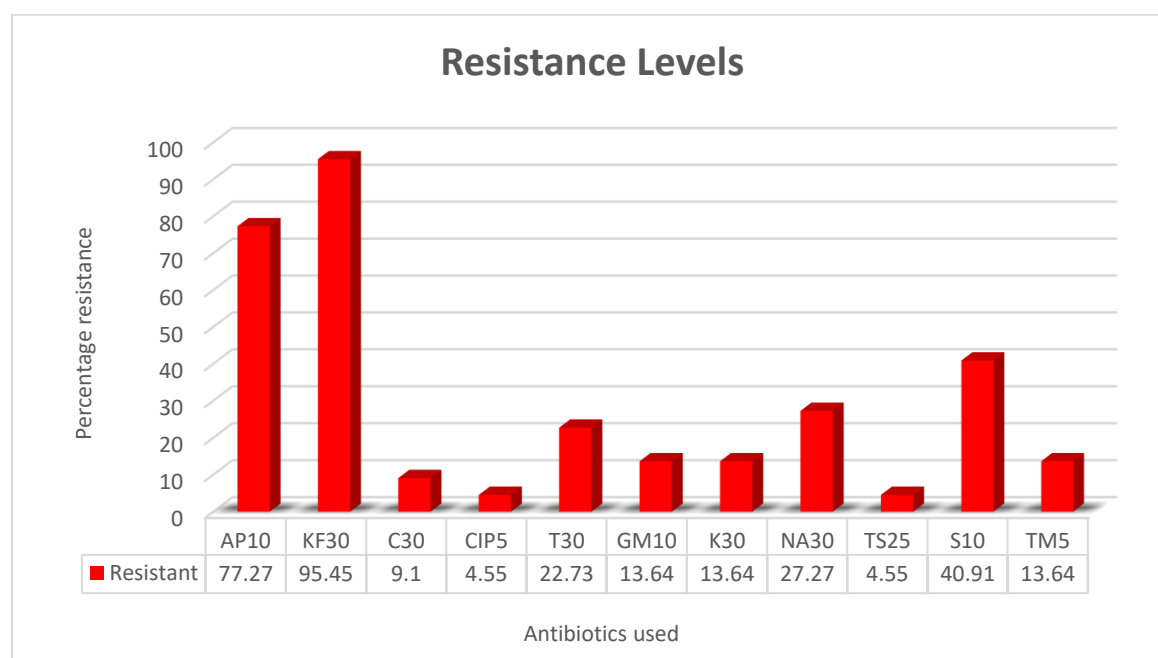


Figure 4. 7: Resistance pattern of isolates obtained from water samples.

AP10: Ampicillin, KF30: Cephalothin, C30: Chloramphenicol, CIP5: Ciprofloxacin, T30: Tetracycline, GM10: Gentamycin, K30: Kanamycin, NA30: Nalidixic acid, TS25: Trimethoprim 1.25 µg sulfamethoxazole 23.75 µg, S10: Streptomycin and TM5: Trimethoprim.

4.8 CLUSTER ANALYSIS OF *VIBRIO* ISOLATES BASED ON BACTERIA GROWTH INHIBITION ZONE DIAMETER DATA

Twenty-two *Vibrio* isolates obtained from water samples were subjected to eleven (11) therapeutic discs in order to determine their resistance profiles. Bacteria Growth Inhibition Zone Diameter Data of the isolates was used to perform a cluster analysis using Statistica software (version 12). This was mainly performed in order to determine the relatedness of these isolates based on antibiotic exposure histories. A dendrogram comprising two clusters (Cluster 1 and Cluster 2) were generated and this is shown in Figure 4.9. Cluster 1 was subdivided into two sub-clusters (Cluster 1A and Cluster 1B). Furthermore, sub-clusters 1A and 1B, including Cluster 2, were analysed for patterns of association of isolates from different sources and/or locations (Table 4.4). As indicated in Figure 4.9, the largest sub-cluster (sub-cluster 1B) contained isolates from 10 out of the 11 sampling areas. Large proportions (10% and 20%) of isolates in this sub-cluster were derived from borehole and tap water samples respectively. None of the isolates obtained from dam water samples was present in either sub-cluster 1A or cluster 2 but they were present in sub-cluster 1B.

Table 4. 4: The percentage representation of *Vibrio* isolated from different species within the various clusters

Sampling site	Source	Cluster 1A N=1	Cluster 1B N=20	Cluster 2 N=1
Pudimoe	Borehole	1(100%)	0(0%)	0(0%)
Choseng	Borehole	0(0%)	1(5%)	0(0%)
	Tap	0(0%)	1(5%)	0(0%)
Lonely park	Tap	0(0%)	1(5%)	0(0%)
	Borehole	0(0%)	1(5%)	1(100%)
Seweding	Borehole	0(0%)	1(5%)	0(0%)
Ramosadi	Borehole	0(0%)	2(10%)	0(0%)
Ntswanahatshe	Borehole	0(0%)	4(20%)	0(0%)
	Tap	0(0%)	1(5%)	0(0%)
Vryburg	Tap	0(0%)	2(10%)	0(0%)
Marico Bosveld	Dam	0(0%)	1(5%)	0(0%)
Pella	Dam	0(0%)	2(10%)	0(0%)
	Tap	0(0%)	1(5%)	0(0%)
Coligny	Dam	0(0%)	1(5%)	0(0%)
Ventersdorp	Tap	0(0%)	1(5%)	0(0%)

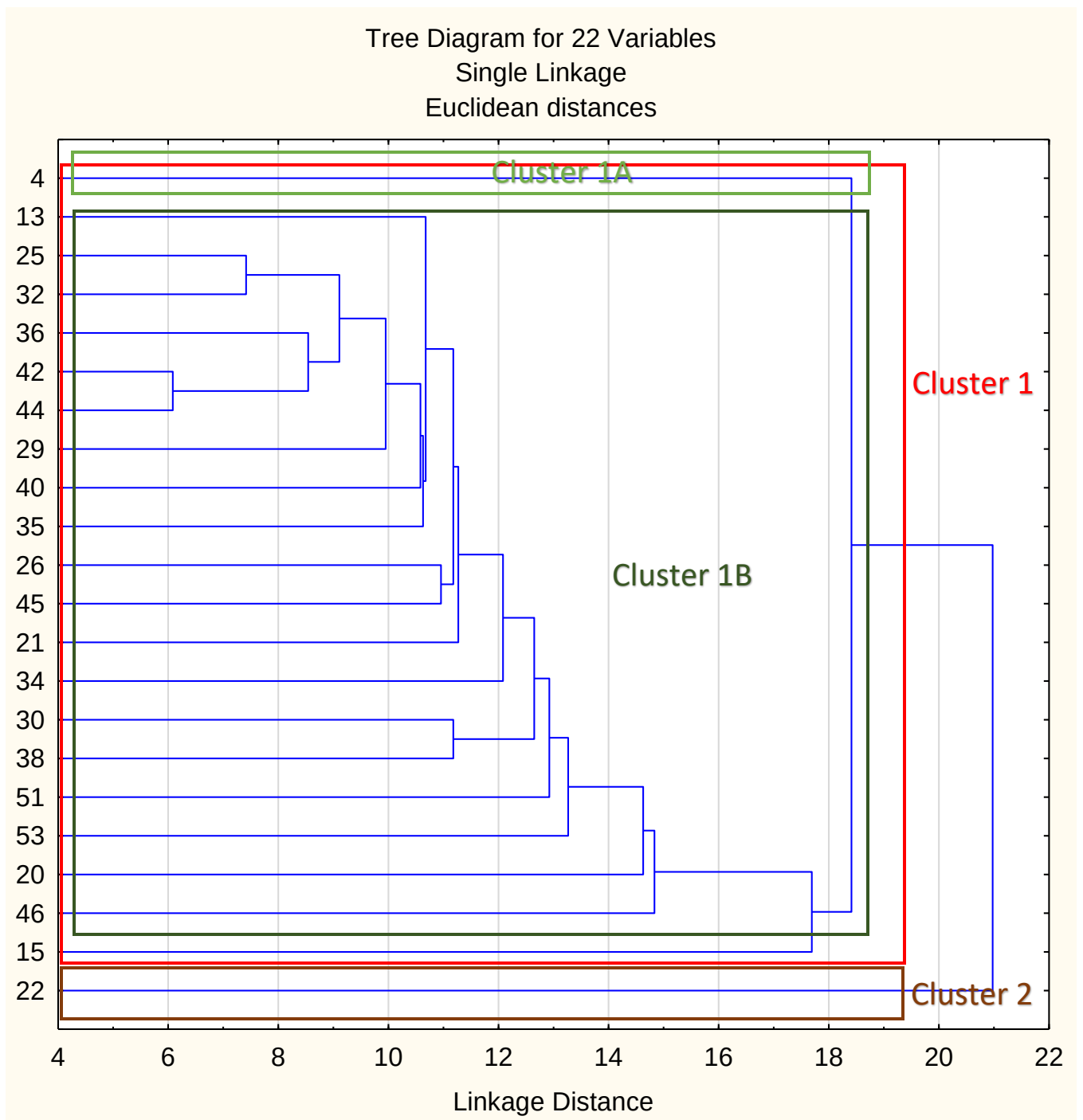


Figure 4. 8: Dendrogram showing the relationship between isolates from water samples based on antimicrobial inhibition zone diameter data.

4.9 PLASMID PROFILES

Plasmid DNA was extracted from all *Vibrio* isolates that showed resistance to two or more antibiotics. Out of the 21 isolates only 8 (38.10%) harboured plasmids. Three strains possessed single plasmids with a size of 5997 bp. In addition, three possessed two plasmids with sizes of 2961 bp and 2000 bp respectively while one strain harboured three plasmids with sizes of 5997 bp, 2961 bp and 1600 bp. An overview of the plasmid profiles of the *Vibrio* species isolated in the study are shown in Figure 4.10.

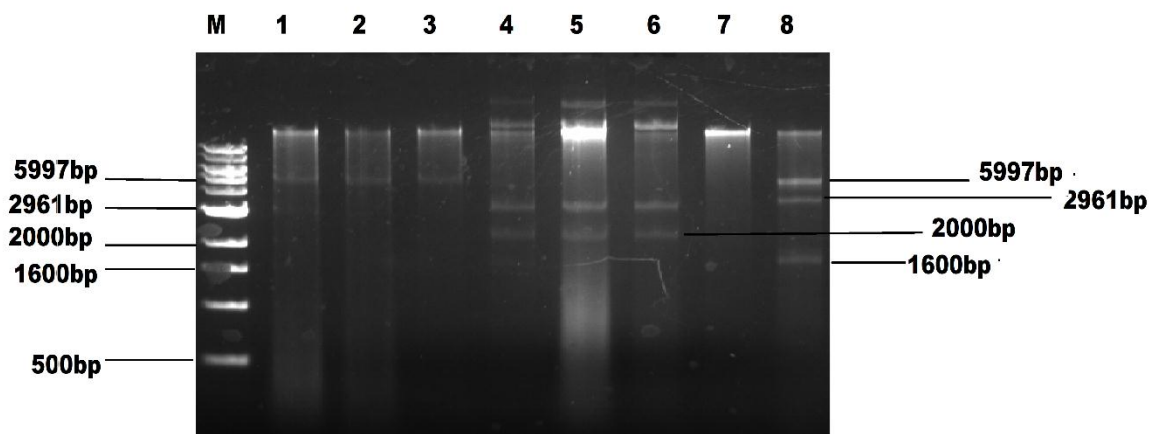


Figure 4. 9: Agarose (2% w/v) gel image depicting plasmid profiles of tested *Vibrio* species. Lane M= DNA marker (1 kilo base DNA ladder), Lanes 1-8= Plasmid DNA fragments amplified from *Vibrio* isolates.

4.10 PROPORTIONS OF *VIBRIO* ISOLATES POSITIVE FOR THE DIFFERENT VIRULENCE GENES

Twenty-two (22) isolates derived from water samples were further screened for the presence of *tdh*, *trh*, *ctxAB*, *zot*, *flrA* and *vpsR* virulence genes using specific PCR assays. The results obtained revealed that 4 (18.18%) isolates that comprised 3 and 1 *Vibrio*

parahaemolyticus from Lonely Park and Ramosadi respectively harboured the *tdh* virulence gene. Similarly, 4 (18.18%) *Vibrio parahaemolyticus* comprising 2 each from borehole water in Ramosadi and Lonely Park were positive for the *trh*. A similar proportion 4 (18.18%) of the isolates possessed the *zot* virulence gene and this comprised 3 *Vibrio cholerae* from Pella and 1 *Vibrio mimicus* from Seweding. Figure 4.11 is a 2% agarose gel image of different virulent genes amplified from the *Vibrio* species in the study. Despite the fact that isolates in this study did not harbour all of the 6 virulence genes targeted, the detection of 3 different virulence genes in isolates obtained from water that was intended for human consumption was a cause for concern. Moreover, the detection of more than one virulence gene in some isolates indicate that these species may be of great public health and clinical significance in the area

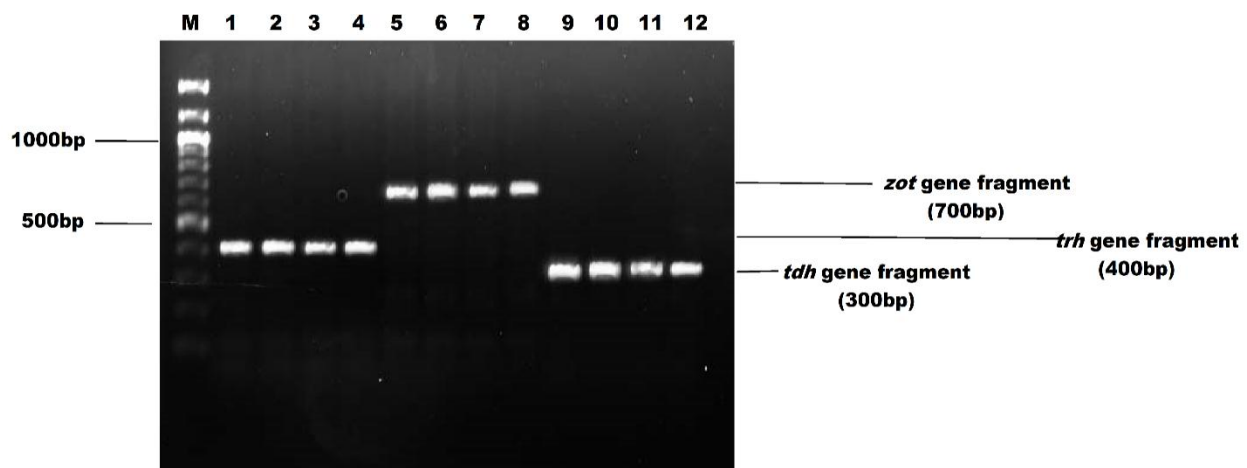


Figure 4. 10: Agarose (2% w/v) gel image depicting the virulence gene fragments of *zot*, *trh* and *tdh* gene. Lane M= DNA marker (100 base pairs DNA ladder), Lanes 1-4= *trh* gene fragments, lanes 5-8= *zot* gene fragments and lanes 9-12= *tdh* gene fragments.

4.11 BIOFILM FORMATION CAPACITY OF *VIBRIO* SPECIES AT DIFFERENT TEMPERATURES

All the 22 *Vibrio* isolates were screened in order to determine their potential to form biofilms on polystyrene plates at three different temperatures (37 °C, 35 °C and 25 °C). Figure 4.12 represents a general overview of the proportion of isolates that were classified under the different biofilm formation categories when exposed to the different temperatures. Figures 4.13, 4.14 and 4.15 indicate the biofilm production potentials of the 22 *Vibrio* isolates at 37 °C, 35 °C and 25 °C, respectively. Results obtained indicated that large proportion (63.64%) of the isolates were not able to form biofilm at 37 °C while eleven (50 %) isolates produced visible form biofilm at 25 °C. However, at 35 °C (36.36%) of isolates were unable to form biofilm. Furthermore, none of the isolates were classified as strong biofilm producers at 37 °C, while a significantly large proportion (27.27%) of the isolates revealed strong biofilm production potentials at 35 °C. On the contrary, only small proportion (4.55%) of isolates showed strong biofilm production potentials at 25 °C.

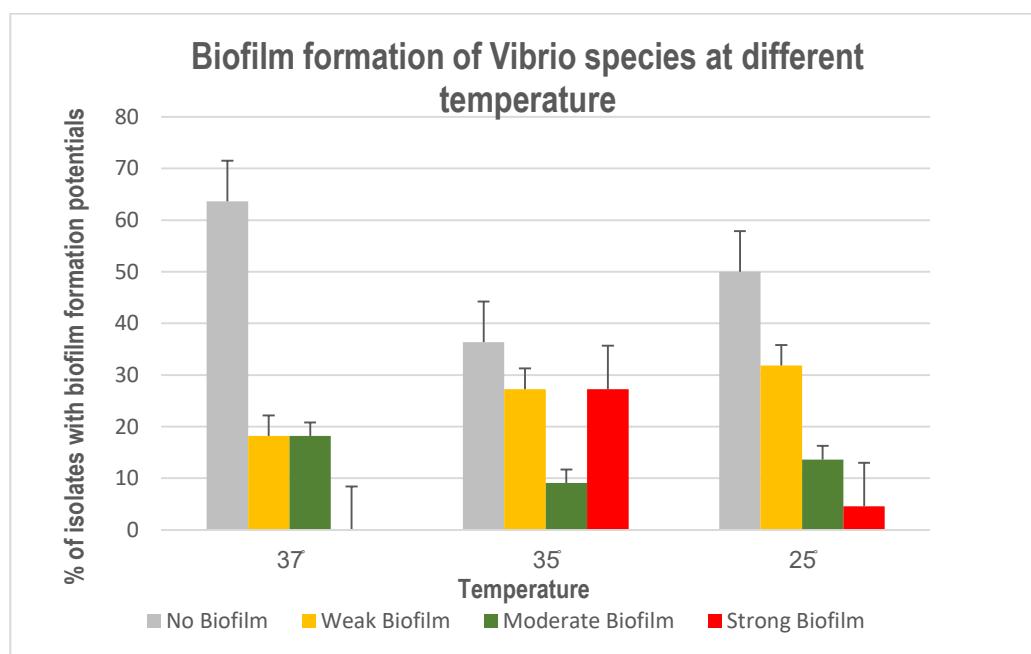


Figure 4. 11: Biofilm formation potentials of *Vibrio* species at different temperatures.

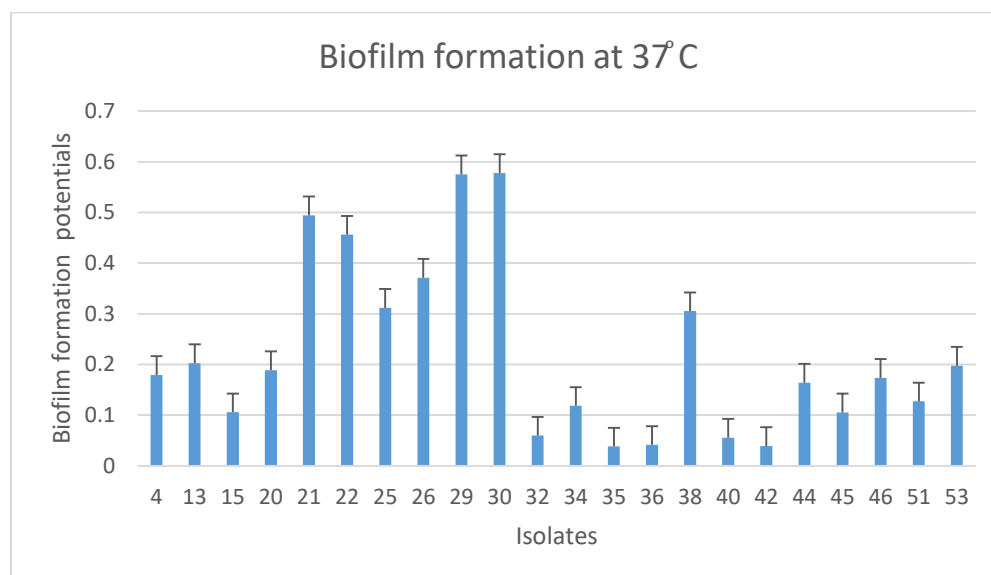


Figure 4. 12: Biofilm formation potentials of the 22 *Vibrio* isolates at 37 °C.

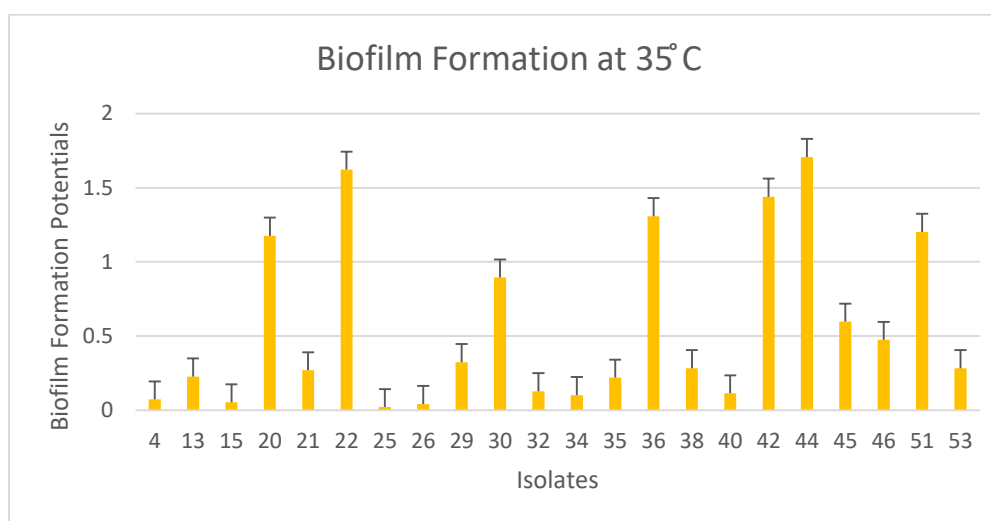


Figure 4. 13: Biofilm formation potentials of the 22 *Vibrio* isolates at 35 °C.

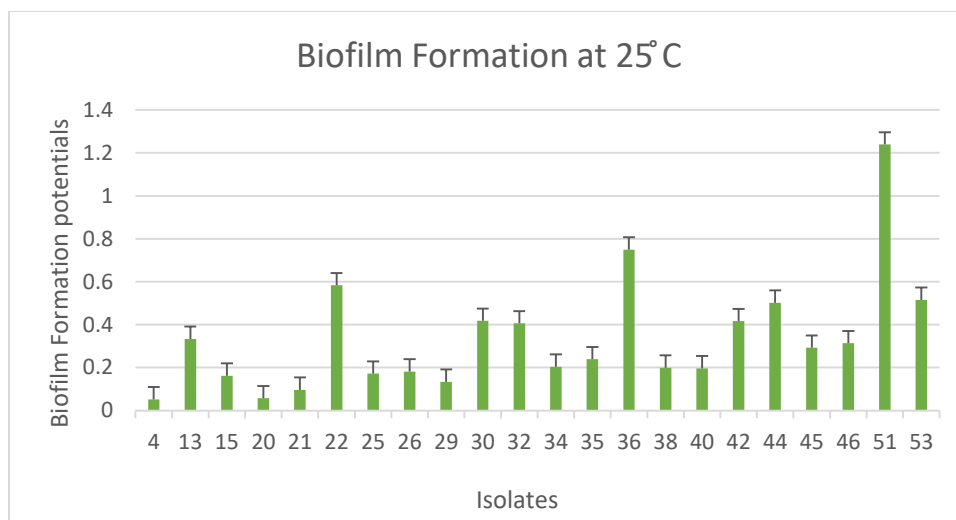


Figure 4. 14: Biofilm formation potentials of the 22 *Vibrio* isolates at 25 °C.

4.12 CHARACTERIZATION OF *VIBRIO* SPECIFIC BACTERIOPHAGES

4.12.1 Transmission electron microscopy

Bacteriophages isolated from sewage water using environmental *Vibrio* host strains obtained in this study were subjected to morphological characterization using Transmission electron microscopy. Transmission electron micrographs of the phages are shown in Figure 4.16. Morphological classification of the phages was achieved using previous guidelines (Ackermann, 2007), and this revealed that the phages may belong to the family *Mycoviridae*.

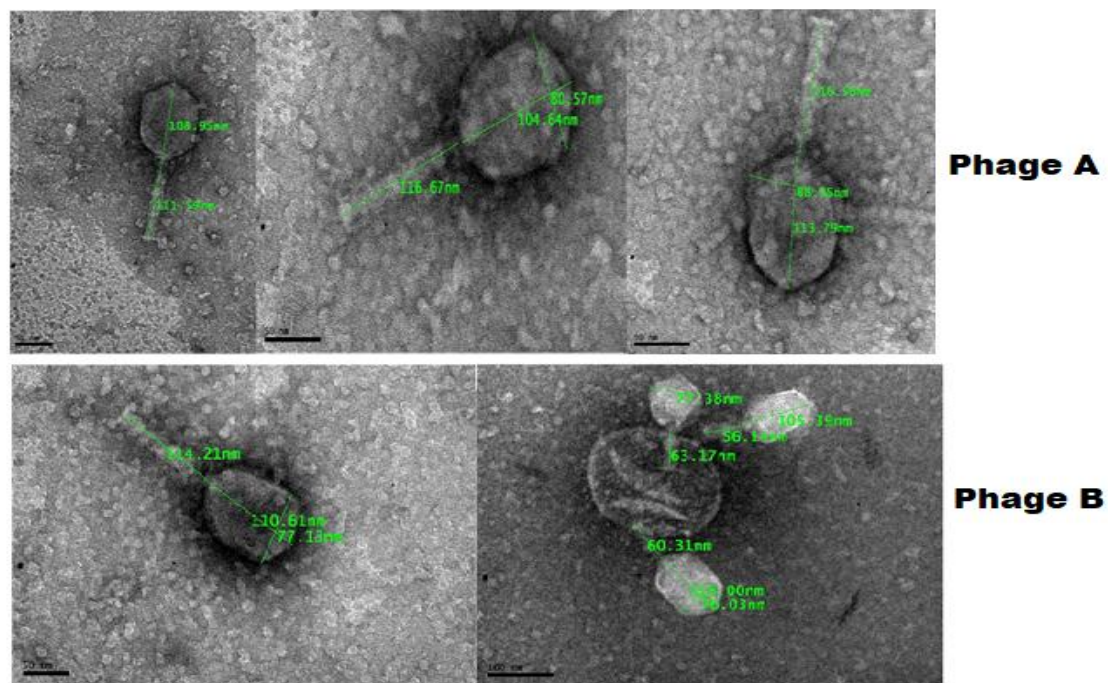


Figure 4.15: Transmission electron micrographs of *Vibrio* specific bacteriophages isolated from sewage water.

4.12.2 Bacteriophage virulent assay

Two bacteriophages designated Phage A and Phage B were used to assess their potentials to serve as bio-control agents against twenty-two environmental *Vibrio* isolates that were isolated in this study based on the virulence assay technique. Results were interpreted based on a previous report and presented as positive (+) when there was inhibition of bacterial growth or negative (-) when bacterial growth was not inhibited. Phage A was biologically active against 19 of 22 *Vibrio* strains while Phage B was active against 18 environmental *Vibrio* strains. Interestingly, both bacteriophages were active against the same sets of bacterial strains. A summary of the bio-active potentials of the

phages against the individual bacteria isolates is shown in Table 4.5. Detailed results of the microplate assays are shown in the Appendix Tables 1D - 10D.

Table 4. 5: Virulence potentials of bacteriophages against environmental resistant bacteria *Vibrio* isolates. The acronym Phage A and Phage B indicates bacteriophages isolated using a *Vibrio* isolate as the host bacteria strain. Isolates with the superscripts “*” were susceptible to Phage A while those with the superscripts “**” were susceptible to Phage B. Isolates with the superscript “***” were susceptible to both Phage A and Phage B.

Bacteria strains	Bacteriophage ID and plaque morphology*/ virulence potentials	
	Phage A (Small*)	Phage B (Small*)
4***	++	++
13	-	-
15	-	-
20***	++	++
21**	+	-
22***	+	-
25***	++	++
26***	++	++
29***	++	++
30***	++	++
32***	++	++
34***	++	++
35***	++	++
36***	++	++
38***	++	++
40***	++	++
42***	++	++
44***	++	++
45***	++	++
46***	++	++
51***	++	++
53***	-	+

Phage A and Phage B = Bacteriophages; + = bacteria growth inhibited; - = bacteria growth not inhibited.

4.12.3 Stability of phage at different conditions

The stability of the phages to heat and pH was assessed and the results are presented in Figures 4.17 and 4.18 for pH and Figures 4.19 and 4.20 for temperature. The results indicated that both Phages A and B were stable at pH values ranging from 6.3 – 10.0. On the contrary, both phages could not survive at pH value of 3.0. The optimum temperature at which both phages were stable was 37 °C while their growth and survival rates reduced with an increase in temperature.

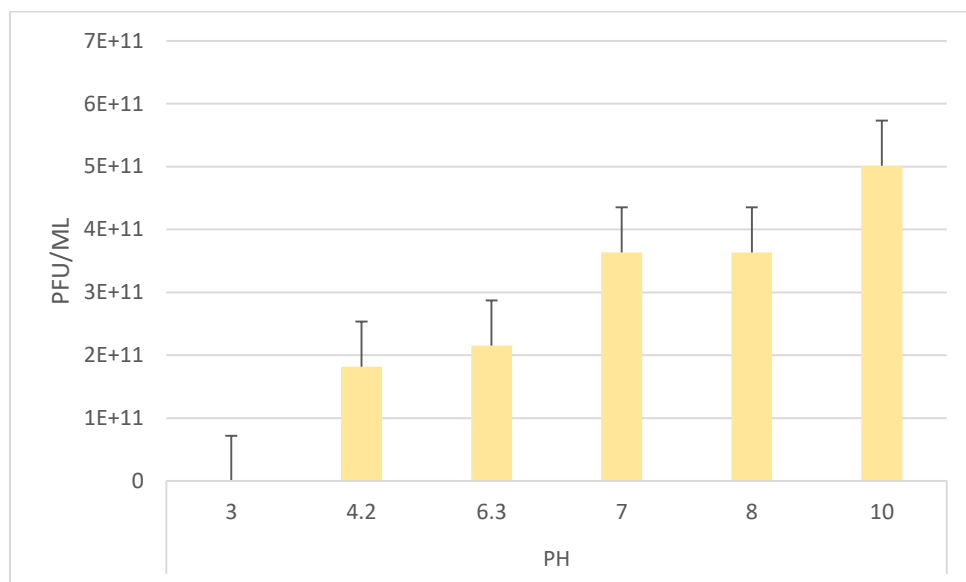


Figure 4. 16 Stability of Phage A at different pH levels

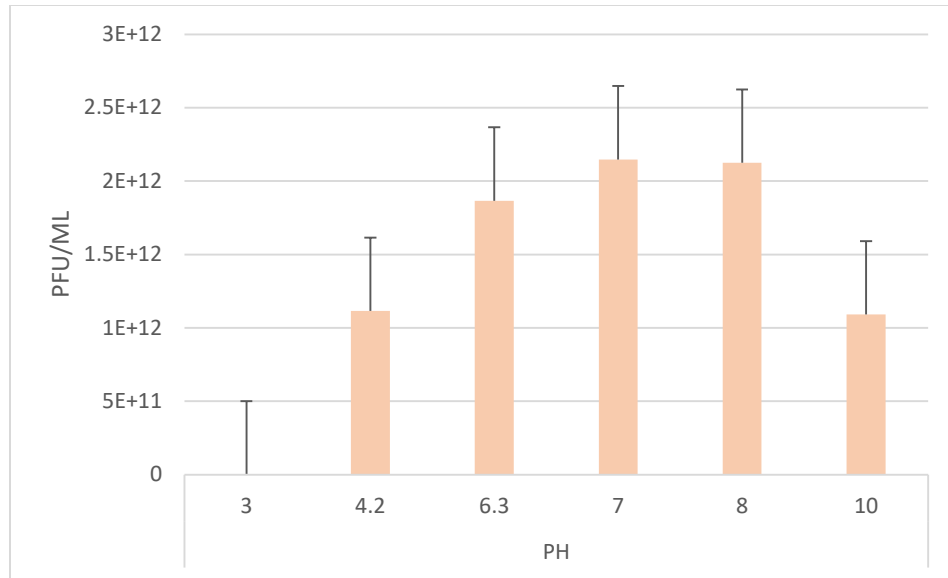


Figure 4. 17 Stability of Phage B at different pH levels

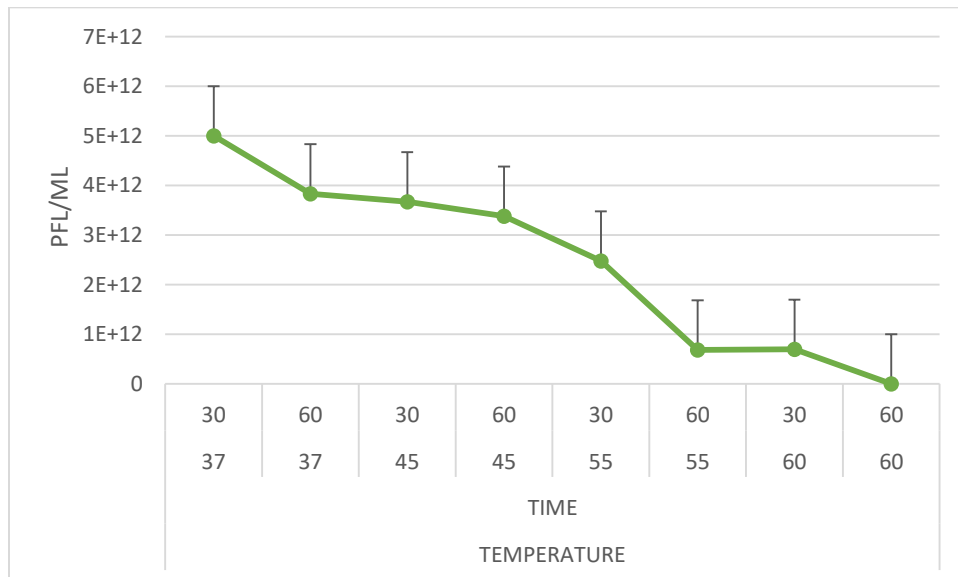


Figure 4. 18 Stability of Phage A at different temperatures

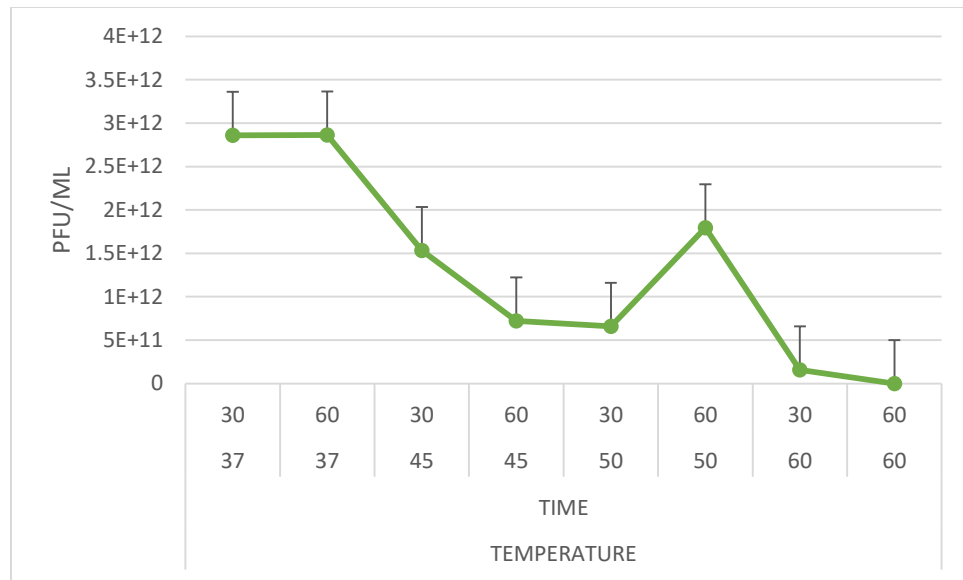


Figure 4. 19 Stability of Phage B at different temperatures

CHAPTER 5
DISCUSSION

CHAPTER 5

DISCUSSION

5. GENERAL DISCUSSION

Water is an important resource for humans and it is very important to sustain life. Water is a very important resource for life and access to safe drinking water is a fundamental human need and therefore a basic right of every individual (WHO, 2011). Safe drinking water should not contain high concentrations of chemicals, minerals as well as pathogenic micro-organisms (Smith, 2009). South Africa is located in a semi-arid region and receives very less rainfall and this results in shortage of water especially potable water. In addition, increased industrialization and frequent establishment of informal settlements also affects the quality of water especially in rural areas. Due to scarcity of potable water most individuals revert to water from other unprotected sources such as boreholes, rivers and dams for daily activities. These unprotected sources of water can be easily be contaminated with faecal matter from human and animal sources.

The presence of microbial contaminants in water that is intended for human consumption as well as recreational water bodies have been reported to cause health complications on consumers (Hurst, 1996; Fong and Lipp, 2005; Toranzos *et al.*, 2007). The present study was also motivated by the numerous recent reports that revealed the presence of pathogenic microorganisms in water that is consumed by individuals in rural communities

in the North West Province of South Africa (Ateba and Maribeng, 2011; Ateba and Phokela, 2011; Ateba *et al.*, 2013; Mulamattathil *et al.*, 2014).

The main aim of this study was to isolate and confirm the identities of *Vibrio* in water samples obtained from different sources, using both phenotypic and molecular assays. A total of 136 water samples were collected from areas around North West Province, South Africa and analysed for presumptive *Vibrio* species by culturing on TCBS agar that is both a selective and differential medium for *Vibrio*. Based on colonial appearance, 57 presumptive isolates were identified and all these isolates were Gram-negative rod-shaped bacteria. Preliminary identification tests that included Oxidase test, Triple Sugar Iron Test, Simmons Citrate agar test, Salt tolerance and Motility tests revealed that a large proportion of the isolates were potentially *Vibrio* species. A number of studies have also used the cellular morphology, oxidase test, Triple Sugar Iron Test, Simmons Citrate agar test, Salt tolerance and Motility tests for presumptive identification of *Vibrio* species (Baffone *et al.*, 2000; Gopal *et al.*, 2005; Igbinosa and Okoh, 2008). Results obtained in this study are similar to those previously reported (Srinivasan and Saranraj, 2017).

All the 57 isolates were screened for *Vibrio* species specific PCR analysis to achieve confirmed identification. This was motivated by the fact that PCR is considered a golden standard tool for identifying bacterial isolates since it is highly specific and sensitive (Song, 2005; Ateba and Mbewe, 2011). This explains why it is always important to supplement preliminary identification tests with this highly specific genotypic assay.

Bacterial 16S rRNA gene fragments were amplified in all the 57 presumptive isolates and a large proportion 22 (38.60%) of the isolates were positively identified as *Vibrio* species through amplification of the *ompW*, *rfbO1*, *flaE*, *sodB* (1), *hsp*, *sodB* and *vhh* gene fragments. In this study, out of 22 *Vibrio* species that were positively identified by PCR analysis, the proportion of *Vibrio harveyi* was higher 10 (45.45%) than *Vibrio parahaemolyticus* 5 (22.73%), *Vibrio mimicus* 3 (13.64%), *Vibrio vulnificus* 2 (9.09%) and *Vibrio cholerae* 2 (2.09%). Despite this, the isolates that were not positive for these species-specific PCR may belong to other *Vibrio* species that were not investigated in this study. Similar observations have been documented in a previous study in which these *Vibrio* species were detected but in Fish and shrimps (Raissy *et al.*, 2015). The implication of these *Vibrio* species in water that is intended for human consumption indicates that water from these sources may present significant public health complications on consumers. Moreover, their presence in tap water that has undergone treatment highlights the urgent need to improve and adhere to standard operational procedures of waste water and water purifications plants. This will greatly contribute towards meeting the World Health Organization proposal that an estimate of 35% diarrhoea cases can be reduced, if basic water hygiene and sanitation are provided to individuals particularly in rural areas (Brick *et al.*, 2004).

The presence of pathogenic microorganisms that are associated with both self-limiting and life-threatening water-borne infections in water bodies most often results from pollution with human and animal faeces and this is more common in rural communities and other informal settlements (Teklehaimanot *et al.*, 2015). Water treatment plants are

designed to treat wastewater and provide safe drinking water to consumers at an affordable cost (Smith, 2009). Poor management of wastewater treatment plants and uncontrolled discharge sewage have been identified as the two major sources of microbial pollution of water sources in South Africa (Teklehaimanot *et al.*, 2015). Therefore, it is of necessity to monitor both treated water and wastewaters for various types of microbial pathogens since this may provide an indication of the risks with water from these sources. An assessment of adherence to standard treatment protocols and the implementation of proper management practices will therefore improve the quality of the finished product. It has previously been reported that water quality assessment parameters that affect optimum performance as well as the quality of the final product may include reliability of the plant, nature of raw materials used, by-product management, safety, human resources, economic and financial resources, infrastructure and maintenance (Zhang *et al.*, 2012). These parameters are very important and should be to be included in any water treatment plant operational performance assessment tool (Zhang *et al.*, 2012) since they directly affect the quality of the finished product. In order to reduce associated health risks on consumers the limits for microbial contaminants in domestic water as determined by the South African National Standard (SANS) 241 of 2015 requires that the levels must not fall outside these limits. This actually means no *E. coli*, total coliform and heterotrophic bacteria should be detected in 100 mL of samples. However, *Vibrio* species may be part of the heterotrophic bacteria load thus indicating that the water does not meet the SANS 241 standards and considered unsafe for either drinking or household use.

A further objective of the study was to determine the antibiotic resistant profiles of *Vibrio* species isolated from water samples that were analysed. A large percentage (40.91% to 95.45%) of *Vibrio* species obtained in this study were most often resistant to Streptomycin, Ampicillin and Cephalothin. Despite the fact that most of the isolates in this study displayed high sensitivity to trimethoprim-sulfamethoxazole, trimethoprim, chloramphenicol, gentamycin, tetracycline and ciprofloxacin, in previous studies large proportion of *Vibrio* isolates from aquaculture environments were resistant to chloramphenicol (Igbinosa, 2016). Similar to the findings of this study in which a large proportion (63.64%) of the isolates were susceptible to ciprofloxacin, in a previous study isolates were most often sensitive to this drug (Yu *et al.*, 2012).

Drug resistance is currently an issue of severe public health concern worldwide mainly due to the fact that the occurrence of resistant determinants in environmental bacteria pathogens is increasing at an alarming rate (Slama *et al.*, 2005; Ansari and Raissy, 2010). This is even aggravated by the negative impact of resistant strains on antibiotic therapy thus affecting disease control and management strategies (Slama *et al.*, 2005; Ansari and Raissy, 2010). Moreover, improper discharge of municipal and industrial wastewater as well as water from aquaculture systems have been reported to play significant roles in dissemination of resistance genes within aquatic ecosystems (Singer *et al.*, 2011; Zhang *et al.*, 2015; Verlicchi and Zambello, 2016). This is as a result of a number of clinically used antibiotics that are used by humans being released in an active biological form via faeces and urine (Singer *et al.*, 2011; Zhang *et al.*, 2015; Verlicchi and Zambello, 2016). If wastewater is not properly treated and these metabolites are present in treated water,

they can be easily transmitted to consumers (Li and Zhang, 2010; Rivora-Ultrilla *et al.*, 2013; Luo *et al.*, 2014; Ahmed *et al.*, 2015; Chen *et al.*, 2015).

Another objective of the study was to determine the pathogenicity of the *Vibrio* isolates obtained through the amplification of genus-specific virulence gene determinants. From these results three different virulence genes were detected amongst the isolates that were tested. *Vibrio parahaemolyticus* isolated possessed both the *tdh* (18.18%) and *trh* (18.18%) while 3 *Vibrio cholerae* isolates and 1 *Vibrio mimicus* isolate harboured *zot* (18.18%). However, these results do not concur with those reported in a previous study (Khouadja *et al.*, 2014) since the isolates did not harbour any *zot* virulence genes. Despite the fact that some species such as *Vibrio harveyi* and *Vibrio vulnificus* isolated in the current study are generally considered to be nonpathogenic for humans, it is known that most virulence determinants in bacteria species are located in mobile genetic elements (Gennari *et al.*, 2012). This therefore implies that ease with which nonpathogenic bacteria strains acquire mobile genetic elements that harbour resistant determinants may result in the development and appearance of virulent strains that may pose severe public health complications (Gennari *et al.*, 2012). Contrary to the findings of this study, isolates belonging to the Harveyi clade that comprise *Vibrio harveyi* and *Vibrio campbellii* possessed both typical and atypical virulence genes *vhh*, *chiA*, *vhpA*, *toxR* (Vh), *luxR* and serine protease and it is suggested that they might acquire these virulence determinants from other *Vibrio* species in the aquatic environments through horizontal gene transfer (Ruwandepika *et al.*, 2010). This therefore implies that the virulence potential of an isolates cannot be determined mainly by virtue of its species nomenclature thus indicating

the need for constant monitoring even in species that were previously considered to be non-pathogenic.

Another objective of the study was to determine the biofilm forming potentials of *Vibrio* species isolates from water samples. Biofilms that are composed primarily of microbial cells and extracellular polymeric substance (EPS) can be defined as an assemblage of the microbial cells that are irreversibly associated with a surface and usually enclosed in a matrix of polysaccharide material (Kokare *et al.*, 2009). In this study, *Vibrio* strains from water samples displayed the ability of bind to surfaces and form biofilms. Despite the fact that there was some variability in the potential to form biofilms at different temperatures, the ability of this isolates to form biofilm presents a significance public health concern since biofilms provide cells with the enhanced opportunity to resist destruction by antimicrobial agents and thus persistent in tissues during associated infections (Hall-Stoodley *et al.*, 2004). Moreover, when in biofilms, bacteria cells are known to resist antibiotics, conserve and absorb nutrients and also produce favourable associations for the proliferation of other bacteria cells (Hall-Stoodley *et al.*, 2004). This increases their effect on humans hosts when present as bacteria pathogenic contaminants.

A further objective of the study was to isolate and determine the morphologies of *Vibrio* specific bacteriophages using electron microscopy and also determine the virulence capabilities of *Vibrio*-specific endemic bacteriophages against antibiotic resistant *Vibrio* isolates using the virulent micro-plates assay. This was motivated by the recent crisis

faced with the ever increasing occurrence of multidrug-resistant bacteria as well as their negative impact on therapeutic processes in both humans and animals (Schwarz *et al.*, 2017). Although bacteriophages may lead to the transmission of antibiotic resistance determinants, these agents have important ecological and evolutionary impacts on their bacterial hosts that has resulted in their use as therapeutic or bio-control agents against a variety of bacteria species (Torres-Barceló, 2018). *Vibrio* species-specific bacteriophages that were isolated from sewage water samples using environmental antibiotic resistant *Vibrio* strains TEM photos of the isolated phage indicated that it belongs to the Mycoviridae family based on its conserved and highly characteristic morphology. Classification of these phages was based on their morphologies comparing them with the morphologies described by (Ackermann, 2007). Bacteriophages belonging to the family Mycoviridae are found anywhere in the biosphere; they reside in places such as top-soils, plants, animals or water bodies (Lopes *et al.*, 2014). Despite the fact that both phages that were analysed were capable of lysing more than one antibiotic resistant *Vibrio* strain not all may be suitable for use in therapeutic processes or bio-control applications without appropriate screening of phage genomes for the presence of undesirable determinants that may render them unsafe (Wei *et al.*, 2015). Bacteriophages isolated in this study possessed the potential to be used as valuable bio-control agents *Vibrio* species. These phages provide alternative strategies for controlling these bacterial contaminants in water given the fact that most bacteria contaminants are exhibiting high levels of resistance to conventional water purification chemicals (Kalatzis *et al.*, 2015). However, phages are known to be highly specific at the species and strain level and determination of their bacteria host range is critical in determining their potential to be

utilized as antimicrobial agents. Against this background, it is hereby suggested that the virulence potential of these phages be assessed against other related bacteria species that may occur as microbial contaminants in water.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

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From this study, water samples were collected from a variety sources in different locations in the North-West Province of South Africa. Upon analysis, 22 *Vibrio* isolates belonging to five species that comprised *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Vibrio mimicus*, *Vibrio harveyi* and *Vibrio cholera* were successfully isolated and identified using both preliminary and confirmatory tests. Despite the fact that MIC values for the different antibiotics were not determined, large proportions of the isolates from different sources and/or locations possessed multi-drug resistant phenotypes.

Despite this, isolates were most often sensitive to Trimethoprim, Trimethoprim-sulfamethoxazole, Chloramphenicol, Gentamycin, Kanamycin and Ciprofloxacin. In addition, cluster analysis of bacteria growth inhibition zone diameter data revealed very close similarities among isolates from different sources and locations indicating similar antibiotic exposure histories and this was an issue of severe public health concern. Therefore, there is a need for constant monitoring of the antibiotic profiles of *Vibrio* species. Given that misuse of antibiotics is currently known to be one of the major factors responsible for the emergence of drug resistant bacteria worldwide, there is need to implement strategies to control access to antimicrobial agents over the counter. Presence of resistance to antibiotics and coupled with virulence factors in some isolates enhances

their pathogenicity. This indicates the need to provide portable water to humans in these communities.

Bacteriophages isolated in this study showed great antibacterial potentials against antibiotic resistant *Vibrio* strains. Two of the employed bacteriophages Phage A and Phage B were able to inhibit bacterial growth of more than one *Vibrio* strain. From these results, these phages may serve as valuable alternatives in the fight against antibiotic resistant bacteria strains in environmental sources. An assessment of the bacteria host range may greatly improve their chances of being used in commercial processes.

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APPENDICES

APPENDIX A

Appendix 1. A: Details of all the materials; culture media, chemicals enzymes and reagents used in this study.

Culture media

Triple Sugar Iron (TSI)	g/L
Meat extract	3.0
Yeast extract	3.0
Tryptone	18.0
Meat peptone	2.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferric Ammonium Sulphate	0.2
Sodium Chloride	5.0
Sodium Thiosulphate	0.3
Phenol Red	0.024
Agar	10.5

Sixty-three grams (63 g) of the above components were dissolved in 1L of distilled water. The media was distributed into McCartney bottles and autoclaved at 121°C for

15 minutes. TSI agar (Biolab, Merck Diagnostic, South Africa) was used as a medium for preliminary identification of *Vibrio* based on its biochemical profile.

Simmons Citrate Agar	g/L
Ammonium Dihydrogen Phosphate	1
Dipotassium Phosphate	1
Sodium chloride	5
Sodium citrate	2
Magnesium Sulphate	0.2
Bromothymol Blue	0.08
Agar	15

Twenty-two grams (22 g) of the above components were dissolved in 1L of distilled water. The medium was distributed into McCartney bottles and autoclaved at 121°C for 15 minutes. Simons Citrate agar (Biolab, Merck Diagnostic, South Africa) was used as a medium for preliminary identification of *Vibrio* based on its biochemical profile.

Crystal Violet	g/L
Crystal violet	2
Ethanol (95%)	20
Ammonium oxalate	0.8

Distilled water

80

Crystal violets and ethanol were mixed together (solution1) and ammonium oxalate was dissolved in distilled water (solution 2). Solution 1 and Solution 2 were mixed together and allowed to stand for at least 24 hours. The solution was filtered before use. This solution was used during gram staining.

Buffered Peptone Water

g/L

Peptone

10.0

Sodium chloride

5.0

Di-sodium Hydrogen Phosphate

3.5

Potassium Dihydrogen Phosphate

1.5

20g of these materials were added in 1L of distilled water and dissolved. It was autoclaved at 121°C for 15minutes, then after cooling off it was poured into sterile falcon tubes. Buffered peptone water (Biolab, Merck Diagnostic, South Africa) was used for serial dilution when plating isolates on TSA for colony count.

Nutrient Broth

g/L

Meat extracts

1.0

Yeast extract

2.0

Peptone	5.0
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Sodium Chloride	8.0
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16g of these materials were added in 1L of distilled water and dissolved. It was autoclaved at 121°C for 15minutes, then after cooling off it was poured into sterile falcon tubes. Nutrient Broth (Biolab, Merck Diagnostic, South Africa) was used as a pre-enrichment medium for DNA extraction overnight cultures.

Muller-Hinton Agar	g/L
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Meat Infusion	5.0
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Casein Hydrolysate	17.5
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Soluble starch	1.5
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Agar	14.0
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38g of these materials were added in 1L of distilled water and dissolved. The media was autoclaved at 121°C for 15minutes, then after cooling off it was poured into sterile petri-dishes. The medium was used to determine the antimicrobial susceptibility profiles of isolates.

Thiosulphate Citrate Bile Salt Sucrose Agar	g/L
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Sucrose	20.0
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Dipeptone	10.0
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Sodium Citrate	10.0
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Sodium Thiosulfate	10.0
Sodium Chloride	10.0
Yeast Extract	5.0
Oxbile (Oxgall)	5.0
Sodium Cholate	3.0
Ferric Citrate	1.0
Bromothymol Blue	0.04
Thymol Blue	0.04
Agar	15.0

Suspended by swirling 90.0g of these materials in 1L of distilled or deionized water. The media was allowed to stand for 15 minutes. The media was boiled (100°C) to completely dissolve the agar and then allowed to cool to 50 to 55°C before pouring. The media was mixed well before pouring into sterile petri dishes and plates were allowed to dry before using them. Thiosulfate citrate bile salt sucrose agar (Davies Diagnostics (Pty) Limited) was used as differential medium for isolation of *Vibrio*.

Luria Bertani (LB) Broth	g/L
Tryptone	10.0

Yeast extract	5.0
Sodium chloride	10.0

25g of these materials were added in 1L of distilled water and allowed to soak 10 minutes then it was swirled to dissolve. The media was autoclaved at 121°C for 15minutes, then after cooling off it was poured into sterile falcon tubes. LB broth was used in the as a pre-enrichment medium plasmid DNA extraction overnight cultures.

Luria Bertani Medium	g/L
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	5.0

20g of these materials were added in 1L of distilled water. The media was heated until it was completely dissolved then autoclaved at 121°C for 15minutes. Later after cooling off it, filtered 2 mL of ampicillin solution was added to the media. The media was poured in preparation of plasmid DNA extraction.

SIM Medium	g/L
Agar	3.0
Ammonium ferric citrate	0.2

Casein peptone	20.0
Meat peptone	6.6
Sodium thiosulfate	0.2

30g of these materials were suspended in 1L of distilled water heating in a boiling water bath or in a current steam, the media was poured into tubes up to about 4cm. The media was autoclaved at 121°C for 15minutes, then allowed to solidify in a vertical position. The media (Biolab, Merck Diagnostic, South Africa) was used for the motility test.

Modified Nutrient Agar (MNA)	g/L
Nutrient Broth	20.0
NaCl	8.5
Agar	10.0
CaCl ₂	8.3mg
FeCl ₃	1.1mg
MgSO ₄	0.5
30% Glucose (added after autoclaving)	10.0ml

These materials were added in 1L of distilled water and dissolved. The media was autoclaved at 121°C for 15minutes. Later after cooling off glucose was added to the media then it was poured into sterile petri-dishes. The medium was used for plaque development during phage work.

Top Agar	g/L
UltraPure Agarose	6.0
MgSO ₄ . 7H ₂ O	2.46

These materials were added in 1L of distilled water and dissolved. The top agar was autoclaved at 121°C for 15minutes. Later after cooling off, it was stored at 4°C and melted every time it was to be used for plaque development during phage work.

Tris-Cl (1M)	g/L
Tris Base	121.1

Tris-base was dissolved in 800ml of distilled water then adjusted pH to desired value by adding HCl (For a pH of 7.4, **7.5**, 8.0 amount of HCl 70ml,**60ml**, 42ml was added respectively). The volume was then adjusted to 1L by adding distilled water and autoclaving the solution at 121°C for 15minutes. The solution was used during phage work.

Lambda Diluent	g/L
1M Tris-Cl (pH 7.5)	10.0ml

MgSO ₄ . 7H ₂ O	2.0
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These materials were added in 1L of distilled water and dissolved. The diluent was autoclaved at 121°C for 15minutes. Later after cooling off, it was stored at room temperature and it was used for plaque development during phage work.

Tryptic Soy Broth (TSB)	g/L
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TSB powder	30.0
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The powder was added to 1L of distilled water and dissolved. Then it was autoclaved at 121°C for 15minutes, then after cooling off it was stored at 4°C. The broth was used as a pre-enrichment broth during phage work.

CHEMICALS

Buffers (50X TAE)

Thermo scientific 50 X TAE-Electrophoresis-buffer (40mM Tris, 20mM Acetic acid and 1mM EDTA) stock solutions was supplied to by Thermo Scientific, Johannesburg, South Africa. Resolving DNA or Amplified PCR products, a 1X TAE buffer was prepared for gel electrophoresis.

Sodium Hypochlorite

10% (v/v) sodium hypochlorite working solution was prepared by aliquoting 10ml of stock solution sodium hypochlorite into 1L Duran bottle with 900ml of distilled water.

The solution was kept at room temperature and used as a disinfectant of the working station.

70% Ethanol

Absolute ethanol (90% v/v) which was supplied for by Merck, Diagnostics, South Africa was used to prepare a 70% (v/v) working solution by; aliquoting 750ml of absolute ethanol into 1L Duran bottle with 300ml of distilled water. The solution was kept at room temperature and used as a disinfectant of the working station.

Ethidium Bromide

In a 5ml Duran bottle a stock solution of 10mg/ml was prepared by dissolving the powder into distilled water and a masking tape was used to protect the solution from light, and then stored at room temperature. A final concentration of 1 μ l was used for staining the gel for electrophoresis for a clear visual of DNA or PCR products amplicons.

DNA Loading Dye (6X)

0.25% (w/v) bromophenol blue

0.25% (w/v) xylene cyanol FF

30% (w/v) glycerol

The above contents were mixed into a 50ml Duran bottle. The solution was filter sterilized using 0.45 μ m filter and stored at room temperature. The solution was used for agarose gel electrophoresis of extracted DNA.

DNA Ladder/Marker

The standard DNA ladders GeneRuler 100 base pairs and O'GeneRuler 1 Kilo base pairs were used to determine the relative amplicon sizes after the gel electrophoresis. The ladders were supplied for by Thermo Scientific, Johannesburg, South Africa.

Oligonucleotide Primers

The primer (forward and reverse) sets used in this study to amplify the *Vibrio* specific genes, and virulence genes were synthesized and supplied for by the Inqaba Biotechnical Industries (Pty) Ltd, Sunnyside, Pretoria, South Africa. All primers were supplied in a lyophilized state and provided a synthesis report on how to reconstitute a 100µM stock solution. A 1.5µl sterile eppendorf tube was used to create and prepare a working solution by aliquoting the required volume of both the forward and reverse primers sets. The primer sets were stored in their separate tubes at -4°C for future use.

ENZYMES

Master Mix (2X DreamTag Green)

2X Dream tag master mix (0.4mM dATP, 0.4mM dCTP, 0.4mM dGTP and 0.4mM dTTP, 4mM MgCl₂ and loading buffer) was used for PCR amplification of target genes and stored at -20 °C for future use. The enzyme was produced by Fermentas, USA and supplied for by Inqaba Biotechnical Industries (Pty) Ltd, Sunnyside, Pretoria, South Africa.

APPENDIX B

Appendix 1. B: Antibigram data of all isolates derived from the inhibition zone data

SAMPLE ID	AP	KF	C	CIP	T	GM	K	NA	TS	S	TM
4	Susceptible	Intermediate	Susceptible	Resistance	Susceptible	Intermediate	Intermediate	Intermediate	Susceptible	Intermediate	Susceptible
13	Resistant	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Intermediate	Intermediate	Susceptible	Resistant	Susceptible
15	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
20	Susceptible	Resistant	Intermediate	Intermediate	Susceptible	Resistant	Intermediate	Resistant	Susceptible	Intermediate	Intermediate
21	Resistant	Resistant	Susceptible	Intermediate	Susceptible	Susceptible	Susceptible	Susceptible	Intermediate	Intermediate	Susceptible
22	Resistant	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Intermediate	Intermediate	Resistant	Susceptible	Resistant
25	Resistant	Resistant	Susceptible	Intermediate	Susceptible	Intermediate	Resistant	Intermediate	Susceptible	Resistant	Susceptible
26	Resistant	Resistant	Susceptible	Intermediate	Susceptible	Susceptible	Susceptible	Intermediate	Susceptible	Susceptible	Susceptible
29	Resistant	Resistant	Susceptible	Intermediate	Resistant	Susceptible	Susceptible	Intermediate	Susceptible	Resistant	Susceptible
30	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Intermediate	Susceptible
32	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
34	Intermediate	Resistant	Susceptible	Susceptible	Intermediate	Susceptible	Intermediate	Susceptible	Susceptible	Resistant	Susceptible
35	Resistant	Resistant	Susceptible	Intermediate	Susceptible	Resistant	Intermediate	Susceptible	Susceptible	Resistant	Susceptible

36	Resistant	Resistant	Susceptible	Intermediate	Susceptible	Susceptible	Intermediate	Susceptible	Susceptible	Intermediate	Susceptible
38	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Intermediate	Resistant
40	Resistant	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Intermediate	Susceptible	Resistant	Susceptible
42	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible
44	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible
45	Intermediate	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Intermediate	Susceptible	Susceptible	Susceptible
46	Resistant	Resistant	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible
51	Resistant	Resistant	Intermediate	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible
53	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible

Appendix 2. A: Percentage Antibiotic resistance pattern of the isolates from water samples.

Area	Antibiotics Resistance (%)										
	AP10	KF30	C30	CIP5	T30	GM10	K30	NA30	TS25	S10	TM5
Pudimoe				4.55 %							
Choseng	4.55%	9.09%	4.55 %		4.55 %			4.55%		4.55%	4.55 %
Lonely park	9.09%	13.64 %				4.55%		4.55%	4.55 %		4.55 %
Seweding	4.55%	4.55%					4.55%			4.55%	
Ramosadi	4.55%	4.55%									
Ntswanahatshe	18.18 %	27.73 %				9.09%	4.55%	4.55%		9.09%	
Vryburg	9.09%	9.09%			4.55 %			4.55%		4.55%	4.55 %
Marico Bosveld	4.55%	4.55%								4.55%	
Pella	9.09%	13.64 %	4.55 %		4.55 %					9.09%	
Coligny	4.55%	4.55%						4.55%			
Ventersdorp	4.55%	4.55%					4.55%	4.55%			

APPENDIX C

Appendix 1. C: Interpretation of biofilm formation results

37					35				25			
SAMP LE ID	No Biofilm	Weak Biofilm	Intermedia te Biofilm	Strong Biofilm	No Biofil m	Weak Biofil m	Intermediat e Biofilm	Strong Biofil m	No Biofilm	Weak Biofilm	Intermedi ate Biofilm	Strong Biofilm
4	No				No				No			
13		Weak				Weak			No			
15	No				No				No			
20	No							Strong	No			
21			Moderate			Weak			No			
22			Moderate					Strong		Weak		
25		Weak			No				No			
26		Weak			No				No			
29			Moderate			Weak			No			
30			Moderate				Moderate			Weak		
32	No				No					Weak		
34	No				No				No			
35	No					Weak				Weak		
36	No							Strong			Moderate	
38		Weak				Weak			No			
40	No				No				No			
42	No							Strong		Weak		
44	No							Strong			Moderate	
45	No						Moderate			Weak		
46	No					Weak				Weak		
51	No							Strong				Strong
53	No				No						Moderate	

APPENDIX D

Appendix 1. D: Virulent assay detailed results

Shenzhen Huisong Technology Development Co., Ltd

Plate No.: PLATE-1 CutOff: 0 Test Mode: Plate Test
 WL1: 630 LP Range 0 Test Doctor:
 WL2: None Determine Symbol: > Test Date: 2018-10-03

	1	2	3	4	5	6	7	8	9	10	11	12	
A	BLK 0.0328	NC 0.6630	0001 0.5791 +	0002 0.6672 +	0003 0.9321 +	0004 0.9496 +	0005 0.9804 +	0006 0.9704 +	0007 0.7203 +	0008 0.7092 +	0009 0.8772 +	0010 0.8799 +	Sample OD Result S/CO
B	BLK 0.0327	NC 0.8930	0011 0.7095 +	0012 0.7425 +	0013 0.9650 +	0014 0.9686 +	0015 0.9794 +	0016 0.9754 +	0017 0.6852 +	0018 0.6869 +	0019 0.9197 +	0020 0.9130 +	Sample OD Result S/CO
C	BLK 0.0369	NC 0.9037	0021 0.7291 +	0022 0.7584 +	0023 0.9777 +	0024 0.9803 +	0025 0.9985 +	0026 0.9882 +	0027 0.7488 +	0028 0.7453 +	0029 0.9287 +	0030 0.9215 +	Sample OD Result S/CO
D	BLK 0.0354	NC 0.9064	0031 0.7140 +	0032 0.7483 +	0033 0.9677 +	0034 0.9749 +	0035 1.0051 +	0036 0.9966 +	0037 0.8343 +	0038 0.8362 +	0039 0.9169 +	0040 0.9072 +	Sample OD Result S/CO
E	BLK 0.0286	NC 0.9147	0041 0.7183 +	0042 0.7258 +	0043 0.9685 +	0044 0.9731 +	0045 1.0053 +	0046 1.0067 +	0047 0.8655 +	0048 0.8823 +	0049 0.9173 +	0050 0.8907 +	Sample OD Result S/CO
F	BLK 0.0341	NC 0.9213	0051 0.6570 +	0052 0.7136 +	0053 0.9847 +	0054 0.9837 +	0055 0.9748 +	0056 0.9778 +	0057 0.8480 +	0058 0.8601 +	0059 0.9018 +	0060 0.9074 +	Sample OD Result S/CO
G	BLK 0.0366	NC 0.9243	0061 0.6493 +	0062 0.6500 +	0063 0.9907 +	0064 0.9937 +	0065 0.9800 +	0066 0.9864 +	0067 0.8470 +	0068 0.8515 +	0069 0.9083 +	0070 0.9190 +	Sample OD Result S/CO
H	BLK 0.0380	NC 0.9085	0071 0.5829 +	0072 0.5888 +	0073 0.9596 +	0074 0.9765 +	0075 0.9788 +	0076 0.9823 +	0077 0.8200 +	0078 0.8294 +	0079 0.9038 +	0080 0.9011 +	Sample OD Result S/CO

Review Doctor: Jack

Review:

Report Time: 2018-10-03 12:56:51 PM

Appendix 2. D: Virulent assay detailed results

Shenzhen Huisong Technology Development Co., Ltd

Plate No.: PLATE-2 CutOff: 0 Test Mode: Plate Test
 WL1: 630 LP Range 0 Test Doctor:
 WL2: None Determine Symbol: > Test Date: 2018-10-03

	1	2	3	4	5	6	7	8	9	10	11	12	
A	BLK 0.0243	NC 0.9079	0081 0.8540 +	0089 0.8733 +	0097 0.8351 +	0105 0.8310 +	0113 0.8027 +	0121 0.7909 +	0129 0.8051 +	0137 0.6920 +	0145 0.6966 +	0153 0.7830 +	Sample OD Result S/CO
B	BLK 0.0286	NC 0.9245	0082 0.9129 +	0090 0.9295 +	0098 0.8733 +	0106 0.8659 +	0114 0.8304 +	0122 0.8442 +	0130 0.7260 +	0138 0.7352 +	0146 0.8268 +	0154 0.8198 +	Sample OD Result S/CO
C	BLK 0.0297	NC 0.9203	0083 0.9129 +	0091 0.9194 +	0099 0.8726 +	0107 0.8711 +	0115 0.8545 +	0123 0.8435 +	0131 0.7548 +	0139 0.7070 +	0147 0.8214 +	0155 0.8037 +	Sample OD Result S/CO
D	BLK 0.0274	NC 0.8556	0084 0.8427 +	0092 0.8652 +	0100 0.8270 +	0108 0.8585 +	0116 0.7618 +	0124 0.7774 +	0132 0.6756 +	0140 0.6640 +	0148 0.7985 +	0156 0.7951 +	Sample OD Result S/CO
E	BLK 0.0227	NC 0.9232	0085 0.9043 +	0093 0.9068 +	0101 0.8773 +	0109 0.8942 +	0117 0.7805 +	0125 0.7859 +	0133 0.6526 +	0141 0.7417 +	0149 0.8064 +	0157 0.7876 +	Sample OD Result S/CO
F	BLK 0.0273	NC 0.9300	0086 0.9171 +	0094 0.9271 +	0102 0.8851 +	0110 0.8880 +	0118 0.9011 +	0126 0.8985 +	0134 0.6116 +	0142 0.6409 +	0150 0.8038 +	0158 0.7851 +	Sample OD Result S/CO
G	BLK 0.0343	NC 0.9536	0087 0.9323 +	0095 0.9432 +	0103 0.9048 +	0111 0.9066 +	0119 0.9431 +	0127 0.9104 +	0135 0.5778 +	0143 0.6173 +	0151 0.8201 +	0159 0.8009 +	Sample OD Result S/CO
H	BLK 0.0361	NC 0.9358	0088 0.9303 +	0096 0.9193 +	0104 0.8968 +	0112 0.8989 +	0120 0.9498 +	0128 0.9308 +	0136 0.6501 +	0144 0.6898 +	0152 0.8105 +	0160 0.7888 +	Sample OD Result S/CO

Review Doctor: Jack

Review:

Report Time: 2018-10-03 01:10:41 PM

Appendix 3. D: Virulent assay detailed results

Shenzhen Huisong Technology Development Co., Ltd

Plate No.: PLATE-3 CutOff: 0 Test Mode: Plate Test
 WL1: 630 LP Range 0 Test Doctor:
 WL2: None Determine Symbol: > Test Date: 2018-10-03

	1	2	3	4	5	6	7	8	9	10	11	12	
A	BLK 0.0363	NC 1.0718	0081 0.9168 +	0082 0.9229 +	0083 0.8413 +	0084 0.8492 +	0085 0.8998 +	0086 0.9151 +	0087 0.8527 +	0088 0.8439 +	0089 0.5292 +	0090 0.5666 +	Sample OD Result S/CO
B	BLK 0.0374	NC 1.0667	0091 0.9607 +	0092 0.9396 +	0093 0.8556 +	0094 0.8832 +	0095 0.9299 +	0096 0.9282 +	0097 0.8808 +	0098 0.8893 +	0099 0.5448 +	0100 0.6428 +	Sample OD Result S/CO
C	BLK 0.0427	NC 1.0870	0101 0.9697 +	0102 0.9713 +	0103 0.8845 +	0104 0.8972 +	0105 0.9290 +	0106 0.9337 +	0107 0.8975 +	0108 0.8598 +	0109 0.4817 +	0110 0.5019 +	Sample OD Result S/CO
D	BLK 0.0394	NC 1.0714	0111 0.9506 +	0112 0.9466 +	0113 0.8951 +	0114 0.9000 +	0115 0.9567 +	0116 0.9516 +	0117 0.8474 +	0118 0.8285 +	0119 0.5235 +	0120 0.5031 +	Sample OD Result S/CO
E	BLK 0.0344	NC 1.0697	0121 0.9590 +	0122 0.9425 +	0123 0.9019 +	0124 0.9064 +	0125 0.9637 +	0126 0.9714 +	0127 0.9460 +	0128 0.8997 +	0129 0.5425 +	0130 0.5617 +	Sample OD Result S/CO
F	BLK 0.0390	NC 1.0458	0131 0.9245 +	0132 0.9404 +	0133 0.9058 +	0134 0.9036 +	0135 0.9676 +	0136 0.9593 +	0137 0.9520 +	0138 0.9557 +	0139 0.5664 +	0140 0.6432 +	Sample OD Result S/CO
G	BLK 0.0453	NC 1.0149	0141 0.9693 +	0142 0.9612 +	0143 0.9106 +	0144 0.9131 +	0145 0.9716 +	0146 0.9702 +	0147 0.9610 +	0148 0.9685 +	0149 0.5114 +	0150 0.5093 +	Sample OD Result S/CO
H	BLK 0.0465	NC 0.9620	0151 0.9420 +	0152 0.9546 +	0153 0.9105 +	0154 0.9126 +	0155 0.9640 +	0156 0.9750 +	0157 0.9646 +	0158 0.9501 +	0159 0.4076 +	0160 0.4289 +	Sample OD Result S/CO

Review Doctor: Jack

Review:

Report Time: 2018-10-03 01:25:40 PM

APPENDIX 4D: Virulent assay detailed results

Shenzhen Huisong Technology Development Co., Ltd

Plate No.: PLATE-4 CutOff: 0 Test Mode: Plate Test
 WL1: 630 LP Range: 0 Test Doctor:
 WL2: None Determine Symbol: > Test Date: 2018-10-03

	1	2	3	4	5	6	7	8	9	10	11	12	
A	BLK 0.0299	NC 0.9187	0001 0.7443 +	0002 0.7382 +	0003 0.8769 +	0004 0.8881 +	0005 0.9246 +	0006 0.9308 +	0007 0.7492 +	0008 0.7479 +	0009 0.6782 +	0010 0.6346 +	Sample OD Result S/CO
B	BLK 0.0302	NC 0.9655	0011 0.7812 +	0012 0.7707 +	0013 0.8406 +	0014 0.8504 +	0015 0.8942 +	0016 0.8904 +	0017 0.7791 +	0018 0.7835 +	0019 0.6798 +	0020 0.6537 +	Sample OD Result S/CO
C	BLK 0.0345	NC 0.9369	0021 0.7615 +	0022 0.7457 +	0023 0.8422 +	0024 0.8386 +	0025 0.7686 +	0026 0.8070 +	0027 0.7473 +	0028 0.7657 +	0029 0.6827 +	0030 0.7632 +	Sample OD Result S/CO
D	BLK 0.0326	NC 0.9200	0031 0.7570 +	0032 0.7251 +	0033 0.8650 +	0034 0.8753 +	0035 0.7565 +	0036 0.7726 +	0037 0.7419 +	0038 0.7346 +	0039 0.6966 +	0040 0.6760 +	Sample OD Result S/CO
E	BLK 0.0278	NC 0.8941	0041 0.7471 +	0042 0.6511 +	0043 0.8890 +	0044 0.8837 +	0045 0.7409 +	0046 0.7877 +	0047 0.6107 +	0048 0.6273 +	0049 0.7567 +	0050 0.7861 +	Sample OD Result S/CO
F	BLK 0.0350	NC 0.8878	0051 0.6435 +	0052 0.6090 +	0053 0.9082 +	0054 0.9077 +	0055 0.7525 +	0056 0.7778 +	0057 0.6201 +	0058 0.5971 +	0059 0.8219 +	0060 0.8471 +	Sample OD Result S/CO
G	BLK 0.0386	NC 0.9161	0061 0.7364 +	0062 0.6265 +	0063 0.9517 +	0064 0.9403 +	0065 0.7782 +	0066 0.8114 +	0067 0.8104 +	0068 0.8335 +	0069 0.8993 +	0070 0.8975 +	Sample OD Result S/CO
H	BLK 0.0405	NC 0.9283	0071 0.7682 +	0072 0.6946 +	0073 0.9397 +	0074 0.9256 +	0075 0.8005 +	0076 0.8326 +	0077 0.8539 +	0078 0.8701 +	0079 0.8490 +	0080 0.8822 +	Sample OD Result S/CO

Review Doctor: Jack

Review:

Report Time: 2018-10-03 01:46:13 PM

APPENDIX 5D: Virulent assay detailed results

Shenzhen Huisong Technology Development Co., Ltd

Plate No.: PLATE-5 CutOff: 0 Test Mode: Plate Test
 WL1: 630 LP Range: 0 Test Doctor:
 WL2: None Determine Symbol: > Test Date: 2018-10-03

	1	2	3	4	5	6	7	8	9	10	11	12	
A	BLK 0.0441	NC 0.8918	0001 0.8585 +	0002 0.8832 +	0003 0.9072 +	0004 0.8928 +	0.0387	0.0361	0.0385	0.0387	0.0412	0.0570	Sample OD Result S/CO
B	BLK 0.0427	NC 0.8698	0005 0.8735 +	0006 0.9366 +	0007 0.9492 +	0008 0.9414 +	0.0393	0.0373	0.0390	0.0401	0.0392	0.0384	Sample OD Result S/CO
C	BLK 0.0488	NC 0.8997	0009 0.8898 +	0010 0.8904 +	0011 0.9409 +	0012 0.8574 +	0.0393	0.0395	0.0426	0.0385	0.0377	0.0370	Sample OD Result S/CO
D	BLK 0.0440	NC 0.9205	0013 0.9187 +	0014 0.8856 +	0015 0.9301 +	0016 0.9401 +	0.0437	0.0431	0.0412	0.0408	0.0405	0.0504	Sample OD Result S/CO
E	BLK 0.0370	NC 0.9370	0017 0.9083 +	0018 0.9194 +	0019 0.9547 +	0020 0.9390 +	0.0382	0.0388	0.0359	0.0347	0.0449	0.0432	Sample OD Result S/CO
F	BLK 0.0391	NC 0.9264	0021 0.9303 +	0022 0.8754 +	0023 0.9653 +	0024 0.9481 +	0.0370	0.0390	0.0369	0.0376	0.0370	0.0552	Sample OD Result S/CO
G	BLK 0.0431	NC 0.9375	0025 0.9225 +	0026 0.9317 +	0027 0.9688 +	0028 0.9642 +	0.0428	0.0413	0.0418	0.0420	0.0391	0.0528	Sample OD Result S/CO
H	BLK 0.0452	NC 0.9543	0029 0.8865 +	0030 0.9066 +	0031 0.9601 +	0032 0.9553 +	0.0393	0.0414	0.0412	0.0405	0.0406	0.0409	Sample OD Result S/CO

Review Doctor: Jack

Review:

Report Time: 2018-10-03 02:01:37 PM

APPENDIX 6D: Virulent assay detailed results

Shenzhen Huisong Technology Development Co., Ltd

Plate No.: PLATE-6 CutOff: 0 Test Mode: Plate Test
 WL1: 630 LP Range: 0 Test Doctor:
 WL2: None Determine Symbol: > Test Date: 2018-10-03

	1	2	3	4	5	6	7	8	9	10	11	12	
A	BLK 0.0287	NC 0.6917	0001 0.6814 +	0002 0.7170 +	0003 0.9558 +	0004 0.9542 +	0005 1.0362 +	0006 1.0673 +	0007 0.5506 +	0008 0.6701 +	0009 0.9416 +	0010 0.6495 +	Sample OD Result S/CO
B	BLK 0.0285	NC 0.9843	0011 0.8130 +	0012 0.8041 +	0013 0.9957 +	0014 1.0019 +	0015 1.0467 +	0016 1.0517 +	0017 0.6726 +	0018 0.5494 +	0019 0.9699 +	0020 0.9812 +	Sample OD Result S/CO
C	BLK 0.0319	NC 0.9867	0021 0.8230 +	0022 0.8207 +	0023 1.0102 +	0024 1.0177 +	0025 1.0390 +	0026 1.0602 +	0027 0.8352 +	0028 0.8463 +	0029 0.9923 +	0030 0.9949 +	Sample OD Result S/CO
D	BLK 0.0282	NC 0.9507	0031 0.8096 +	0032 0.8477 +	0033 0.9967 +	0034 0.9821 +	0035 1.0379 +	0036 1.0530 +	0037 0.8792 +	0038 0.8629 +	0039 0.9670 +	0040 0.9893 +	Sample OD Result S/CO
E	BLK 0.0233	NC 0.9619	0041 0.7096 +	0042 0.8593 +	0043 1.0054 +	0044 0.9946 +	0045 1.0224 +	0046 0.9637 +	0047 0.8720 +	0048 0.8932 +	0049 0.9815 +	0050 0.9886 +	Sample OD Result S/CO
F	BLK 0.0362	NC 0.9495	0051 0.7966 +	0052 0.7133 +	0053 1.0036 +	0054 1.0010 +	0055 1.0267 +	0056 1.0258 +	0057 0.8348 +	0058 0.8644 +	0059 0.9793 +	0060 0.9353 +	Sample OD Result S/CO
G	BLK 0.0319	NC 0.9445	0061 0.8055 +	0062 0.7369 +	0063 1.0274 +	0064 1.0133 +	0065 1.0456 +	0066 1.0621 +	0067 0.8353 +	0068 0.8465 +	0069 1.0007 +	0070 0.9555 +	Sample OD Result S/CO
H	BLK 0.0336	NC 0.9541	0071 0.6444 +	0072 0.6407 +	0073 0.9697 +	0074 0.9682 +	0075 1.0946 +	0076 1.1041 +	0077 0.7974 +	0078 0.8074 +	0079 0.9451 +	0080 0.9291 +	Sample OD Result S/CO

Review Doctor: Jack

Review:

Report Time: 2018-10-03 02:30:01 PM

APPENDIX 7D: Virulent assay detailed results

Shenzhen Huisong Technology Development Co., Ltd

Plate No.: PLATE-7 CutOff: 0 Test Mode: Plate Test
 WL1: 630 LP Range: 0 Test Doctor:
 WL2: None Determine Symbol: > Test Date: 2018-10-03

	1	2	3	4	5	6	7	8	9	10	11	12	
A	NC 0.0416	NC 0.9894	0001 0.9221 +	0002 0.9324 +	0003 0.8713 +	0004 0.8692 +	0005 0.8417 +	0006 0.8342 +	0007 0.4909 +	0008 0.4779 +	0009 0.3735 +	0010 0.3671 +	Sample OD Result S/CO
B	NC 0.0303	NC 1.0113	0011 1.0177 +	0012 1.0147 +	0013 0.9123 +	0014 0.9165 +	0015 0.8806 +	0016 0.8738 +	0017 0.7518 +	0018 0.6213 +	0019 0.4329 +	0020 0.3820 +	Sample OD Result S/CO
C	NC 0.0345	NC 1.0147	0021 1.0277 +	0022 1.0396 +	0023 0.9337 +	0024 0.9303 +	0025 0.8452 +	0026 0.8321 +	0027 0.8055 +	0028 0.6373 +	0029 0.8758 +	0030 0.8594 +	Sample OD Result S/CO
D	NC 0.0517	NC 1.0389	0031 1.0577 +	0032 1.0001 +	0033 0.9095 +	0034 0.9125 +	0035 0.8190 +	0036 0.8059 +	0037 0.9505 +	0038 0.7731 +	0039 0.9038 +	0040 0.8541 +	Sample OD Result S/CO
E	NC 0.0243	NC 1.0398	0041 1.0311 +	0042 0.9939 +	0043 0.9153 +	0044 0.9211 +	0045 0.7900 +	0046 0.6626 +	0047 0.7623 +	0048 0.8538 +	0049 0.9190 +	0050 0.8894 +	Sample OD Result S/CO
F	NC 0.0344	NC 1.0214	0051 1.0114 +	0052 1.0309 +	0053 0.8805 +	0054 0.9061 +	0055 0.7848 +	0056 0.7655 +	0057 0.7518 +	0058 0.7888 +	0059 0.9106 +	0060 0.8736 +	Sample OD Result S/CO
G	NC 0.0456	NC 1.0514	0061 1.1037 +	0062 1.0616 +	0063 0.9080 +	0064 0.9326 +	0065 0.9027 +	0066 0.8240 +	0067 0.6938 +	0068 0.8315 +	0069 0.8973 +	0070 0.8802 +	Sample OD Result S/CO
H	NC 0.0382	NC 1.1208	0071 1.1831 +	0072 1.1246 +	0073 0.9292 +	0074 0.9262 +	0075 0.8176 +	0076 0.8238 +	0077 0.6557 +	0078 0.8118 +	0079 0.8829 +	0080 0.8778 +	Sample OD Result S/CO

Review Doctor: Jack

Review:

Report Time: 2018-10-03 03:00:14 PM

APPENDIX 8D: Virulent assay detailed results

Shenzhen Huisong Technology Development Co., Ltd

Plate No.: PLATE-8 CutOff: 0 Test Mode: Plate Test
 WL1: 630 LP Range: 0 Test Doctor:
 WL2: None Determine Symbol: > Test Date: 2018-10-03

	1	2	3	4	5	6	7	8	9	10	11	12	
A	BLK 0.0332	0001 1.1124 +	0009 0.9696 +	0010 0.9537 +	0011 0.8562 +	0012 0.8917 +	0013 0.9127 +	0014 0.9009 +	0015 0.7999 +	0016 0.7079 +	0017 0.2730 +	0018 0.2821 +	Sample OD Result S/CO
B	BLK 0.0331	0002 1.1013 +	0019 1.0072 +	0020 1.0229 +	0021 0.8809 +	0022 0.9273 +	0023 0.9521 +	0024 0.9426 +	0025 0.8541 +	0026 0.8013 +	0027 0.2468 +	0028 0.2635 +	Sample OD Result S/CO
C	BLK 0.0387	0003 1.1507 +	0029 1.0359 +	0030 1.0308 +	0031 0.9419 +	0032 0.9499 +	0033 0.9738 +	0034 0.9483 +	0035 0.8868 +	0036 0.8549 +	0037 0.2238 +	0038 0.3125 +	Sample OD Result S/CO
D	BLK 0.0359	0004 1.1838 +	0039 1.0346 +	0040 1.0181 +	0041 0.9408 +	0042 0.9580 +	0043 0.9534 +	0044 0.9335 +	0045 0.8136 +	0046 0.9452 +	0047 0.5943 +	0048 0.6459 +	Sample OD Result S/CO
E	BLK 0.0297	0005 1.1605 +	0049 1.0234 +	0050 0.9869 +	0051 0.9548 +	0052 0.9724 +	0053 0.9572 +	0054 0.9359 +	0055 0.9404 +	0056 0.9013 +	0057 0.7296 +	0058 0.6329 +	Sample OD Result S/CO
F	BLK 0.0345	0006 1.1499 +	0059 1.0187 +	0060 1.0057 +	0061 0.9447 +	0062 0.9657 +	0063 0.9896 +	0064 0.9699 +	0065 0.9589 +	0066 0.9236 +	0067 0.7072 +	0068 0.6893 +	Sample OD Result S/CO
G	BLK 0.0404	0007 1.1597 +	0069 1.0687 +	0070 1.0877 +	0071 0.9579 +	0072 0.9827 +	0073 1.0032 +	0074 0.9927 +	0075 0.9861 +	0076 0.9614 +	0077 0.5725 +	0078 0.5654 +	Sample OD Result S/CO
H	BLK 0.0404	0008 1.1222 +	0079 0.9975 +	0080 1.1118 +	0081 0.9561 +	0082 0.9847 +	0083 0.9861 +	0084 0.9733 +	0085 1.0027 +	0086 0.9531 +	0087 0.7252 +	0088 0.6425 +	Sample OD Result S/CO

Review Doctor: Jack

Review:

Report Time: 2018-10-03 03:29:58 PM

APPENDIX 9D: Virulent assay detailed results

Shenzhen Huisong Technology Development Co., Ltd

Plate No.: PLATE-9 CutOff: 0 Test Mode: Plate Test
 WL1: 630 LP Range: 0 Test Doctor:
 WL2: None Determine Symbol: > Test Date: 2018-10-03

	1	2	3	4	5	6	7	8	9	10	11	12	
A	BLK 0.1091	0001 0.7227 +	0009 0.4107 +	0010 0.4582 +	0011 0.4824 +	0012 0.4673 +	0013 0.4780 +	0014 0.4508 +	0015 0.4974 +	0016 0.5006 +	0017 0.4162 +	0018 0.4865 +	Sample OD Result S/CO
B	BLK 0.0994	0002 0.7245 +	0019 0.4912 +	0020 0.5734 +	0021 0.5129 +	0022 0.5144 +	0023 0.4908 +	0024 0.5380 +	0025 0.6179 +	0026 0.6744 +	0027 0.4957 +	0028 0.5817 +	Sample OD Result S/CO
C	BLK 0.0980	0003 0.7818 +	0029 0.4867 +	0030 0.5068 +	0031 0.5329 +	0032 0.5199 +	0033 0.5223 +	0034 0.5219 +	0035 0.6448 +	0036 0.6836 +	0037 0.4929 +	0038 0.6006 +	Sample OD Result S/CO
D	BLK 0.1030	0004 0.7520 +	0039 0.4801 +	0040 0.7214 +	0041 0.5797 +	0042 0.5277 +	0043 0.4846 +	0044 0.5384 +	0045 0.6429 +	0046 0.6644 +	0047 0.5779 +	0048 0.5918 +	Sample OD Result S/CO
E	BLK 0.0959	0005 0.8089 +	0049 0.5480 +	0050 0.6882 +	0051 0.5688 +	0052 0.5931 +	0053 0.5825 +	0054 0.5942 +	0055 0.6519 +	0056 0.6254 +	0057 0.5539 +	0058 0.5608 +	Sample OD Result S/CO
F	BLK 0.0983	0006 0.7574 +	0059 0.4303 +	0060 0.6658 +	0061 0.6273 +	0062 0.7255 +	0063 0.5699 +	0064 0.5704 +	0065 0.6724 +	0066 0.6605 +	0067 0.6512 +	0068 0.5799 +	Sample OD Result S/CO
G	BLK 0.1106	0007 0.7651 +	0069 0.4225 +	0070 0.4403 +	0071 0.6576 +	0072 0.8602 +	0073 0.6482 +	0074 0.6333 +	0075 0.6239 +	0076 0.7138 +	0077 0.6636 +	0078 0.6928 +	Sample OD Result S/CO
H	BLK 0.1225	0008 0.7740 +	0079 0.5720 +	0080 0.6634 +	0081 0.8035 +	0082 0.8015 +	0083 0.6746 +	0084 0.6867 +	0085 0.5576 +	0086 0.0589 +	0087 0.7424 +	0088 0.7347 +	Sample OD Result S/CO

Review Doctor: Jack

Review:

Report Time: 2018-10-03 03:58:40 PM

APPENDIX 10D: Virulent assay detailed results

Shenzhen Huisong Technology Development Co., Ltd

Plate No.: PLATE-10 CutOff: 0 Test Mode: Plate Test
 WL1: 630 LP Range: 0 Test Doctor:
 WL2: None Determine Symbol: > Test Date: 2018-10-03

	1	2	3	4	5	6	7	8	9	10	11	12	
A	BLK 0.0408	0001 0.9248 +	0010 0.9089 +	0011 0.8669 +	0012 0.9335 +	0013 0.9066 +	0.0388	0.0437	0.0415	0.0446	0.0370	0.0370	Sample OD Result S/CO
B	BLK 0.0425	0002 0.9161 +	0014 0.9206 +	0015 0.8866 +	0016 1.0057 +	0017 0.9378 +	0.0440	0.0387	0.0431	0.0415	0.0428	0.0394	Sample OD Result S/CO
C	BLK 0.0457	0003 0.8729 +	0018 0.9075 +	0019 0.9645 +	0020 1.0008 +	0021 1.0005 +	0.0385	0.0383	0.0380	0.0387	0.0385	0.0389	Sample OD Result S/CO
D	BLK 0.0424	0004 0.9073 +	0022 0.8860 +	0023 0.9596 +	0024 1.0379 +	0025 0.9613 +	0.0371	0.0385	0.0391	0.0402	0.0413	0.0401	Sample OD Result S/CO
E	BLK 0.0375	0005 0.8550 +	0026 0.9015 +	0027 1.0038 +	0028 0.9543 +	0029 0.9904 +	0.0339	0.0333	0.0346	0.0361	0.0353	0.0318	Sample OD Result S/CO
F	BLK 0.0402	0006 0.8991 +	0030 0.8997 +	0031 0.9323 +	0032 0.9839 +	0033 1.0074 +	0.0337	0.0344	0.0345	0.0346	0.0354	0.0321	Sample OD Result S/CO
G	BLK 0.0458	0007 0.8803 +	0034 0.9114 +	0035 0.8970 +	0036 0.9719 +	0037 0.9492 +	0.0385	0.0374	0.0390	0.0380	0.0403	0.0359	Sample OD Result S/CO
H	BLK 0.0454	0008 0.8538 +	0038 0.9110 +	0039 0.8545 +	0040 0.9669 +	0041 0.9655 +	0.0391	0.0414	0.0383	0.0386	0.0383	0.0383	Sample OD Result S/CO

Review Doctor: Jack

Review:

Report Time: 2018-10-03 04:05:06 PM