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ANTIBIOTICS COMBINATION THERAPY OPTION FOR THE CONTROL OF ANTIMICROBIAL RESISTANT NON-CHOLERA CAUSING *VIBRIO* SPECIES RECOVERED FROM ENVIRONMENTAL NICHES IN THE EASTERN CAPE, SOUTH AFRICA

BY

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A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE DEGREE

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ALICE

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APRIL, 2021

DECLARATION

I, Ayodele Oluwakemi Victoria, declare that this dissertation entitled "Antibiotics combination therapy option for the control of antimicrobial-resistant non-cholera causing *Vibrio* species recovered from environmental niches of Eastern Cape, South Africa" submitted to the University of Fort Hare for the degree of Master of Science in Microbiology in the Faculty of Science and Agriculture is my work and that it has not been submitted to any other University in part or entirely for the award of any degree.

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Date: 20-4-2021

DECLARATION ON RESEARCH ETHICAL CLEARANCE

I, Ayodele Victoria Oluwakemi, student number: 201816567 hereby declare that I am fully aware of the University of Fort Hare's policy on research ethics and I have taken every precaution to comply with the regulations. I have noted that my research does not comply with Rule G17. 6. 10.5 and an ethical certificate with reference number **OKO041SAYO01** was obtained.

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CERTIFICATION

The dissertation titled "Antibiotics combination therapy option for the control of resistant Non-cholera causing *Vibrio* species recovered from Environmental niches in the Eastern Cape, South Africa" comply with the regulation that governs the award of Master of Science (MSc) of the University of Fort Hare and it was approved for its contribution to scientific knowledge.

…… ……

Prof A.I. Okoh Date

Supervisor

DEDICATION

This project work is dedicated to my parents, Prof. and Mrs. S.M. Ayodele. Thank you for your being my source of strength, for your love and moral support towards my academic success.

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LIST OF ABBREVIATIONS

AEMREG: Applied and Environmental Microbiology Research Group

AMR: Antimicrobial Resistance

cAMP: Cyclic Adenosine Monophosphate

CDC: Centers for Disease Control and Prevention

CFU: Colony Forming Unit

CLSI: Clinical and Laboratory Standards Institute

CPKP: Carbapenemase-Producing *Klebsiella pneumoniae*

CPS: Capsular Polysaccharide

CT: Cholera Toxin

DNA: Deoxyribonucleic Acid

DWA: Department of water affairs

DWARF: Department of Water Affairs and Forestry

FIC: Fractional Inhibitory Concentration

ICU: Intensive Care Unit

MAMs: Multivalent Adhesion Molecule

MARI: Multiple antibiotic resistance index

MARP: Multiple antibiotic resistance phenotypes

MBC: Minimum Bactericidal Concentration

MDR: Multi-Drug Resistance

MHA: Mueller Hinton Agar

MHB: Mueller Hinton Broth

MIC: Minimum Inhibitory Concentration

NaCl: Sodium Chloride

NNDSS: National Notifiable Disease Surveillance System

NS: Normal Saline

PCR: Polymerase Chain Reaction

SABC: South African Broadcasting Corporation

T3SS: Type III Secretion Systems

TCP: Toxin-co-regulated pilus

TDH: Thermostable direct hemolysin

UNCTAD: United Nations Conference on Trade and Development

UNEP: United Nations Environment Programme

VCG: Virulence-correlated Gene

WHO: World Health Organization

WRC: Water Research Commission

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GENERAL ABSTRACT

Increased rate of antibiotic resistance (AR) poses a serious threat with a resultant notion of a possible end of the antibiotics era, making it a problem of concern to public health and a great implication on the world economy and human society. Despite many approaches developed to curb this menace, antibiotics resistance is still a challenge worldwide. This has made the use of combined therapy as one of the options in many cases. This study was conducted to assess antibiotics combination therapy as an option for the control of antimicrobial-resistant non-cholera causing *Vibrio* species that were recovered from the environment in the Eastern Cape, South Africa. Two hundred and twenty-eight *Vibrio* species were recovered from the environment in the Province, and these were deposited in the archive of AEMREG. PCR was used to identify target *Vibrio* species. Disc diffusion method was used to evaluate the antibiotic susceptibility profile of the confirmed isolates against 11 antibiotics commonly used against infections. MIC and MBC were determined using antibiotics (imipenem, tetracycline, and nalidixic acid) that high resistance was discovered. Checkerboard assay was used to carry out antibiotics combination assay, and the FICI was calculated. Rate of kill was also determined using ½ × MIC, 1 × MIC, and 2 × MIC concentrations of the combined antibiotics at 2 hr intervals.

One hundred of the isolates were confirmed to be *Vibrio parahaemolyticus*, 82 were *Vibrio vulnificus* and 46 were *Vibrio fluvialis*. Twenty-two (22) percent of the *Vibrio parahaemolyticus* isolates showed resistance against tetracycline and their resistance against other antimicrobials is as follows; nalidixic acid (16 %), ampicillin (14 %), cefotaxime (14 %), chloramphenicol (12 %) and amikacin (11 %). For *Vibrio vulnificus*, prevalence of resistance was as follows: imipenem (40 %), tetracycline (22 %), ampicillin (18 %), meropenem (15 %), and chloramphenicol (11 %). *Vibrio fluvialis* showed the following resistance profile: nalidixic acid (28 %), tetracycline (28%), ampicillin (20 %), chloramphenicol (15 %), amikacin (11 %) and cefotaxime (11 %). About 38 multiple

<antibiotic resistance phenotypes (MARP) were recorded in all species that were evaluated. About 23 % were resistant to over 3 antibiotics used. The multiple antibiotic resistant indices (MARI) ranged between 0.3 and 0.8. MIC and MBC were carried against isolates that were resistant to the two most common antibiotics tested.

MIC and MBC were determined in the following order: tetracycline and nalidixic acid at concentrations ranging from 16 µg/ml to 1024 µg/ml for *Vibrio parahaemolyticus* and 32 µg/ml to 2048 µg/ml for *Vibrio fluvialis*. Also, the MIC and MBC of imipenem and tetracycline at concentrations ranging from 8 µg/ml to 256 µg/ml for *Vibrio vulnificus* were determined. Antibiotics combination therapy was carried out and synergistic activity was observed in 3 of the 16 resistant *V. parahaemolyticus* isolates, 3 of the16 resistant *V. vulnificus* isolates and 2 of the 13 resistant *V. fluvialis* isolates. Antagonism was not observed across all the drug combinations. Rate of kill was also determined and at 6 hr exposure time, the highest concentration (2 × MIC) exhibited bactericidal effect across all three *Vibrio* species. The result derived in this research, therefore, propose that combination therapy is a promising solution to antimicrobial resistance in *Vibrio* species.

Key-words: Synergistic activity, antibiotics susceptibility testing, *Vibrio* species, combination therapy, rate of kill.

CHAPTER ONE

BACKGROUND OF STUDY

1.0. Introduction

Water is an essential natural resource of the earth that supports the lives of humans and animals. Seventy percent of the water used by humans is for agricultural, domestic, recreational, and industrial purposes (UNEP, 2010). Despite the necessity of water, freshwater sources are becoming increasingly contaminated by agricultural run-off, wastewater or industrial waste, and other anthropogenic pollutions regularly (UNEP, 2010). In many developing countries like South Africa, the release of agricultural run-off and wastewater (treated and untreated) from communities and industries into lakes and rivers has led to an increased level of polluted water, making it a hazard to public health as it has led to outbreaks of water-related diseases (UNCTAD, 2010). Over the years, about eighty percent of the South African population has been reported to be dependent on surface water for their day-to-day needs, but recently, this dependence has increased owing to a rise in population and economic growth. This has given rise to high numbers of cases of waterborne disease (Raja *et al.*, 2008). In 2014, the Green Drop report indicated a high level of inefficiency in 75% of sewage treatment plants across South Africa. This report stated that 403 of the 852 wastewater treatment plants in South Africa were not competent enough to be accessed, while 203 had a green drop assessment score to be greater than 50% (Green Drop Report, 2014). The deteriorating level of wastewater treatment plants is a major contributing factor to health problems and water pollution in the South Africa. The World Health Organization has reported waterborne diseases to be responsible for about 1.5 million deaths yearly, and 52% of those deaths are by limited access to clean drinking water and inadequate hygiene (WHO,

2014). The aquatic environment serves as a natural habitat to some pathogenic and non-pathogenic microorganisms such as *Vibrio* species, and these organisms are transmitted to humans and animals through various routes such as ingestion of contaminated water, seafood, and vegetables (Gugliandolo *et al.*, 2005).

Vibrio is a Gram-negative, comma-shaped bacteria, that causes foodborne and waterborne infections. Many *Vibrio* species are amongst those enteric pathogens that cause human infections; hence, they are pathogenic (Madigan and Martinko, 2005). *Vibrio* species can also cause cholera disease, septicaemia, wound infections, and acute gastroenteritis in humans and animals when contaminated water or raw seafood is consumed (Austin and Zhang, 2006). *Vibrio* species that are pathogenic are known to possess some virulence factors that aids them to cause infections, these factors include; cholera toxin (CT) commonly found in *Vibrio cholerae*, thermostable direct hemolysin (TDH) found in *V. parahaemolyticus*, virulence-correlated gene (VCG) found in *V. vulnificus*, heme utilization protein gene amongst others found in *V. fluvialis* (Fri *et al.*, 2017). *Vibrio* species can also be found in environmental water, vegetables, and seafood where they occur as free-living organisms or are found in association with biofilms (Thompson *et al.*, 2005). *Vibrio* infections are self-limited and do not require the use of antimicrobials, except in complicated cases. Antimicrobials are the most successful agents for treating illnesses or diseases which serve as a threat to the human population (Das and Patra, 2017). Antimicrobials such as fluoroquinolones, tetracycline, cephalosporin, trimethoprim-sulfamethoxazole, and imipenem are commonly used to prevent complicated cases of *Vibrio* infections (CDC, 2013). Over the years, cholera causing *Vibrio* species has been the area of focus for many researchers because of their ability to cause severe disease in humans, but recently, researchers are paying attention to other non-cholera causing *Vibrio* species because of their ability also to cause severe infection in both animals and humans (Tantillo *et al.*, 2004).

Since the discovery and use of antibiotics against microbial infections, many microorganisms have developed resistance to some of the available antibiotics of medical importance (Džidić *et al.,* 2008). *Vibrio* species are commonly sensitive to antibiotics; nevertheless, species such as *V. vulnificus* and *V. parahaemolyticus* are reported to exhibit resistance to many antibiotic agents (Elmahdi *et al.,* 2016). Antibiotics misuse has caused bacteria to adapt and develop resistance to many antibiotics in the world today. This microbial adaptation is considered a natural process that may occur in organisms that are resistant to some antibiotics or are acquired through genetic mutation or via transfer of resistant genes from one bacterial cell to another, vertically or horizontally (Munita and Arias, 2016). Antimicrobial resistance amongst microorganisms has been an important health challenge in medical and environmental settings as it has made infections difficult to treat, leading to a high rate of mortality. Hence, posing a threat that could bring an end to the era of antibiotics (Frieri *et al.,* 2017). Antibiotic-resistant bacteria can spread from humans and animals into the environment via manure application on farms, discharge of improperly treated wastewater effluent, faeces, eating and drinking of food and water that are already contaminated by this organism (Alam *et al.,* 2013).

There are few treatment options against infections that are triggered by antibiotic-resistant bacteria because these organisms are usually resistant to almost all antibiotic groups (Carmeli *et al.,* 2010). In other to proffer a solution to the rising issue of resistance, researchers have come up with the use of combinational therapy (the use of two or more antibiotics combined for treatment) as a promising approach for treatment (Cotteral and Wiezdowski, 2007). Recent articles have reported that compared to monotherapy, combination therapy is associated with an increased human-survival rates (Bass *et al.,* 2015). The antibiotic resistance effect on the ability to effectively manage the common infectious disease and the devastating associated impact can only be limited once the true prevalence and extent of resistance are known.

1.1. Justification for the study

Disease outbreaks caused by microorganisms found in water is on the increase globally and these pathogens' presence in the environment has contributed to an increase in water-related infections in humans and animals (Igbinosa and Okoh, 2008). Foodborne and water-related diseases have been reported to be responsible for about one-third of the intestinal infections globally, and water pollution is accountable for 40% of all global deaths out of which 5.7% are pathogen-related (Hunter 1997; Pruss *et al.*, 2002). The incidence of diarrhea amongst children and adults is mainly caused by pathogens like *Vibrio* species which can lead to sickness and death when not treated (Pegram *et al.,* 1998). South Africa is a country with an increased number of immuno-compromised populations especially in poor provinces such as Limpopo, Northern Cape, Free State, and Eastern Cape and the effect of this low water quality on humans and animals has caused waterborne disease outbreaks in the country (Grabow, 1996). Between 1980 and 1989 in South Africa, an estimated 25,251 cases of *Vibrio* infections were reported and communities with a high rate of poverty, poor sanitation, and low domestic supply were severely affected (DWAF, 2002). In the year 2000, there was an epidemic of *Vibrio* infections in South Africa where 106,389 people were infected, and this resulted in about 232 deaths (Okeyo *et al.,* 2018). In 2007, 80 deaths were reported in children and this was said to be caused by diarrhea-related infections. In 2014, the SABC news reported a diarrhoea outbreak in Fort Beaufort, which resulted in many deaths and hospitalizations, and this outbreak was said to be a result of the supply of poor water quality (Okoh, 2018).

Antimicrobial resistance is no longer a laboratory concern but a global threat as it has led to series of life-threatening infections and increased death rate. In Europe, 25,000 people die yearly from multi-drug resistant bacterial infections (Zaman *et al.*, 2017). In Africa, information on the extent of antimicrobial resistance is limited because monitoring is only

done in a few African countries. However, a public health report discovered some level of weakness in the antibiogram testing across many African countries (Frean, 2012). Despite limited capacity to monitor the rate of antimicrobial resistance in Africa, available data indicates that Africa is also experiencing this trend of drug resistance in bacteria from both clinical and environmental settings (Frean, 2012). Scientists have resolved to use antibiotic combination therapy for treatment. Old antimicrobial agents like colistin, Fosfomycin, and polymyxins, which were not often used because of their efficiency and level of toxicity is now used in combination with other antimicrobial agents including carbapenem (Bass *et al.*, 2015). These combinational therapy options are being re-evaluated and have become treatment options of last-resort (Morrill *et al.*, 2015). Hence, the need for alternative antimicrobial combination therapy options for the control of resistant bacteria cannot be overemphasized as it attempts to provide baseline data for clinical management and epidemiological surveys.

1.2. Hypothesis

This study is based on the null hypothesis that antimicrobial combination therapy is not an option for the control of resistant non-cholera causing *Vibrio* species.

1.3. Aim

This study is aimed at determining the antibiotics susceptibility pattern and antimicrobial combination therapy options for the control of antimicrobial-resistant non-cholera causing *Vibrio* species recovered from environmental niches in the Eastern Cape, South Africa.

1.4. Objectives of the study

1. To determine the phenotypic antibiotic susceptibility pattern of isolates belonging to the three pathogenic non-cholera causing *Vibrio* species.
2. To determine MIC and MBC of selected antibiotics against resistant variants.
3. To evaluate effect of antimicrobial combination on the susceptibilities and rate of kill of the antimicrobial-resistant isolates.

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CHAPTER TWO

LITERATURE REVIEW

2.0. Introduction

Vibrio are facultative, comma-shaped, Gram-negative bacteria genus that belongs to the family *Vibronaceae*. They are halophilic anaerobic bacteria (Thompson *et al.,* 2005). They are oxidase-positive bacteria that possess polar flagella which are used for motility (Drake *et al.,* 2007). *Vibrio* are inhabitants of aquatic environments that are not necessarily linked to faecal contaminants as they can be released into the water through water discharge. However, factors such as; temperature, salinity, nutrient availability amongst other factors are associated with their prevalence in water (Summer *et al.,* 2001). *Vibrio* tend to grow well in alkaline environments (some *Vibrio* species grow better within pH 6.5 and 9.0) and high temperatures (20 °C to >40 °C) (Percival *et al.*, 2014). When the temperature of the aquatic environments is low, isolation of *Vibrio* species of clinical interest becomes less successful (Heath and Nelson, 2002). Salinity is also a factor that affects the distribution of these organisms in an aquatic environment. *Vibrio* species exist in an environment with salinity between 5 ppm and 30 ppm, hence, they are halophilic.

Over 100 species of *Vibrio* are known to exist, but its taxonomy is continually and frequently updated as new species are being discovered (Igbinosa *et al.,* 2009). These species are pathogenic and are capable of causing waterborne and foodborne infections when contaminated food and water are consumed (Madigan and Martinko, 2005). *Vibrio* species can cause gastroenteritis, cholera, wound infections, and septicemia in humans. They are also zoonotic, causing infections and even death in animals (Austin and Zhang, 2006). *Vibrio* species are grouped into two, cholera causing *Vibrio* species and non-cholera causing *Vibrio*.

2.1. Cholera causing *Vibrio* species

2.1.1. *Vibrio cholerae*

Vibrio cholerae are halophilic, curved-shaped, facultatively anaerobic, Gram-negative bacteria that possess both flagellum and pili. They are commonly found in high numbers in aquatic environments (Weil and Harris, 2015). It is one of the most pathogenic species with *Vibrio cholerae* O1 and O139 serogroup as the common cause of cholera worldwide, while *Vibrio cholerae* Non-O1 and Non- O139 is not cholera causing, but can cause isolated cases of mild gastroenteritis (Waldor and Ryan, 2015). *Vibrio cholerae* is highly sensitive to high temperatures (greater than 45 ˚C) and disinfectants (ICMSF, 1996). The only known natural host for cholera-causing *Vibrio* species are humans. In 1849, Sir John Snow first linked contaminated water to cholera, and he used that information as a strategy for infection control (Snow, 1855). The epidemic of *Vibrio cholerae* emerged from Celebes, now Sulawesi in 1961, and spread severely to Asia, Africa Europe and North America, where it was relatively restricted and self-limited due to increased level of sanitation (Finkelstein, 1996). This organism also caused an outbreak in Peru in 1991 where over a million cases were confirmed (Finkelstein, 1996). *Vibrio cholera* is considered the most important *Vibrio* species because of its association with diarrhea outbreaks in many regions globally (Cavallo *et al.,* 1998).

2.1.2. *Vibrio cholerae* O1 and O139

Vibrio cholerae O1 is a serogroup of *Vibrio cholerae* that is known to cause cholera epidemic in Africa and other countries of the world except for Asia. *Vibrio cholerae* O139, another serogroup of *Vibrio cholerae,* is only known to cause cholera outbreaks in Asian countries and reports of its occurrence in other countries have not been reported (du Preez *et al.*, 2010). Due to limited reports on the isolation of *Vibrio cholerae* O139, it has been generally accepted that in aquatic environments, it behaves similarly as *Vibrio cholerae* O1 (du Preez *et al.*, 2010).

2.2. Pathogenesis of cholera causing *Vibrio* species

Cholera disease is usually transmitted through the fecal-oral route. When a surface attachment has been initiated, *Vibrio cholerae* forms biofilms that can withstand the acidity of the human stomach to enable colonization (Zhu and Mekalanos, 2003). However, studies have shown that *Vibrio cholerae* biofilm is required during the bacteria's movement through the stomach acidic environment (i.e biofilm is used as a protective shield) and not during colonization (Zhu and Mekalanos, 2013). *Vibrio cholerae* is known to possess factors that contribute to its ability to cause diseases, some of these factors include; toxin co-regulated pilus (TCP), cholera toxin (CT); and flagella which are used for motility. Without one or more of these virulence factors, pathogenesis will not take place (Spagnuolo *et al.*, 2011).

Pathogenesis of *Vibrio cholerae* begins after it successfully established itself in the bowel of the host, thus colonizing the epithelial layer of the small intestine (Sharmila and Thomas, 2018). *Vibrio cholerae* then produces a mucolytic enzyme that crumbles the mucous layer of the intestine. The bacteria uses its polar flagella to move across the thick mucosal layer and successfully colonize the small intestine (Sharmila and Thomas, 2018). It then secrets an endotoxin called Cholera toxins which is its major factor for virulence. This toxin is the source of severe watery diarrhea also known as "rice-water stool" (Finkelstein, 1996). Cholera Toxin binds to the ganglioside (GM1), a plasma membrane component that regulates the process of cell signal transduction. The receptor-toxin complex becomes endocytosed and channeled to the endoplasmic reticulum. Cholera Toxin binds to only one GM1 subunit in other to enter into the host cell (Holmgren *et al.*, 1973). When in the endoplasmic reticulum, the disulphide bond in the "A" subunit reduces, this reduction leads to the detachment of the "A" subunit from the complex and move into the cytoplasm where it binds an ADP ribosylation factor. This binding activates the adenylate cyclase, which produces cyclic adenosine monophosphate (cAMP) from ATP. An increase in cAMP alters the trans-mural ion flux, thus triggering the intestine to secret Cl^- and inhibits the absorption of NaCl. This

action causes the intestine to reach an abnormal level which leads to watery diarrhea and causing loss of electrolytes and dehydration in infected humans (Speelman *et al.,* 1986).

Cholera disease is often mild and asymptomatic but sometimes severe and life-threatening (CDC, 2013). The incubation period for *Vibrio cholerae* is within 6 to 48 hours but it may be up to 5 days (Finkelstein, 1996). Cholera in its early stages exhibits the following symptoms: profuse watery diarrhea, vomiting, loss of skin elasticity, low blood pressure, increased thirst level, muscle cramp, and restlessness or irritability (CDC, 2013). In severe cholera cases, acute renal failure, electrolyte imbalance and even coma can be observed and when left untreated, it can result in the death of the patient (CDC, 2013).

2.3. Non-cholera causing *Vibrio* species

2.3.1. *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is a species of the genus *Vibrio* that are Gram-negative, comma-shaped bacteria and are non-spore-forming. They possess a polar flagellum which it uses for movement (Shinoda, 2011). It can cause wound infections, septicaemia, and acute gastroenteritis in humans (Morris and Black, 1985). Incidences of *Vibrio parahaemolyticus* infections across countries in all continents have raised a public health issue of seafood safety (Vugia *et al.,* 2004). *Vibrio parahaemolyticus* is halophilic requiring 3 %-10 % NaCl for growth and is known to be isolated only in summer when the water temperature is 19-20 ˚C, hence, it is mesophilic in nature (ICMSF, 1996). This organism was first reported during an outbreak of gastroenteritis due to people consuming undercooked or raw seafood, leading to 272 cases of infections of which 20 deaths were recorded in 1951 in Osaka, Japan (Gugliandolo *et al.,* 2005). A study conducted in Thailand and Malaysia reported the prevalence of antimicrobial-resistant *Vibrio parahaemolyticus* from the white leg and black leg shrimps (Al-Othrubi *et al.,* 2011; Yano *et al.,* 2014). In 1997, outbreaks of gastroenteritis caused by *Vibrio parahaemolyticus* were reported in France and Spain, leaving 44 patients

and 64 patients infected respectively. These outbreaks were reported to occur due to people consuming undercooked seafood. In 2004, an outbreak of gastroenteritis occurred, involving 80 cases among wedding guests in a restaurant due to the consumption of cooked crab prepared under unsanitary environments in Spain (Letchumanan *et al.*, 2014).

In 1971 in the US, infections caused by *Vibrio parahaemolyticus* were first reported after three different outbreaks of gastroenteritis resulted in 425 cases (Molenda *et al.*, 1972). CDC recorded about forty outbreaks of gastroenteritis between 1973 and 1998 in the United States and 4 of these 40 outbreaks resulted in about 700 cases. These outbreaks were confirmed to be caused by *V. parahaemolyticus*. (CDC, 1998). In 2004 in Alaska, 14 passengers that were on a cruise ship were reported to manifest symptoms of gastroenteritis after consuming raw seafood in Alaska (McLaughlin *et al.*, 2005). In 2006, an outbreak of gastroenteritis in Washington and British Columbia involving 177 cases was confirmed to occur as a result of people eating oysters infected with this organism (CDC, 2006).

2.3.2. *Vibrio vulnificus*

Vibrio vulnificus is a comma-shaped halophilic, motile bacterium of the genus *Vibrio*, commonly found in aquatic environments (Oliver, 2005). *Vibrio vulnificus* is an emerging pathogen as it causes acute gastroenteritis, wound infections, septicemia and foodborne diseases in humans (Baker-Austin *et al.*, 2010). This *Vibrio* specie has an average mortality rate of 50% after one to two days of symptoms emergence (Jones and Oliver, 2009). *Vibrio vulnificus* has been reported to cause 95% of foodborne deaths, resulting from the consumption of contaminated seafood (Oliver, 2005). People who are immune-compromised with severe liver disease are at higher risk of getting infected when exposed to contaminated water and food (Hsueh *et al.*, 2004). *Vibrio vulnificus* was first reported in1976 in the United States by CDC and has since been the major cause of deaths due to seafood-related infections. CDC has reported that there are estimates of 95 cases and 35 deaths occur

annually due to infections caused by *Vibrio vulnificus* across the globe (CDC, 2013). Between 1988 and 1996, 422 cases of infections caused by *Vibrio vulnificus* were reported to the CDC by 23 states. About 45% of those cases were wound infections, 43% were septicemia. This led to a 38.4% mortality rate (Amaro and Biosca, 1996). In Israel, between May 1996 and December 1997, 62 cases of *V. vulnificus* infections were reported, where only 33 of those cases were confirmed to be caused by this organism and the remaining 29 lacked laboratory confirmation so they were reported to be suspected cases (Bisharat *et al*., 1999). Between 2000 and 2010 in Korea, The NNDSS confirmed about 588 cases of *Vibrio vulnificus* infections, 285 of the confirmed cases were led to a high mortality rate of 48.5% (Lee *et al*., 2014).

2.3.3. *Vibrio fluvialis*

Vibrio fluvialis is a comma-shaped, halophilic bacteria that is found in aquatic environment. *Vibrio fluvialis* possesses a single polar flagellum which aids their motility (Kothary *et al.,* 2003). It is capable of causing gastroenteritis in humans. They can also cause wound infections although in rare cases in humans when wounds are exposed to water contaminated by this species. *Vibrio fluvialis* was first reported in Bahrain in 1977 from humans with severe diarrhea. Isolation of *Vibrio fluvialis* has been reported not to occur in humans with diarrhea cases only, but also from aquatic environments (Igbinosa *et al.,* 2009). Between 1982 and 1988 in Florida, there were 10 reported cases of gastroenteritis due to *Vibrio fluvialis* from people who consumed seafood that is contaminated by this organism (Klontz and Desenclos, 1990). In 1988 in India, a report showed less rate (0.6%) of occurrence of *Vibrio fluvialis* among children that have diarrhea, while in 1989, there was an increase of >2 % in the occurrence of *Vibrio fluvialis* among hospitalized patients that have acute diarrhea (Chowdhury *et al.,* 2012). Between 1996 and 1998 in North Jakarta, the prevalence of *Vibrio fluvialis* in hospitalized diarrhea patients was 9.4 %, while in China, it was 12%, making it the second most occurring pathogen among acute diarrhea cases (Ramamurthy *et al.,* 2014).

In Russia, acute enteric infections by *Vibrio fluvialis* have been reported to reach up to 30% in summer due to frequent consumption of water other than seafood (Ramamurthy *et al.,* 2014).

2.3.4. *Vibrio alginolyticus*

Vibrio alginolyticus is a curved-shaped, Gram-negative bacterium that can be found in an aquatic environment and is capable of causing food spoilage, otitis, gastroenteritis (in rare cases) and wound infections in both humans and animals. *Vibrio alginolyticus* is a highly halophilic organism that requires salt concentration as high as 10 % for their growth (Schmidt and Cobbs, 1979). This species of *Vibrio* is the most isolated specie in the world as its prevalence in water is influenced by a rise in water temperature. It was first documented to be a human pathogen in 1973 (Zen-Yoji *et al.*, 1973). Diseases by *Vibrio alginolyticus* were reported in 1997 in Russia, during an outbreak of acute enteric illnesses (Smolikova *et al.,* 2001). In 2004, 96 cases of *Vibrio alginolyticus* infections in China were reported after the consumption of brine shrimps (Xie *et al.,* 2004).

2.4. Pathogenesis of non-cholera causing *Vibrio* species

Infections caused by non-cholera causing *Vibrio* species, occur through many routes such as the fecal-oral route and open wounds when exposed to saltwater. *Vibrio parahaemolyticus* strains have different factors which help them in their pathogenesis, some of which include: adhesins, thermostable direct hemolysin (*tdh*), TDH related hemolysin (*trh*) and two type III secretion systems (T3SS1 and T3SS2) (Letchumanan *et al.,* 2014). Type III secretion systems enable protein translocation from bacteria cells into the host cells where it disrupts the normal host cell functions (Zhou *et al*., 2008). T3SS1 encoded in *Vibrio parahaemolyticus* is known to ensure the survival of this organism in an environment and causing host cell lysis that is characterized by swelling of the host cell and formation of the vacuole in the cytosol, while T3SS2 contributes directly to bacteria invasion of the host cells and thus causing fluid

accumulation in the intestines of humans (Zhou *et al.*, 2008). Multivalent Adhesion Molecules (MAMs) are used by *Vibrio parahaemolyticus* during an infection to attach themselves to the epithelial cells of the host and breaking the barriers between the host cells, therefore, allowing the free passage of this organism (Lim *et al.*, 2014). MAMs also help to initiate the transfer of virulence determinants from the bacterial to the host (Stones and Krachler, 2015). TDH and TRH are major factors used by *V. parahaemolyticus* for virulence. These virulence factors bind and form pores on the host cell membrane, leading to the flow of water and ions within the membrane (Raghunath, 2015). This causes cell toxicity by inducing an increase in the concentration of the extracellular Ca^{2+} and secretion of Cl^-. Increased osmotic pressure leads to morphological and pathological changes which result in cell expansion and cell death (Letchumanan *et al.*, 2014).

The ability of *Vibrio vulnificus* to cause infection is mainly dependent on the host susceptibility (Gulig *et al.*, 2005). This organism is known to possess multiple factors that aid its pathogenesis (Strom and Paranjype, 2000). In other to fully understand the pathogenesis of *V. vulnificus*, their interaction with various host defense mechanisms has been studied by researchers. When *Vibrio vulnificus* comes in contact with the acidic environment in the digestive tract of their host, they are capable of neutralizing the acidic environment by converting amino acids to amines and carbon dioxide (CO_2) (Jones and Oliver, 2009). When *Vibrio vulnificus* successfully bypass the digestive tract of the host into the bloodstream and comes in contact with the primary innate immune factor, they block the complement cascade and eliminate the bactericidal effect of the serum (Jones and Oliver, 2009). The relationship between the acquisition of excess iron and *Vibrio vulnificus* remains unclear, but researchers have been able to come up with two theories. Some researchers have deduced that an increase in iron level increases the growth rate of *Vibrio vulnificus* (Starks *et al.*, 2006), while other researchers stated that, excess iron concentration reduces neutrophil activity, therefore resulting in a weakened immune response (Hor *et al.*, 2000). This non-cholera causing *Vibrio*

species are known to exhibit symptoms such as watery diarrhea, vomiting, fever, dehydration, headache, and abdominal cramps (Ryan and Ray, 2004). Skin lesions of severe cellulitis with ecchymosis and bullae are developed within 24 hours of symptom manifestation in patients with wound infections (Bross *et al.*, 2007).

Vibrio fluvialis can produce haemolysin, protease, and cytotoxins which serve as factors contributing to their pathogenicity. However, despite possessing these virulence factors, there is a paucity of information on the pathogenicity of this organism (Liang *et al.*, 2013).

2.5. Treatment and prevention of *Vibrio* infections

Infections due to *Vibrio* species are self-limited, hence, treatment is necessarily not required. In adults, antibiotics such as third-generation cephalosporin, fluoroquinolone, tetracycline, and imipenem can be used not necessarily as part of treatment but to reduce the duration of diarrhea (Malcolm *et al.*, 2018). While children in whom fluoroquinolones and tetracycline are contraindicated, trimethoprim-sulfamethoxazole with an aminoglycoside can be administered (CDC, 2013). Zinc supplements are also used to reduce diarrhea duration in children with cholera disease. Oral rehydration salts in water are used to reduce dehydration by replacing lost fluids and electrolytes in infected patients. Intravenous fluids can be used in severely dehydrated people (CDC, 2013). In cases of wound infection due to *Vibrio* species, surgical debridement and sometimes, amputation can be done to reduce mortality rate (Liu *et al.*, 2006). *Vibrio* infections can be avoided by ensuring raw seafood consumption is prohibited, gloves should be worn when handling raw seafood but in the absence of gloves, washing of hands after handling raw seafood should be done. Open wounds should not be exposed to saltwater or brackish water, but if exposure occurs, wounds should be thoroughly washed (CDC, 2019a).

2.6. Antimicrobial resistance in *Vibrio* species

The discovery of penicillin in the 1940s represent the start of antibiotics era, and this was regarded as one of the biggest breakthroughs in therapeutic medicine (WHO, 2014). Following Alexander Fleming's discovery in 1928, several antimicrobial agents were produced and classified into groups which include: quinolones and oxazolidinones among others (Walsh, 2003). Treatment of bacterial infection with antimicrobials in the medical discipline has become a great challenge as many microorganisms have developed resistance to a wide range of antimicrobials available today (WHO, 2018).

Antimicrobial resistance occurs when a microbe is able to withstand the activity of an antimicrobial agent that was effective against it (Hayek *et al.*, 2015). This resistance may occur in organisms that are naturally resistant, or by transfer of genes coding for resistance from one cell to another horizontally or vertically (Hayek *et al.*, 2015). Horizontal and vertical gene transfer involves the transfer of resistance gene between bacteria of the same species or of different species through transformation, transduction, or conjugation as shown in Figure 2.1 below (Hayek *et al.*, 2015).

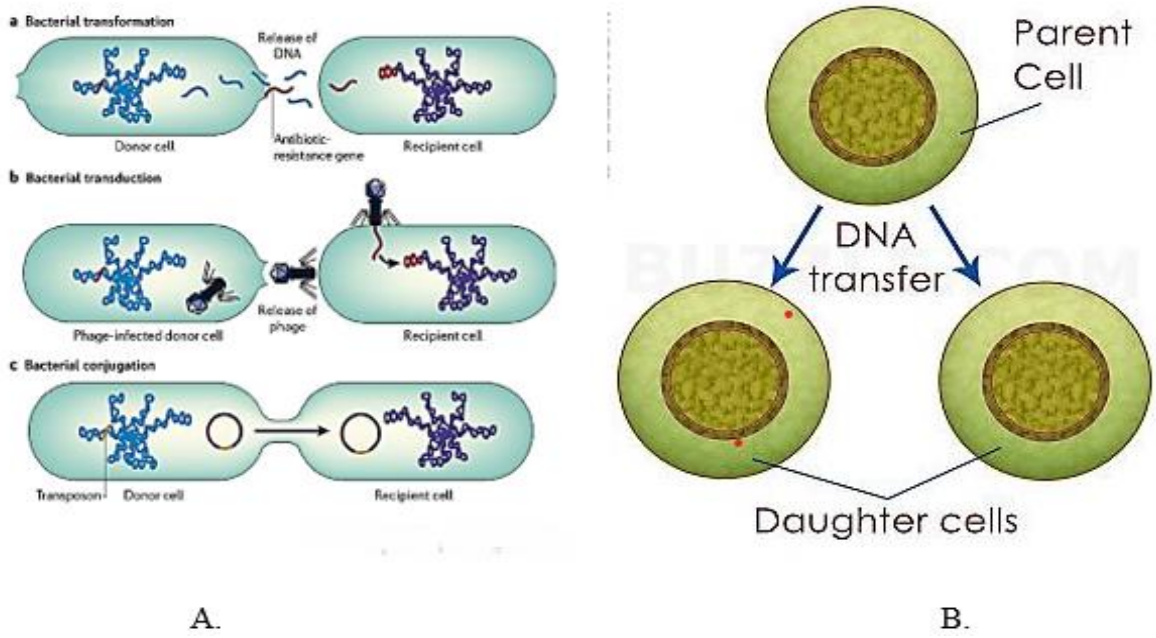

Figure 2.1: (A) The horizontal method of gene transfer and (B) vertical method of gene transfer (Source: Furuya and Lowy, 2006).

Antimicrobial resistance is not a new issue in public health, but recently, the prevalence of resistant organisms and the affected geographical regions are drastically increasing (Levy and Marshall, 2004). In 1961, Methicillin was introduced as the first semi-synthetic penicillinase-resistant penicillin to fight against penicillinase-producing strains of *Staphylococcus aureus*, but soon after the initiation of this drug, resistance by *Staphylococcus aureus* was reported, giving rise to Methicillin-Resistant *Staphylococcus aureus* (Zaman *et al.*, 2017). In the 1980s, fluoroquinolones were introduced as an effective treatment plan against Gram-negative bacteria, but their effectivity against Gram-positive bacterial infection was later discovered. Quinolone resistance occurred as a result of chromosomal mutation, especially among the methicillin-resistant bacteria strains.

Antibiotics are highly effective against *Vibrio* species as susceptibility of this organism have been reported by several studies. However, there have been several reports on the resistance of *Vibrio* species to some of these antibiotics (Elmahdi *et al.,* 2016). Antibiotic resistance has become more widespread among a numerous of infectious agents, serving as a threat to the public at large in many countries (WHO, 2014). In The United States, about 23,000 deaths out of 2 million infected people occurred as a result of infections caused by antibiotic-resistant bacteria strains (CDC, 2014). Reports from a study conducted between 2005 and 2006 on *Vibrio* species recovered from retail oysters in Louisiana Gulf showed that *Vibrio* species were sensitive to almost all antibiotics evaluated (Han *et al.,* 2007). Another study conducted in South Carolina and Georgia estuaries on environmental and clinical isolates subjected to 26 antibiotics showed that 45% of the isolates from the environmental were resistant to 3 antibiotics, while 17.3 % of the clinical isolates showed resistance to eight of the test antibiotics. (Baker-Austin *et al.,* 2009). Ottaviani *et al.* (2013) examined the susceptibility pattern of 127 *Vibrio* isolates from both clinical isolates and shellfish in Italy. The study reported about 62% of resistance to four antibiotics. All the isolates were resistant to ampicillin and amoxicillin and susceptible to doxycycline and chloramphenicol.

A study conducted in Indonesia on the antibiotic-resistant profile of *Vibrio* species reported 98% resistance to ampicillin 100% susceptibility to chloramphenicol, gentamicin, tetracycline and trimethoprim-sulfamethoxazole (Lesmana *et al.,* 2001). Okoh and Igbinosa (2010) carried out a similar study in South Africa. The study reported a high rate of resistance to penicillin and sulfamethoxazole and sensitivity to imipenem, meropenem, and norfloxacin. Numerous findings have reported the resistance of *Vibrio* species to azithromycin, furazolidone, and other antibiotics that are recommended for treating *Vibrio* infections (Igbinosa *et al.,* 2011; Hossain *et al.,* 2012; Al-Othrubi *et al.,* 2014; Silvester *et al.,* 2015; Igbinosa, 2016; Mechri *et al.,* 2017, Tan *et al.,* 2017). Despite the increase in resistance level of *Vibrio* species to antibiotics often used for effective treatment, high susceptibility pattern

to these drugs have also been reported (Oh *et al.*, 2011; Shaw *et al.*, 2014; Kang *et al.*, 2017). Consequently, this implies that the choice of antibiotics in a geographical location for the treatment of *Vibrio*-related infections should depend on the susceptibility pattern of the antibiotics in that environment.

2.6.1. Mechanism of Antimicrobial Resistance

There are several classes of antibiotics with each exhibiting their antimicrobial actions differently. Also, there are different mechanisms by which bacteria can resist the activity of antibiotics (Lin, 2015). Some antimicrobials need to enter into the bacterial cell in other to get to their target sites, but organisms such as Gram-negative bacteria, prevent this from happening by changing their cell membrane porin channel frequency, size, and selectivity (Blair *et al.*, 2015). Bacteria such as *Enterobacteriaceae* are also capable of resisting antimicrobials by destroying the active components of the antimicrobials e.g hydrolytic deactivation of beta-lactam ring by the production of beta-lactamase enzyme, preventing the antimicrobial from binding to the PBPs, and protecting cell wall synthesis (Blair *et al.*, 2015).

Some bacteria can resist antimicrobials by camouflaging target sites in other to avoid recognition, thus, preventing inhibition from taking place. This can be found in *Staphylococci*, *Mycobacterium* species, *Enterococci* e.t.c (Blair *et al.*, 2015). Some bacteria possess efflux pumps used to eject out antibiotics from the cell wall immediately it is detected these mechanisms are described in Figure 2.2 below. This results in an insufficient concentration of antibiotics in the bacteria making it less effective as some antimicrobials require sufficiently high concentrations for their effectivity. This can be seen in *Vibrio* species, *Staphylococcus aureus* and *Streptococcus pneumonia* (Kumarasamy *et al.*, 2010). *Vibrio* species possess multi-drug efflux pumps for exporting antibiotics outside their cells. These multi-drug efflux pumps in *Vibrio* species are also used for the expression of virulence genes (Paulsen *et al.*, 1996).

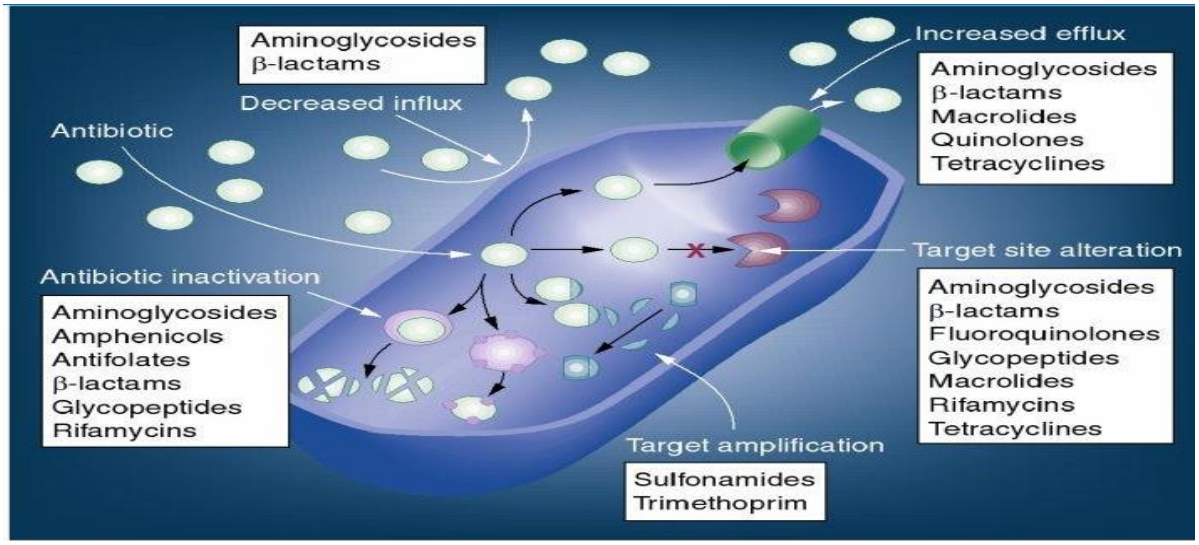

Figure 2.2: Mechanism of antibiotic resistance against some antibiotics (Source: Schmieder and Edwards, 2012).

2.6.2. Causes and Spread of Antibiotic Resistance

Many factors have been known to induce resistance of bacteria to almost all classes of antibiotics, amongst which include drug misuse, mutation, drug prescription, and so on (Michael *et al.*, 2014). During microbial replication, microbes are capable of evolving rapidly and mutation may occur. This mutation may cause bacteria to become resistant to antibiotics (Aminov, 2010). Antibiotic-resistant genes can also be acquired during gene transfer from one cell to another (Hayek *et al.*, 2015). Inappropriate prescription of broad-spectrum antibiotics by doctors when a specific antibiotic can be prescribed can increase the risk of antibiotic resistance. Also, incomplete use of drugs or drug abuse can increase the chances of resistance (Aminov, 2010). The use of antimicrobials as supplements in animal feed can promote drug resistance. In the United States, over half of the antibiotics produced are utilized for agricultural purposes and researchers have discovered resistant bacteria in animal meats and farm produce that have been exposed to fertilizers and polluted water (Mellon *et al.*, 2001). However, the debate is still ongoing as to whether drug-resistant microbes in animals pose a significant burden to public health. Antimicrobials frequently used in hospitals where patients are critically ill and in close interaction with medical staff and others, thus creating an environment that makes it easy for microbes to spread from one human to another (Aminov, 2010).

Propagation of resistant bacteria from environmental and clinical environments can be achieved in several ways, such as hospital wastewater, health care staff, excretion of urine, and feces excretion. As mentioned in Figure 2.3 below, resistant bacteria transmit to humans and other animals through poorly prepared food, proximity, and poor hygiene. It can also spread by water polluted by feces or by animals to the environment and food. Antibiotics can also be given to patients in hospitals and as a result of drug misuse, will develop resistance and begin to spread to people in the environment (Alam *et al.*, 2013).

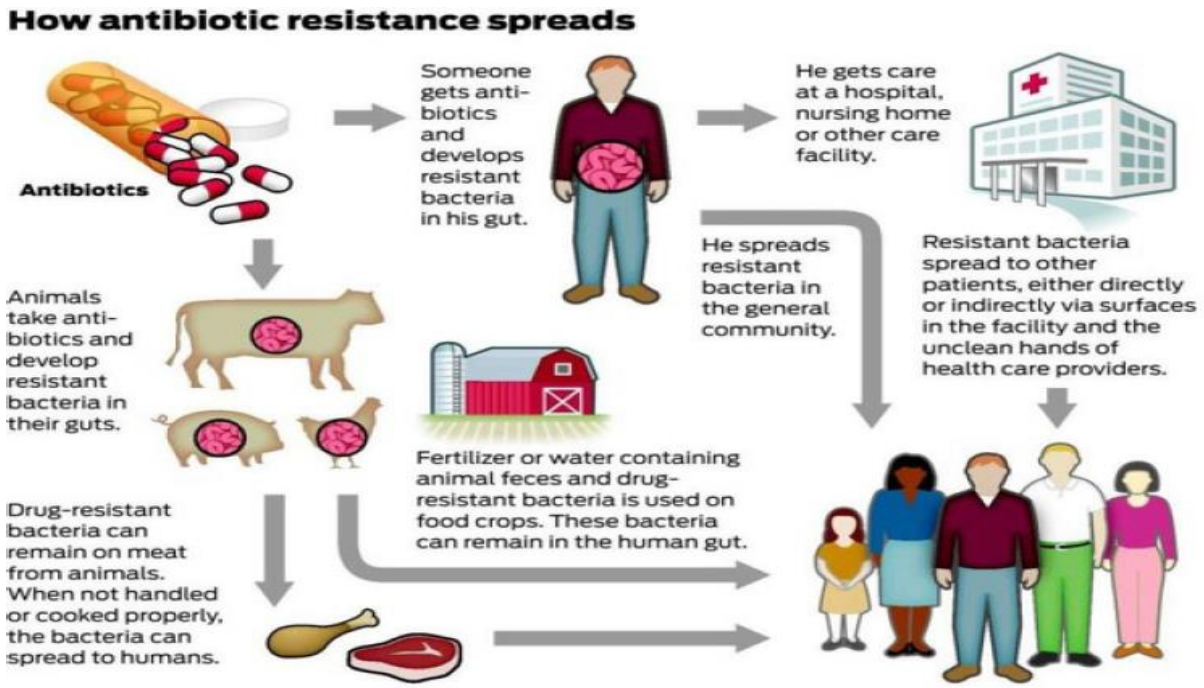

Figure 2.3: Spread of Antibiotic resistance through various channels (Source: CDC, 2019b).

2.6.3. Treatment options for antibiotic resistance bacteria

With high mortality rate related to infections caused by these bacteria, the problem of antibiotic resistance among Gram-negative bacteria is on the rise globally, posing a health risk to the public (Righi *et al.*, 2017). Only few medications are available against bacteria because they have been documented to be resistant to almost all antibiotics, including beta-lactams (Bass *et al.*, 2015). Researchers have therefore proposed the use of combination therapy as a possible treatment option to enhance the activity of these antimicrobials.

2.7. Antibiotics combination therapy

Antibiotics combination therapy is a treatment option that involves combining two or more antibiotics to increase the efficiency of both drugs against bacteria that were resistant to an antibiotic used as monotherapy (Cotteral and Wiezdowski, 2007). It is commonly used in patients that are critically ill as a result of multi-drug resistant organisms (Ahmed *et al.*,

2014). Successful use of antibiotics combination therapy has been reported against multi-drug resistant Gram-negative bacteria such as *Enterobacteriaceae*, *Klebsiella pneumoniae*, *Acinetobacter baumanii* amongst others that have a high mortality rate from 30% - 70% (Tamma *et al.*, 2012). Combination therapy is currently used as a treatment option in other to (i) broaden the antibacterial spectrum of the empirical therapy, (ii) in cases of poly-microbial infections require the use of more than one antibiotic for treatment, and (iii) to curb the issue of drug resistance as chances of resistance against two or more drugs are lowered compared to when drugs are used alone (Ahmed *et al.*, 2014). Despite so many reasons for using antimicrobial combination therapy as an option for treatment, there have been arguments for and against its use. One report stated that the use of a second antibiotic for the treatment of Gram-negative bacteria that is susceptible to a single agent may lead to an increased rate of resistance, adverse effect and costs (Tamma *et al.*, 2012).

Studies have reported that the use of combination therapy for treatment is a promising approach to health as it has shown a tremendous success rate in the elimination of these organisms. A study involving 200 patients with *Pseudomonas aeruginosa* bacteremia, evaluated the effectiveness of combination therapy against monotherapy. The mortality rate was observed to be 27% in patients treated with combination therapy, while a mortality rate of 47 % occurred in patients receiving monotherapy (Hilf *et al.*, 1989).

Rifampicin in combination with colistin was used on 210 patients with CRE *Acinetobacter baumanii* infection in another study, wherein the combination resulted in an increased microbiological eradication of the infection compared to when each drug was used alone (Durante-Mangoni *et al.*, 2013).

Resistance against tigecycline is rapidly increasing with a mortality rate of 41.1% (Morrill *et al.*, 2015). However, reports have shown its activity against many infections caused by resistant Gram-negative bacteria when the concentration is increased or combined with other

antimicrobial agents (Morrill *et al.*, 2015). Also, a high dose of tigecycline has been reported to cause increased plasma concentration, increased intracellular accumulation, and tissue distribution (Cunha, 2009). Tigecycline in use with other antimicrobial agents has been reported to have a 92% success rate in ICU patients treated for KPC (*Klebsiella pneumonia* carbapenemase*)* infections (Morrill *et al.*, 2015).

Studies have reported 40% mortality rate related with carbapenem monotherapy, hence, there is a need to combine carbapenems with other active agents as it may increase clinical response (Tzouvelekis *et al.*, 2014). Two carbapenem combination therapy might be an efficient treatment option for infections caused by carbapenem-resistant bacteria. However, there have been arguments against the use of dual carbapenems for treatment, where a high-affinity carbapenem is used to attach and dissipate the pathogen's carbapenemase allowing a second carbapenem can have antibacterial activity (Fritzenwanker *et al.*, 2018). Experiments conducted indicated that KPC enzyme have high affinity for ertapenem compare to other carbapenems, hence, when ertapenem is administered in combination with another carbapenem, KPC enzyme deactivates ertapenem, hindering degradation and improving the effectiveness of the other carbapenem (Bulik and Nicolau, 2011). Ertapenem combined with either doripenem or meropenem has been effectively used against pan drug-resistant and colistin-resistant bacteria.

A cohort study conducted on 27 patients with carbapenemase-producing *Klebsiella pneumoniae* (CPKP) infections using dual carbapenem therapy recorded a high success rate of 77.8% (Souli *et al.* 2017). Another study conducted on 36 patients with carbapenem-resistant *Klebsiella pneumoniae* bacteremia reported that, 18 patients received ertapenem and doripenem (dual carbapenem combination therapy), while the other 18 patients received doripenem and colistin combination (control group). The dual carbapenem combination was

reported to have a significantly high cure rate of 72% compared to the control group which had a 39% cure rate (Venugopalan *et al.,* 2017).

Several studies involving the use of dual antibiotics combination therapy and a combination of plant extract and an antibiotic against *Vibrio* species have been carried out and successful use of combination therapy as an option for treatment was reported. One of those studies is the study of Kim *et al.,* (2005), whose study reported successful use of combination therapy when an *in-vitro* combination of ciprofloxacin and cefotaxime was carried out against *Vibrio vulnificus.* Mandal *et al.,* (2009) also reported successful use of combined therapy against *Vibrio* species. Hang *et al.,* (2016) carried out a similar study involving an *in-vitro* and *in-vivo* test against *Vibrio vulnificus,* using tigecycline in combination with other antimicrobial agents. The increased survival rate of the mice understudy was reported compared to when the antibiotic was used as a monotherapy.

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CHAPTER THREE

Molecular confirmation and susceptibility pattern of non-cholera causing *Vibrio* species from environmental niches in the Eastern Cape, South Africa.

(The pre-print of this chapter has been published on research square)

Abstract

The problem of antimicrobial resistance is an important global concern, as it has an increased mortality rate. The susceptibility patterns of these target *Vibrio* species were evaluated against 11 antibiotics recommended for the treatment in this research. Two-hundred and twenty-eight (228) isolates sampled from different environmental niches were used in this research. The isolates were confirmed using molecular methods, while the standard disc diffusion method was used to determine the antibiotic susceptibility pattern of the confirmed isolates. MARPs and MARI were measured using standard methods. While MIC and MBC were evaluated against antibiotics to which the highest resistance were observed.

Of the 228 isolates, 100 were confirmed to be *Vibrio parahaemolyticus*, while 82 and 46 were confirmed to be *V. vulnificus* and *V. fluvialis* respectively. Twenty-two percent of the *V. parahaemolyticus* isolates were resistant against tetracycline, while resistance against other antibiotics is in the following order: nalidixic acid (16 %), ampicillin (14 %), cefotaxime (14 %), chloramphenicol (12 %) and amikacin (11 %). Against *Vibrio vulnificus* isolates, the frequency of resistance followed the order: imipenem (40 %), tetracycline (22 %), ampicillin (18 %), meropenem (15 %) and chloramphenicol (11 %); whereas for *V. fluvialis* isolates, the frequency of resistance is in the following order: nalidixic acid (28 %), tetracycline (28%), ampicillin (20 %), chloramphenicol (15 %), amikacin (11 %) and cefotaxime (11 %). A total of 38 MARP patterns were detected in all evaluated isolates and approximately 23 % were resistant against over 3 antibiotics. MARI was between 0.3 and 0.8. MIC and MBC were carried against isolates that were resistant to the two most common antibiotics tested.

Against isolates of *V. parahaemolyticus* and *V. fluvialis*, the MIC concentration used was from 16 µg/ml to 2048 µg/ml. Nalidixic acid inhibited the most bacterial growth at 128 µg/ml concentration, inhibiting four of the sixteen resistant isolates tested. Tetracycline inhibited the most *Vibrio parahaemolyticus* isolates, with seven out of sixteen resistant isolates inhibited at 32 µg/ml. The MIC concentrations observed against *Vibrio vulnificus* ranged from 8 µg/ml to 256 µg/ml for both imipenem and tetracycline. The most bacteria inhibitions was observed at 16 µg/ml imipenem concentration, where six of the resistant sixteen isolates were inhibited. While for tetracycline, 64 µg/ml inhibited the growth of 9 of the resistant 16 isolates. MBC was further carried out and concentrations ranging from 2048 µg/ml to 8192 µg/ml were the most effective concentrations that eliminated the growth of *Vibrio parahaemolyticus* and *Vibrio fluvialis* isolates. MBC for *Vibrio vulnificus* was observed to range from 128 µg/ml to 1024 µg/ml of imipenem and tetracycline. According to the findings of this study, these organisms are resistant to antimicrobials normally used for the management of *Vibrio* bacterial infections.

Key-words: Non-cholera causing *Vibrio* species, antibiogram signature, antibiotic resistance.

3.0. Introduction

Vibrio species are commonly found in large numbers in aquatic environment. Majority of *Vibrio* species can cause severe infections especially in those with underlying conditions (Elhadi, 2013). Numerous studies focused on the isolation and spread of *Vibrio* species around the world, as infections caused by these bacteria continues to be a public health concern when contaminated water or food is consumed (Maje *et al.*, 2020). *V. cholerae* and *V. parahaemolyticus* are common human pathogenic *Vibrio* species, while *V. vulnificus, V. fluvialis, V. algynolytics, V. mimicus* amongst others are still emerging pathogens, though still capable of causing severe infections in human (Maje *et al.*, 2020). Globally, clinically safe antibiotics such as quinolones, tetracycline, imipenem among others, has helped in limiting the rate of mortality associated with bacterial infections (CDC, 2013; Odjadjare and Igbinosa, 2017). However, most bacteria are developing resistance to these antimicrobial agents. This is as a result of drug misuse which has contributed greatly to resistance in the environment (Aarestrup *et* al., 2008; Finley *et al.*, 2013).

Antimicrobial resistance has been a challenge over the years, the number of resistant species, and affected geographic areas are on are unprecedented and rapidly growing (Stuart and Bonnie, 2004). Antibiotics have been highly effective in successful treatment of *Vibrio* infections, however, antimicrobial resistance have been reported by several studies (Elmahdi *et al.,* 2016). In South Africa, polluted water consumption is a major cause of *Vibrio* infection because a large number of the populace are dependent surface water for their day-to-day need (Osunla and Okoh, 2017). Surface water has been confirmed to be a cause of antimicrobial-resistant bacteria in numerous studies over the years, owing to various human activities and discharge of poorly treated effluent into the setting (Devarajan *et al.*, 2016). The studies of Igbinosa *et al.* (2011); Hossain *et al.* (2012); Al-Othrubi *et al.* (2014); Silvester *et al.* (2015); Igbinosa, (2016); Mechri *et al.* (2017), Tan *et al.* (2017) recorded an increase in *Vibrio* species resistance to antibiotics recommended for. Given *Vibrio* species' high tolerance to

these prescribed drugs for treatment, other reports has shown a high sensitivity of *Vibrio* species against these drugs (Oh *et al.,* 2011; Shaw *et al.,* 2014; Kang *et al.,* 2017). As a result, the goal of this research is to establish the susceptibility profiling of target *Vibrio* species from niches in the Eastern Cape.

3.1. Materials and methods

3.1.1 Culture collection

In this present study, non-cholera *Vibrio* species used were obtained from AEMREG culture collection, University of Fort Hare. These species were first isolated from several environmental niches in Eastern Cape Province.

3.1.2. Revalidation and DNA extraction of target *Vibrio* species

To ensure the viability of the archived isolates collected, glycerol stocks of the isolates preserved at -80℃ in cryo-tubes and thawing was done at room temperature. A bacteria loopful was suspended in a sterile NB and at 37℃, it was incubated for 24 h. Thereafter, a bacterial loopful was extracted from the culture and added into 200 µl sterile dH_2O. It was homogenized and heated on an Accu block for 15 min at 100℃. The cell suspension was centrifuged for 5 min at 15,000 rpm and the supernatant was used as a DNA template in a PCR assay (Maugeri *et al.*, 2004).

3.1.3. Molecular identification of *Vibrio* species

As revealed in Table 3.1, specific primer sets and PCR cycling conditions used to confirm the *Vibrio* species of interest in a polymerase chain reaction (PCR) are summarized. A 25µl final reaction mixture was used (Fri *et al.,* 2017). The amplicons were then resolved in 1.5 % agarose gel electrophoresis and stained with ethidium bromide. The result was viewed under a UV trans-illuminator and photographed.

Table 3.1: Specific primers and cycling conditions for confirming target *Vibrio* species.

Vibrio specie	Primer	Sequence (5'–3')	Thermal cycling condition	Basepair Size	References
Vibrio parahaemolyticus	toxR F	TGTACTGTTGAACGCCTAA	5 minutes Initialization at 94℃, then 35 cycles of Denaturation for 30 seconds at 94℃, 30 seconds of Annealing at 55℃, 30 seconds of Elongation at 72℃ and 10 minutes of Final extension at 72℃.	503	Neogi *et al.*, (2010)
	toxR R	CACGTTCTCATACGAGTG			
Vibrio vulnificus	VvhA F	ACTCAACTATCGTGCACG		410	Neogi *et al.*, (2010)
	VvhA R	ACACTGTTCGACTGTGAG			
Vibrio fluvialis	toxR F	GGATACGGCACTTGAGTAAGACTC	5 minutes Initialization at 94℃, then 30 cycles of Denaturation at 94℃ for 30 seconds, 1 minute Annealing at 57℃, 90 seconds of Elongation at 72℃ and 7 minutes Final extension at 72℃.	217	Chakraborty *et al.*, (2006)
	toxR R	GACCAGGGCTTTGAGGTGGACGAC			

3.1.4. Antibiotics susceptibility testing

As described by CLSI, (2018), antibiogram testing was performed on the identified isolates. To change the turbidity to 0.5 McFarland standard, colonies were extracted from a pure bacteria culture and inserted in a 5 ml sterile NS. The bacteria suspension was evenly spread on MHA, thereafter, antibiotic discs were impregnated on the plate. At 37 °C, they were incubated for 24 hours. This test used a panel of 11 antibiotics, whose breakpoint values are described in Table 3.2 below. The findings were described as resistant, intermediate, or susceptible based on the zones of inhibition assessed (CLSI, 2018).

Table 3.2: Breakpoint zone diameter for *Vibrio* species (CLSI, 2018).

Antimicrobial class	Antibiotics	Disc Content (µg)	Breakpoint Diameter		
			S	I	R
Penicillins	Ampicillin	10	≥ 17	14-16	≤13
	Augmentin	20/10	≥18	14-17	≤13
Cephems	Cefotaxime	30	≥26	23-25	≤22
Carbapenems	Imipenem	10	≥23	20-22	≤19
	Meropenem	10	≥23	20-22	≤19
Tetracycline	Tetracycline	30	≥15	12-14	≤11
Aminoglycosides	Amikacin	30	≥ 17	15-16	≤14
Fluoroquinolones	Ciprofloxacin	5	≥ 21	16-20	≤15
	Nalidixic Acid	30	≥ 21	16-20	≤15
Folate Pathway Inhibitors	Trimethoprim-sulfamethoxazole	1.25/23.75	≥ 16	11-15	≤10
Phenicols	Chloramphenicol	30	≥18	13-17	≤12

3.1.5. MAR index and MAR phenotypes of *Vibrio* species

Based on the description of Titilawo *et al.*, (2015), MARP was evaluated for isolates resistant to a minimum of 3 antibiotics, while MARI was calculated using the mathematical expression calculated as follows:

$$MARindex = a / b,$$

The total number of antibiotics each isolate is resistant to indicated by *a*, the average number of antibiotics tested on each isolate is indicated by *b* (Krumperman 1983).

3.1.6 Determination of MIC

The least concentration at which an antibiotic hinders detectable bacterial growth is known as MIC value (McKinnon and Davis, 2004). The MIC against resistant isolates was calculated using the micro-broth dilution method defined by Edziri *et al.*, (2012), in accordance with CLSI recommendations (2018). The optimal concentration of antibiotics was prepared using a two-fold serial dilution method (i.e. 512 µg/ml was the concentration range used, so the prepared concentration was 1024 µg / ml). Three to five colonies were suspended in NS and attuned to 0.5 McFarland standards from an overnight pure culture. Each standardized isolate was diluted to 100-fold and this was achieved by taking 100 µl of the standardized isolates and dispensed into 9.9 ml of MHB. This suspension was regarded as the standard inoculum. A 96 well microtitre plate was used for this experiment. About 100 µl of the highest concentration of antibiotics was added to column 1, while 50 µl of Mueller Hinton broth was put into columns 2-11. About 100 µl of the Mueller Hinton broth only was put into column 12. Fifty microlitres of the antibiotics in column 1 were withdrawn and serially diluted into column 2, thorough mixing was done by pipetting up and down 4-6 times. This process was repeated until column 10 where 50 µl was withdrawn and discarded. Thirty microlitres were withdrawn from the standard inoculum and dispensed into each well from columns 1 to 11. The microtitre plates were incubated for 18-24 hr at 37 °C.

Forty microlitres of 2, 3, 5-Triphenyltetrazolium chloride (TTC dye) was added to each well after 24 hr incubation and further incubated for 20-30 min, after which each well was observed for color change (from colorless to pink). The color change observed indicated the presence of bacteria growth in the wells and all reactions were carried out in triplicates. The MIC value was calculated using the least concentration that did not cause bacteria growth (no color change).

3.1.7. Determination of MBC

This is the least concentration at which an antibiotic completely kills a microorganism. The MBC was evaluated according to Sudjana *et al.* (2009). Using a sterile glass spreader, 40 µl from the microtiter well that had shown no growth after 24 hours of incubation was spread on sterile nutrient agar plates that contained no antimicrobial agent. At 37 ^{o}C, incubation was done for 24 hours. The MBC value was determined using the least concentration which resulted in no growth after incubation.

3.2. Results

3.2.1. Identification of *Vibrio* species using PCR

Approximately 228 isolates of *Vibrio* were recovered from the AEMREG archive collection. A 503, 410 and 217 base pair gene markers was used to validate 100 *V. parahaemolyticus*, 82 *V. vulnificus*, 46 *V. fluvialis* isolates respectively, as shown in Figure 3.1, 3.2 and 3.3 below. The isolation of these pathogenic bacteria from the environment revealed that several pathogenic bacteria could be present in the environment.

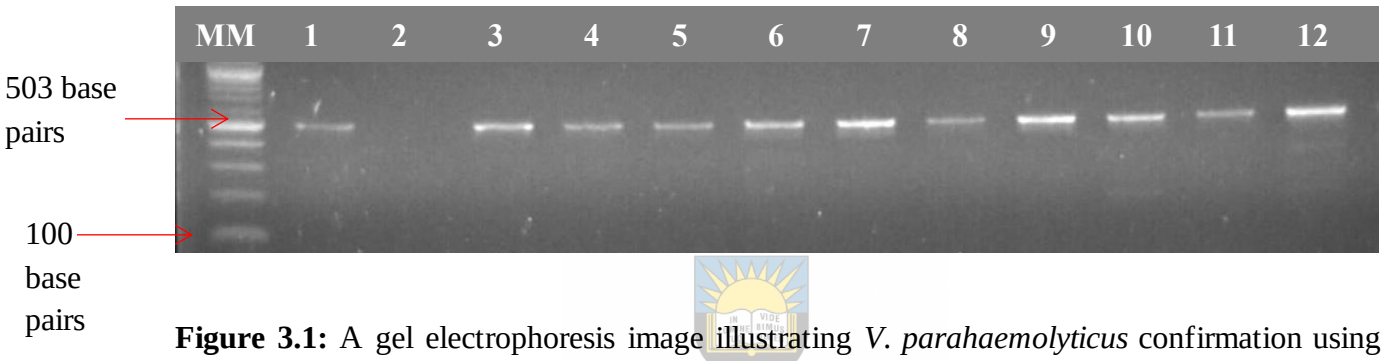

Figure 3.1: A gel electrophoresis image illustrating *V. parahaemolyticus* confirmation using the *toxR* gene. Lane MM indicates a 100 bp molecular marker; lane 1 indicates a positive control (DSM 10027); lane 2 indicates a negative control; and lane 3 to 12 indicates positive isolates.

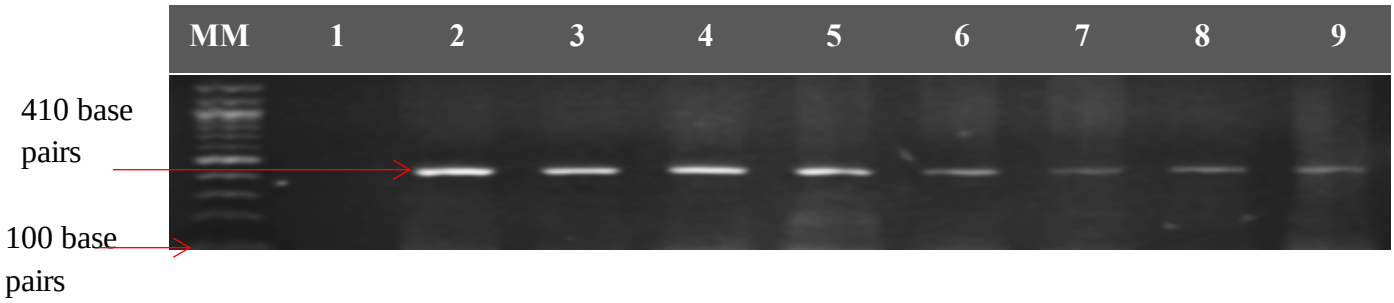

Figure 3.2: A representative gel image of the confirmed *V. vulnificus* using *hsp60* gene. Lane MM indicates a 100 bp molecular marker; lane 1 indicates a negative control; lane 2 indicates positive control (DSM 10143); and lane 3 to 9 indicates Positive isolates.

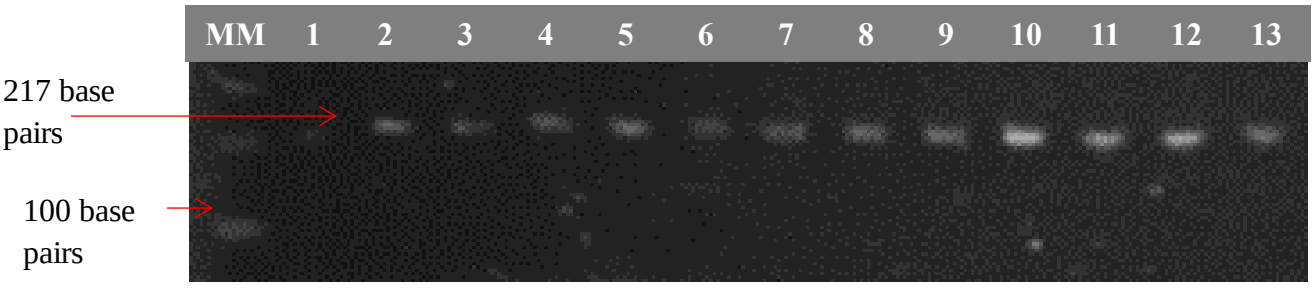

Figure 3.3: A gel image of the confirmed *V. fluvialis* using *toxR* gene. Lane MM indicates a 100 bp molecular marker; lane 1 indicates negative control; lane 2 indicates positive control (DSM 19283); and lane 3 to 13 indicates positive isolates.

3.2.2. Antibiogram profiling of identified *Vibrio* species

Antibiotic susceptibility testing was performed on all 228 confirmed *Vibrio* organisms. Tetracycline (22%) resistance was found to be the most common in *Vibrio parahaemolyticus*. High resistance against imipenem (40%) was detected in *Vibrio vulnificus*, while highest resistance was against Nalidixic acid (28%) and tetracycline (28%) in *Vibrio fluvialis*. The summarized findings are revealed in Table 3.3.

Table 3.3: Antibiotic susceptibility profile for the identified *Vibrio* species.

Antibiotics Class	Antibiotic (µg)	*V. parahaemolyticus* n = 100 (#/%)			*V. vulnificus* n = 82 (#/%)			*V. fluvialis* n = 46 (#/%)		
		S	I	R	S	I	R	S	I	R
Carbapenems	Imipenem (10)	98/98	2/2	0	31/38	18/22	33/40	39/ 85	7/15	0
	Meropenem (10)	96/96	4/4	0	64/78	6/7	12/15	46/100	0	0
Penicillins	Ampicillin (10)	82/82	4/4	14/14	57/70	10/12	15/18	35/76	3/7	8/20
	Augmentin (20/10)	90/90	4/4	6/6	79/96	0	3/4	39/85	5/11	2/4
Cephems	Cefotaxime (30)	74/74	12/12	14/14	68/83	9/11	5/6	38/83	3/6	5/11
Aminoglycosides	Amikacin (30)	87/87	2/2	11/11	77/94	4/5	1/1	32/70	9/19	5/11
Fluoroquinolones	Ciprofloxacin (5)	88/88	8/8	4/4	68/83	12/15	2/2	38/83	6/13	2/4
	Nalidixic acid (30)	78/78	6/6	16/16	60/73	16/19	7/9	29/63	4/9	13/28
Phenicols	Chloramphenicol (30)	80/80	8/8	12/12	53/65	20/24	9/11	30/65	9/20	7/15
Tetracycline	Tetracycline (30)	69/69	9/9	22/22	59/72	4/5	18/22	32/70	1/ 2	13/28
Folate pathway inhibitors	Trimethoprim-sulfamethoxazole (1.25/23.75)	92/92	1/1	7/7	73/89	9/11	0	41/89	1/ 2	4/9

3.2.3. MARI and MARP

For the *Vibrio* species studied, approximately 38 MARP trends were discovered. Approximately 23% identified *Vibrio* isolates showed resistance to a minimum of 3 antibiotics. The isolates' MARI was over 0.2, with the highest value being 0.8 and the lowest being 0.3. Table 3.4 shows a description of the findings.

Table 3.4: MARP and MARI patterns of target *Vibrio* species.

***Vibrio* species Evaluated**	**Number observed**	**MAR Phenotypes**	**Number of MARP**	**MARI**
Vibrio parahaemolyticus	1	T-TS-AP-C-NA-CTC-AG-CIP-AK	9	0.8
	1	NA-AG-TS-T	4	0.4
	1	AP-T-C-AK	4	0.4
	1	T-NA-AG	3	0.3
	1	CIP-AP-NA-AG-TS-AK-T	7	0.6
	1	CTX-T-AP-C	4	0.4
	1	NA-CTX-AP-T	4	0.4
	1	T-CTX-AP-AK-NA	5	0.45
	1	AK-NA-T	3	0.3
	1	AP-C-CTX-AK	4	0.4
	1	NA-TS-AK-T-AP-CTX	6	0.55
	1	TS-NA-T-AK	4	0.4
	1	T-AP-C-NA-TS-CTX	6	0.55
	2	AP-AG-CTX-NA-AK-CIP-C-T	8	0.7
	2	NA-T-TS	3	0.3
	2	C-AK-T-NA-CTX	5	0.45
	3	AP-C-CTX-T	4	0.4
Vibrio vulnificus	1	IMI-MEM-AP-CTX-T	5	0.45
	1	MEM-CTX-IMI-C-AP-AK-CP-T	8	0.7
	1	IMI-NA-C	3	0.3
	1	IMI-CIP-AP-NA-T	5	0.45
	1	IMI-AG-NA-AP	4	0.4
	1	MEM-CTX-C-IMI-T	5	0.45
	1	CTX-AP-MEM-C-IMI-T	6	0.55
	2	IMI-T-C	3	0.3
	2	AP-IMI-MEM-T	4	0.4
	3	IMI-T-AP	3	0.3
	3	AP-IMI-NA-T-MEM-C	6	0.55
Vibrio fluvialis	1	AG-T-NA-AP	4	0.4
	1	AP-NA-T-C-AK	5	0.45
	1	CIP-T-AP-CTX-AK-NA	6	0.55
	1	AG-NA-T-AP	4	0.4
	1	T-AK-C-NA	4	0.4

1	T-C-NA	3	0.3
1	CIP-T-TS-NA	4	0.4
1	C-AP-CTX-NA-T	5	0.45
2	T-AK-NA	3	0.3
3	TS-T-NA-AP-CTX-C	6	0.55

3.2.4. MIC of the antibiotics

The antibiotics with the highest resistance rate among *Vibrio* isolates had their MICs determined. The commonest antibiotics *V. parahaemolyticus* and *V. fluvialis* are resistant in this report are tetracycline and nalidixic acid, as revealed in Tables 3.3 and 3.4. *V. vulnificus* appears to be sensitive to imipenem and tetracycline, which tend to be the most effective antibiotics. Tables 3.5, 3.6 and 3.7 below indicate the outcomes.

Table 3.5: MIC of antibiotics against *Vibrio parahaemolyticus*.

Antibiotics	Total resistant isolates	MIC range (µg/ml)									
		4	8	16	32	64	128	256	512	1024	2048
Nalidixic acid	16	0	0	2	2	2	4	3	2	1	0
Tetracycline	16	0	0	1	7	3	5	0	0	0	0

Table 3.6: MIC of antibiotics against *Vibrio vulnificus.*

Antibiotics	Total resistant isolates	MIC range (µg/ml)									
		1	2	4	8	16	32	64	128	256	512
Imipenem	16	0	0	0	2	6	5	3	0	0	0
Tetracycline	16	0	0	0	0	0	2	9	4	1	0

Table 3.7: MIC of antibiotics against *Vibrio fluvialis*.

Antibiotics	Total resistant isolates	MIC range (µg/ml)									
		4	8	16	32	64	128	256	512	1024	2048
Tetracycline	13	0	0	0	0	0	0	5	4	3	1
Nalidixic acid	13	0	0	0	1	-	1	1	3	1	6

3.2.5. MBC of the antimicrobial agents

Beginning with concentrations that inhibited bacterial growth, the MBC was assessed on the resistant *Vibrio* organisms. Resistant *Vibrio parahaemolyticus* species were eliminated at higher concentrations of nalidixic acid and tetracycline, ranging from 256 µg/ml to 4096 µg/ml. The concentration was increased against *V. fluvialis* due to high resistance to the test antibiotics (nalidixic acid and tetracycline). At 8192 µg/ml, both antibiotics killed 46.2 percent and 69.2 percent of the *V. fluvialis* isolates. Imipenem was bactericidal against *V. vulnificus* at a concentration range of 32 µg/ml to 1024 µg/ml, whereas MBC of tetracycline ranged from 256 µg/ml to 4096 µg/ml. The summarized result is expressed in Table 3.8, 3.9, and 3.10.

Table 3.8: MBC of test antibiotics against *Vibrio parahaemolyticus*.

Antibiotics	Total resistant isolates	MBC range (µg/ml)							
		32	64	128	256	512	1024	2048	4096
Nalidixic acid	16	-	-	-	1	-	4	3	8
Tetracycline	16	-	-	-	-	3	5	6	2

Table 3.9: MBC of test antibiotics against *Vibrio vulnificus*.

Antibiotics	Total resistant isolates	MBC range (µg/ml)							
		32	64	128	256	512	1024	2048	4096
Imipenem	16	1	-	5	3	5	2	-	-
Tetracycline	16	-	-	-	2	4	7	2	1

Table 3.10: MBC of test antibiotics against *Vibrio fluvialis*.

Antibiotics	Total resistant isolates	MBC range (µg/ml)							
		64	128	256	512	1024	2048	4096	8192
Tetracycline	13	-	-	-	-	1	-	5	6
Nalidixic acid	13	-	-	-	-	-	1	3	9

3.3. Discussion

The presence of other microorganisms that cause disease in humans in the environment is demonstrated by the isolation of *Vibrio* species from the environment. This may be as a result of human-caused groundwater pollution or the release of untreated effluent into the environs (Igbinosa, 2016). Antibiotic susceptibility testing was performed on these PCR confirmed *Vibrio* species, and the results displayed that these organisms were sensitive to most antibiotics used in this research. Antibiotic resistance is a health problem owing to its direct connection to disease control (Ramamurthy, 2008).

Antibiogram testing was performed on the identified *Vibrio* species, and this revealed that these organisms were resistant to most antibiotics examined in this research. Tetracycline resistance occurred in 29% of the isolates, whereas tetracycline susceptibility was found in 71% of the isolates. This report is in accord with results of Quilici *et al*., (2010); Raissy *et al.*, (2012); Osuolale and Okoh, (2018). Contrary to this research, Mandal *et al.* (2012) and Singh *et al.* (2014), discovered that *Vibrio* species had an increased tetracycline resistance rate. In comparison to Srinivasan *et al.* (2006), who discovered 27 % resistance to nalidixic acid, while 73 % were susceptible. The high rate of nalidixic acid resistance is worrying, as bacteria sensitive to nalidixic acid are possibly resistant to other fluoroquinolones, according to findings of Nelson *et al*., 2011; Kumar *et al*., 2014. In *V. parahaemolyticus* and *V. fluvialis*, no resistance to imipenem and meropenem, whereas *V. vulnificus*, was sensitive to Trimethoprim-sulfamethoxazole. According to Baron *et al.* (2016), *Vibrio* species are more susceptible to imipenem, ampicillin, amikacin, and trimethoprim-sulfamethoxazole. An exemption is imipenem, which *Vibrio vulnificus* was susceptible to, this present study was similar to that finding. Okoh and Igbinosa (2010) also documented similar result.

MARP analyzed in this study showed that 38 different trends were found in all isolates tested, and the majority of the isolates were sensitive over three antibiotics. The correct threshold

value for distinguishing low-risk and high-risk antibiotic use regions is 0.2 when MARI was assessed. MARI in this study ranged from 0.3 to 0.8, classifying it as a high-risk source of pollution. A MAR index of less than 0.2 was found in none of the isolates examined. This therefore, indicates means that antibiotics are being used improperly in the environment. Increased MARI values, such as those found in this report, may be caused by a variety of anthropogenic activities in the area, implying that the environment is heavily contaminated with antibiotics (Adefisoye and Okoh 2016). The MARI value discovered support the research of Okoh and Igbinosa (2010), which also indicated a ≥0.3 threshold value.

Table 3.5 shows that at MIC concentrations 128 µg/ml, nalidixic inhibited the most bacteria. Tetracycline inhibited the most *Vibrio parahaemolyticus* isolates at 32 µg/ml, MBC was then performed, and as shown in Table 3.8, most of the isolates were eliminated at tetracycline and nalidixic acid concentrations of 2048 µg/ml and 4096 µg/ml respectively. MBC values were determined using these concentrations.

Table 3.6 shows that the MIC concentrations for imipenem and tetracycline against *Vibrio vulnificus* was from 8 µg/ml to 256 µg/ml. At a concentration of 16 µg/ml, imipenem prevented the most bacteria development, with 6 (37.5%) being inhibited. At a concentration of 64 µg/ml, the highest number of bacteria inhibitions were detected, with 9 (56.3 percent) of the 16 resistant isolates being inhibited. MBC was performed on *Vibrio vulnificus* isolates that were sensitive to antibiotics. As shown in Table 3.9, imipenem concentrations of 128 µg/ml and 512 µg/ml had the highest levels of bactericidal activity, whereas tetracycline concentrations of 1024 µg/ml had the most bactericidal impact.

Against *Vibrio fluvialis*, MIC concentration was from 32 µg/ml to 2048 µg/ml for both antibiotics. The two antibiotics tested inhibited the most growth at 256 µg/ml and 2048 µg/ml, inhibiting 5 (38.5 %) and 6 (46.2%) of the resistant isolates as shown in Table 3.7. MBC was performed on resistant *Vibrio fluvialis* isolates, and the largest number of

bactericidal activities was at 8192 µg/ml concentration. As resistance to these 3 antibiotics were reported in both studies, the MIC and MBC results discovered agree with the results from the susceptibility test. Chandrakala *et al.* (2014) conveyed susceptibility to tetracycline and nalidixic acid at low MIC values in their research. This is in contrast to the results of this report.

3.4. Conclusion

Optimal combination and concentration of antimicrobials used to inhibit the different *Vibrio* species were determined. Antibiotic overuse and misuse have played a prominent part in the growth and spread of antimicrobial resistance. *Vibrio* species isolation from the environment suggest that other organisms that can endanger human and animal health exist in the environment. As a consequence, it's likely that resistant species can be found in the environment. Bacteriostatic antibiotics have the potential to be bactericidal at higher concentrations, according to the MIC and MBC findings. As a result, constant observation and regulation of drug use in the community is necessary to ensure successful and adequate infection treatment. To avoid an outbreak of infection, members of the community should also practice environmental hygiene.

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CHAPTER 4

The effect of the combination of antibiotics on their activities against selected *Vibrio* species from environmental niches of the Eastern Cape, South Africa.

Abstract

Antimicrobial resistance amongst Gram-negative organisms has been reported to be on the increase globally as they have shown resistance to available antibiotics including carbapenems, a broad-spectrum antibiotic. This has contributed to the use of double antibiotics by physicians for efficient therapy. In this study, the impact of antibiotics combination on antibiotics resistant *Vibrio* species was assessed. Checkerboard assay was used to carry out antibiotics' combination assay and the FIC index was calculated. A combination of tetracycline and Nalidixic acid at different concentrations was carried out against *V. parahaemolyticus* and *V. fluvialis*, while tetracycline and imipenem combination at different concentrations was assessed against *V. vulnificus*. Rate of kill was also determined using ½ × MIC, 1 × MIC, and 2 × MIC concentrations of the combined antibiotics at 2 hr intervals.

Synergy was observed against 3 *Vibrio parahaemolyticus* and *Vibrio vulnificus* isolates respectively, while synergy was observed against only 2 *Vibrio fluvialis* isolates. None of the combinations had an antagonistic effect on the isolates and most of the combinations were observed to have indifferent interactions. The rate of kill of the combined antibiotics was further determined and 2 × MIC at the 6th hr, eliminated bacteria growth. The result from this study indicates that the percentage of bacteria cell growth is dependent on the concentration of antibiotics combined and the extent of exposure time. This, therefore, suggest that combination therapy (tetracycline-nalidixic acid and tetracycline-imipenem) is a promising solution to antimicrobial resistance in *Vibrio* species.

Key-words: antibiotics combination, rate of kill, synergism, antagonism, exposure time.

4.1. Introduction

Vibrio species are Gram-negative bacteria capable of causing waterborne and foodborne infections. Cholera-causing and non-cholera-causing *Vibrio* species are two groups of *Vibrio* that cause severe infections in humans especially those who are immune-compromised (Hogan, 2010). Non-cholera-causing *Vibrio* species are capable of causing septicemia, acute gastroenteritis, and wound infections when contaminated food is ingested (Lee *et al*., 2019). Several outbreaks of infections by this group of bacteria have been confirmed across all continents in the world (Vugia *et al.,* 2004). Infections by *Vibrio* species are self-limited, however, antibiotics are used in patients with an underlying medical condition (Malcolm *et al*., 2018). Fluoroquinolones, tetracycline, carbapenems, aminoglycosides are a few examples of accepted antibiotics for the treatment of *Vibrio* infections due to the high sensitivity of *Vibrio* species to these antimicrobial agents (Malcolm *et al*., 2018). Inappropriate use of antibiotics is a factor that spreads antibiotic resistance (Aminov, 2010). The failure of antibiotics to effectively treat or manage infections serves as a danger to human lives and economic development. This has led scientists to the use of combination therapy as a possible solution to antibiotic resistance.

Combination therapy involves the combination of more than two antibiotics for the effective treatment. This therapy is often used to evade the rise of resistance and boost activity of both antibiotics for effective treatment (Omoya and Ajayi, 2016). Successful use of antibiotics combination therapy against resistant bacteria have been reported to be associated with increased survival rate (Tamma, *et* al., 2012). When used appropriately, combination therapy can improve treatment rate, lower fatality ratio, and slows down resistance development (Bozic *et al*., 2013). There are different methods available for the *in-vitro* evaluation of synergy between antibiotics, however, checkerboard assay and rate of kill are the most used methods. Checkerboard assay uses a method that is similar to that of the MIC determination

and unlike the rate of kill method, checkerboard assay is merely an evaluation of the inhibitory impact of antibiotics, whereas the rate of kill assesses the killing activity of antibiotics with respect to time (White *et al.*, 1996).

Tetracycline is often used for treating various bacterial infections. It is known to block the ribosomal attachment of charged aminoacyl-tRNA, thus inhibiting protein synthesis. Bacteria possessing efflux pumps always eject tetracycline from their cell as a mechanism of resistance (Chopra and Roberts, 2001). Nalidixic acid is a quinolone antibiotic used mainly for the treatment of Gram-negative bacterial infections. It is bacteriostatic when used in lower concentration but can be bactericidal when its concentration is increased. It is known to inhibit DNA synthesis (Emmerson and Jones, 2003). Imipenem is known to hinder cell wall synthesis by binding to the penicillin-binding proteins (PBPs) of bacteria (Breilh *et al.*, 2013). Combination therapy involving fluoroquinolone, carbapenem or tetracycline has been reported to increase survival rates in patients (Morrill *et al.*, 2015). Therefore, this research examined the synergistic effect of combinations of tetracycline-imipenem and tetracycline-nalidixic acid against antibiotic-resistant non-cholera causing *Vibrio* species.

4.2. Materials and methods

4.2.1. Antimicrobial combination therapy.

Combination therapy was calculated using checkerboard assay as described by White *et al.* (1996). Checkerboard assay is used to assess the efficacy of antibiotics combination in contrast to their individual behaviors. About 100 µL of desired concentration of compound A was added to column 1 to 11 horizontally, and 100 µL of compound B was added to row A to G vertically. Column 12 contain a serial dilution of compound B alone, while row H contains a serial dilution of compound A alone. Column 12 and row H were used as the control to determine the MIC value of each test compound. About 50 µL of the bacteria inoculum was added to all the wells and microtiter plates were incubated at 37 °C for 24 hr after which the MIC was interpreted. To enumerate the interaction between the antibiotics being tested, the FICI was calculated using:

$A/MIC_A + B/MIC_B = FIC_A + FIC_B = FIC\ Index$

A and B indicate the MICs of individual drug in combination, and MIC_A and MIC_B indicate MICs of individual drug separately.

Results were considered synergistic if FICI value is < 0.5, antagonistic if FICI value is > 4 and additive or indifference if FIC value is > 0.5 - ≤ 4 (Kim *et al.*, 2005). A synergistic effect is when the effect of the two combined antibiotics increases the inhibitory activity of both antibiotics than the drug alone, antagonism is when the effect of the combined antibiotics is lesser than each drug alone and additive or indifference is when the activity of the combination is the same as the activity of each drug alone (Mandal *et al.*, 2004).

4.2.2. Determination of rate of kill

The killing activity of the combined antibiotics was calculated using a method described by Eliopoulos and Moellering, (1996). Desired antibiotics were dissolved in 10 MHB in McCartney bottles at three different concentrations which are 1/2 × MIC, 1 × MIC, and 2 × MIC. A bottle was set aside as positive control (Mueller Hinton broth with test microorganisms but without antibiotics). The bottles were incubated at 37 ^{o}C on a shaker at 120 rpm. At every 0 h, 2 h, 4 h, 8 h, 10 h, 12 h, and 24 h, 1000 µL aliquot of the culture medium was removed and serially diluted in sterile normal saline (10^{-1} to 10^{-6} dilution factors) to ensure there are no residual antibiotics. Colony forming unit (CFU/mL) was calculated by plating out 100 µL of each dilution and incubated at 37 ^{o}C for 24 hr. Bacteria colonies observed after incubation were counted, CFU/mL was evaluated and compared with counts from the growth control.

4.3. Result

4.3.1. Antibiotics combination therapy

Antibiotics combination was carried out on isolates that expressed resistance to the two antibiotics studied. Against *V. parahaemolyticus* and *V. fluvialis*, the combination of tetracycline and nalidixic acid at different concentrations exhibited synergistic activity on 3 and 2 isolates respectively. Whereas against *V. vulnificus*, the combination of imipenem and tetracycline had synergistic activity on 3 isolates. None of the antibiotic combinations had antagonistic activity against isolates tested. Most of the combinations were indifference/additive. The results are expressed in Table 4.1, 4.2, and 4.3 below.

Table 11: The FIC index (∑FIC) of tetracycline and nalidixic acid in combination against selected *Vibrio parahaemolyticus* species.

Number of isolates tested	MIC of individual drug (µg/ml)		MIC of combined drugs (µg/ml)		FIC		∑FIC (FIC_A+FIC_B)	Remark
	Tet	Na	Tet	Na	Tet (FIC_A)	Na (FIC_B)		
1	32	128	8	4	0.25	0.125	0.375	Synergy
2	32	32	8	16	0.25	0.5	0.75	Indifference
3	64	16	8	4	0.125	0.25	0.375	Synergy
4	32	256	32	128	1	0.5	1.5	Indifference
5	128	256	32	64	0.25	0.25	0.5	Indifference
6	64	64	16	16	0.25	0.25	0.5	Indifference
7	128	512	64	512	0.5	1	1.5	Indifference
8	32	32	8	16	0.25	0.5	0.75	Indifference
9	32	16	8	4	0.25	0.25	0.5	Indifference
10	16	256	8	64	0.5	0.25	0.75	Indifference
11	64	128	64	128	1	1	2	Indifference
12	128	1024	64	512	0.5	0.5	1	Indifference
13	32	128	8	16	0.25	0.125	0.375	Synergy
14	32	64	8	16	0.25	0.25	0.5	Indifference
15	128	128	64	128	0.5	1	1.5	Indifference
16	128	512	128	256	1	0.5	1.5	Indifference

FIC index = FIC_A +FIC_B. tet = tetracycline, na= nalidixic acid

As observed in the result above, the combination of tetracycline and nalidixic acid exhibited a synergistic effect against 3 (highlighted in red) out of the sixteen resistant *V. parahaemolyticus* isolates.

Table 12: The FIC index (∑FIC) of tetracycline an imipenem in combination against selected *Vibrio vulnificus* species.

Number of isolates tested	MIC of individual drug (µg/ml)		MIC of combined drugs (µg/ml)		FIC		∑FIC (FIC_A+FIC_B)	Remark
	Tet	Imi	Tet	Imi	Tet ($FIC_{A)}$	Imi (FIC_B)		
1	64	8	16	4	0.25	0.5	0.75	Indifference
2	32	16	16	8	0.5	0.5	1	Indifference
3	256	32	64	16	0.25	0.5	0.75	Indifference
4	128	32	32	16	0.25	0.5	0.75	Indifference
5	64	32	8	8	0.125	0.25	0.375	Synergy
6	64	16	8	2	0.125	0.125	0.25	Synergy
7	128	16	32	8	0.25	0.5	0.75	Indifference
8	64	64	32	64	0.5	1	1.5	Indifference
9	64	16	8	8	0.125	0.5	0.625	Indifference
10	32	8	4	2	0.25	0.25	0.5	Indifference
11	64	16	32	16	0.5	1	1.5	Indifference
12	64	16	16	8	0.25	0.5	0.75	Indifference
13	128	64	64	32	0.5	0.5	1	Indifference
14	64	16	8	4	0.125	0.25	0.375	Synergy
15	128	64	64	32	0.5	0.5	1	Indifference
16	64	16	16	8	0.25	0.5	0.75	Indifference

FIC index = FIC_A +FIC_B. tet = tetracycline, imi= imipenem

As observed in the result above, the combination of tetracycline imipenem exhibited a synergistic effect against 3 (highlighted in red) out of the sixteen resistant *V. vulnificus* isolates.

Table 13: The FIC index (∑FIC) of tetracycline and nalidixic acid in combination against selected *Vibrio fluvialis* species.

Number of isolates tested	MIC of individual drug (µg/ml)		MIC of combined drugs (µg/ml)		FIC		∑FIC (FIC_A+FIC_B)	Remark
	Tet	Na	Tet	Na	Tet (FIC_A)	Na (FIC_B)		
1	256	512	32	128	0.125	0.25	0.375	Synergy
2	1024	128	1024	64	1	0.5	1.5	Indifference
3	512	256	256	128	0.5	0.5	1	Indifference
4	256	1024	256	512	1	0.5	1.5	Indifference
5	256	2048	128	512	0.5	0.25	0.75	Indifference
6	256	32	32	8	0.125	0.25	0.375	Synergy
7	2048	512	2048	512	1	1	2	Indifference
8	1024	2048	512	1024	0.5	0.5	1	Indifference
9	512	2048	128	1024	0.25	0.5	0.75	Indifference
10	512	2048	512	2048	1	1	2	Indifference
11	1024	512	1024	256	1	0.5	1.5	Indifference
12	256	2048	64	512	0.25	0.25	0.5	Indifference
13	512	2048	256	1024	0.5	0.5	1	Indifference

FIC index = FIC_A +FIC_B. tet = tetracycline, na= nalidixic acid

As observed in the result above, the combination of tetracycline and nalidixic acid exhibited a synergistic effect against 2 (highlighted in red) out of the 13 resistant *Vibrio fluvialis* isolates.

4.3.2. Determination of rate of kill

The killing activity of the test organism by the combined antibiotics was assessed by determining the colony count of surviving bacteria over time. This method can access both the rate and extent to which antibiotics can kill bacteria test organisms. This is an advantage it has over checkerboard assay (Eliopoulos and Moellering, 1996). Isolates to which the two test antibiotics in combination showed synergistic activity were used in this study. Two *Vibrio fluvialis* isolates were used, while three isolates of *V. parahaemolyticus* and *V. vulnificus* were also used. Figure 4.1 to 4.3 shows killing rate of *Vibrio* species by the test antibiotics. As the combination concentrations and exposure time increased, the number of viable bacteria cells decreased.

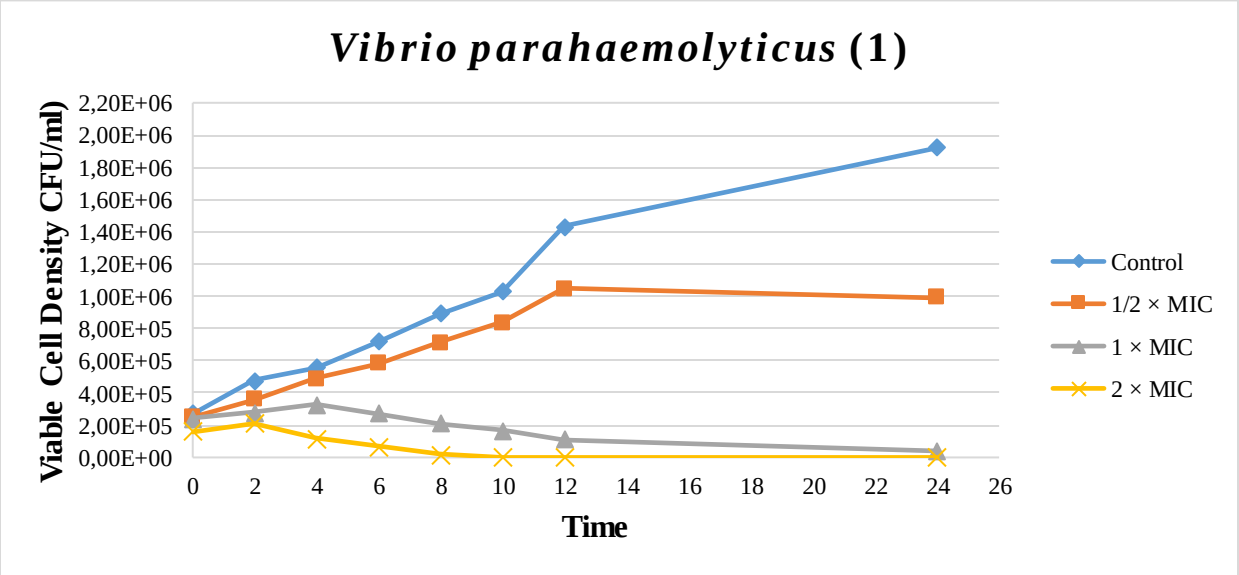

Figure 4.1a: Rate of kill of *Vibrio parahaemolyticus* by Tetracycline (32 µg/ml) and Nalidixic acid (128 µg/ml) combination. This chart is showing changes in the viable cell density of exponentially growing *Vibrio parahaemolyticus* (isolate 1).

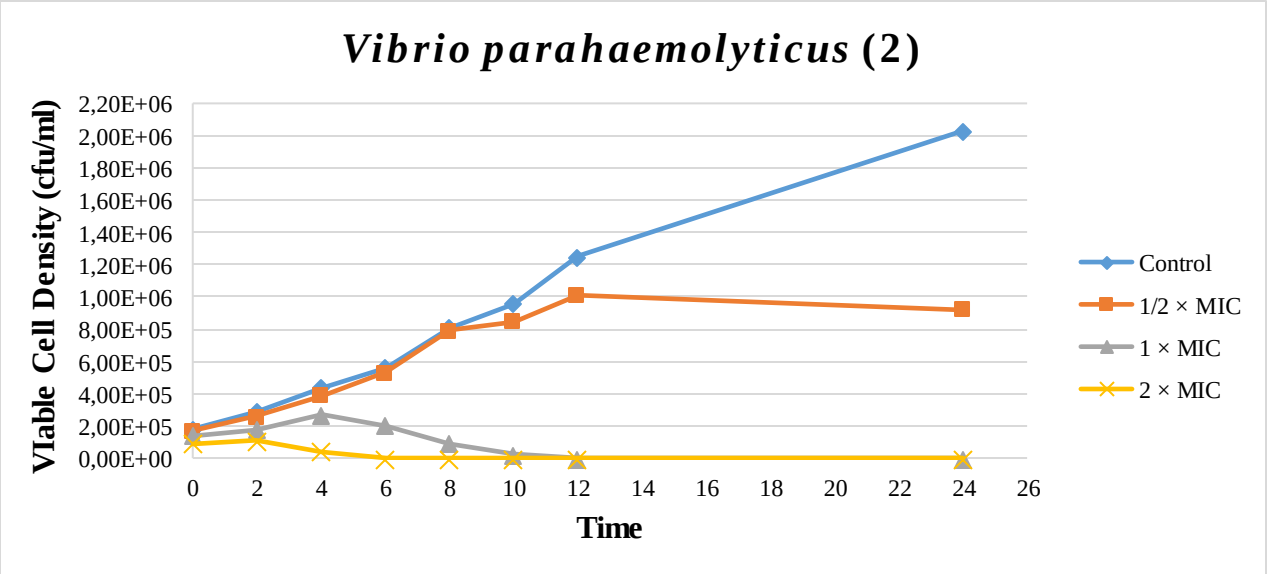

Figure 4.1b: Rate of kill of *Vibrio parahaemolyticus* by Tetracycline (64 µg/ml) and Nalidixic acid (16 µg/ml) combination. This chart is showing changes in the viable cell density of exponentially growing *Vibrio parahaemolyticus* (isolate 2).

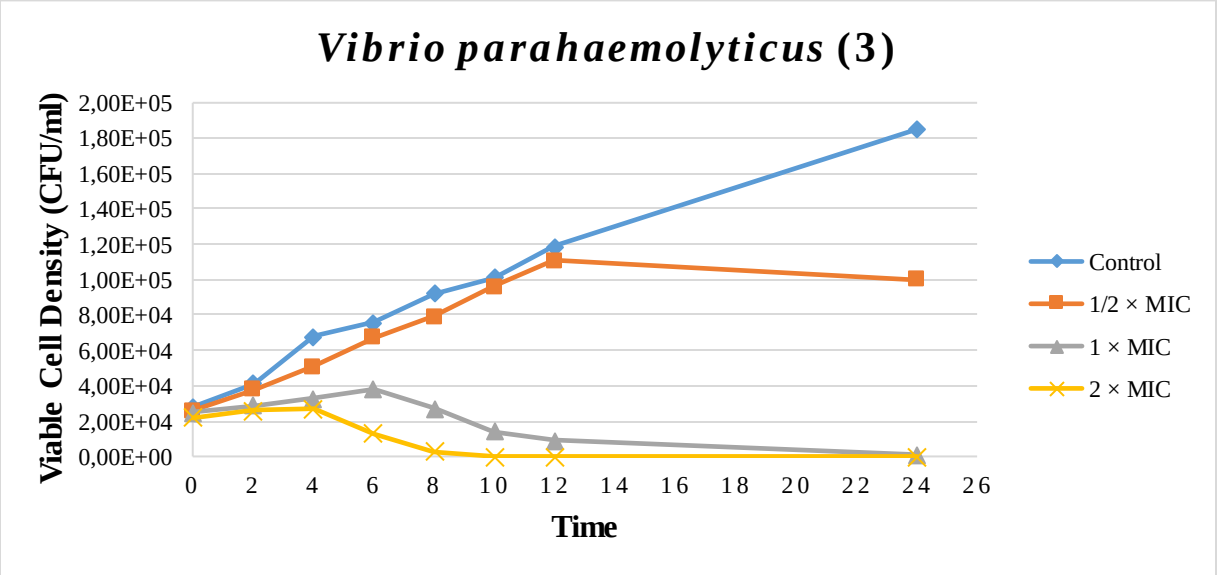

Figure 4.1c: Rate of kill of *Vibrio parahaemolyticus* by Tetracycline (32 µg/ml) and Nalidixic acid (128 µg/ml) combination. This chart is showing changes in the viable cell density of exponentially growing *Vibrio parahaemolyticus* (isolate 3).

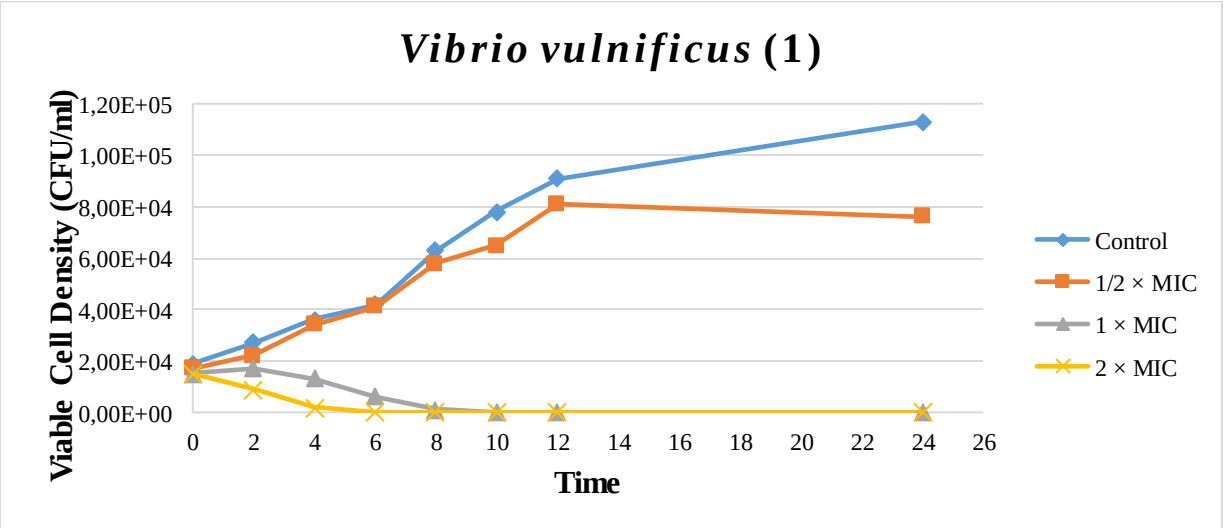

Figure 4.2a: Rate of kill of *Vibrio vulnificus* by Tetracycline (64 µg/ml) and Imipenem (32 µg/ml) in combination. This chart is showing changes in the viable cell density of exponentially growing *Vibrio vulnificus* (isolate 1).

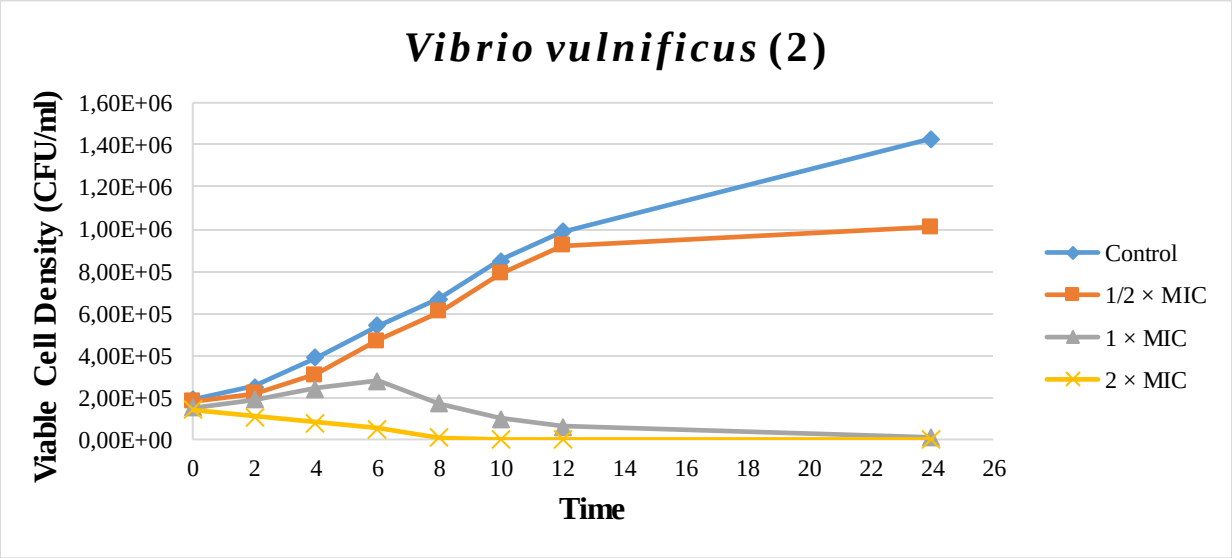

Figure 4.2b: Rate of kill of *Vibrio vulnificus* by Tetracycline (64 µg/ml) and Imipenem (16 µg/ml) in combination. This chart is showing changes in the viable cell density of exponentially growing *Vibrio vulnificus* (isolate 2).

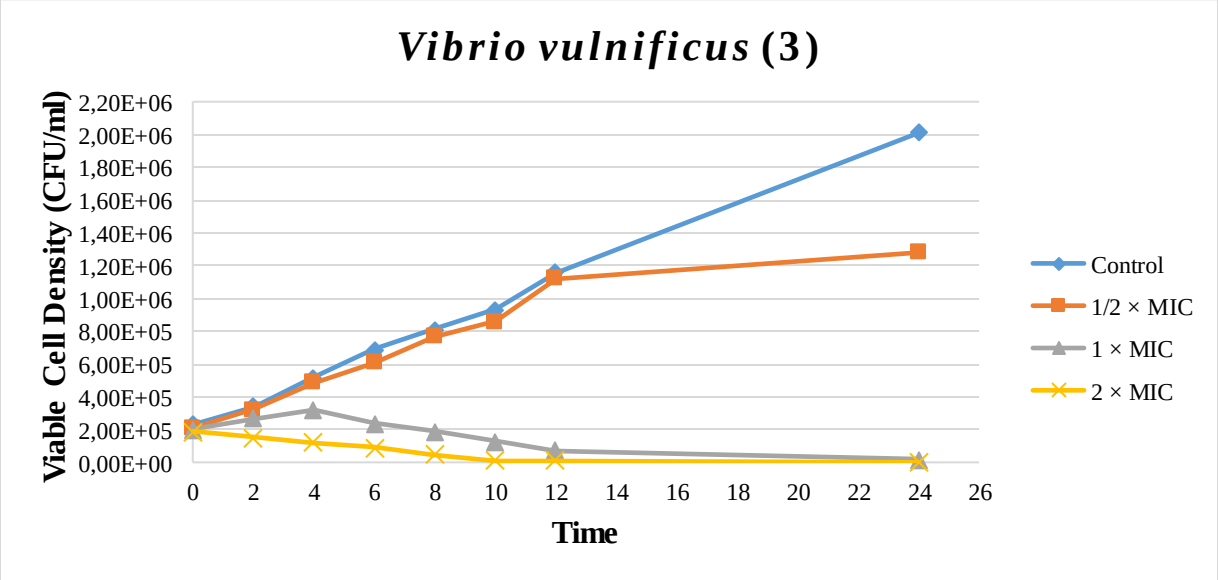

Figure 4.2c: Rate of kill of *Vibrio vulnificus* by Tetracycline (64 µg/ml) and Imipenem (16 µg/ml) in combination. This chart is showing changes in the viable cell density of exponentially growing *Vibrio vulnificus* (isolate 3).

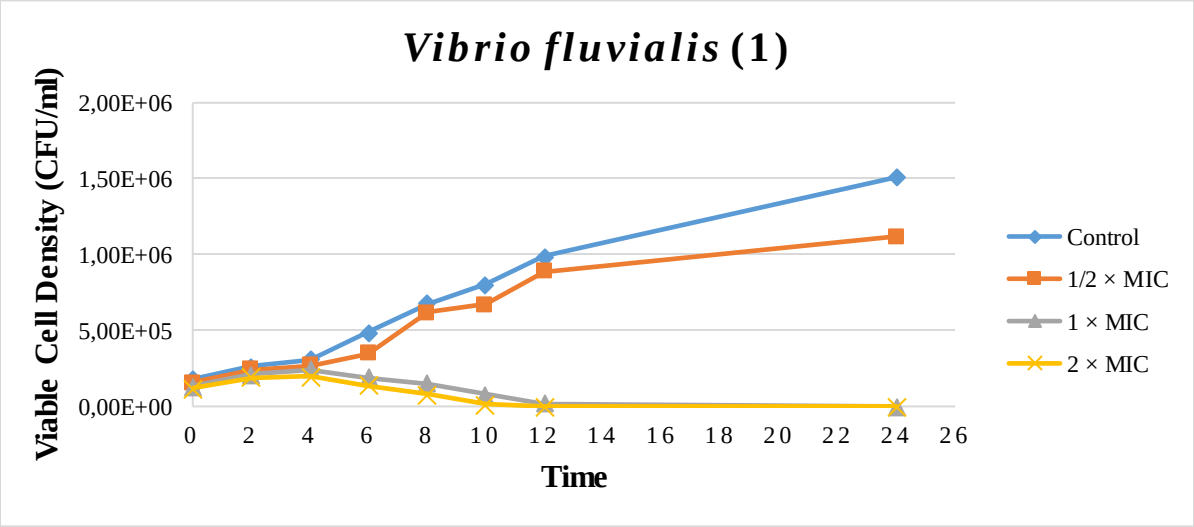

Figure 4.3a: Rate of kill of *Vibrio fluvialis* by Tetracycline (256 µg/ml) and Nalidixic acid (512 µg/ml) combination. This chart is showing changes in the viable cell density of exponentially growing *Vibrio fluvialis* (isolate 1).

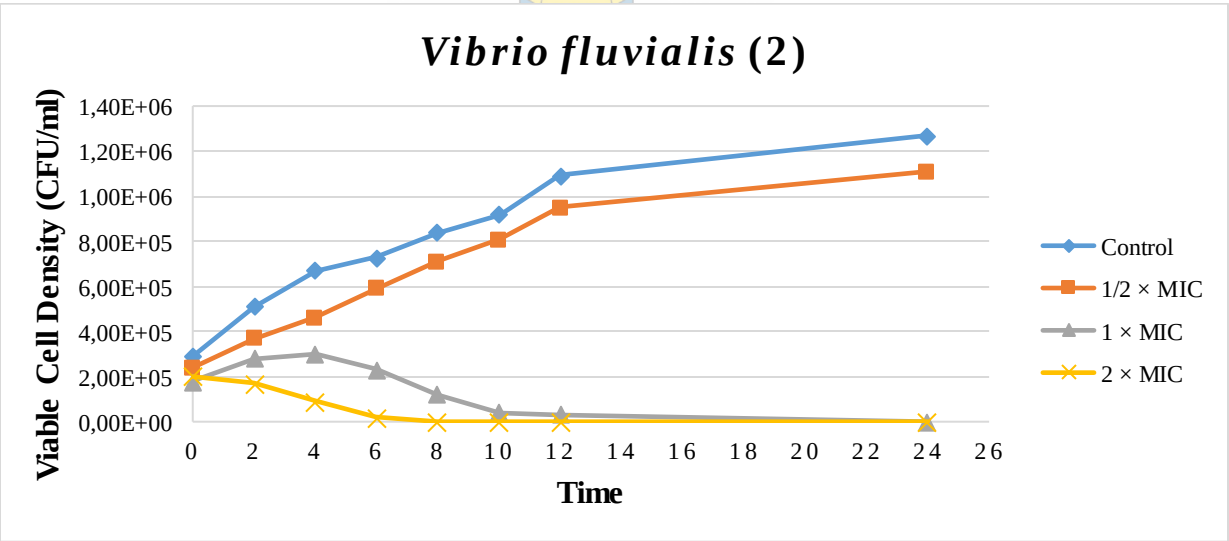

Figure 4.3b: Rate of kill of *Vibrio fluvialis* by Tetracycline (256 µg/ml) and Nalidixic acid (32 µg/ml) combination. This chart is showing changes in the viable cell density of exponentially growing *Vibrio fluvialis* (isolate 2).

4.4. Discussion

Gram-negative organisms that are multi-drug resistant have been reported to have a high mortality rate of 30 % to 70 %, serving as a problem to human health (Tamma *et al.*, 2012). Combination therapy is considered an option because of few supply of antimicrobial agents for the treatment of these organisms. The report of Bliziotis *et al.* (2005) stated that the combination of a β-lactam and aminoglycoside or fluoroquinolone was highly effective against Gram-negative organisms. Related research on *Vibrio* species by Wong *et al.* (2015) also stated that low mortality is always associated with treatment options that involve the use of quinolones. However, in this study, nalidixic acid and tetracycline were combined against *Vibrio parahaemolyticus* and this resulted in a synergistic activity against 3 out of the 16 isolates tested as shown in Table 4.1. At those varying concentrations, the antibiotics in combination were bactericidal. The other combinations had an indifferent or additive activity against the remaining isolates. None of the combinations had antagonistic activity. The same result was also observed against *Vibrio fluvialis*, where different concentrations of Nalidixic acid and tetracycline were combined. Meletiadis *et al.* (2010) stated that an in-vitro antibiotics combination that resulted in an additive or indifference interaction (0.5 to 4) could be as a result of each antibiotics acting differently as monotherapy while attacking the same or different target sites.

Many studies have reported the combinatory effect of plant extracts and an antimicrobial agent against *Vibrio* species. However, limited reports are available on dual or triple antibiotics combination against *Vibrio* species. Amongst the few, are studies conducted by Omoya and Ajayi, (2016); Trinh *et al.*, (2017), whose studies reported a significant decrease in bacteria growth when combination therapy was used. Their reports further concluded that the most successful treatment choice is combination therapy. The effect of antibiotics combination therapy against the three target *Vibrio* species carried out in this study was only successful against 18.8 % resistant *Vibrio parahaemolyticus* and *Vibrio vulnificus* isolates,

and 15.4 % resistant *Vibrio fluvialis* isolates. Olajuyigbe, (2012) carried out a study to observe the synergistic influence of tetracycline and amoxicillin against different resistant bacteria using checkerboard assay, and 87.5 % synergy was observed when antibiotics combination therapy was used compared to when used as monotherapy. Also, the *in-vivo* and *in-vitro* studies of Lin *et* al., (2016) and Tang *et al.*, (2018) revealed that antibiotics combination therapy is linked with high survival rate compared to when each drug is used as monotherapy, thus further stating that the combination of two or more antibiotics is a promising approach to curbing antibiotics resistance. Kim *et al.*, (2019) reported 75 % synergy when a tetracycline and fluoroquinolone combination was carried out against *Vibrio vulnificus.* This report is not in agreement with this present study where synergy was observed against 3 (18.8 %) out of the 16 tested *Vibrio parahaemolyticus and Vibrio vulnificus* isolates, and 2 (15.4 %) out of the 13 *Vibrio fluvialis* isolates. The study further stated that the combination of tetracycline and fluoroquinolone is a potent treatment for invasive *Vibrio vulnificus* infection.

The rate of kill analysis showed a reduction in the bacterial cell number. This analysis was carried out on the 8 *Vibrio* isolates against which synergy was observed. The trend of the kill was observed to rely on the concentration of antibiotics and exposed time. As observed in Figure 4.1b, 4.2a, and 4.4b, the highest number of bacteria cells killed was observed with the 2 x MIC (the highest concentration tested) after 6 hours of exposure time. CLSI, (2018) documentation revealed that about 99.9% of antibiotics killing impact after twenty-four hours of exposure is often used as a typical measurement of bactericidal efficacy of an antibiotic. In Figure 4.1a and 4.1b, against *Vibrio parahaemolyticus*, at 1 × MIC value of tetracycline and nalidixic acid combination showed an increase in the number of bacteria cells up until 4 hr of exposure time before the antibiotics began to take effect leading to a gradual reduction in the number of viable cells and at 24 hr of exposure time, the bacteria cells were eliminated. The same trend was also observed in Figure 4.1c, but a decline in the number of viable cell death

began to occur at the 6th hr. Against *Vibrio fluvialis*, as shown in Figures 4.3a and 4.3b, the combination of tetracycline and nalidixic acid at 1 × MIC was effective against the 2 bacteria isolates as the antibiotics began to take effect by showing a decline in the growth curve at the 4th hour of exposure time.

Against *Vibrio vulnificus*, different growth trends were observed. In Figure 4.2a, 4.2b, and 4.2c, tetracycline and imipenem combination at 1 × MIC had a different effect on the 3 isolates tested. the most effective was observed against isolate 1, where cell death began to occur at the 2nd hr, while against the remaining 2 *Vibrio vulnificus* isolates, a decline in the growth curve was observed at the 6th and 4th hr respectively. The combination of tetracycline and imipenem at 1× MIC was the most effective combination across all the tested combinations in this report. The results obtained revealed the bactericidal potentials of the combined antibiotics. This result was not compared with other studies due to a lack of similarities in tested antibiotics and bacteria of interest.

4.5. Conclusion

This research was carried out on the hypothesis that antibiotics combination therapy is not an option for the control of resistant non-cholera causing *Vibrio* species. The resistance to most monotherapy use of antibiotics of clinical importance as observed in this study can lead to difficulties in treating *Vibrio* infections. However, the results obtained in this study indicate that combination therapy is considered an option for treatment. The ability of these antibiotics' combination to inhibit the growth of some of the *Vibrio* isolates is an indication that tetracycline-nalidixic acid and the tetracycline-imipenem combination could be a promising treatment option compared to when used as monotherapy. Also, timely administration of the antibiotics is required to cease the progression of bacteria growth. To

find out whether the synergistic interactions obtained in this *in-vitro* study are clinically effective, a follow-up study involving an *in-vivo* test is further recommended.

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CHAPTER 5

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1. General Discussion

Vibrio species have been reported to play major roles in diarrhoea disease and their direct link to septicemia, gastroenteritis and wound infections have been established globally (Thompson *et al.*, 2004; Jay *et al.*, 2005). The increased number of environmental studies has resulted in a greater understanding of *Vibrio* species (Romalde *et al.*, 2014).

This research investigated the antibiotic combination therapy option for the control of resistant non-cholera causing *Vibrio* species. Farming is an activity most people in this region practice, therefore, the spread of resistant organisms in the ecosystem is possible because humans and animals carrying these resistant organisms are capable of spreading them to the environment. Water contamination as a result of urban waste is an important environmental issue in South Africa (Osode and Okoh, 2009). Akanbi *et al.* (2017) stated that humans and animals are key sources of antibiotic-resistant organisms in the environment. For technical and economic reasons, the evaluation of water safety by checking for the existence of many pathogens is usually impractical, so indicator species are mostly used for routine monitoring of pathogens present in water (DWAF, 2012).

About 228 environmental isolates of *Vibrio* were confirmed and the most prevalent species observed was *Vibrio parahaemolyticus*, where 100 (44 %) isolates were confirmed. 82 (36 %) isolates were confirmed to be *Vibrio vulnificus* and 46 (20 %) were confirmed to be *Vibrio fluvialis*. The isolation and confirmation of these *Vibrio* species confirms the existence of potentially pathogenic bacteria in the environment. Antimicrobial resistance is one of the significant health dilemmas as it is directly related to disease control (Ramamurthy, 2008). Several pathogens are infamous for resistance to many antibiotics, including several species of *Vibrio* (Ahmed *et al.*, 2004; Rowe-Magnus *et al.*, 2006). Waterborne infections that are

associated with *Vibrio* species have resulted in an increased mortality rate worldwide. Despite the availability of antibiotics for disease management, antibiotics resistance is a limiting factor to effective treatment (Baquero *et al*., 2008).

Originally, tetracycline was the appropriate antibiotic for treating severe *Vibrio* infections because of its ability to bind to the 30S ribosomal subunit of an organism and inhibit protein synthesis. However, studies have reported increased resistance to tetracycline (Labella *et al*., 2013). In recent times, fluoroquinolones, carbapenems and aminoglycosides amongst other antibiotics have been recommended as another treatment option (CDC, 2013). An antimicrobial susceptibility test carried out in this study revealed that a high level of resistance to tetracycline, nalidixic acid, and imipenem was observed. Resistance to these antimicrobial agents is not a recent development as the result observed in this study is in corroboration with the findings of Quilici *et al.* (2010); Mandal *et al.* (2012); Choudhury *et al.* (2012); Mahmud *et al.* (2014); Baron *et al.* (2016); Okoh and Igbinosa, (2010); Osuolale and Okoh, (2018), where the resistance of *Vibrio* species to tetracycline, imipenem, and nalidixic acid was observed. However, the findings of Srinivasan *et al*., (2006); Singh *et al*., (2014) revealed a result that is contrary to the findings of this present study. MARP and MARI was evaluated and the result obtained revealed 38 different MARP patterns across all the *Vibrio* isolates tested. Most of the isolates were resistant to over 3 antibiotics with a MAR index value that ranged from 0.3 to 0.8. This MAR index value is greater than the 0.2 acceptable threshold value used to determine both low-risk and high-risk regions of antibiotics usage. MIC and MBC were carried out using tetracycline, nalidixic acid, and imipenem at varying concentrations to determine the concentration that was able to inhibit and eliminate bacteria growth. These varying concentrations successfully inhibited bacteria growth. MBC value from 256 µg/ml to 1024 µg/ml were observed against *V. parahaemolyticus* and *V. fluvialis*, while MBC from 32 µg/ml to 4096 µg/ml against *V. vulnificus*.

Combination therapy is when two or more antibiotics are used together in combination for effective treatment of antibiotic-resistant bacterial infections. Checkerboard assay and rate of kill are two methods used in this research to find out the synergistic impact of combined antibiotics (tetracycline-nalidixic acid and tetracycline-imipenem). The combination of tetracycline and imipenem yielded a synergy on 3 of the 16 resistant *V. vulnificus* isolates. Synergy was also observed against 3 of the 16 resistant *V. parahaemolyticus* isolates and 2 out of 13 resistant *Vibrio fluvialis* isolates when nalidixic acid and tetracycline were combined. The rest of the combinations had an indifferent interaction against other isolates. Several Scientists have associated combination therapy with decreased mortality rate while others have argued against its use as a therapy option. However, findings from this report are following the studies of Olajuyigbe, (2012); Morrill *et al.* (2015); Tang *et* al., (2016); Omoya and Ajayi, (2016). Their findings and result from this present study revealed that the combination of dual or triple antibiotics for the treatment of bacterial infection is a promising approach to antibiotic resistance as this has proven to be more effective than when antibiotics are used as monotherapy.

The rate of kill analysis showed a decline in the number of viable bacterial cells with respect to time. This analysis was carried out on the 8 *Vibrio* isolates against which synergy was observed. The number of viable cells was calculated after every 2 hr interval up until the 24th hr. At 2 × MIC, a decline in cell number occured across the *Vibrio* species tested after 6 hr of antibiotics exposure time, while at 1 × MIC, the number of viable cell death began to occur at varying time intervals ranging from 2 hr to 6 hr exposure time. CLSI, 2018 documentation stated that ninety-nine percent killing impact of antibiotics is often used as a typical measurement of the bactericidal efficacy of an antibiotic. The trend of kill in this study was observed to be dependent on the concentration of antibiotics with respect to the exposure time.

5.2. Conclusion

The working hypothesis of this study is that antibiotics combination therapy is not an option for the control of resistant non-cholera causing *Vibrio* species from the environment. The environment plays a significant role in the growth and spread of resistant bacteria. The environment is easily contaminated as a result of pollution and other activities caused by humans, causing selective pressure for resident bacteria by making them evolve different mechanisms for resistance. The resistance to most antibiotics of clinical importance as discovered in this research can lead to difficulties in treating *Vibrio* infections. The result from this study revealed the therapeutic potential of tetracycline-imipenem and tetracycline-nalidixic acid combination. The bactericidal activity exhibited by the combined antibiotics in this study serves as a potential treatment option for infection control. Therefore, it can be concluded that combination therapy is considered an option for treatment.

5.3. Recommendation

- To find out whether the synergistic interactions obtained in an *in-vivo* test correlate with the findings of this *in-vitro* study, a follow-up study involving an *in-vivo* test is recommended.
- There is a need to investigate the bactericidal potential of tetracycline-imipenem and tetracycline-nalidixic acid on a wider range of environmental bacteria isolates for an accurate evaluation of the therapeutic potential of the combined antibiotics.
- The prescription of antibiotics should be regularly monitored by health officials to avoid the development of resistance by these organisms in humans and animals.
- The use of two or more antibiotics for treatment may help clinicians choose effective antibiotics for empirical therapy and it may help reduce the development of resistance to antibiotics when used as monotherapy.
- Wastewater treatment plants should be monitored regularly to avoid the release of improperly treated effluents into receiving water bodies in the environment and also to ensure their compliance with the existing laws and regulations guiding their operations.

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