UNIVERSITY OF NAIROBI

DIVERSITY OF *VIBRIO CHOLERAE* BACTERIOPHAGES FROM LAKE VICTORIA, COASTAL AND CENTRAL REGIONS OF KENYA AND ASSESSMENT OF THEIR POTENTIAL AS BIO CONTROL AGENTS AGAINST THE PATHOGEN

BY

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DECLARATION

I declare that this thesis is my original work and has not been submitted elsewhere for examination or award of a degree. Where other people's work has been used, this has been properly acknowledged and referenced in according with the University of Nairobi's requirements.

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DEDICATION

This Thesis is dedicated to my mother Monika Njoki and my family members. Thank you for your incredible support throughout my Academic life.

God richly bless you

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Ad	-	Adaptor
AGP	-	Antibiotic growth promoters
APW	-	Alkaline peptone water
AWD	-	Acute watery disease
BLAST	-	basic local alignment search tool
Bp	-	Base pairs
$CaCl_2$	-	Calcium Chloride
cAMP	-	Cyclic adenosine monophosphate
CDC	-	Centre for disease control
CDS	-	CoDing Sequence
CFR	-	Case Fatality Rates
CSLC	-	carrier state life cycle
CT	-	Cholera Toxin
CTX	-	Cholera toxin
ddH_2O	-	double distilled water
DNA	-	deoxyribonucleic acid
ds	-	double stranded
EDTA	-	Ethylene diamine tetra acetic acid
ESAR	-	Eastern and Southern Africa Region
EU	-	European Union
FEWS NET	-	Famine Early Warning Systems Network
FDA	-	Food and Drug Administration
g	-	Grams
Gbp	-	Glucosamine-binding protein
GTFCC	-	Global Task Force on Cholera Control
GTP	-	Guanosine 5'-triphosphate
h	-	Hour
Hc	-	Head closure
HCl	-	Hydrogen Chloride
HPP	-	High pressure processing
H_2O	-	Water
ICE	-	Integrating conjugative element

Kg	-	Kilograms
KMTC	-	Kenya Medical Training College
L	-	Liters
LB	-	Luria Bertani
LPS	-	Lipopolysaccharide
LT	-	Large terminase
M	-	Molar
MCP	-	Major capsid protein
MDa	-	Mega Diatons
MDRB	-	Multidrug-resistant bacteria
MEGA	-	Molecular Evolutionary Genetics Analysis
$MgSO_4$	-	Magnesium Sulphate
min	-	Minutes
ml	-	Milliliters
mM	-	Millimolar
MTP	-	Major tail protein
NaCl	-	Sodium Chloride
NaOH	-	Sodium Hydroxide
NCBI	-	National Center for Biotechnology Information
Ne	-	Neck protein
ng	-	Nanograms
OCV	-	Oral cholera vaccines
OMP	-	Outer membrane protein
ORS	-	Oral Rehydration solution
ORT	-	Oral rehydration Therapy
PCR	-	Polymerase Chain Reaction
PFU	-	Plaque forming unit
qPCR	-	Quantitative PCR
rRNA	-	Ribosomal RNA
RnR	-	Ribonucleotide reductase
RO	-	Reverse osmosis
SDS	-	Sodium dodecyl sulfate
SM	-	Saline-Magnesium

SSA	-	Sub Saharan Africa
ss	-	Single stranded
TAE	-	Single stranded
TCP	-	Toxin coregulated pili
TEM	-	Transmission Electron Microscopy
VPI	-	*Vibrio* pathogenicity island
WHO	-	World health organization

ABSTRACT

Cholera, a devastating diarrheal disease that accounts for more than 10% of children's deaths worldwide, has persistently remained a health threat to world's population at large. It is endemic in many countries and in Sub-Saharan Africa, prevention has not achieved much because the causative agent, *Vibrio cholerae* has emerged to be resistant to antibiotics that were previously efficacious towards the bacteria. Previous research has shown that phages may offer an alternative plausible biological control method for management of cholera. In this study potential exploitation of bacteriophages as antimicrobials to control toxigenic *Vibrio cholerae* El Tor was evaluated. The goal of the study was to isolate novel lytic *Vibrio cholerae* bacteriophages from environmental water sources of Kenya that included: Lake Victoria region, Coastal region, Nairobi and Central regions. Pathogenic *Vibrio cholerae* El Tor was isolated from these environmental water sources and used as the respective propagating strain for isolation of phages. Polymerase chain reaction amplification and sequencing of partial 16S ribosomal RNA gene was used for identification of the bacteria. In total, 140 surface water samples were obtained from ponds, rivers, lake, beaches, boreholes, springs, wells and Indian Ocean. Lytic spectrum confirmed that all the 15 phages displayed a lytic activity against different environmental *Vibrio cholerae* strains as well as a clinical strain. Further characterization by Transmission Electron Microscope assigned the vibriophages to order *Caudovirales* of *Myoviridae* family owing to their icosahedral capsids and contractile tails. From a total of 15 vibriophages, 4 were selected for complete nucleotide genome sequencing using various bioinformatic tools and restriction digestion profiles. The four full, linear phage genomes: vB_Vch4M_Ke, vB_Vch6M_Ke, vB_Vch18M_Ke, and vB_Vch24M_Ke had highly similar genome sizes of 148180bp, 148181bp, 148179, and 148179bp respectively, 4 tRNAs each, same G+C content of 36.4% and similar molecular weight of approximately 91.54MDa. The four genomes carried the *phoH* gene, which is

overrepresented in marine cyanophages. The bacteriophage, vB_VchM_Kuja complete genome sequence was deposited in the NCBI nucleotide databank and assigned; MN718199.1 as the accession number and corresponding Refseq record is: NC048827.1. It was assigned to the subfamily *Ackermannviridae* according to the current classification of viruses. These novel lytic vibriophages represent potential biocontrol candidates for water decontamination against pathogenic *Vibrio cholerae* and ought to be considered for future studies of phage therapy.

Key words**:** *phoH; Vibrio cholerae;* vB_VchM_Kuja; vibriophages; *Myoviridae; Ackermannviridae.*

CHAPTER ONE: INTRODUCTION

1.1: Historical background of bacteriophages

Bacteriophages or "phages", most abundant forms of life in the biosphere are viruses that recognize and infect bacteria specifically. They outnumber bacteria in order of magnitude by an estimated factor of one (Cobián-Güemes et al., 2016). The history of bacteriophages dates back in 1896 when a British bacteriologist, Ernest Hanbery Hankin, demonstrated the presence of a biological principle in the waters from rivers Ganga and Yamuna in India that destroyed the bacteria and caused cholera infection. Larger microorganisms such as bacteria would be retained but the substance passed through millipore filters (Summers, 2014). However, Hankin did not pursue his dream.

In 1915, a British bacteriologist, Fredrick Twort noted that pure culture of bacteria may be associated with a filter–passing transparent material that may entirely breakdown bacteria of a culture into granules. The Author described the substance secreted by microbes a transparent material found to be unable to grow in absence of bacteria as ferment (Summers, 2014). The results of Fredrick Twort were published but due to shortage of funds and the First World War, the work was disrupted.

Independently, in 1917 similar experimental findings were described by Felix d' Herelle. Through filtering stools that were incubated for 18 hours, the Author isolated 'anti-Shiga' microorganism from stools of patients recovering from shigellosis. He was undertaking a study of patients suffering or recovering from bacillary dysentery. He described his discovery as a microbe that was 'veritable' microbe of immunity and an obligate bacteriophage (Summers, 2014). It was d'Herelle who coined the term bacteriophage meaning bacteria eater. In order to ascertain their safety, in 1926 he carried out self-ministration of phage preparations, gave to his colleagues and family members before ministering the phage preparations to human patients. He was invited for cholera treatment studies by British government to the Haffkine Institute, Bombay, India after four patients were treated of bubonic plague in Egypt, a visit that resulted in establishment of 'The bacteriophage inquiry'. Due to deficiency understanding of the phage-host interactions, low quality of some of the scientific studies, coupled with introduction of antibiotics, there was discontinuation of phage therapy in the Western world (Summers, 2014). The Eliava Institute was opened in Tbilisi, Georgia in 1923 for study of phages and development of bacteriophage therapy. The study on

the application of bacteriophages in medical field was nearly wiped out in 1930s when antibiotics were discovered and found to be effective.

However, production of some bacteriophage based prophylactic and therapeutic preparations on commercial basis continued in Western Europe, Africa and the United States through the 1950s and 1960s despite introduction of antibiotics (Sulakvelidze & Alavidze, 2001). Phages self-restraint their numbers at the location of infection by the host bacteria contrary to antibiotics as they replicate on the undesirable pathogen disintegrating into their protein subunits when the bacterial host is lysed (Rohde & Wittmann, 2020). Bacteriophages have reemerged as a promising legitimate alternative to fight this international predicament in the antibiotic dilemma in which bacterial multi-drug resistance as well as pan-drug resistance is increased adequately. However, the implication of the discovery is that bacteriophages have received center of interest for prevalent research afresh. In spite of bacteriophages having been used for more than a centennial, state-of-the-art bacteriophage research is essential in addition to the case of systematic clinical trials as well as in all fields of phage administration. More knowledge on bacteriophage diversity as well as taxonomy, their mode of host interaction, their structure–function interrelationship and their genotype–phenotype association will benefit research (Rohde & Wittmann, 2020). While discussing bacteriophage therapy, sole important point that has to be kept in mind is that bacteriophages complement their actions and finally strengthen the antibacterial effect on the whole but should not generally replace antibiotic drugs in therapy (Rohde & Wittmann, 2020).

Phage diversity is huge as already revealed by projects analysing the metagenomes of different habitats; there is always something new to discover with regard to habitat diversity and to bacterial species still underrepresented in terms of bacteriophage research. Bacteriophages have also played a considerably crucial role in the development of molecular biology on top of their impact on ecology and evolution. In order to find new ways to efficiently use bacteriophage diversity for different applications and phages as tools, research is going on. Bacteriophages have served as appreciated model systems and are a success story of nature, entailing for human disease like for example Alzheimer's disease, acquired immunodeficiency syndrome (AIDS) and in the year 2020, the SARS-CoV-2 pandemic (Rohde & Wittmann, 2020). Apart from anti-bacterial objectives, bacteriophages are once again in the focus in light of the current Covid -19 pandemic. Automation vaccines producible in a period of a few weeks are indispensable in a virus pandemic as well as the vaccine

response to unfolding RNA viruses like SARS-CoV-2 might be improved through application of bacteriophages (Rohde & Wittmann, 2020). According to Serwer (2020), application of coronavirus-like bacteriophages as pathogen homologs for development of model platforms; bacteriophages can be evolved which bind to particular antibodies against membrane-covered RNA viruses.

1.2: Chronology of cholera

In 1817, the first cholera pandemic begun from South East Asia, Bengal region of India, Calcutta, spreading across India by 1820 thereafter to other parts of the globe. In the course of the past 200 years, seven cholera pandemics have occurred. Six worldwide cholera pandemics occurred between 1817 and 1923. The documented or presumed etiological agent of the first six pandemics was *Vibrio cholerae* O1, the classical type. The causative agent of the seventh pandemic that appeared in 1961 in Indonesia since spreading worldwide particularly to South Asia in 1963, Africa in 1970, Latin America in 1991 and the Caribbean (Haiti) in 2010, *Vibrio cholerae* El Tor biotype has been described (Weill et al., 2017). Accordingly, recent high definition genome documentation and isolated historical records stipulate Bay of Bengal as the major hub linking the spread of cholera around the globe during the 19th and early 20th centuries (Moore et al., 2014). There were worldwide outbreaks in the early 1990s in South and Central America. For more than three years, cholera has persisted in Haiti with seasonal exacerbation of the epidemic occurring during the rainy season. Historically, the disease had never been documented previously in the country and the epidemic constitutes the largest national epidemic of the seventh pandemic. The epidemic strain in Haiti is O1 biotype El Tor serotype Ogawa, a variant of *Vibrio cholerae* (Moore et al., 2014). The continent of Africa is mostly affected by the present seventh cholera pandemic. The first Ogawa strain was introduced in West Africa in 1970 (Guinea, Sierra Leone, Liberia and Ghana) (Weill et al., 2017).

1.3: The cholera disease

Acute infectious diarrheal diseases remain among the most frequent causes of childhood deaths, accounting for 10-12% of the death toll for children under the age of five years and around 1.4-1.9 million fatalities worldwide (Levy et al., 2016). The disease cholera is devastating, causing watery diarrhea that can lead to severe dehydration and if untreated, death. It is mainly caused by O1 and O139 toxigenic *Vibrio cholerae* serotypes. The disease is

spread through faecal-oral route and hence strongly associated with poverty, poor hygiene, clean water shortage and lack of adequate sanitation facilities (Deen et al., 2020). *Vibrio cholerae* is a motile single flagellated, Gram-negative curved rod-shaped bacterium belonging to *Vibrionaceae* family. Naturally, worldwide, *Vibrio* species inhabit the coastal, estuarine as well as marine environment (Letchumanan et al., 2015a). Occurrence of this bacterium in the marine environment is perturbation of human on food safety owing to the potential leading to cholera outbreaks depending on the surrounding conditions (Ceccarelli et al., 2013). Worldwide, each year approximately 2.9 million cholera cases occur (Somboonwit et al., 2017). An estimated 1.8 billion are still at risk for cholera due to lack of potable water, inadequate sanitation and hygiene (Somboonwit et al., 2017). However, the World Health Organization (WHO) estimates that only 5–10% of the cases occurring annually are officially reported (Ali et al., 2012).

In Asia, South America and Africa, cholera is endemic, coincidence of outbreaks usually corresponding to and/or emerging from conflict and natural catastrophe (Martinez et al., 2010). Outbreaks, in South America and African countries take place after summer rains or floods (Emch et al., 2008).

In Africa from 2010, there has been cholera outbreaks and statistics show that the highest number of cases was in 2017 with 5654 deaths and 1,227,391 cases were revealed in accordance with the cholera yearly review 2017 (WHO, 2018). According to Cholera Bulletin (Africa, 2019), roughly, 1,681 cholera / Acute watery disease (AWD) cases including thirteen deaths were announced in seven arising out of the twenty one countries in Eastern and Southern Africa Region (ESAR); with 0.8% as an average Case Fatality Rate (CFR), from the onset of the year 2019. Angola, Burundi, Kenya, Tanzania, Somalia, Uganda and Zimbabwe are among these countries. Outbreaks from the rest of these countries spilled over from 2018, apart from Kenya. Out of the total case load recorded in the year, 2019, Kenya accounted for 51.3 % (863) followed by Somalia at 31.2% (524). In 2019 the highest case fatality rate was recorded in Zimbabwe (6.2%), followed by Uganda (5.7%) then Tanzania (2.9%). In many African countries, cholera remains a heavy disease burden and it is mostly escalated during heavy rains that occur every year when cholera springs up killing many.

Kenyan populace has suffered from cholera outbreaks for the last decade due to heavy rains, poor sanitation coupled with inadequate availability of decontaminated, potable water for

consumption especially for poor communities. Since December 2014, a cumulative total of 10,568 cases were reported in 2015 and 6,448 in 2016. Kenya endured a gush in cholera cases traversing the territory in the year 2017 with an inclusion of urban outbreaks in Nairobi the capital city. During the said period of time, there were 76 deaths with a total of 3,967 cases reported across twenty of forty-seven counties (43%) in the entire nation of Kenya. Seven counties continued to experience active cholera outbreaks throughout the year 2017. There have also been cholera outbreaks in refugee camps in Kenya like Dadaab, Kakuma and Kalobeyei refugee camps (WHO, 2018).

In 2018, March to May long rains in Kenya were the heaviest in the past 55 years (FEWS NET, 2019:p.1). During the period, the report by the World health organization indicated that in total cases of cholera were 5719 in addition to 78 deaths revealed (WHO, 2019). Out of 47 counties, 20 were affected including: Mombasa, Kirinyaga, Garissa, Siaya, Tharaka Nithi, Meru, Tana River, and Turkana.

In the course of the eighth week of January 2019, in Kenya, nineteen new cholera cases were reported in comparison to forty-two cases that included one death (Case Fatality Rate, 2.4%) as demonstrated in the seventh week. Emergence of new cases was from the county of Kajiado. The total cases accumulated to 863 among them 34 were confirmed while the deaths were three (Case Fatality Rate, 0.4%) that was the report since the emergence of the state of the art surge of the cholera outbreak in the year 2019 on second January. Owing to shortage of clean potable water from alternative sources, inadequate or no sanitation, dependency on river water from Ewaso Nyiro for domestic purposes which is already contaminated and unhygienic behavior like open defecation, such are factors that escalate transmission risks.

Cholera mostly affects poor communities with low socioeconomic status. The residential populace most vulnerable is the one residing in congested zones with scanty safe water and sanitation services, among others undesirable mass expansion. Worldwide, according to 2017 World Health Organization- United nations Childrens' fund collective yearly report on water and sanitation (based on 2015 data), the indication was that the population that lack access to potable water include 663 million as well as above two billion people consuming water that has been contaminated with faucal matter (WHO, 2018).

About 58% populace living in sub-Saharan Africa, approximately 159 million individuals fetch water from environmental sources that are unreliable. Frequent cholera outbreaks have

had a significant impact on the livelihoods of communities' in spite of the efforts that have been made towards reduction in transmission through improvement in accessing potable water. Since cholera remains a global threat, the world urgently requires an alternative solution to prevent these outbreaks especially in Africa. The worst epidemics in terms of suspected case totals are happening in the Democratic Republic of Congo, Zambia, Mozambique, Kenya and Uganda.

Figure 1.1 shows 'cholera cases reported in Africa in 2017' (WHO, 2018:p.492). Among the seven Sub-Saharan African countries that reported outbreaks of cholera between the years 2017 and 2018 included, Cameroon, Democratic Republic of Congo, Tanzania, Kenya, Mozambique, and Zambia. Recently, Zimbabwe has also experienced cholera outbreak.

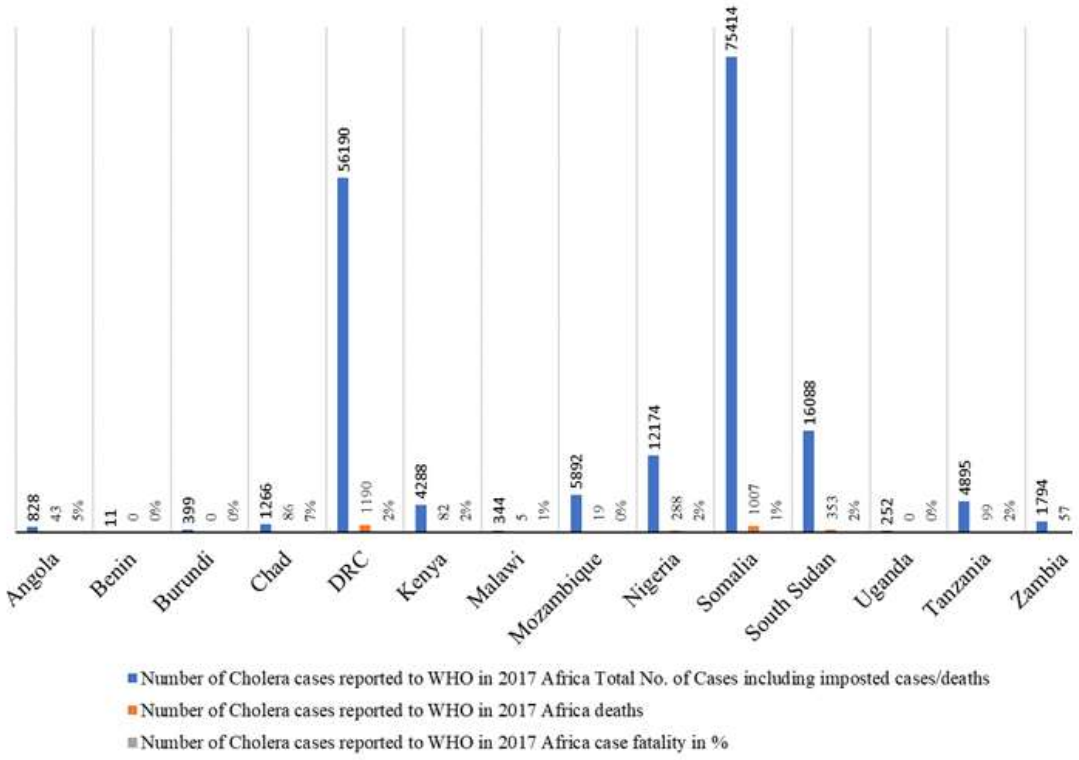


Figure 1.1: Cholera cases reported in Africa in 2017.
Source: WHO (2018:p.492)

An initiative entitled "ending cholera" was commenced in the month of October 2017 by the Global Task Force on Cholera Control (GTFCC). A comprehensive guideline for 2030 was set with a target of bringing down loss of livelihood due to cholera by 90% globally and eradicate cholera in more than twenty countries.

An all-inclusive preference list of pathogens that took into account twelve species of bacteria designated into medium, high and critical on the basis of their resistance level as well as accessible therapeutics was issued by the WHO in 2017 (Rello et al., 2019).

1.4: Statement of the problem

The pathogenic strain of the bacterium *Vibrio cholerae* is the causative agent of the devastating diarrheal disease cholera. Cholera disease is linked to poverty, insufficient enough potable water and proper sanitation (Faruque et al., 2005a). Diarrheal diseases including cholera are the second leading in terminating lives of children under five years and killing nearly 525,000 children on yearly basis (Weill et al., 2017). Published reports (Mutonga et al., 2013; Oyugi et al., 2017) show that communities in Lake Victoria, coastal and some parts of central regions of Kenya frequently suffer from diarrheal diseases, including cholera outbreaks especially after heavy rains. Domestic sources of water get contaminated with the pathogenic bacterium Vibrio cholerae. These communities lack clean, potable water for domestic purposes and other basic essential amenities of life. In Kenya, rainfall patterns have become erratic and unpredictable due to climate change. Water availability for both farming and domestic use is a great challenge. During heavy rains, overflow from pit latrines and runoff in areas with few or no pit latrines are common causes of contamination of water bodies. This causes multiplication of Vibrio cholerae if present, in these water bodies that end up being consumed by humans. Some cases are not reported and therefore the data may not be accurate on deaths due to cholera infections. Some communities do not gain access to basic health treatment because of infectious diseases. Furthermore, the bacteria become resistant to antibiotics, commonly available and Multi drug resistance (MDR) in Vibrio cholerae has been reported in Kenya (Onyuka et al., 2011). This therefore requires an alternative treatment of water bodies both in the reservoir as well as in the infected individuals. Availability of clean and potable water throughout the year is a big challenge to the poor communities in Kenya. According to the Kenya National Bureau of Statistics, the Kenya Population and Housing Census data in 2019 revealed total enumerated population was 47,564,296 of which 23,548,056 were Males, 24,014,716 were Females and 1,524 were Intersex. These figures translate to an increase number of people who do not have access to clean potable water and other essential amenities.

Generally, in Africa unpolluted water fit for human consumption is inaccessible to all inhabitants and existence of poor sanitation hence, cholera is prevalent. From 1995 to 2005,

Africa reported the largest and most frequent cholera outbreaks followed by Asia. Therefore, cholera is threatening in most developing countries in Africa and Asia. In the year 2005, estimation was done that showed above 1.8 million human beings passed on because of diarrheal diseases, the world health organization reported in the March 2007 fact sheet (Sillankorva et al., 2012). According to various reports, there has been an upsurge of cholera epidemics for the past ten years in Kenya (Cowman et al., 2017; Mutonga et al., 2013; Maina et al., 2014; Oyugi et al., 2017; Saidi et al., 2014).

1.5: Justification of the study

Multi drug resistance (MDR) in *Vibrio cholerae* has been reported frequently, usually after acquisition by strains of a conjugative plasmid (Das et al., 2019). Previous reports have showed that other genetic elements such as class 1 and SXT element carry genetic determinants for antimicrobial resistance (Das et al., 2019).

Bacteriophages that infect *Vibrionaceae* family of bacteria are known as vibriophages. Phages that lyse the *Vibrio* species acknowledged as crucial motivation towards the adaptation of *Vibrionaceae* giving rise to the development of disease causing and genus ecological-succession. The pandemic disease cholera is caused by the bacterium *Vibrio cholerae* while the virulence of the bacterium is encoded by viral genes of the *Vibrio cholerae* bacteriophages (Chibani et al., 2019). Due to its medical and economic importance, the *Vibrionaceae* family is an excellent test for sequence based characterization method and potential identification (Chibani et al., 2019). Therefore, to isolate, characterize and identify both the bacterium and the bacteriophages are of ultimate importance in the current study.

However, little is known about biocontrol of *Vibrio cholerae* application of vibriophages. Use of vibriophages for biocontrol of *Vibrio cholerae* has not been adequately assessed. Therefore, use of vibriophages as alternative biocontrol agents against the pathogen will help prevent cholera outbreaks in Kenya. This will provide clean and potable water to poor communities residing in cholera prone areas, hence prevention of outbreaks.

Looking into this contest, the current research work took place in conjunction of an overall goal to isolate as well as characterize vibriophages in the regions where cholera is endemic. The study was to investigate the potential of the phages for use as biocontrol agents against the bacterium. The results obtained in this study may help to prevent major cholera outbreaks

in the future by providing a way of controlling *Vibrio cholerae* populations in domestic sources such as wells for communities residing in these regions.

1.6: Research questions

1. What are the characteristics of pathogenic *Vibrio cholerae* bacterium isolated from the environmental waters of Kenya?
2. What is the identity and the phenotypic characteristics of the diverse vibriophages to be used as biocontrol agents against *Vibrio cholerae*?
3. What are the genomic characteristics of isolated vibriophages?
4. What are the lytic characteristics of isolated vibriophages against different pathogens?

1.7: Objectives

1.7.1: General objective

To isolate a diversity of *Vibrio cholerae* bacteriophages from four regions of Kenya and explore their use as bio control agents against the pathogen. These regions included: Lake Victoria region (Kisumu, Kisii and Migori), Siaya region (Bondo and Rarieda), Coastal region (Kaloleni, Ukuda, Ribe, Rabai, Maliakani, Likoni and town center), and Central region (Nairobi, Kiambu and Murang'a). Environmental water samples were taken from water bodies: ponds, rivers, Lake Victoria, beaches, boreholes, springs, wells and Indian Ocean.

1.7.2: Specific objectives

a. To isolate and characterize pathogenic *Vibrio cholerae* bacterium from the environmental waters of Kenya.
b. To isolate, identify and characterize a diversity of vibriophages for use as biocontrol agents against the pathogen.
c. To carry out genomic characterization of candidate vibriophages.
d. To conduct host range profiles of isolated vibriophages.

1.8: Hypothesis

There are no vibriophages in the environmental waters of Kenya able to lyse *Vibrio cholerae* strains isolated from the same regions.

CHAPTER TWO: LITERATURE REVIEW

2.1: Characteristics of *Vibrio cholerae* bacterium

Vibrio cholerae is a Gram negative, coma shaped rod, motile with a single polar flagellum and facultative anaerobe bacterium. It requires salt, Sodium Chloride (NaCl) for its growth, exists naturally in aquatic environments and may enter in a viable but non-culturally form (Alam et al., 2007).

The disease-causing strains of *Vibrio cholerae* are grouped into two serogroups: O1 and O139. This grouping is based on their lipopolysaccharide (LPS) O antigens. Serogroup O1 is further divided into three serotypes: Inaba, Ogawa and Hikojima and two biotypes Classical and El Tor. Serogroup O1 and O139 pandemic strains exist as natural inhabitants of aquatic ecosystems, making them facultative human pathogens (Yen & Camilli, 2017).

An enterotoxin (CTX) that causes severe, fluid rice diarrhea is produced by the bacterium *Vibrio cholerae*, the etiological agent of cholera that is attributed to the consumption of faecally contaminated food or water. The patient loses as much as 20 liters of fluid per day and if not treated on time may lead to death due to dehydration. Virulence factors of *Vibrio cholerae* are encoded within mobile genetic elements and were horizontally acquired by the pathogenic strains. Cholera toxin (CT), the source of profuse watery rice diarrhea, is encoded within the CTX lysogenic phage and toxin-coregulated pilus (TCP), an essential colonization factor, is encoded within vibrio pathogenicity island 1 (VPI-1) (Sakib et al., 2018). Other factors, such as N-acetylglucosamine-binding protein A (GbpA), an adhesin involved in attachment to intestinal epithelial cells, and the inner membrane-localized virulence regulator ToxR, are encoded in the core genome of both clinical and environmental strains (Sakib et al., 2018).

2.2: Cholera pathogenesis

Upon attachment during infection, the bacterium induces cholera toxin that causes diarrheal discharge. The resulting increased chloride ion secretion and net water flow into the gut lumen and decreased sodium ion absorption into the tissues via the blood stream is attributed to cholera toxin. As a consequence, there is speedy deprivation of fluid into the lumen in addition to chloride ions causing enormous diarrhea and electrolyte disproportion (Hodges & Gill, 2010).

The channels of chloride ion and sodium change due to high levels of cyclic adenosine monophosphate (cyclic AMP) an intracellular mediator that signals cAMP synthesis and pathways through catalysis by enzyme adenyl cyclase. The G proteins (Guanosine nucleotide-binding proteins) regulate the activity of adenyl cyclase, the intracellular signaling proteins (Neves, 2002). By active G proteins the CT keeps adenyl cyclase activated continuously. The G proteins in the gastrointestinal track are in a functioning condition when they are GTP (guanosine 5'-triphosphate) confined also upon hydrolysis of GTP by GTPase they are no longer functional (Alberts et al., 2002). A toxin stops GTP hydrolysis when CT affects the gastrointestinal tract such that the G proteins are operational throughout (Hodges & Gill, 2010). After sometime, the levels of cAMP are increased that modify ion channels generating an enormous effluence of chloride ions along with fluid into the intestinal track giving rise to acute diarrhea.

The disease commences with manifestation such as muscle and stomach cramps accompanied by fever and vomiting. Enormous dehydration is caused by the prolonged absorption of fluid as well as electrolytes that lead to the cholera clinical symptoms (Sharmila & Thomas, 2018). As the disease progresses, there is change of the skin of the affected individual that changes to blue discoloration because of massive dehydration thus the term "blue death" (Sharmila & Thomas, 2018).

2.3: Treatment of cholera

Treatment of cholera patients involves administering oral or intravenous fluids to prevent dehydration of the patient and restore body fluids for normal functioning. Over the last 20 years, oral rehydration therapy (ORT) has become one of the greatest success stories in the annals of medicine (Echenberg, 2011). Oral rehydration is a concoction containing sugars, salts and other products combining to restore life-giving fluids that is simple, effective and inexpensive (Echenberg, 2011). Sufficient rehydration is the pivotal to therapy until the disease has finished its course (generally within a period of one to six days with exclusion of antibiotics). Just like other cases of dysentery and diarrhea, process of restoring the body fluid can be achieved by oral fluid restoration with oral rehydration solution (ORS). Infected patients, in extreme conditions with modest sickness and dehydration, can obtain oral fluid substitution to make up for the water and electrolyte exhaustion. For moderate situations, the recommendation by the world health organization is 50 millimeters of oral rehydration solution per kilogram of body weight be administered initially at the 4h. The quantity should

be escalated twice, for moderate dehydration in other words one hundred millimeters per kilogram mixture of fluid will be essential.

Intravenous rehydration is done instantly for grown-ups; two liters is dispensed the first thirty minutes. The rate of infusion can be lowered to 100ml/kg of the body weight initially at four hours of treatment if the condition of the patient indicates an improvement. During the first hour, children should be injected with thirty millimeters of the fluid per kilogram of body weight plus an additional forty millimeters per kilogram in the after two hours.

Even though the oral rehydration therapy is available throughout the world, devastating deaths still occur today due to cholera especially the disadvantaged population (Echenberg, 2011). Treatment of cholera is a challenging endeavor. A combination of antibiotics with rehydration therapy relieves the symptoms of cholera and shortens the disease duration. Unfortunately, environmental drug-resistant *V. cholerae* strains have been recently reported, hampering the treatment option for cholera and urgently calling for adjunct or alternative approaches (Loo et al., 2020), such as bacteriophage therapy.

2.4: Antibiotic therapy and resistance in *Vibrio cholerae*

Multidrug resistance as well as antibiotic resistance are rising problems worldwide as more species of microorganisms such as protozoan, bacteria, virus, yeasts and moulds acquire resistance to presently accessible medicinal alternatives. In particular, issues increasingly arise associated with prolonged communicable illness, raising fatality, biosecurity issues, animal health, and outbreaks of foodborne disease (Garvey, 2020). The antimicrobial resistance (AMR) crisis is like a virus pandemic advances sweeping all boundaries (Rohde & Wittmann, 2020). The use of antibiotics in treatment of cholera patients with severe dehydration is supported by several studies that show antibiotics can reduce the diarrhea spell by one plus half a day, decrease the portion of feces down to fifty percent thus lessening length of discharging *Vibrio cholerae* to one to two days (Leibovici-Weissman et al., 2014). Synergetically, combination of antibiotics produces effect of cholera treatment (Mandal et al., 2009).

One major concern that governs recommendations for antibiotic use is the risk of antibiotic resistance resulting from overuse in those patients who are unlikely to benefit with an illness that is self-limited and, in the vast majority of cases, resolves with appropriate supportive care

alone. This is particularly important now that resistance to all classes of drugs used for *Vibrio cholerae* has been documented and no new antibiotics are currently in development for *Vibrio cholerae*. At the same time, the effect of targeted antibiotic use in cholera on the resistance profile of those potentially pathogenic enteric bacteria also present at the time of treatment is unascertained (Centers for Disease Control and Prevention (CDC), 2018).

Existence of multiple drug resistance bacteria to clinically-proven antibiotics is a major health concern and a great obstacle to the global drug discovery plan of action (Alanis, 2005). Development of multiple resistant bacteria at the moment is a common distress. Some bacterial strains are resistance to almost all available antibiotics despite the speedy victory of antimicrobials (Domingo-Calap & Delgado-Martínez, 2018).

There is documentation that shows that both environmental and clinical *Vibrio* species have retained antibiotic resistance (Letchumanan et al., 2016; Letchumanan et al., 2015a; Zavala-Norzagaray et al., 2015). On yearly basis, toxigenic *Vibrio* species have increased; moreover, there is documentation that there has been development of elevated resistance towards most of the clinically proven antimicrobial (Letchumanan et al., 2015b). Environmental *Vibrio* strains, in recent years have been studied in detail for its prospects as a reservoir for the wide spread of antibiotic resistance (Zhang et al., 2012).

Isolation of multidrug resistant *Vibrio* strains has been documented either from environmental sources or clinical samples (Letchumanan et al., 2016; Zavala-Norzagaray et al., 2015). Bacterial pathogens have increasingly acquired resistance to currently used antibiotics subsequently leading to resurfacing use of phages as potential biological control as well as phage therapy applications (Cohen et al., 2013; Orlova, 2012; Parisien et al., 2008). Anti-microbial resistance (AMR) has been recognized as a fundamental threat to human health and one of the greatest challenges to our civilization; it is estimated that by 2050 ten million people may be dying annually and the economic burden may hit $100 trillion (Międzybrodzki et al., 2018).

In African region key enteric pathogens including, *Salmonella* spp, *Shigella* spp, *Escherichia coli, Klebsiella* and *Vibrio cholerae* have shown negative trends in multidrug resistance genes (Ekwanzala et al., 2018). In Kenya, multidrug resistance of *Vibrio cholerae* both environmental and clinical has been reported (Saidi et al., 2014; Kiiru et al., 2013; Weill et al., 2017).

2.5: Cholera vaccine

Clinical trials for an effective cholera vaccine have been underway since 1990s but by far the most important change has been the ability to rehydrate patients quickly and effectively by means of an inexpensive and effective treatment as oral rehydration therapy. Vaccines that can help prevent cholera are available with their effectiveness ranging from 50 to 90 percent, provided by documentation of the study. Intravenous vaccines have not proved to be very effective and so these vaccines are oral cholera vaccines. Presently, there exists three WHO pre-qualified oral cholera vaccines: Euvichol®, Dukoral® and Shanchol™. These three vaccines entail two doses for complete protection. Dukoral® is distributed with a buffer solution which for grown-ups, requires one hundred and fifty millimeters of potable water. Dukoral can be administered to every individual above two years of age. A minimum of seven days is required and no more than six weeks, interval between each dose. A third dose is mandatory for children between two to five years of age. Generally, travelers use Dukoral®. Protection against cholera for two years is provided by two doses of Dukoral®. Essentially, Euvichol® and Shanchol™ are the same vaccine processed by two different manufacturers. A buffer solution is not required for their administration. These two vaccines can be administered to any individual as long as they are more than one-year-old. At minimal, an interval of two weeks between each dose of these vaccines is required. Two doses of Euvichol and Shanchol give protection against cholera for three years, while a one dose present short term protection. More than 20 million doses of OCVs have been used in mass vaccination campaigns. There is limited supply of these vaccines unfortunately; their recommended use is for those travelling to cholera endemic areas presumably the individual could possibly be exposed to cholera infection (Weill et al., 2017). The oral cholera vaccines should not be taken in isolation from other measures as sole preventive measures in control of cholera disease (Lopes et al., 2014). There are suggestions by some researchers that this limited oral vaccine availableness should be changed and cite data that oral vaccine may help curb outbreaks, even after commencement.

2.6: Biology of bacteriophages

Bacteriophages also known as phages in short, are precisely bacterial obligate parasites but other kind of organisms are not infected (Rhode et al., 2018). They are bountiful, divergent biologic existence on the globe, found in coexistence with host bacteria while carrying out a vital task on their evolution and dynamics (Fernandes & São-José, 2018). Under adverse

conditions bacteriophages can remain viable (Jończyk et al., 2011). Bacteriophages are much smaller than bacterial cells and they consist of nucleic acids enclosed in a protein envelope i.e. the capsid (head). Phages also possess tails that are can either be non-contractile or contractile. Functionally, the tail adsorbs to the receptors on the bacterial cell surface. The collar, basal plate, spikes and tail fibers are auxiliary systems also present and are involved in adsorption to the bacterium plus consequently, injecting nucleic acid into the cell.

Figure 2.1 shows structural features of phage vB_Vch4M_Kuja which was isolated from river Kuja in Migori County in 2015 in this study. The coat (capsid) protects the genome which is hidden inside the capsid and additional features that provide interface with the host bacterium membrane for release of genome.

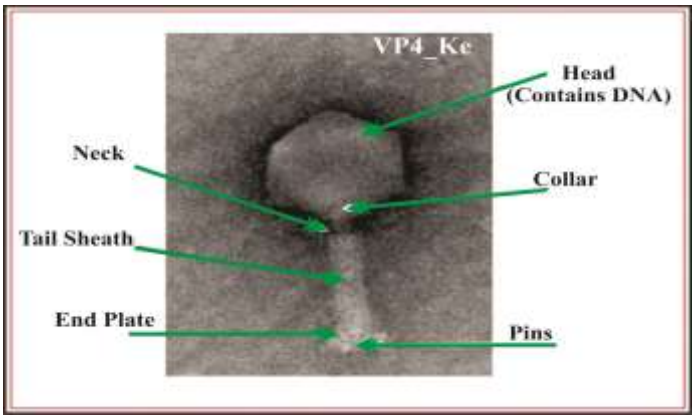


Figure 2.1: Vibriophage, vB_Vch4M_Kuja isolated from river Kuja in Migori County

The tail of the phage is attached to the capsid through a connector which serves as an adaptor between these two components of the phage (Leiman et al., 2010). The tail serves both as a signal transmitter and subsequently a pipeline through which deoxyribonucleic acid (DNA) is delivered to the host cell during infection. The tails have outer appendages attached to the distant end of the tail and often include a base plate with several fibers and a tip or a needle that has specificity to the membrane receptors of the bacterium (Leiman et al., 2010). Bacteriophage life cycle can either the lytic or the lysogenic stage. The lytic cycle involves infection of the host cells by the phage causing multiplication and subsequent release of virions after the lysis of the bacterium (Faruque & Makalanos, 2013). Lysogenically the viral genome integrates onto host DNA either replicating along with it or may even lead to formation of a plasmid. Phage becomes dormant and when the host situations worsen then endogenous phages (known as prophages) actively flatter. Initiation of the reproductive cycle takes place at such a time resulting in host cell killing. Since lysogenic cycle allows the host

cell to continue to survive and reproduce, the virus is reproduced in all of the cell's offspring. New properties can be conferred to the host bacterium by the bacteriophages for example genes of interest such as resistance to antimicrobials subsequently leading propagation either within species or from a species to the other. Diversification of bacterial species contributed by the bacteriophages is the end result (Klieve, 2005). Whatever the case, the bacterial virus is capable of reviving the lytic cycle either in response to stress or spontaneously. There are several options to the key scheme of either lysogeny or lysis that have been documented. Their inherent instability is a common feature among alternate life cycles complicating the study of phages. The two most documented types are pseudolysogeny (false lysogeny) and the carrier state life cycle (CSLC) but terminology related to these variant life cycles is often confused (El-Shibiny & El-Sahhar, 2017). Usually pseudolysogeny is associated with starvation conditions. In this state the phage undergoes neither lysogeny nor lytic cycle instead it remains in an inactive condition (Thung et al., 2018). Upon replenishment of nutrient supply, the phage genomes establish either true lysogeny or become active again to produce and subsequent release viable bacteriophage following the bursting of the host cell.

In carrier state life cycles (CSLC) bacterial cells and the phages are in equilibrium but a proportion of bacterial cells are resistant while others are sensitive to the phages thereby sustaining the phage population such that both thrive. Recently, there has been a renewed interest in the use of bacteriophages (phages) for environmental and clinical applications (Wittebole et al., 2014). In contrast to antibiotics, phages are specific to their targets and because they are replicating viruses, are capable of auto-dosing, a phenomenon where phage replication increases their number and contributes to the dose (Yen et al., 2017).

Globally, prophages and lytic bacteriophages are keys in modulation of bacterial equilibrium as well as animal and healthy human microbiome. An assumption is made that our constant intake from natural food and water resources contain a considerable amount of bacteriophages. It is a varied reason why allergies to preparations of natural bacteriophage do not result in human beings, and why the innate immune system does not notably give a response to bacteriophages; biological microbiome balance would be disrupted (Rhode et al., 2018). Bacteriophages that are lytic destroy their host bacterial cells through lysis process and are virulent. After adsorption to the receptor of the bacterial cell, injection of nucleic acid into the host bacteria takes place, and genetic replication follows, resulting to death of the host cell

through lysis. These obligatory lytic phages in general opinion, for various reasons are suitable for complementing bacteriophage therapy implementation (Rhode et al., 2018).

The virulent bacteriophage takes over control mechanism of the host cell in the course of lytic cycle, replication process of new phages begins and there is destruction of the host bacterial cell finally. A lytic cycle of bacteriophages consists of five stages: The first stage is adsorption to the host cell by the phage in the infection process where there is interaction of the phages with the receptors of the host bacterium cell surface (such as outer membrane protein C (OmpC) on host surfaces and lipopolysaccharides). The host range spectrum for most bacteriophages is narrow and may infect one strain within a species or one species of bacteria. This unique recognition can be exploited for targeted treatment of bacterial infection by phage therapy or for phage typing to identify unique bacterial subspecies or strains. Penetration or entry is the second stage of a bacteriophage infection cycle. The process takes place through contraction of the tail sheath, which acts like a hypodermic needle such that the phage genome is injected through the cell wall and membrane of the host cell while the head and remaining components of the bacteriophage are left outside the cell of the host bacterium.

Replication of the viral components takes place during the third stage of phage infection of the bacterial cell. The phage synthesizes virus-encoded endonucleases to degrade the bacterial chromosome upon entering the bacterium host cell. The phage afterwards takes over the host cell machinery to replicate, transcribe, and translate the required bacteriophage constituents (viral enzymes, sheath, capsomeres, base plates and tail fibers) for the assembly of new bacteriophages. Usually, of early expression in the replication cycle are the polymerase genes and later on capsid and tail proteins are expressed. Maturation phase entails the creation of new virions. Virions are released to the environment upon lysis of the host bacterium cell.

2.7: Phage application in food safety

Application of bacteriophages in food products in Europe, Australia and the United States of America, is already documented (Hudson et al., 2005). Different bacteriophage preparations are available commercially and have been given approval in the United States of America. Such phage preparations include: ListshieldTM, SALMONELEXTM, LISTEX P100; LMP-102TM, ECP-100TM (EcoshieldTM), AgriPhageTM, and Biophage-PA (Ly-Chatain, 2014)

To improve food safety and livestock in particular intensively reared livestock, use of bacteriophages has been reviewed (Endersen et al., 2014; Fernández et al., 2018; Sarhan & Azzazy, 2015) and various approaches have been employed (Moye et al., 2018). Approaches such as: high pressure processing (HPP) that exposes foods to high pressure for inactivation microbes, heat pasteurization, food irradiation and finally chemical sanitizers such as chlorine (Sulakvelidze & Alavidze, 2001) have been applied. Unfortunately, these techniques destroy microorganisms indiscriminately, that is to say, both the pathogenic as well as potentially beneficial microbiota are equally targeted. In addition, even with the availability of diverse methods, there is relatively frequent occurrence of foodborne outbreaks. A combination of these factors is a demonstrated need for a targeted antimicrobial approach, one that can be used alone or in combination with the techniques described above, to establish additional barriers in a multi-hurdle approach to prevent foodborne bacterial pathogens from reaching consumers.

The use of lytic bacteriophages for targeting specific foodborne bacteria in the foods, without a deleteriously impact on their normal and often beneficial microbiota is one such technique. The approach is known as either “phage biocontrol” or “bacteriophage biological control”. Among the most harmless antibacterial approaches available towards food safety perspective are the lytic bacteriophages (Sillankorva et al., 2012). In order to safeguard the food chain, bacteriophage biological control is increasingly gaining acceptance as green technology or natural and is effective at specifically targeting bacterial pathogens in various foods, (Moye et al., 2018). Application of bacteriophages as biocontrol agents is an intervention available today, arguably the most environmentally-friendly antimicrobial. Currently, most if not all available commercial bacteriophage biocontrol products contain natural phages. These are bacteriophages isolated from the environment without any genetic modification. There are no food additives included in most of these preparations or even preservatives; typically, they are water-based solutions consisting of purified bacteriophages and low salt levels. The cost of applying bacteriophages is relatively low in comparison to other food safety interventions (Moye et al., 2018).

Controlling the levels of microbial pathogens in the food industry is essential to avoid public health issues and for the safety of the products in order to prevent recalls/withdrawals. Food products have also been sprayed with phages and phage subcomponents (Galarce et al., 2014) to increase food safety as well as pet foods (Squires, 2018). Direct application of phages on

foods has been given approval by the United States Food and Drug Administration (USFDA) and in some cases by the European Union through the use of products such as the bacteriophage-based ListShield and Listex (Mcauliffe, 2018). Table 2.1 shows a list of commercialized bacteriophages that have been given regulatory approval by different agencies since 2006 (Sillankorva et al., 2012).

Table 2.1: Food safety regulatory approvals

Date	Agency	Phage preparation	Target application
2006, August	FDA, 21 CFR 172.785	ListShield	RTE meats
2006, October	FDA, GRN 198	Listex	Cheese
2007, January	USDA, FSIS Directive 7120.1	*E. coli* O157:H7 targeted	Hides of livestock
2007, March	USDA, FSIS Directive 7120.1	*Salmonella*-targeted	Hides of livestock
2007, June	FDA, GRN 218	Listex	Foods, generally
2008, July	USDA, FSIS Directive 7120.1	*Salmonella*-targeted	Feathers of live poultry
2010, September	Health Canada	Listex	RTE meat, dairy, fish
2011, February	FDA, FCN 1018	EcoShield	Ground beef
2012, August	FSANZ	Listex	Meat, seafood, cheese, RTE foods
2013, February	FDA, GRN 435	SalmoFresh	Poultry, fish, fruits, vegetables
2013, December	FDA, GRN 468	Salmonelex	Pork and poultry
2014, August	Health Canada	SalmoFresh	Poultry, fish, fruits, vegetables
2014, August	Israel Ministry of Health	SalmoFresh	Poultry, fish, fruits, vegetables
2014, August	Israel Ministry of Health	ListShield	RTE meats
2014, August	Israel Ministry of Health	EcoShield	Ground beef
2014, December	FDA, GRN 528	ListShield	Fruits, vegetables, dairy, fish
2016, July	FDA, GRN 603	SalmoPro poultry	Poultry

Source: Sillankorva et al. (2012:p.4)

2.7.1: Phages in agriculture

In agriculture, use of antimicrobials may result in reduced efficacy of antibiotics due to facilitated emergence of antibiotic resistant human pathogens, increased human morbidity and mortality, increased healthcare costs, and increased potential for carriage and dissemination of

pathogens (Svircev et al., 2018). Removal of antibiotics from agricultural use in certain jurisdictions and popularity of organic products have resulted in exploration of options because of consumer's demand for products that have not received treatment with antibiotics (Svircev et al., 2018). A recent study on phages showed promising results by displaying the ability of phages to control the development of soft rot disease in potatoes caused by *Pectobacterium carotovorum* (Muturi et al., 2019)

2.7.2: Phages in veterinary medicine

For the last 70 years, there has been an innovation drug period, implementation in animal and human as well as in agriculture (Jassim & Limoges, 2014). At different periods in the life cycle of swine, poultry and cattle, some of the antibiotics used are: lincosamides, polyethers, beta-lactams, quinoxalines, arsenicals, polypeptides, glycolipids, tetracyclines, elfamycins, streptogramins, sulfonamides and macrolides (Sarmah et al., 2006). The four broad categories of these antibiotics include: Therapy which is the treatment administered to sick animal that has undergone diagnosis, Metaphylactics: the presence of clinical illness in one animal triggers drug treatment of the whole herd or flock, Prophylaxis: antibiotics in the absence of clinical illness are used for elimination of symptomatic because of sharing feed troughs as well as water by both livestock and poultry. The spread of illness among these animals can be rapid because they are in close contact with one another through laying on each other, licking and even rubbing snouts and noses. At times when livestock is at risk, antibiotics are used for disease prevention for example in particular at times when young ones wean from the mothers. Animals' reared for food products are also given antibiotics to facilitate the production and rate of growth, phenomenon known as Growth promotion.

For effective treatments of bacterial infections, the entire globe urgently requires an alternative (Jassim & Limoges, 2014) of the last century to replace the antibiotics. The sub-therapeutic use of antibiotic growth promoters (AGPs) in animal feeds was discontinued in the European Union because antibiotic resistance has become increasingly widespread (Goode et al., 2003). Through the application of well-controlled animal models, it has been demonstrated that use of smart bacteriophages can treat or prevent animals that have been infected with disease causing bacteria and may be good alternatives for the treatment of infections with multi-drug resistant bacteria (MDRB) (Jassim & Limoges, 2014; Fernández et al., 2018).

Biological control applications and Veterinary therapy call for proper administration, with a target of particular bacteria, a comprehensive methodology strategy, the phage-host interactions description, dose optimization and accounting for all chemical and physical factors (Jassim & Limoges, 2014). Poultry products are the most widely-used meats to study the efficacy of phage-mediated biocontrol in food products with bacteriophages targeting *Campylobacter* and *Salmonella* because of their importance as contributors to food-borne disease especially in chicken meat (Jassim & Limoges, 2014).

The growth promoting effects of antibiotics may be replaced by bacteriophages to control enteric bacteria, the food energy competitors. The bacteriophage must be able to remain active during the digestive processes if they are to be used to target enteric bacteria was a suggestion put forward (Bull & Gill, 2014). There were two experiments that were carried out in order to determine the effects of supplementing diet with bacteriophages, probiotics plus their combination on growth performance, apparent total tract digestibility, fecal bacteria populations and serum immunoglobulins in growing pigs (Kim et al., 2019). It was proposed, after the end results were obtained, that different aspects of a pig's growth performance were improved by both bacteriophages and probiotics. However, bacteriophages were more effective than probiotics and would appear to offer an alternative to antibiotic type growth promoters. The required level that was suggested which could be used as an antibiotic alternative for growing pigs, was 0.5 grams per kilogram (Yan et al., 2012). Pigs fed a diet containing anti-*Salmonella typhimurium* phage, at 3×10^9 plaque forming units (PFUs)/kg of feed, showed improved performance throughout the 28-day study. This was in agreement with the previous results (Gebru et al., 2010). However, to better understand their mode of action in this class of pigs, it was suggested that further work needed to be carried out.

2.7.3: Phage therapy

For the last 10 years there have been intensified studies on phage therapy because of a surge in resistance due to antibiotics strains for both animal and humans (Jassim & Limoges, 2014). Bacteriophage therapy has been shown to have efficacy to treat many diseases of plants, animals, and humans. In broiler chickens, the potential of bacteriophages for treatment of bacterial respiratory infections caused by *Escherichia coli* have been explored (Huff et al., 2002; 2003). Aerosol and intramuscular injection have given the best results in bacteriophage delivery methods over addition into drinking water/feed or oral administration (Sillankorva et al., 2012). It has also been documented that bacteriophage therapy reduced colonization of

broiler chickens with *Campylobacter jejuni* (Wagenaar et al., 2005). Application of bacteriophages to reduce *Campylobacter* and *Salmonella* concentrations in poultry has also been addressed in several studies (Kittler et al., 2013). Bacteriophage therapy in poultry and pigs can be effective against *Salmonella typhimurium* rapid selection of phage and administration of the phage and high bacterial loads (Allen, 2013). During the last 30 years, the focus for manufacturers of pharmaceuticals that deal with antibiotics has been mainly on the development of new products derived from the known classes of antibiotics. Unfortunately, there has been a major concern because no new antibiotics have been found (Sulakvelidze & Alavidze, 2001). As a worthwhile task, exploitation of alternative approach for production of antibacterial products and re-examining the potential of promising older methods might be of value. A possible substitution for antibiotics is the use of bacteriophages as antimicrobial agents (Vinodkumar et al., 2008).

2.7.4: Bacteriophages for water decontamination

Government all around the globe have been struggling to remove the pathogens, chemicals and other pollutants from wastewater. The major sources of these pollutants are unplanned urbanization, lack of education, increase in industries, introducing the sewage directly into the river without any prior treatment (Gautam et al., 2019). Presence of pathogenic bacteria in water causes public health concern due to morbidity and mortality that they cause, the high cost of disinfecting wastewater by using physical and chemical methods in treatment plants and they also bring about environmental damage (Jassim et al., 2016).

There has been persistence as well as existence of *Vibrio cholerae* in various maritime systems like lakes and rivers. The bacterium, *Vibrio cholerae* is released into the environment directly during an outbreak via the excreta from human beings and waste water discharge from municipal councils (Gwenzi & Sanganyado, 2019). Considering that cholera is a waterborne disease, maritime establishments provide a sink of bacterium as well as the source (Gwenzi & Sanganyado, 2019). Since the bacterium persists and proliferates in these environmental waters through intake of spoilt food and contaminated water, the bacterium gets into the body of the human beings. Viewed in this way, water used for human consumption becomes a transitional zone between the body of human beings as well as the and maritime systems (Ntema et al., 2010) and this forms the linkage between the human and environment that plays a major role in sustaining and promoting the persistence of cholera outbreaks (Gwenzi & Sanganyado, 2019). Bacteriophages have a huge influence on the

environment because they play a vital role in maintaining microbial balance (El-Shibiny & El-Sahhar, 2017).

Antibiotic resistance from clinical and environmental origin in strains of the genus *Vibrios* has been reported (Plaza et al., 2018). The lack of effective treatments to control pathogenic *Vibrios* resistant to antibiotics has led to the exploration of new alternatives and one of the most promising options is the use of lytic bacteriophages to kill pathogenic bacteria (Clark, 2015). Phages also will remain in the environment only if the host bacteria are present (Clark, 2015). According to WHO 2018, as cholera continues to be a major public health concern to developing countries, it is also re-emerging in countries where it had disappeared long time ago. Therefore, coupled with the occurrence of multidrug resistance strains of *Vibrio cholerae* in the environment, there has been a renewed search for an alternative source of treatment such as the lytic bacteriophages (Letchumanan et al., 2016). Although phages isolated from different environmental sources show same lytic activity towards bacteria, they possess advantages over the conventional antibiotics (Al-Fendi et al., 2014). Since lytic phages replicate quickly and rapidly cause death and lysis of the host, they are ideal for the development of phage therapies for use in treating animal infections and in reducing pathogens in various foods and the environment (Richards, 2014). The bactericidal effects of bacteriophages have long been studied for their usefulness in treating gastrointestinal infections. Early studies originating from former Soviet Union, Eastern Europe and Western Asia suggested bacteriophages could present and treat *Vibrio cholerae* infections (Bielke et al., 2012). For use as decontaminants, several studies have been conducted to evaluate the efficacy of phages as biocontrol agents against food and beverage borne pathogens (Kazi & Annapure, 2016).

2.7.5: Re-emergence of phages and challenges in phage therapy

Since 1920s after their discovery, bacteriophages have been used for treatment of bacterial infections in humans and other animals for over 100 years. Bacteriophages can kill their host bacteria regardless of whether or not the host is resistant to multiple antimicrobial compounds (Squires, 2018). After introduction of antibiotics in 1940s, phage therapy ceased in most countries. In some countries however, use of phages in clinical treatment of wounds continued in Eastern Europe and Soviet Union (Morozova, Vlassov & Tikunova, 2018). Recently, there has been a marked increase in the number and quality of research publications dealing with phage therapy (Squires, 2018).

Phage preparations approved for clinical application have been produced in the Russian Federation, Republic of Georgia, Poland and a large number of studies on phage therapy have been reported in these countries (Al-Fendi et al., 2014; Górski et al., 2012; 2018; Myelnikov, 2018; O'Flaherty, Ross & Coffey, 2009). Worldwide there has been a rapid increase in multidrug resistance bacteria which has led to a renewed interest in phage therapy as a complimentary approach in treatment of bacterial disease infections (Morozova et al., 2018). Recently, the results of bacteriophage and phage cocktail application for the treatment of various infections have been reported in a number of clinical cases, case series and clinical trials (Wright, 2009). Scientists today are faced with the threat of superbugs that is pathogenic bacteria resistant to most of all available antibiotics (Livermore, 2004; Fischetti, Nelson & Schuch, 2006; Fischetti, 2005). There is wide distribution of bacteriophage therapeutic medicine in Russia and Georgia and are made available in various pharmaceutical industries. The approval of these bacteriophage products has been carried out and regulatory processes have been followed where production at commercial level has been achieved, these typically apply to ready-made preparations (Chanishvili & Sharp, 2009).

Application of bacteriophages as biocontrol agents' offer advantage to low and middle income establishments (LMICs). Bacterial infections are predisposing factors in such set ups where food or water has been contaminated rendering it unfit for human consumption, resistance to antimicrobials, hygiene and sanitation problems (Nagel et al., 2016). Above this the population is made more vulnerable by malnutrition and HIV-mediated immunosuppression. Potential of bacteriophages in these low and middle income establishments need to be unleashed because there is an urgent need for supplementary treatment modalities in these areas (Nagel et al., 2016).

Nagel et al. (2016) have explained that bacteriophages availed for use particularly to low and middle income establishments because phages display several characteristics that make them suitable for such. Bacteriophages are highly specific, when applied, the microbiota is preserved a significant benefit for malnourished and immunocompromised individuals are some of the benefits among others. Additionally, bacteriophage isolation and production done locally are not complicated and basic tools as well as technical knowledge are readily available for such establishments.

However, personalized medicinal products based on natural phages are not prone to strong patent protection and broad market distribution (Minssen, 2014; Todd, 2019). Therefore, bacteriophage therapy is not suitable for the market model which prevails for pharmaceutical industries (Pirnay et al., 2011; 2012). It is not a surprise that the little interest in this field has been acquired by mainstream pharma. Preferably, bacteriophage therapy practice is confined to hospitals or, to a lesser extent; some niche biotech companies and not-for-profit phage therapy centres (Fauconnier et al., 2020).

Bacteriophage manufacturing requirements is another hindrance to phage therapy medicinal products. Although there is no question about need for a quality system, the good manufacturing practices GMP, are typically out of reach for potential phage therapy medicinal products manufacturers as currently applicable for standard industry-made medicines. Indeed, full-blown good manufacturing practices for phage therapy medicinal products would imply major investments, out of proportion with the very small-scale production of named patient therapeutic phages (Fauconnier et al., 2020).

2.7.6: History of *Vibrio cholerae* bacteriophages

Relationship between *Vibrio cholerae* and bacteriophages dates back in 1920s where Felix d' Herelle found that after onset of an outbreak bacteriophages were widespread in the environment (Jassim & Limoges, 2014; Silva-Valenzuela & Camilli, 2019). Back then, several studies proved the effectiveness of phage treatment against versatile diseases, including cholera, staphylococcal infections, typhoid fever and bacterial dysentery (Sulakvelidze et al., 2001; El-Shibiny & El-Sahhar, 2017). Therapeutic applications from the earliest discovery of the bacteriophage went hand-in-hand with the theoretical investigations and d'Herelle himself used phage to treat dysentery, plague, and cholera. Bacteriophage based products were available at commercial level in France, Britain, Germany, Italy, and the United States of America in the 1930s and 1940s (Myelnikov, 2018).

Mortality decline during a cholera epidemic in India was observed by d'Herelle while carrying out a study on excreta where he made a link between death and recovery from disease with the presence or absence, respectively, of virulent vibriophages in the patients. Based on this insight, vibriophages were given orally to 200 cholera patients and achieved reduction in mortality of 8% compared to 63% observed in controls when the phages were given in early infection (Brüssow, 2017).

It was during a cholera epidemic in an Assam village, where rapid end of the epidemic was as a result of distribution of vibriophage. Another field trial was carried out where vibriophages were distributed in one village and in another village the phages were not distributed. Follow –up was made for four years and the results showed that mortality due to cholera dropped rapidly in the village where phage distribution had been carried out and death rates remained high in the village that had not received any vibriophage distribution. In 1931, scientists from India observed that when vibriophages were widely distributed in the environment the mortality rate fell rapidly (Brüssow, 2017).

Despite this early success, enthusiasm towards phage treatment and research immensely declined with discovery of antibiotics (Abedon et al., 2011). By 1945 after raising hopes in the interwar years, phage therapy had been abandoned almost entirely in the West. It is until recently that revival of interest in response to the crisis of antibiotic resistance has been made. Within Soviet medicine, especially in Georgia the use of phage therapy, however, persisted (Myelnikov, 2018). More recent work has been done proposing that cholera outbreaks are partially driven by a surge in the hyper infectious *Vibrio cholerae* populace, quenched by an increase in virulent phages in infected humans and in surface reservoirs (Silva-Valenzuela & Camilli, 2019). Revisiting phage therapy ought to be taken into consideration given that human and environmental *Vibrio cholerae* populations are naturally controlled by serogroup-specific bacteriophages (Faruque et al., 2005a). Since *Vibrio cholerae* is a natural inhabitant of aquatic environments (Almagro-Mareno & Taylor, 2013; Lutz et al., 2013), these environments are favorable for exploring candidate therapeutic bacteriophages. Indeed, several tailed bacteriophages especially from the family of *Myoviridae*, were detected during environmental surveys in regions where outbreaks were reported eg, Peru (Talledo et al., 2003), Kolakata (Sen & Ghosh, 2005) and Kenya (Maina et al., 2014). Pathogenic *Vibrio cholerae* forms reservoirs in fresh water in the environment and it is endemic in many countries. It persists in aquatic environments in planktonic form, by association with phytoplankton among others. Virulent phages may be of special relevance among the many interactions this pathogen could have in the environment (Silva-Valenzuela & Camilli, 2019). Of note, tailed bacteriophages are the most abundant viruses in the aquatic environment (Rao & Lalitha, 2015; Letchumanan et al., 2016), which makes them a good initial candidate for screening, but will require elaborate efforts for their isolation. Application of bacteriophages as biocontrol agents against *Vibrio cholerae* will therefore be an alternative complimentary measure to control the pathogenic bacterium in the environment or for phage therapy.

2.8: Classification of bacteriophages

For application of bacteriophages as natural biological control agents against the toxigenic bacteria species, the first step involves testing the virulent phages against the target bacterium invitro (Gill & Hyman, 2010).

It is therefore important to understand bacteriophage properties before application. Understanding of the host properties first will help to identify the stages in the growth curve especially exponential phase and generation time. This will help to study different characteristics of the phages like the rationale behind phages to each bacterial cell administration (that is multiplicity of infection), host range profiles, one step growth curve as well as modification of protocols. International Committee on Taxonomy of Viruses (ICTV) is responsible for maintenance of the taxonomic classification of viruses and naming of virus taxa (Adams et al., 2017). The system is based on the evaluation of a variety of phage properties including structure of the virus capsid whether or not it is enveloped, host range, pathogenicity and sequence similarity the molecular composition of virus genome double or single strand that is (ss/ds, DNA (deoxyribonucleic acid) or RNA (ribonucleic acid).

2.8.1: Physical classification

On the basis of their morphology, over 6000 bacteriophages have been classified. With the help of Transmission Electron Microscopy, the classification can be based on their morphological structure (Ntema et al., 2010). All phages are composed of a nucleic acid genome (whether DNA or RNA) encased within a capsid (El-Shibiny & El-Shahhar, 2017). Almost all of the currently classified bacterial viruses are, however, assigned to just three families of tailed phages, namely the *Myoviridae* with long contractile tail, *Podoviridae* with short tail and *Siphoviridae* with long non-contractile tail, all the three families belong to the order *Caudovirales* (Simmonds & Aiewsakun, 2018).

2.8.2: Genomic classification

Modern advance in sequencing technology and metagenomics has facilitated whole genome sequencing of bacteriophage virions and prophages offering an exciting new avenue for understanding phages and their impacts on host bacteria. To date, approximately 8,300 complete bacteriophage genomes have been sequenced (Ha & Denver, 2018). Bacteriophages must undergo whole-genome sequencing as a security measure in their use as antimicrobials

so as to ensure that the genome is free of genes encoding known bacterial virulence factors and potential immunoreactive allergens. This also helps understand the multiplicative cycle of the phages at molecular level, and also other important biological traits (Bardina et al., 2016).

Phage genome comprises of four types of genomic material namely; single stranded Deoxyribonucleic acid (DNA), double DNA single stranded or double stranded Ribonucleic acid (RNA). Genetic material varies widely among phages: genome length ranges from 3405 bp to 497513 bp, gene density ranges from 0.29 to 1.36 and number of encoded proteins ranges from 1 to 675 (Ali et al., 2012; 2015; Lopez et al., 2014). In phage therapy applications, primary determinants for therapeutic selection include the antibacterial virulence, lifestyle, and the absence of deleterious genes. Besides these genome quality, human and animal safety is of major importance (Philipson et al., 2018).

CHAPTER THREE: MATERIALS AND METHODS

3.1: Area of study

Environmental water samples were collected from four regions of Kenya: Lake Victoria region, Coastal region, Nairobi and Central regions. Tables 3.2 to 3.5 displayed the specific sources of the environmental water samples.

3.2: Study period

To search for the pathogenic *Vibrio cholerae* bacterium and respective bacteriophages in the environmental waters of Kenya, sampling was carried out during both dry season (when there were no rains at all) and warm rainy seasons (when there were heavy rains) in Kenya. The study commenced in 2015 from March through August, 2018. The initial environmental water sampling took place in Kisumu, Migori and Kisii. This was after following media reports of a cholera outbreak in Migori County. According to the media report, consumption of contaminated water from a certain river (River Kuja) in Migori County was associated with the cholera outbreak. In Kenya, March is a dry spell period of time but due to climate change, sometimes rains begin during this month. At the time of sampling in the month of March 2015, it was a dry spell period. Sampling of the environmental water from the Coastal region took place in June 2016 while sampling in Siaya County took place in October 2016. Sampling at different seasons was important because when water level fluctuates, microbial levels are affected. Functional dynamics of *V. cholerae* are important in wet and dry seasons because they affect vibriophages as well. Following heavy rains that were experienced in central and Nairobi regions of Kenya in 2018, sample collection of environmental water was carried out from these two regions. This was done from the months of May to August same year.

3.2.1: Sampling sites

Figure 3.1 shows a map of Kenya displaying the different sampling points of environmental water in this study.

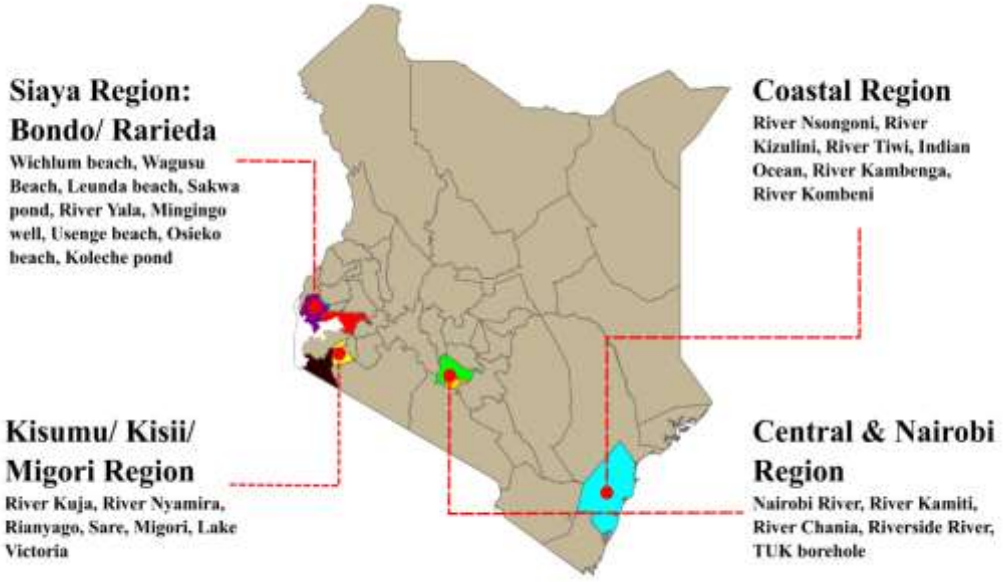


Figure 3.1: A Map of Kenya showing sampling sites
Source: MapSVG (2014)

A total of 140 samples were collected from various environmental water bodies that included; rivers, boreholes, springs, wells, ponds, beaches, lake and Indian Ocean. The sampling regions were selected based on previous records of cholera outbreaks. The target group was the low income poor communities without accessibility to neither treated nor piped water. The main focus was on surface waters used for domestic purposes. Interestingly, in Siaya County, the main source of water used for domestic purposes was mainly ponds, beaches and wells unlike other regions where the source of water was mainly rivers, boreholes and wells. In Mombasa County especially Likoni, boreholes and wells were mostly the main sources of drinking waters for the residential communities. In most of the sampling sites, human activities were very common like washing clothes, bathing; cleaning dishes, swimming, feeding cattle and other activities were taking place around these water bodies. Beaches in Siaya are man-made water bodies that form the main entrances to the lake. Originally, they were shallow water bodies where Hyacinth and lichens grew but the residential communities' uprooted those weeds leaving a depression in which water from the lake diverged and accumulated. The accumulated water that formed a water body/source is what the residential communities' referred to as beach. These beaches form the main sources of water used for domestic purposes by the surrounding communities who do not have access to piped water. Houses are also constructed near the beaches because of the availability of water; shopping

centers are also constructed near these beaches. The summary of water collected from the various regional sources is displayed in Tables 3.2 to 3.5.

3.2.2: Sample collection and processing

At the time of collecting the environmental water samples, surface water temperature, pH levels were measured at the sampling sites. For measurement of pH, a portable field pH meter, (EZDO 5011A) was used. For recording of temperatures, a portable thermometer, (PAICO DELUXE) was also used. The environmental water samples were collected from the surface using sterile plastic 500ml bottles that had a narrow mouth. The environmental water samples were transported to the Medical Microbiology laboratory department, University of Nairobi, College of Health Sciences in a cool box containing frozen packs at the bottom to maintain contact with the water bottles. Analysis of the environmental water samples was performed within a period of 12 to 24 hours after collection. After sampling it took between 6 hours (Central and Nairobi regions) to 72 hours (Kisumu, Migori and Kisii regions) for samples to be transported to the laboratory for analysis

3.2.3: Enrichment, selective cultures and bacterial identification

Conventional and Molecular methods were used for isolation and identification of the bacterial isolates in this study.

3.2.4: Conventional identification of bacterial strains

Alkaline Peptone Water (APW) was used to enrich the growth of *Vibrio cholerae* bacterial cultures. For enrichment 10ml of APW sterile broth was mixed with 100ml of the environmental water sample, cultures were incubated for 12 hours at 37 degrees centigrade. Subsequently, sub-culturing was done on a selective media, TCBS (Hi media Mumbai, India) which is a selective media for *Vibrio cholerae*. The positive yellow sucrose fermenting colonies were aseptically transferred using a sterile disposable loop onto TSA plates to attest for growth. Additionally, gram staining was performed to ascertain the gram reaction of the isolates. Other bacteria isolated from the different environmental waters as shown in Table 3.1 were cultured on TSA. The colonies of *Vibrio cholerae* from TSA were then subjected to Oxidase test (Oxidase strips, Himedia, India). Triple sugar Iron (TSI) test was also a biochemical test carried out to identify *Vibrio cholerae.* For further identification of the bacterial strains isolated from the environmental waters of Kenya in this study, sequencing of

16S rRNA partial gene was performed according to the method described by (Rainey & Stackerbrandt, 2000).

3.2.5: Molecular identification of bacterial isolates

Bacterial DNA was extracted for polymerase chain reaction (PCR) in order to amplify the 16S rRNA gene using the universal primers 27F (5'-AGA GTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTAC CTTGTTACGACTT-3'). Specific primers, Y1 (5'-TTACCGG ACGCCGAGCTGTGGCGT-30) and Y2 (5'-CAGGAAGA TGCGTTATCGCGAGT-3')

For extraction of bacterial DNA, boiling method was used where one big colony or several small colonies were mixed with 50µl of ddH_2O and subjected to 4 cycles of 98°C for 2 min and 4°C for 1min. PCR in both cases was prepared using 50µl mixture containing 10X Taq buffer, dNTP Mix (2mM each), Primers (10µM), 25mM $MgCl_2$, Template DNA, 1.25U Taq DNA polymerase and ddH_2O. PCR conditions for the universal primers were: 95°C for 3 min, followed by 34 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min with final extension been set at 72°C for 5 min. Similar conditions were used for Y1/Y2 primers except for the annealing temperature which was adjusted to 60°C. DNA was detected by gel electrophoresis in 1% Tris-acetate-EDTA (TAE) buffer and stained with Gel Red. The PCR products were sequenced by Sangon Biotech Co. Ltd (Shanghai, China), China, followed by analysis of the sequences on NCBI Standard Nucleotide (BLAST) basic local alignment search tool. Appendix 2 displays results of the partial 16S rRNA sequences of the different strains of *Vibrio cholerae* and other bacteria identified in the study. The sequencing of 16S ribosomal RNA gene was performed according to the method described by (Rainey & Stackebrandt, 2000).

3.2.6: Frozen storage of *Vibrio cholerae*

For future reference samples, it was important to preserve the bacterial strains at low temperatures. To obtain a culture for preservation at low temperatures, *Vibrio cholerae* was subcultured fresh on TCBS media overnight. Using a sterile wire loop an isolated pure colony was then suspended onto 10 millimeter sterile Tryptose Soy Broth thereafter allowed to grow up to mid-exponential phase for 4 to 6h at 37 degrees centigrade. Then 800µl of the culture was added onto a sterile cryovial (German) and 400µl of sterile 30% Glycerol added. This was thoroughly mixed, thereafter transferred to frozen storage at minus 80°C or –20°C for future references.

A *Vibrio cholerae* clinical isolate was kindly provided by the department of medical Microbiology of the University of Nairobi, Kenya (serial No. VCM). The pathogen had been isolated from a patient suffering from cholera infection. A total of 21 bacterial isolates were used in the study as summarized in Table 3.1.

1. Lake Region: Kisumu, Migori and Kisii.
   Environmental *Vibrio cholerae* serial Nos. 1-5 was isolated from these regions specifically from River Kuja. Other environmental bacterial strains isolated from these regions were: *Providencia sneebia, Escherichia coli* 083*, Ralstonia solanacearum* and *Proteus mirabilis.*

2. Lake region: Bondo and Rarieda
   Vibrio cholerae, serial Nos. 6-11 was isolated from environmental waters of either ponds or beaches in these regions.

3. Coast region: Kaloleni, Ukuda, Likoni, Rabai, Ribe, Mariakani and Town center
   Vibrio cholerae, serial Nos.12 and 13 was obtained from this region.

4. Central region: Nairobi, Thika and Murang'a
   Vibrio cholerae, serial Nos. 14 and 15 was isolated from these regions

5. University of Nairobi, Medical Microbiology Department.
   Vibrio cholerae, serial No. 16 was a clinical isolate kindly provided by the department of Medical microbiology, University of Nairobi. The pathogen had been isolated from a patient suffering from cholera infection.

The 16S rRNA partial sequences of some of these bacterial isolates are provided in appendix 2. Serial numbers 1-15 were environmental pathogenic *Vibrio cholerae* isolates; Serial number 16 was a clinical isolate while serial numbers 17 to 21 were other bacterial strains isolated from respective environmental water sources other than *Vibrio cholerae* strain.

Table 3.1: Sources of bacterial strains used for this study

Se. No.	Code	Bacteria	Strain	Region	Specific source/site
1	Vc_ke	*Vibrio cholerae* 01biovar	El Tor	Migori	River Kuja
2	Vc_ke	*Vibrio cholerae* 01biovar	El Tor	Migori	River Kuja
3	Vc_ke	*Vibrio cholerae* 01biovar	El Tor	Migori	River Kuja
4	Vc_ke	*Vibrio cholerae* 01biovar	El Tor	Migori	River Kuja
5	Vc_ke	*Vibrio cholerae* 01biovar	El Tor	Migori	River Kuja
6	Vc_Koleche	*Vibrio cholerae* 01biovar	El Tor	Bondo	Koleche Pond
7	VH1	*Vibrio cholerae* 01biovar	El Tor	Bondo	Kotonde Pond
8	VH1	*Vibrio cholerae* 01biovar	El Tor	Bondo	Owira Pond
9	VH1	*Vibrio cholerae* 01biovar	El Tor	Rarieda	Usenge Beach
10	VH1	*Vibrio cholerae* 01biovar	El Tor	Rarieda	Osieko Beach
11	VH1	*Vibrio cholerae* 01biovar	El Tor	Rarieda	Lauda Beach
12	Vc_Nsongoni	*Vibrio cholerae* 01biovar	El Tor	Coast	River Nsongoni
13	VH2	*Vibrio cholerae* 01biovar	El Tor	Coast	River Kizulini
14	VH3	*Vibrio cholerae*	NP	Central	River Kamiti
15	VH3	*Vibrio cholerae*	NP	Central	Riverside River
16	VCM	*Vibrio cholerae*	Clinical	UoN	Medical Microbiology
17	PROS_ke	*Providencia sneebia*	-	Migori/ Kisii	River Nyambira, Rianyago, Kuja
18	PREM_ke	*Proteus mirabilis*	-	Migori/ Kisii	River Nyambira, Rianyago, Kuja
19	RALS	*Ralstonia solanacearum*	NP	Migori, Kisumu	Rivers: sare, migori, riayago, nyambira, stella well, mwamogesha spring and lake Victoria
20	EC_ke	*Escherichia coli* :083	-	Central	Rivers: Nairobi, Kamiti.
21	Ec_Kuja	*Escherichia coli* :083		Migori	Kuja

KEY: Vc_ke-*Vibrio cholerae* isolates all from river Kuja, Migori, Vc_Koleche- *Vibrio cholerae* isolate from Koleche pond in Siaya, Vc_Nsongoni, - *Vibrio cholerae* from river Nsongoni in Coast region, VH3- *Vibrio cholerae* isolates from Central and Nairobi regions, RALS- *Ralstonia solanacearum*, VCM- *Vibrio cholerae* a clinical isolate from the department of medical microbiology, University of Nairobi, NP- Not provided

3.3: Regional sources of the environmental water samples for phage isolation

To isolate bacteriophages, environmental water samples from different regions previously explained were collected in 500ml sterile plastic bottles with a narrow mouth.

3.3.1: Environmental water samples from the lake region: Kisumu, Migori and Kisii Counties

Sampling from this region was carried out in March 2015 which was a dry period. Environmental water samples were obtained from rivers, wells, boreholes, lakes and springs. These are the water bodies the surrounding communities obtain their water for domestic purposes. A total of 63 environmental water samples were collected from this region, summary is as shown in Table 3.2.

The environmental water temperature at the time of sampling were characterized by a broad pH range of 5.8 (Mwamogesha spring) which was the lowest to 7.8 (river Kuja) which was the highest. Temperatures however had a very narrow range of between 23 to 24°C. Collection of the environmental water samples took place during a dry spell period.

Table 3.2: Environmental water samples from lake region: Kisumu, Migori and Kisii Counties

S/No	pH	Temp (°C)	Specific Source	Abbreviation	No. of samples collected	Sampling period
1	7.2±2	23±1	Lake Victoria	LV	9	March 2015
2	7.3±2	24±1	River Sondu	SR	4	March 2015
3	7.3±2	23±1	Stella well	SW	4	March 2015
4	7.1±2	24±1	River Nyasare	NR	4	March 2015
5	7.8±2	24±1	River Kuja	KR	9	March 2015
6	7.7±2	23±1	River Migori	MR	7	March 2015
7	7.4±2	24±1	River Nyangumbo	Nyangumbo	2	March 2015
8	7.5±2	23±1	River Sare	Sare	4	March 2015
9	5.8±2	23±1	Mwamogesha spring	MWS	4	March 2015
10	6.9±2	24±1	River Nyambira	Yam R	4	March 2015
11	7.1±2	23±1	Ndaranja moja Spring	Ndaranja MS	4	March 2015
12	7.3±2	24±1	River Rianyago	Rianyago, RR	4	March 2015
13	7.4±2	23±1	River Awach	RA	4	March 2015

Figure 3.2 shows one of the sites where water sample collection took place along river Kuja. The river is located in Migori County in Nyanza region of Kenya. It originates in the highlands of Kiabonyoru in Nyamira County, passing through the heart of Gucha District runs west through Migori County where it joins the Migori River.

Figure 3.2: Water sample collection at river Kuja.

3.3.2: Environmental water samples from Bondo and Rarieda Sub-counties in Siaya county

The sources of the water samples used for domestic purposes by the surrounding communities in this region were rivers, ponds, beaches and wells. Sample collection of the environmental waters was done in the month of October in the year 2016. The propagating bacterial strain of bacteriophages was also isolated from the same environmental waters. Table 3.3 shows the sources of the environmental water samples from these regions. A total of 32 environmental water samples were collected from this region. The pH ranged from the lowest 7.8 to the

highest 8.1 while the temperatures ranged between 25℃ (River Yala) to 29℃ (for some beaches). The main source of water used for domestic purposes in this region was mainly shallow beaches and ponds which is a unique source of water. These sampling sites are among regions affected by cholera, frequently (Mutonga et al., 2013).

Table 3.3: Environmental water samples from Bondo and Rarieda Sub-counties in Siaya County

S/No.	pH	Temp (°C)	Specific Source	Region	No. of samples collected	Sampling period
1	8.1±2	27±1	Wichlum beach	Bondo	2	Oct 2016
2	7.9±2	29±1	Wagusu beach	Bondo	2	Oct 2016
3	7.9±2	27±1	Leuda beach	Bondo	2	Oct 2016
4	7.9±2	28±1	Sakwa pond	Bondo	2	Oct 2016
5	7.8±2	25±1	River Yala	Bondo	2	Oct 2016
6	8.0±2	27±1	Mingingo well	Bondo	1	Oct 2016
7	8.1±2	29±1	Usenge beach	Bondo	3	Oct 2016
8	8.1±2	27±1	Osieko beach	Bondo	2	Oct 2016
9	7.8±2	29±1	Kolango beach	Bondo	2	Oct 2016
10	7.9±2	29±1	Kamaringa beach	Rarieda	2	Oct 2016
11	7.9±2	29±1	Osindo beach	Rarieda	2	Oct 2016
12	8.0±2	28±1	Koleche pond	Rarieda	2	Oct 2016
13	8.1±2	28±1	Kotonde pond	Rarieda	2	Oct 2016
14	7.9±2	27±1	Owira pond	Rarieda	2	Oct 2016
15	8.1±2	26±1	Luanda beach	Rarieda	2	Oct 2016
16	8.1±2	29±1	Misori beach	Rarieda	2	Oct 2016

3.3.3: Environmental water samples from the coastal region

Environmental water sampling from this region was done in June 2016. The sources of water used for domestic purposes by the communities from the region are wells, boreholes and rivers. A total of 27 water samples were collected in 500ml sterile plastic bottles from this region. Summary is shown in table 3.4. A broad range of temperatures from 23 to 29℃ was recorded while the pH ranged from to 7.1 to 8.2. The region is characterized by salty water and hot temperatures throughout the year. There is also history of frequent cholera outbreaks in the region (Mohamed et al., 2012).

Table 3.4: Environmental water samples from coastal region

Se. No	pH	Temp (ºC)	Specific Source	Region	No. of samples collected	Sampling period
1	7.8±2	28±1	River Magutwa	Kaloleni	2	June 2016
2	7.6±2	29±1	River Kombeni	Kaloleni	2	June 2016
3	7.9±2	24±1	Nour Hundaa borehole	Ribe	2	June 2016
4	7.6±2	28±1	River Mrenji	Rabai	2	June 2016
5	8.2±2	25±1	Heaven gate borehole	Kaloleni	2	June 2016
6	8.0±2	27±1	River Kizulini	Kaloleni	2	June 2016
7	7.9±2	29±1	River Kambenga	Kaloleni	2	June 2016
8	7.8±2	27±1	River Nsongoni	Kaloleni	2	June 2016
9	7.5±2	29±1	River Mwatsuma	Maliakani	2	June 2016
10	7.1±2	25±1	Likoni borehole	Likoni	1	June 2016
11	7.9±2	29±1	River Tiwi	Ukuda	1	June 2016
12	7.5±2	23±1	Kwamburu borehole	Ukuda	1	June 2016
13	7.3±2	24±1	Noor borehole	Ukuda	1	June 2016
14	7.6±2	23±1	Masjid Abdalla Al Mazruiy borehole	Ukuda	1	June 2016
15	7.9±2	25±1	Al-MabruQ borehole	Ukuda	1	June 2016
16	7.6±2	24±1	Istijaaba borehole	Town centre	1	June 2016
17	8.2±2	29±1	Indian Ocean	Town centre	1	June 2016
18	7.9±2	24±1	Kingorani borehole	Town centre	1	June 2016

3.3.4: Environmental water samples from Nairobi and central regions of Kenya

Environmental water samples were collected from Thika, Murang'a and Nairobi. The propagating strain was as well isolated from the respective region. Table 3.5 summarizes the environmental samples from this region. Sampling was done between May to August 2018

and a total of 18 environmental water collected. A total of 18 environmental water samples were collected from this region. Temperature at the time of sample collection ranged between 23 to 26℃ while the pH ranged from the lowest pH 6.8 to pH 8.2 (Kamiti river) which was the highest. The source of water for domestic purposes was mainly rivers.

Table 3.5: Environmental water samples from the central regions: Murang'a, Thika and Nairobi regions

Se. No.	pH	Temp (ºC)	Specific source	Region	No. of samples collected	Sampling period
1	6.8±2	25±1	River Nairobi	Nairobi	2	2018
2	6.9±2	26±1	River Ngong	Nairobi	2	2018
3	7.3±2	23±1	TUK borehole	Nairobi	1	2018
4	7.6±2	25±1	River Thiririka	Thika	1	2018
5	7.7±2	25±1	River Theta	Thika	1	2018
6	7.5±2	24±1	River Chania	Thika	1	2018
7	7.0±2	23±1	River Ruiru	Thika	1	2018
8	8.2±2	24±1	River Kamiti	Thika	2	2018
9	7.6±2	24±1	River Riverside	Nairobi	1	2018
10	8.1±2	25±1	Juja well	Thika	1	2018
11	7.0±2	23±1	River Arboretum	Nairobi	1	2018
12	6.8±2	23±1	River KMTC	Nairobi	2	2018
13	7.0±2	24±1	River Nyanjara	Murang'a	1	2018
14	7.0±2	23±1	River Cathanda	Murang'a	1	2018

Key: KMTC-Kenya Medical Training College, TUK-Technical University of Kenya

3.4: Isolation of bacteriophages

Bacteriophages can be isolated either directly or through the enrichment methods described by van Twest and Kropinski (2009). In this study the enrichment method was followed in the isolation of vibriophages from the environmental water bodies.

3.4.1: Enrichment of the environmental water samples

Isolation of *Vibrio cholerae* bacteriophages was carried out using protocol as explained by van Twest and Kropinski (2009) but slight adjustments were made. These modifications

included: use of Enrichment of the environmental water samples instead of direct method, the environmental water samples were not centrifuged prior to enrichment, use of 0.45µm filters instead of 0.22µm since the larger virions may not be able to pass through, TSB was used instead of Luria Bertani (LB) broth and Chloroform was not employed when preserving the phage stocks.

Enrichment of the environmental water samples was done following the protocol as described by van Twest and Kroprinski (2009) with some modifications. This was carried out by adding 10 ml of *Vibrio cholerae* overnight culture (12h) grown in single strength TSB sample into 10ml of the environmental water sample in a 250ml Erlenmeyer flask. This was mixed with 20ml double strength TSB that had been supplemented with 2mM $CaCl_2$. Incubation of the reaction mixture was done at 37°C for 48h in a shaker water bath at 100 revolutions per minute (RPM).

After 48h, approximately, 30 ml of the solution was dispensed onto a 50 ml falcon tube and centrifugation was done at 3400 × g for 15minutes. The supernatant was filtered through a sterile syringe mounted on 0.45µm pore sized filter get a free filtrate from any bacterial cell contamination onto small screw capped glass bottles that were labeled “crude lysate”. This was suspected to contain bacteriophages lytic to *Vibrio cholerae* and was preserved at 4°C for further testing.

3.4.2: Spot assay

The “crude lysate” obtained from the above enrichment procedure was spot-tested on the host cell to ascertain if it contained any lytic phages against the host bacterium.

A spot test was done by spotting 10µl crude lysate in triplicates, on a lawn of *Vibrio cholerae* that had been isolated from the respective regional environmental water samples used for enrichment. Upon incubation for 12h at 37 degrees centigrade, the lawns of bacterial cells were inspected for a plaque that is a clear zone on the lawn of the host bacterium caused by consecutive infection as well as burst cycles of bacteriophage lysis. Zones of clearing indicated presence of lytic phages in the environmental water samples (Carlson, 2005). A control was set where sterile SM buffer instead of the crude lysate was dropped on bacterial host cell lawns. Absence of a clear zone made the sample be considered negative for phage i.e. free of phages against the host bacterium.

3.4.3: Plaque assay

A clear zone from a positive spot assay was picked, suspended in 1ml sterile SM buffer, mixed thoroughly for the phages to dislodge from the soft agar. After allowed to settle for a few minutes, the suspension was passed through 0.45µm filter. Approximately, 400µl filtrate was obtained. Sterile micro centrifuge tubes were set in a row of 9 to carry out a tenfold sequential of phage serial dilutions. Aseptically, 900µl of SM buffer was added onto each of the 9 tubes. To make serial dilutions, 100µl lysate of bacteriophage was dispensed onto a tube labeled one and contained 900µl SM buffer; mixed well, pipette tip changed and 100µl transferred onto the second tube in the series. Ten-fold dilutions were made using a fresh sterile pipette tip for each transfer. Three dilutions were selected for carrying out the plaque assay that was three, five and seven whereby 100µl of the selected dilution of the phage lysates was mixed with 500µl of 12h culture of the host bacteria. This mixture was transferred onto 4ml of the top agar that had been held in a water bath at 45-50°C, mixed well by inversion and the contents poured over the surface of a dried, labeled TSA underlay plate. The overlay was allowed to settle, incubated for 12h at 37°C. The plates were inspected for lysis/clear spots thereafter plates with plaques between 30 and 300 were recorded as plaque forming units (PFUs). If no clear zones were observed, further incubation for 24h was done.

3.4.4: Bacteriophage plaques purification

A sterile pipette tip was used to pick up distinct isolated plaques that were suspended in one millimeter SM buffer. This was mixed well by inversions, passed through 0.45µm sterile filter. The filtrate was serially diluted to 10-fold as described above, 500µl 12h culture of the propagating bacteria added, mixed with 4ml top agar. After mixing by inversion the mixture was poured onto the surface of the plate containing bottom agar. Plates were incubated at 37°C for 12h to get separate plaques. After incubation a single plaque was identified, picked with a sterile pipette tip, suspended in 1ml SM buffer and the purification procedure above repeated three times in order to get clonal phages. Alternatively, purification of phages was done according to the procedure described by (Clokie & Kropinski, 2009).

3.5: Preparations of media and buffer for *Vibrio cholerae* cultivation

For enrichment of *Vibrio cholerae*, alkaline peptone water (APW) broth was used (HiMedia, Mumbai, India). It was prepared according to manufactures' instructions where 10g of APW was added to 500 milliliters (Hounmanou et al., 2019) of distilled water, dispensed into

smaller volumes of approximately 10 to 15 milliliters. This was autoclaved at 121 degrees centigrade (°C) for fifteen minutes (min) for further use and storage was done at room temperature (24°C). Same media was also employed for collection of the environmental water samples.

3.5.1: Tryptose Soy Broth (TSB) broth/agar

Tryptose soy broth was used for routine culturing of *Vibrio cholerae* (CMO 129 Oxoid LTD, Basingstoke, Hampshire, England). The broth media was prepared by adding 30g in one liter of distilled water, dispensed into smaller volumes of 10 ml then autoclaved at 121 degrees centigrade for fifteen minutes. Storage of the media was carried out at approximately 24 degrees' temperature/ambient temperature. Preparations of serial dilutions of the bacterial cultures were made using the same media. It was also used for culturing other bacteria isolated from environmental waters. Tryptose Soy Agar (TSA) was used for culturing *Providencia sneebia*, *Proteus mirabilis* and *Escherichia coli*: O83. Tryptose soy broth (TSB) (Oxoid, Basingstoke, Hampshire, England) was also used for culturing these bacteria where a colony of each bacterium grown on TSA was emulsified in TSB and incubation done at 37℃ for 12-24 hours.

3.5.2: Thiosulphate Citrate Bile Salt Sucrose media (TCBS)

Thiosulfate Citrate Bile Salt Sucrose (TCBS) media was used (HiMedia, Mumbai, India) as a selective media for isolation and culturing of *Vibrio cholerae*. For preparation, according to manufactures' instructions, 88g of Thiosulfate Citrate Bile Salt Sucrose (in powder form) was added onto an Erlenmeyer flask containing one liter of distilled water and brought to boil on a hot plate. Without autoclaving, the media was allowed to cool to approximately 50°C then dispensed onto petri dishes. It was allowed to cool, transferred to an incubator for overnight then preserved at 4°C in the fridge. It was used for a four weeks before more preparations were done.

3.5.3: Triple Sugar Iron (TSI) agar

Triple sugar iron (TSI) agar is a selective media for *Enterobacteriaceae* family and gram negative bacteria based on which sugar is fermented. It contains three sugars: Glucose, Lactose and Sucrose (Oxoid, Basinfstoke, Hamphire, England). This was prepared according to manufactures' instructions where before autoclaving; the media was distributed into tubes

that were 1/3 full. Before solidifying, after autoclaving the tubes were inclined to obtain 4-5 centimeters (cm) slant and 2-3 cm butt. The slants were stored at 4°C till use.

3.6: Preparation of media and buffers for isolation and characterization of bacteriophages

For phage dilutions and their storage, Saline Magnesium (SM) buffer was used. The buffer was prepared by adding 5.8g/L of sodium chloride (NaCl), Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) 2g/L, 5 ml/L of 2 % w/v Gelatin solution and Tris (50ml/L of a sterilized 1M stock solution (pH 7.5) was added to one liter of distilled water. This was dispensed in 30ml aliquots to glass universal bottles, pH adjusted to 7.5 prior to autoclaving at 121°C for 15 minutes and stored at room temperature until use.

3.6.1: Tryptose Soy Basal (TSA) and overlay (top) agar

Supplementation of Basal medium

Upon preparation of Tryptose Soy Agar (TSA) basal medium according to manufacturers' instructions, it was supplemented with 2mM $CaCl_2$ and 2mM $MgSO_4$ for reduction in loss by reabsorption and for attachment of the phage respectively.

Tryptose Soy Broth (TSB) Overlay (Top) 0.6% Agar

Supplementation of Tryptose Soy Broth (TSB) was carried out with bacteriological agar (Fisher) up to 0.6% w/v as final concentration, preparation done according to manufacturers' instructions. For the preparation of TSB basal agar, 0.6g of the bacteriological agar was added to 100ml of TSB broth, autoclaved at 121°C for 15minutes. After sterilization, aliquots of 4ml were dispensed aseptically in sterile universal glass bottles and stored at 4°C for a maximum period of 6 weeks. Prior to use, overlay media was melted either in a hot water bath or in a microwave and held at 45-50 degrees centigrade. The purpose of the top agar was to make bacterial lawns.

3.6.2: Magnesium Sulphate $MgSO_4$ (1M)

Magnesium Sulphate heptahydrate ($MgSO_4.7H_2O$) solution was prepared by dissolving 24.65g in 100 ml distilled water. This was autoclaved at 121°C for 15 minutes then the sterile solution stored at room temperatures for further use.

3.6.3: Calcium Chloride $CaCl_2$ (1M)

Preparation of Calcium chloride ($CaCl_2$) was done by addition of 11g in 100 ml distilled water as a stock solution. To make 2mM, 1 ml of the concentrated solution was diluted to 495 ml distilled water/broth. As stated above in section 3.2.1, this was to supplement the media as described by (Sambrook & Russell, 2001).

3.6.4: Trehalose

Preparation of Trehalose was carried out by dissolving 34.2g of Trehalose powder in 100 ml distilled water. The sterile solution was then stored at 4℃ for future use. This was for stocking the phages as a pilot study.

3.6.5: TAE buffer

The Tris-acetate-EDTA (TAE) buffer was prepared by adding 242g Tris base, 5.7 ml Glacial acetic acid and 100 ml 0.5M EDTA (Ethylene-diamine-tetra acetic acid) (pH 8.0). To make one liter of 1× TAE buffer, 20 ml of 50× TAE buffer was added in 980 ml of Reverse Osmosis (RO) water. The main purpose of Tae buffer was for Gel electrophoresis.

3.7: Growth kinetics of *Vibrio cholerae*

An investigative examination was done in order to determine the growth curve of *Vibrio cholerae*. This was done by streaking the host bacterium on TSA from the frozen storage, incubated overnight for 12h at 37°C. A single colony of this bacterium from the TSA after incubation overnight was picked, sub cultured on TCBS agar then incubated for 12h at 37°C. From the TCBS, a sterile wire loop was used to touch a single yellow colony of *Vibrio cholerae*, aseptically transferred onto 10ml TSB medium. This was considered to be zero time and after mixing well, 10µl was immediately transferred onto a 96 well plate containing 90µl TSB to make the first serial dilution before transferring the 10ml culture of the host strain to a shaker water bath at 100rpm. From each dilution, 10µl was spotted on the surface of TSA plates in triplicates, allowed to settle then incubated at 37°C for 12h. After every 10min, aliquots of 10µl of the bacterium were removed from the 10ml culture, serial dilutions performed, plated on TSA plates, incubated for 12h at 37°C. This was done for approximately 8h. The following day the colonies were counted only the plates that contained 3-30 colonies were considered.

3.8: Characterization of vibriophage isolates

For propagation of vibriophages in liquid broth media, bacterial strain *Vibrio cholerae* was subcultured on fresh TCBS and then incubated for 12h at 37°C. A distinct colony was drawn using a pipette tip that was sterile and suspended in 10ml TSB media, incubation was carried out in a shaker water bath at 100RPM and after 4h, inoculation of the culture with 100µl purified phage stock was done. This was incubated for 12h at 37°C. After 12h the solution was centrifuged at 14000×g for 10min, filtered with 0.45µm syringe mounted filter and the filtrated phage kept at 4°C awaiting titer determination.

3.8.1: Host propagating bacterial lawn preparation

Determination of host range for bacteriophages was accomplished by the spot assay procedure hitherto reported (Kutter, 2009) but with slight modifications. Propagation of bacteriophages requires that a confluent lawn of host bacterial strain be prepared. In lawn preparation, *Vibrio cholerae* was grown for 4h in TSB and 500µl of the host bacterium mixed with 4ml top agar (0.6% TSB media) that had been held at 50°C in a water bath. Following mixing host culture in top agar gently by inversion, it was poured on top of the TSA and swirled gently for production of confluent lawn. It was kept to settle for 20 minutes and used for phage spot assays.

3.8.2: Whole plate lysates preparations

Whole plate lysis was used for the propagation of the vibriophages. The phage stock was diluted and plaque assay was carried out to select the dilution that gave the clear, confluent plaques. An aliquot of the selected phage dilution, 100µl was mixed with 500 µl host culture grown in TSB for 4h. This was mixed with 4ml overlay then dispensed on top of TSA media plates, left briefly for settling before incubation for 12h at 37°C. Harvesting of bacteriophages was carried out by dispensing five millimeter of SM buffer onto the surface of each agar plate, incubation done at 37°C for 12h in a water bath shaker at 50RPM. The SM buffer was recovered and passed through 0.45µm filter. The filtrate was serial diluted 10-fold; spot tested on a lawn of *Vibrio cholerae,* incubated at 37°C for 12h for calculation of the phage titer.

3.8.3: Determination of phage titers

Bacteriophage titer determination was by double layer method as defined by (Kroprinski et al., 2009) however, slight modifications were made. SM buffer was used to make ten-fold

serial dilutions where aliquots of 10µl of each diluted sample of phage dropped on a lawn of host *Vibrio cholerae* bacterium. The spots were left for some time to settle then incubated for 12h at 37°C thereafter examined for plaque formation. Only plates with plaques between 3 and 30 were counted and titer recorded as PFUs/ml. Calculation of titer was done by multiplying dilution factor of the serial dilution and the volume that had been spotted on the lawn of the propagating host bacterium and the number of plaques.

3.8.4: Concentration of the vibriophages

The concentration of the vibriophages was done according to method prescribed by (Green & Sambrook, 2017), however slight changes were made. Approximately, 40 ml of the suspension of the vibriophages that had been filtered through 0.45µm filter was centrifuged for 2h at 40,000 x g at 4°C. Supernatant was discarded and the vibriophage pellet re-suspended in 2ml SM buffer, mixed well, stored at 4°C awaiting further analysis.

3.8.5: Freezing of the phages.

Phages were kept at minus 80°C in cryovials for long term preservation. For prolonged preservation of the phages for future reference, 600µl phage was mixed with 400µl of 50% glycerol, mixed well by inversion and transferred to a freezer at -80 degrees centigrade. More phages were stored at -20°C as well as 4°C for future reference.

3.8.6: Determination of the host range of the phages

Bacteriophage host range determination was done based on the capability of phage to produce clear zones/plaques on a lawn of 15 *Vibrio cholerae* strains isolated from the different environmental waters of Kenya, a clinical isolate of *Vibrio cholerae* that had been isolated from a patient suffering from cholera infection. Host range examination was carried out as described by (Stenholm et al., 2008; Kutter, 2009) however, with slight modifications (TSB with 0.6% agar was used instead of Luria-Bertani agar). Host range was also tested on the other gram negative bacterial strains isolated from different environmental waters during the same period of collection of environmental waters from the described regions. These bacteria included; *Escherichia coli*: 083, *Providencia sneebia* and *Proteus mirabilis*.

A lawn of each of these bacteria was also prepared to test the infectivity of the vibriophages. This was done by culturing each of this bacterium on TSA plates except for *Vibrio cholerae* that was cultured on TCBS. The incubation of the plates was done for 12h at 37°C for all the

bacterial strains. Thereafter, 10ml of TSB was inoculated with a single colony from each of the bacterial strain, incubated for 12h at 37°C. The lawns were prepared from each bacterium by mixing 500µl of the host bacterium with 4ml of soft agar then poured onto the surface of the TSA plates. After the plates were allowed to set, spot-on assay was done where 10µl of phage was spotted on each of the lawns of the propagating bacterial strain. After the spots were allowed to set, plates were incubated for 12h at 37°C. The plates were examined for zones of clearing/lysis where the phage had been spotted (Yu et al., 2013).

If a clear zone was observed, the sample was declared positive for phage; that is the phage was lytic against that bacterial strain and negative where no clear zone was observed. A control was set where, instead of spotting a phage lysate on the lawn of respective bacterial strain; a sterile SM buffer was spotted.

3.8.7: Thermal stability of phages

Thermal stability tests of vibriophages were carried out using the procedure described by (Jamal et al., 2015), with slight modifications. The initial titer of the stock phage was determined before the experiment. Bacteriophage suspensions of known titer were poured onto 1.5ml tubes and treated at different temperatures: 20°C, 30°C, 40°C, 50°C, 60°C and 70°C for 1 and 2h. Optimum temperatures for proliferation of *Vibrio cholerae* were put onto consideration while selecting these temperature ranges. A control was set at 37°C. After the specified time, the surviving phages were tittered using the double layer method as described by (Yu et al., 2013). An aliquot of 10µl of each of the dilutions was spotted on a lawn of the *Vibrio cholerae* bacterial strain.

3.8.8: pH stability of the bacteriophages

The pH stability tests were carried out as described by (Jamal et al., 2015) however, with modifications. Phage suspensions of 1ml was added onto 9ml TSB of different pH levels: 2, 4, 6, 8, 10 and incubated overnight (12h) at 37°C. The pH adjustment of TSB media was done using either 1M HCl or 1M NaOH to obtain between 2-12 ranges of pH. After 12h the phage titer was determined using the double layer method (Yu et al., 2013) and each dilution tested on a lawn of *Vibrio cholerae* host propagating strain.

3.8.9: Shelf life of the phages

Determination of the shelf life of the phages was done for 6 to 12 months. Although the recommended buffer for storage of phages is SM buffer (Clockie & Kroprinski, 2009), as a pilot study, a different solution, Trehalose was also used to determine the shelf life of the phages. This is because Trehalose is a known stabilizer of biologics (Singer & Lindquist, 1998). The phages were stored in SM buffer or Trehalose for a specified period of time after which they were tittered. Thereafter, their lytic activity determined by spotting on a lawn of respective propagating bacterium strain by double layer method.

3.8.10: Phage purification and staining

Before staining the vibriophages for preparation of Transmission Electron Microscope, purification of phages had been done for three rounds with respect to procedure described by (van Twest & Kroprinski, 2009). After three round of purification using the plaque assay, a clear plaque was picked using a sterile pipette tip, suspended in 2ml SM buffer in a falcon tube. Clearing on a lawn of host bacteria was an indication of bacterial cell lysis (Stenholm et al., 2008). After adding 200µl chloroform, the mixture was vortexed for 30 seconds and the contents transferred onto 1.5ml eppendorf tubes. This was centrifuged at 14000rpm for 10min, supernatant removed, filtered through 0.45µm cellulose acetate filter. Approximately, 1ml filtrate was obtained, an overnight sterility test done and samples further purified for further Transmission Electron Microscopy (TEM) analysis.

3.8.11: Transmission electron microscopy

Processing of samples for Transmission Electron Microscopy, (TEM) was kindly done by David Goulding of The Wellcome Sanger Institute, operated by Genome Research Limited in the United Kingdom (UK). Negative staining was performed with 5% of Urany acetate at ratio of 5:1 for each phage sample and magnification of X 60000 (Ntema *et al.,* 2010). Purified and washed phage samples were adhered to freshly glow-discharged carbon-Formvar grids, briefly stained with 5% uranyl acetate and then blotted and air dried. Grids were then viewed on a 120kV FEI Spirit Biotwin and imaged on a Tietz 4.16 CCD. Measurements were taken directly using TVIPS EMTools in EM-Menu. The morphology of bacteriophages was described according by the International committee on Taxonomy of viruses outlined criteria (Ackermann, 1996; Murphy et al., 1995)

3.8.12: One step growth curve of the bacteriophages

For the determination of the burst size and latent period, one step growth curve was employed as defined by Yu et al. (2013), with slight modifications. A colony from an overnight culture of *Vibrio cholerae* was infused in 100 millimeter TSB and incubated for approximately 3hours at 37°C at 200rpm. After 3hours, the culture was mixed with 1ml of phage solution and incubation done at 37°C for 15min to enable adsorption of phages to the propagating bacterial strain. The reaction solution was thereafter centrifuged for 5min at 14000 × g and the supernatant removed. The pellet was then resuspended in TSB broth media and incubated. Approximately, 100µl aliquots of the sample were taken after every 15min for 3h and the titer of the bacteriophage determined by overlay agar method.

3.8.13: Extraction of the phage DNA

The phage genomic DNA was extracted by the phenol-chloroform protocol as earlier on described by Sambrook et al. (2001) and adopted by Shah's study (Shah, 2014), with some slight modifications.

A phage solution of 40ml containing 1M NaCl and 10% w/v PEG 8000 was centrifuged at 10000rpm and 4°C for 20 minutes. Carefully, supernatant was discarded and the pellet resuspended in 500µl SM buffer thereafter transferred into a sterile 2ml eppendorf tube. To the phage solution, 50µl 10×buffer, 2.5µl 30/µl DNase enzyme and 1.5µl 20 mg/mL RNase A, were added then vortexed. The mixture was heated for 1h at 38°C. After 1h, the samples were removed and 60µl 0.2 M EDTA, 30µl 20mg/mL proteinase K and 50µl 10% Sodium Dodecyl Sulfate (SDS) added. The mixture was then vortexed and heated at 56ºC for 1hour. The samples were removed and cooled to room temperature followed by DNA purification by conventional method of phenol-chloroform extraction. On to the contents, 1ml TRIS-phenol was added, mixed well by vortexing, the contents were then centrifuged at 14000rpm for 5min at 4°C. Carefully, the top layer was taken and transferred onto a sterile eppendolf tube. In the meantime, a phenol-chloroform mixture, in the ratio of 10:10ml was prepared. After mixing well, 1ml of the mixed solution was added onto the contents in the eppendolf tube. All the contents were thoroughly mixed then centrifuged at 14000rpm at 4°C for 5minutes. The top layer was transferred onto a sterile eppendolf tube and the above procedure repeated twice. Thereafter, 1ml of either Isopropanol or Isoamyl alcohol was added in the ratio of 1:1 of the contents. Mixing was done gently but thoroughly until a white precipitate was visible.

Contents were placed in ice for 10 minutes, centrifuged at 14000 × g for 20 min at 4^{o}C. The liquid was poured out gently, taking care not to dislodge the white precipitate that settled at the bottom. Onto the white precipitate, 500µl of cold 70% ethanol was added, mixed well and the contents centrifuged at 14000rpm for 20 min at 4^{o}C. Ethanol was discarded as the DNA settled at the bottom as a white precipitate (ppt). To the white precipitate, 500µl of 100% Ethanol was added, thoroughly mixed and centrifuged at 14000rpm for 20 min at 4^{o}C. The supernatant was discarded by pipetting it out, care being taken not to remove the DNA pellet. Any remaining ethanol was aspirated to get dry DNA and 70µl double distilled (dd) water added. The final extracted DNA pellet was kept at 4^{o}C and later sent for sequencing.

3.8.14: Phage DNA restriction patterns and maps

Four restriction digestion endonucleases were used to digest the phage genomic DNA for comparison of the DNA fragments patterns. These restriction enzymes used were: *HindIII, EcoRI, SalI,* and *XhoI.* The identification was done as per description given by (Vincze et al., 2003). The following components were mixed in order at room temperature: 30µl nuclease free water, 5µl of 10Xfast digest buffer, 10µl DNA and 5µl fast digest enzyme. These contents were mixed well and kept at 37°C for 10 minutes after which the contents were loaded onto the Gel electrophoresis (agarose 1%) as described in the next section below. For the generation of restriction maps the raw sequences were De novo assembled using CLC genomics workbench. The restriction map was subsequently generated based on the assembled whole genome sequence for each phage.

3.8.15: Gel electrophoresis

For gel electrophoresis, 0.48g of agarose gel was dissolved in 60ml of TAE buffer in a 250ml Erlenmeyer flask. To completely dissolve the agarose gel, the contents were placed in a microwave and heated for approximately 2 minutes while mixing intermittently. Mixture was then cooled to approximately 45°C, 2.5µl Gel Red added and mixed well. Gel was poured onto the tray taking care not to introduce air bubbles. After placing the comb in position, gel was allowed to set for approximately 30 minutes to 1hour. Combs were then removed gently by lifting upwards and then gel placed in electrophoresis tank that had previously been filled with TAE buffer. Bacteriophage DNA samples either pure or restricted were mixed with loading buffer in the ratio of 5:1 (sample: buffer), and then loaded onto the wells. DNA marker also loaded but without the dye. Gel electrophoresis tank was closed after loading all

the samples, connected to power then run for 30 minutes. Power was then switched off, electrophoresis tank disconnected, gel carefully removed from the buffer, placed on the imaging black tray and the imaging visualized by Agilent Bio analyzer 2100 system (Agilent Technologies, CA, USA).

3.8.16: Phage genome purity, integrity and concentration

The phage genome concentration measurements were done using the Qubit fluorometer (Thermo Fisher Scientific). Phage DNA purity and integrity measurements were done using the agarose gel electrophoresis. The detection parameters included: genomic gel concentration was 1%, voltage 100V and electrophoresis time was 40 minutes. In addition, the PCR-gel concentration was 2%, voltage at 80V and electrophoresis time was 50 minutes.

3.8.17: Procedure for library construction and sequencing

After confirming the correction of the DNA samples, sonication on Covaris sonicator was used to break the genome DNA randomly. DNA fragments were then end-repaired, A-tailed and ligated with adapters, purified and amplified by PCR to prepare the library. After the construction of the library, Qubit3.0 was firstly used for quantitation the library. After the dilution of the library, library quality was assessed on an Agilent Bioanalyzer 2100 system. If the inserted fragments were expected, qPCR was used to determine the concentration of the library to ensure the quality of the library.

After the confirmation of a good quality of the library, the library was pooled to flow cell according to the requirement of the concentration and the quantity. After clustering by cBOT, the sequencing was performed on the high-throughput platform of Illumina Nova-Seq. Below are the instruments and reagents used for the above procedure:

DNA shearing: Covaris (Covaris, USA)

Library construction kit: NEBNext® Ultra™ II DNA Library Prep Kit for Illumina (NEB, USA)

DNA quantifying: Qubit®3.0 Flurometer (Life Technologies, CA, USA)

Detection instrument for fragments: Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA)

Sequencing platform: NovaSeq 6000 (Illumina, USA)

Sequencing reads: PE150

3.8.18: Assembly and annotation of phage genome

After the phage genome was extracted as described in section 3.8.13, the phage genomes were sequenced on NovaSeq 6000 (Illumina, USA) (Navarro-Ocaña et al., 2016). The reads were trimmed using trimmomatic, the quality was assessed using FastQc and assembled using unicycler with standard settings. The quality of the assembled contigs was assessed using Quast. The phages were annotated using VIBRANT v1.2.1 (Kieft et al., 2020) and Rapid annotation using Subsystems technology (RAST) server was used for annotation (Aziz et al., 2008), after adjusting RAST settings to the optimized phage pipeline (Aziz et al., 2018; McNair et al., 2018). Detection of tRNA genes sequences was performed using tRNAscan-SE that is able to identify 99–100% of transfer RNA genes in DNA sequence (Schatter et al., 2005; Lowe & Eddy, 1997) and ARAGON (Laslett & Canback, 2004). For additional elements such as reverse and forward codons, G+C percent content and visualization of the sequences, ARTEMIS comparison tool was used (Carver et al., 2008). Annotations of VP4 were visualized using Geneious Prime 2019.0.3(Biomatters, Ltd., New Zealand) and colored according to the respective annotation category.

3.8.19: Comparative sequence statistics analysis

Further analysis in the protein sequence similarities was carried out using CoreGenes3.5 software (Turner et al., 2013). Additionally, Virfam server used to obtain a comparative sequence statistics analysis (Lopes et al., 2014). The Virfam server uses annotations of the phage genome to predict structural proteins (head-neck-tail modules), then classify the phage. Virfam was also able to predict hits to the major capsid protein, portal protein, large terminase and some other features to look for relationships to known phages (Lopes et al., 2014).

Phage VP4 was aligned by tBlastx identity (Altschul et al., 1990) to two closely related phages according to the phylogenetic trees (MG592609.1 and NC_048709.1) using EasyFig for visualisation (Sullivan et al., 2011) (v2.2.2, minimum blast length: 100, maximum e-value: 1e-5, minimum identity: 50%). Tools of the Pathosystems Resource Integration Center (PATRIC) portal (Davis et al., 2020) were used for comparing predicted proteins of four phages.

3.9: Construction of genomic maps

Before construction of genomic map, the phage genome was annotated using RAST as explained previously by (Aziz et al., 2008; Overbeek et al., 2014) and VIBRANT v1.2.1 (Kieft et al., 2020). Genomic map construction was done using GenomeVX (Melodelima & Lobréaux, 2013; Conant & Woolfe, 2008). GenomeVx was opened as described by (Melodelima & Lobréaux, 2013). To input the annotated file (downloaded gene bank data): The name of the phage was indicated on the chromosome name, auto-colored existing genes (color selected automatically), in diagram orientation clockwise selected from the top and finally genome clicked draw genome. Since the phage genomes of the four selected phages for further characterization revealed similarity, VP4_Ke was selected as a representative for construction of the genomic map using PATRIC tools (Davis et al., 2020), comparing predicted proteins of the four phages, generating a CIRCOS circular map with percentage GC content and GC skew of the phage genome (Krzywinski et al., 2009). For the filamentous phage sequence that is the *Inoviridae* phage it was named VP24-2_Ke to represent the small genome sequence.

3.10: Construction of phylogenetic trees of phages

Phylogenetic trees of the four bacteriophage sequences were done to access their position taxonomically. The annotations from VIBRANT were used to identify proteins of ribonucleotide reductase (RnR) subunits alpha and beta as well as the large terminase (LT) subunit for phylogenetic tree construction. RnR alpha, RnR beta and LT protein sequences from VP4 were used to query the NCBI nr database (accessed August 2020, taxid10239, e-value < 1e-5) for reference virus sequences. The top 100 results from each search were taken and individually dereplicated using CD-HIT v4.6, -aS 0.8 -c 0.9 (Huang et al., 2010). Respective protein sequences for VP4, VP6, VP18 and VP24 were added to the reference sequences and aligned using MAFFT (v7.388) (Katoh & Standley, 2013), with default settings. Phylogenetic trees were constructed using RAxML (v8.2.4) (Stamatakis, 2014), with the command: raxmlHPC-PTHREADS -N 100 -f a -m PROTCATLG. Resulting trees representing 100 bootstrap iterations and rooted by outgroup, were visualized by FigTree v1.4.4 (Rambaut, 2007).

For the filamentous vibriophages, phylogenetic analysis has been performed based on the level of individual genes rather than the whole genome so as to asses evolutionally relatedness and divergence among these phages (Wang et al., 2013). Three essential gene sequences

namely: genes encoding replication proteins, pIII-like receptor-binding coat proteins and Zot-like phage assembly proteins were used for phylogenetic analysis of the filamentous phage VP24-2_Ke. Nine reference genomes of vibriophage were searched on NCBI for comparative analysis. The following steps were used to get phylogenetic tree:

a. Open the software MEGA7; click Edit/Built Alignment under the drop-down menu of "Align".
b. Then click "Open" and choose one of the files built above.
c. After loading the sequences, click "Alignment by Clustal W" under the drop-down menu of "Alignment".
d. After the alignment results present, click "phylogenetic analysis" under the drop-down menu of "Data".
e. Go back to the main menu, and choose the Neighbor-Joining method to construct a phylogenetic tree under phylogeny menu.
f. There was a window to appear. Set "Bootstrap method" as 1000, and then click compute. A phylogenetic tree was completed.

CHAPTER FOUR: RESULTS

4.1: Isolation, characterization and identification of bacterial isolates

A total of 140 different environmental water samples were collected from various sources. The highest number of samples was collected from rivers (75), beaches (21), boreholes (12), Lake Victoria (9), ponds (8), springs (8), wells (6) and one from Indian Ocean.

A total of five different types of bacteria were isolated from the different environmental water sources of Kenya as shown in Table 4.1. These bacteria were all Gram negative, grew well on Tryptose Soy Agar (TSA) media at 37°C for 12hours. Morphologically, these bacteria were all rods except for *Vibrio cholerae* that was comma shaped and *Ralstonia solanacearum.* From a total of 140 water samples collected from various sources of different regions in Kenya, 15 water samples were positive for *Vibrio cholerae* which accounted for 10.7 percent. Nine water samples collected from rivers tested positive for *Vibrio cholerae* (12%). The distribution of the samples that tested positive from rivers was as follows; 5 water samples were collected from River Kuja in Migori county, one from each of the following rivers; Kamiti in central region, Nsongoni in coast, Kizulini also in coast and Riverside river in Nairobi. Three water samples out of a total of 21 collected from beaches tested positive for *Vibrio cholerae*; Osieko, Lauda and Usenge. Three water samples out of a total of 8 collected from ponds also tested positive for *Vibrio cholerae*; Koleche, Owira and Kotonde. The environmental water sources from boreholes, wells, springs, Lake Victoria and Indian Ocean did not test positive for *Vibrio cholerae*. The 16S ribosomal RNA partial sequences of genes of the bacteria isolated in this study showed similarity levels of between 97-99% with the respective known isolates BLAST search that was performed against NCBI. These comparative results are displayed in Table 4.1. Seven of these bacteria 16S ribosomal RNA partial gene sequences were deposited in GenBank and given the accession numbers as depicted in the Table 4.1. The partial sequences of the 16S ribosomal RNA gene of the seven bacteria ranged from 954 to 1226 base pairs. From a total of 15 *Vibrio cholerae* strains isolated from the different environmental water samples, 16S ribosomal RNA partial gene sequences of three of them was deposited in gene bank.

Table 4.1: Bacterial strains, %16S rRNA gene sequence similarity morphology and genbank accession numbers

Se. No.	Bacterial strain code	Bacterial Species	Affiliated to/Closest taxonomic match in BLAST	Similarity of %16S rRNA gene sequence	Gram reaction	Morphology	Genbank accession numbers
1	Vc_ke	*V. cholerae*	Vc strain CIFT MFB VC165 (KP058474.1)	98.10	Negative	Comma	MN467399.1
2	Vc_Koleche	*V. cholerae*	Vc strain NIOT VC 05 (MF692791.1)	99.18	Negative	Comma	MN907464.1
3	Vc_Nsongoni	*V. cholerae*	Vc strain RD1 (KF307775.1)	97.86	Negative	Comma	MN907465.1
4	EC_ke	*E. coli*	Ec strain Huaian_14_2 (MN314160.1)	98.27	Negative	Rods	MN467398.1
5	Ec_Kuja	*E. coli*	Ec strain Sihong_668_2 (MN314257.1)	97.28	Negative	Rods	MN907473.1
6	PROS_ke	*P. sneebia*	Pr JCM 3954 (LC420104.1)	98.12	Negative	Rods	MN467401.1
7	PREM_ke	*P. mirabils*	Pm strain NA-20 (MN882646.1)	99.69	Negative	Rods	MN467400.1
8	RALS	*R. solanacearum*	NP	NP	NP	NP	NP

Key: PREM_ke- *Proteus mirabilis*, PROS_ke- *Providencia sneebia*, Vc -*Vibrio cholerae*, Ec-*Escherichia coli*, NP-Not Provided, Pr-*Providencia rustigianii*, Pm-*Proteus mirabilis*, RALS- *Ralstonia solanacearum*

4.1.1: Selective growth of *Vibrio cholerae* on TCBS

For differentiation of *Vibrio cholerae* from the other bacteria, a selective media, TCBS was used. Figure 4.1 shows the appearance of *Vibrio cholerae* strain isolated from one of the environmental water sources on TCBS media. *Vibrio cholerae* ferments sucrose producing small yellow colonies as seen in the Figure 4.1. The yellow appearance of the colonies on TCBS was an indication of a positive presumptive test for *Vibrio cholerae*. If any other color of colony was observed, this was considered negative for *Vibrio cholerae*, hence ignored. The *Vibrio* strains also showed good growth in TSB and on TSA. Colonies, big enough for sub-culturing, would be seen after 6 hours of incubation at 37°C.

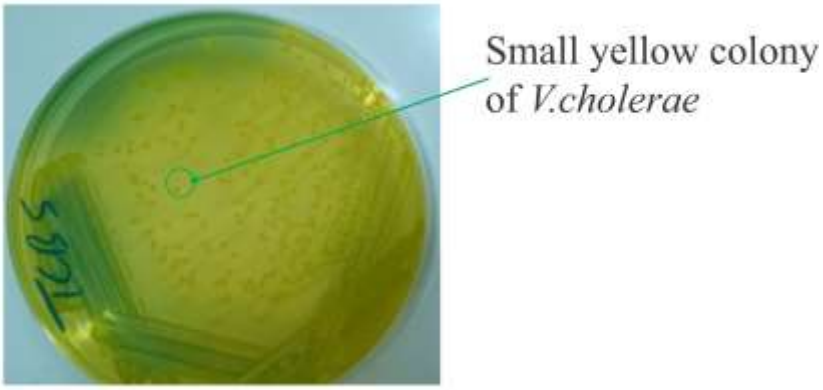


Figure 4.1: Yellow colonies of *Vibrio cholerae* on TCBS media.

4.1.2: *Vibrio cholerae* reaction on TSI

The reaction results of *Vibrio cholerae* on TSI as demonstrated in Figure 4.2 displaying a yellow butt, yellow slant due to production of acid without gas as an indication of reaction for *Vibrio cholerae.* The appearance of yellow color in the slant was an indication that the isolate had the ability to ferment sucrose or /and lactose. The yellow color appearance on the butt was an indication that some fermentation had occurred with production of acid. It was also attested that the isolate was a facultative anaerobe. There were no cracks, no bubbles, breaks or lifting of the gas in the tube an indication that there was no gas production. In addition, there was no black precipitate visible in the butt indicating absence of Hydrogen Sulfide gas formation since the isolate did not reduce sulfur component in the medium.

Figure 4.2: 12 Hour reaction of *Vibrio cholerae* on TSI

4.2: Isolation, biological and physical characterization of phages

Isolation of bacteriophages from the environmental water sources of four regions of Kenya was successful. Five phages were isolated from River Kuja in Migori. The propagating host strain was *Vibrio cholerae* isolated from the same river at different sites. Six phages were successfully isolated from Siaya region specifically Bondo and Rarieda. Two phages were isolated from the environmental waters of the coastal region. From the central region it was successful to isolate two phages. One phage from River Kamiti, Kiambu in Central region and the other phage was from Riverside River Nairobi County. The propagating host, *Vibrio cholerae* was isolated from each of the respective sources where the phage was isolated as depicted in Table 3.1. The results of plaque morphologies of the phages are displayed in Table 4.2.

4.2.1: Plaque description of phages

All the phages had clear, visible plaques after propagation on the respective host strain for 12h at 37°C. The shape of the plaque was circular with regular margins of diameter approximately 0.5-3mm except for phages VP64_Ke and VP68_Ke that had turbid plaques initially. The size of plaques of these two phages was pinpoint, approximately less than 0.5mm. The initial spot assay for all the phages were all clear except for VP64_Ke and VP68_Ke which were relatively clear. However, all the phages underwent three rounds of purification and even the phages VP64_Ke and VP68_Ke had clear and pure plaques after three rounds of purification. Figure 4.3 shows the appearance of spot assay on a lawn of propagating host strain, *Vibrio cholerae* of phages VP4_Ke, VP6_Ke and VP8_Ke. Spotting

of the phage on the lawn of *Vibrio cholerae* was done in triplicates as shown in Figure 4.3. Figures 4.4 and 4.5 represent positive plaque tests on a lawn of propagating *Vibrio cholerae* strain at the initial stages of purification process portraying the different sizes of plaque morphologies.

Table 4.2: Description of plaque morphologies of the isolated phages across Kenya

Se. No.	Phage code	Sample source	Sample location	Sample pH	Plaque description
1	VP4_Ke*	EW	River Kuja	7.8	0.5-3mm diameter, clear, circular, regular margins
2	VP6_Ke*	EW	River Kuja	7.8	0.5-3mm diameter, clear, circular, regular margins
3	VP8_Ke	EW	River Kuja	7.8	0.5-3mm diameter, clear, circular, regular margins
4	VP12_Ke	EW	River Kuja	7.8	0.5-3mm diameter, clear, circular, regular margins
5	VP18_Ke*	EW	River Kuja	7.8	0.5-3mm diameter, clear, circular, regular margins
6	VP24_Ke*	EW	Usenge beach	8.1	0.5-3mm diameter, clear, circular, regular margins
7	VP28_Ke	EW	Leuda beach	7.9	0.5-3mm diameter, clear, circular, regular margins
8	VP42_Ke	EW	Owira pond	7.9	0.5-3mm diameter, clear, circular, regular margins
9	VP56_Ke	EW	Koleche pond	8.0	0.5-3mm diameter, clear, circular, regular margins
10	VP64_Ke	EW	Kotonde pond	8.1	Less than 0.5mm diameter, pinpoint, clear, circular, regular margins
11	VP68_Ke	EW	Osieko beach	8.1	Less than 0.5mm diameter, pinpoint, clear, circular, regular margins
12	VP94_Ke	EW	Kambenga river	7.9	0.5-3mm diameter, clear, circular, regular margins
13	VP124_Ke	EW	Kizulini river	8.0	0.5-3mm diameter, clear, circular, regular margins
14	VP132_Ke	EW	Kamiti river	8.2	0.5-3mm diameter, clear, circular, regular margins
15	VP140_Ke	EW	Riverside river	7.6	0.5-3mm diameter, clear, circular, regular margins

Key: * -further sequenced, EW- Environmental Water

Figure 4.3 shows a lawn of environmental *Vibrio cholerae* with a clear zone where the lysate was spotted. The clear spot was deep on the TSA top agar indicative of complete cell lysis.

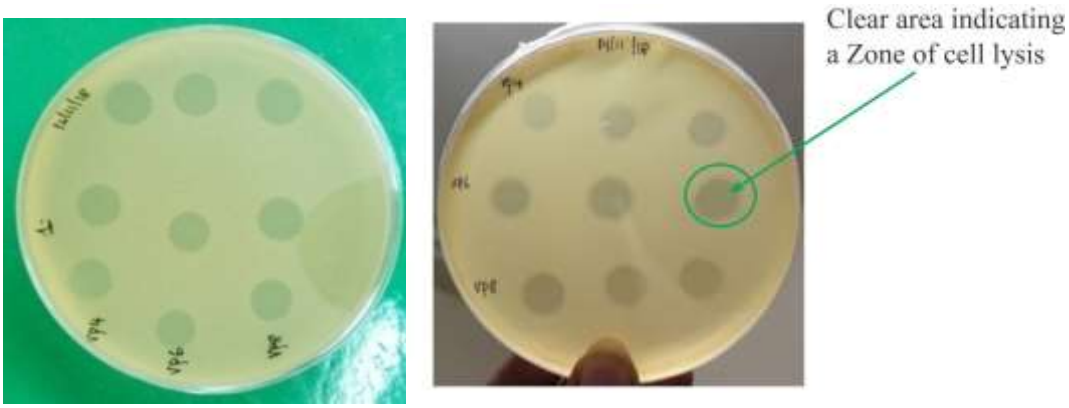


Figure 4.3: A positive spot assay of phages VP4_Ke, VP6_Ke and VP8_Ke.

Figure 4.4 shows clear zones (plaques) of cell lysis on a lawn of *Vibrio cholerae* host bacterium. The plaques were of diameter greater than 1mm, not uniform since it was at early/initial stages of the purification process.

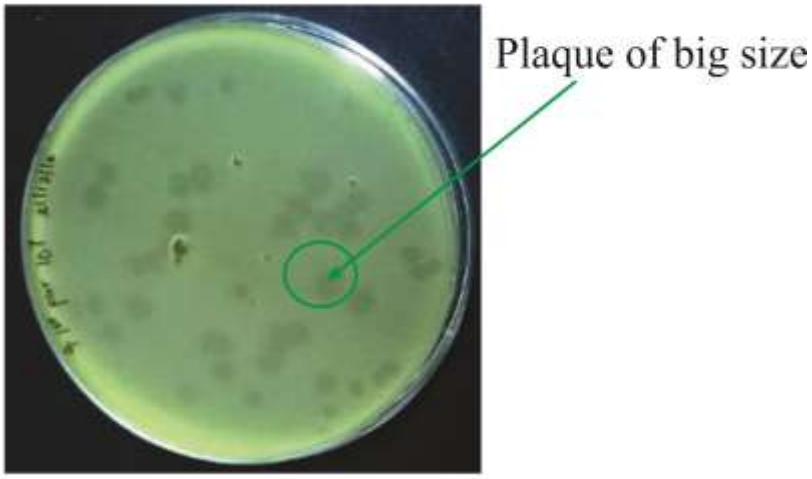


Figure 4.4: A Positive plaque assay of phage with plaques of big size

Figure 4.5 shows positive plaque assay with point plaques of diameter less than 0.5mm on a lawn of *Vibrio cholerae* as the propagating strain. The plaques are zones of bacterial lysis.

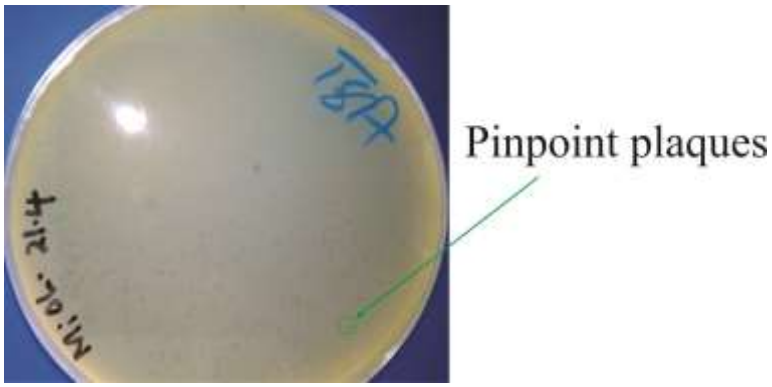


Figure 4.5: A positive plaque assay showing pinpoint plaques

4.2.2: Phage titers

The titers of the phages after overnight propagation (12h) at 37°C on the lawn of *Vibrio cholerae* ranged between 10^9 and 10^{10} PFUs/mL. Out of the fifteen isolated phages, six phages, VP4_Ke, VP6_Ke, VP8_Ke, VP24_Ke, VP68_Ke and VP140_Ke had a titer of 10^{10} PFUs/mL while the other phages had a titer of 10^9.

4.2.3: Thermal stability of phages

Thermal stability tests of phages were done between 20°C to 70°C temperatures. All the phages during the time of experiment were stored at 4°C. A minimal decline in phage titer observed at 20°C to 50°C. All phages having a titer of between 1×10^9 PFUs/mL and 1×10^7 PFUs/mL. There was a drastic reduction in plaques, from 10^3 PFU/mL to Zero between 50°C to 60°C range. Above 60°C, there were no plaques observed on a lawn of propagating *Vibrio cholerae.*

4.2.4: Stability of phages with respect to pH

The isolated and purified phages were subjected to pH stability tests. At pH 2 and 4 no plaques at all were observed on a lawn of *Vibrio cholerae.* Optimum pH was from 8 to 10, where the maximum number of plaques was recorded.

4.2.5: Shelf life of the phages in different solutions

After storing the phages in SM buffer for one year at 4°C, all the five phages isolated from Migori and Kisumu namely: VP4_Ke, VP6_Ke, VP8_Ke, VP12_Ke and VP18_Ke showed a clear zone after spotting on a lawn of *Vibrio cholerae.* All the fifteen phages isolated in this study had a shelf life of 6 months after storing in either SM buffer or Trehalose. However, in

this study of shelf life the titer was not determined only a spot assay was performed after the respective storage periods.

4.3: Growth kinetics of *Vibrio cholerae*

It was important to establish the growth kinetics of the host strain bacteria used for propagation of the phages isolated in this study in order to establish the exponential phase. This in turn was helpful for one step growth curve study of the phages. The growth kinetics of the host strain therefore involved establishment of the growth curve of *Vibrio cholerae.* The representative growth curve of one of the *Vibrio cholerae* host strain used in this study Vc_ke is depicted in Figure 4.6. The generation time was approximately 60 min and the mid exponential time was between 100 and 200 minutes. The viable counts were calculated as CFU/mL and Log_{10} data were plotted against time. The error bars represent the standard error of the means.

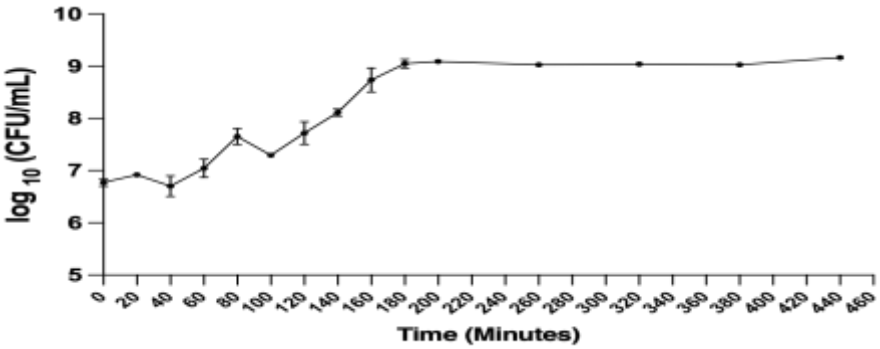
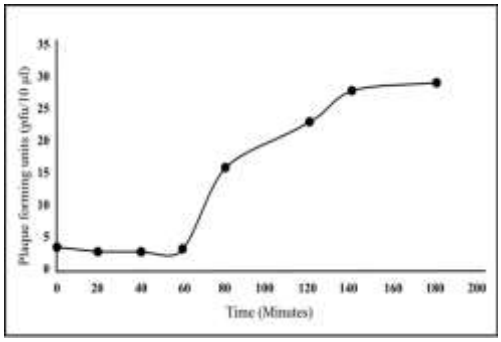


Figure 4.6: Growth curve of *Vibrio cholerae* (Vc_ke) strain

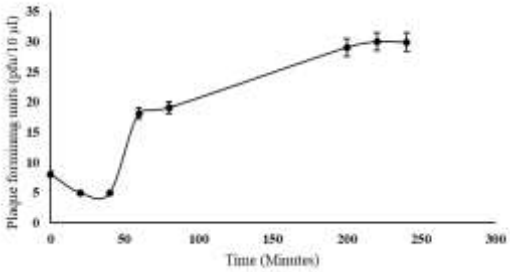
4.3.1: One step growth curves of five phages

To determine burst size of the phages and latent period, one step growth curves of phages were carried out. Latent period of the five phages in this study was found to fall in the range of 40 to 60 min while burst size ranged between 23 and 26 PFUs/10µl. Figure 4.7 (A to E) shows representative one step growth curves of the five phages, VP4_Ke (A), VP6_Ke (B), VP18_Ke (C), VP24_Ke (D) and VP68_Ke (E) isolated from different environmental regions of Kenya in this study. The results of burst size and latent period of the phages selected are shown in Table 4.3. Phage VP4_Ke had, approximately a latent period of 50 minutes and a burst size of 26PFUs as displayed in Figure 4.7A. This phage had been isolated from river Kuja in Migori County. Phage VP6_Ke had a latent period of 40 minutes and a burst size of 23 PFUs, Figure 4.7B, also isolated from river Kuja in Migori County. The latent period for

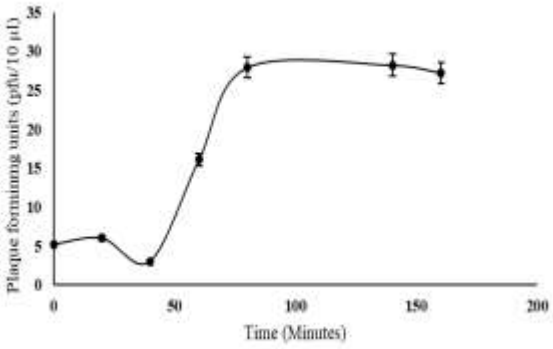
phage VP18_Ke was 30 minutes and the burst size was 26 PFUs as shown in Figure 4.7C. The phage was isolated from river Kuja in Migori. Phage VP24_Ke had a latent period of 40 minutes and a burst size of 24 PFUs as depicted in Figure 4.7D. Approximately, the latent period for phage VP68_Ke was 40 minutes and the burst size was 23 PFUs, (Figure 4.7E).



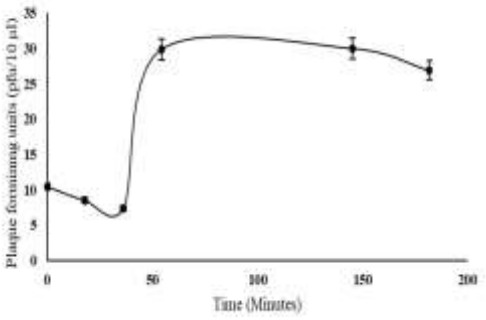
A



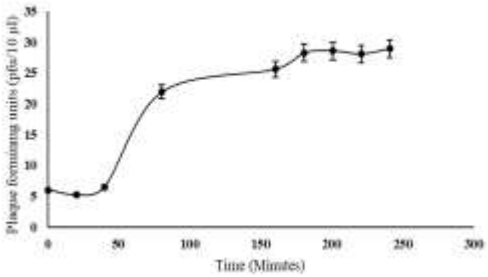
B



C



D



E

Figure 4.7: One step growth curves of five phages

A- One step growth curve of VP4_Ke phage, **B-** One step growth curve of VP6_Ke phage, **C-** One step growth curve of VP18_Ke phage, **D-** One step growth curve of VP24_Ke phage, **E-** One step growth curve of VP68_Ke phage

The results of the Latent period and burst size of the five phages are displayed in Table 4.3. The burst size ranged from 23 to 26 PFUs/mL while the latent period was between 30 to 50 minutes.

Table 4.3: Latent period and burst size of five phages

***Vibrio cholerae* phage**	***Vibrio cholerae* source**	**Latent period (minutes)**	**Burst size (PFU/10µl)**
vB_Vch4M_Ke	River Kuja, Migori	50	26
vB_Vch6M_Ke	River Kuja, Migori	40	23
vB_Vch18M_Ke	River Kuja, Migori	30	26
vB_Vch24M_Ke	Usenge beach, Siaya	40	24
vB_Vch68M_Ke	Osieko beach, Siaya	40	23

4.3.2: Phages' host range profiles

All the fifteen bacteriophages produced clear lysis with both environmental and clinical strains of *Vibrio cholerae* as respective propagating strains. There were no zones of clear lysis observed on a lawn of *Proteus mirabilis* for all the fifteen phages used in the host range profiles as shown in Table 4.4. However, phages VP64_Ke and VP68_Ke displayed opaque lysis on a lawn of *Escherichia coli*: 083 and *Providencia sneebia* bacterial isolates.

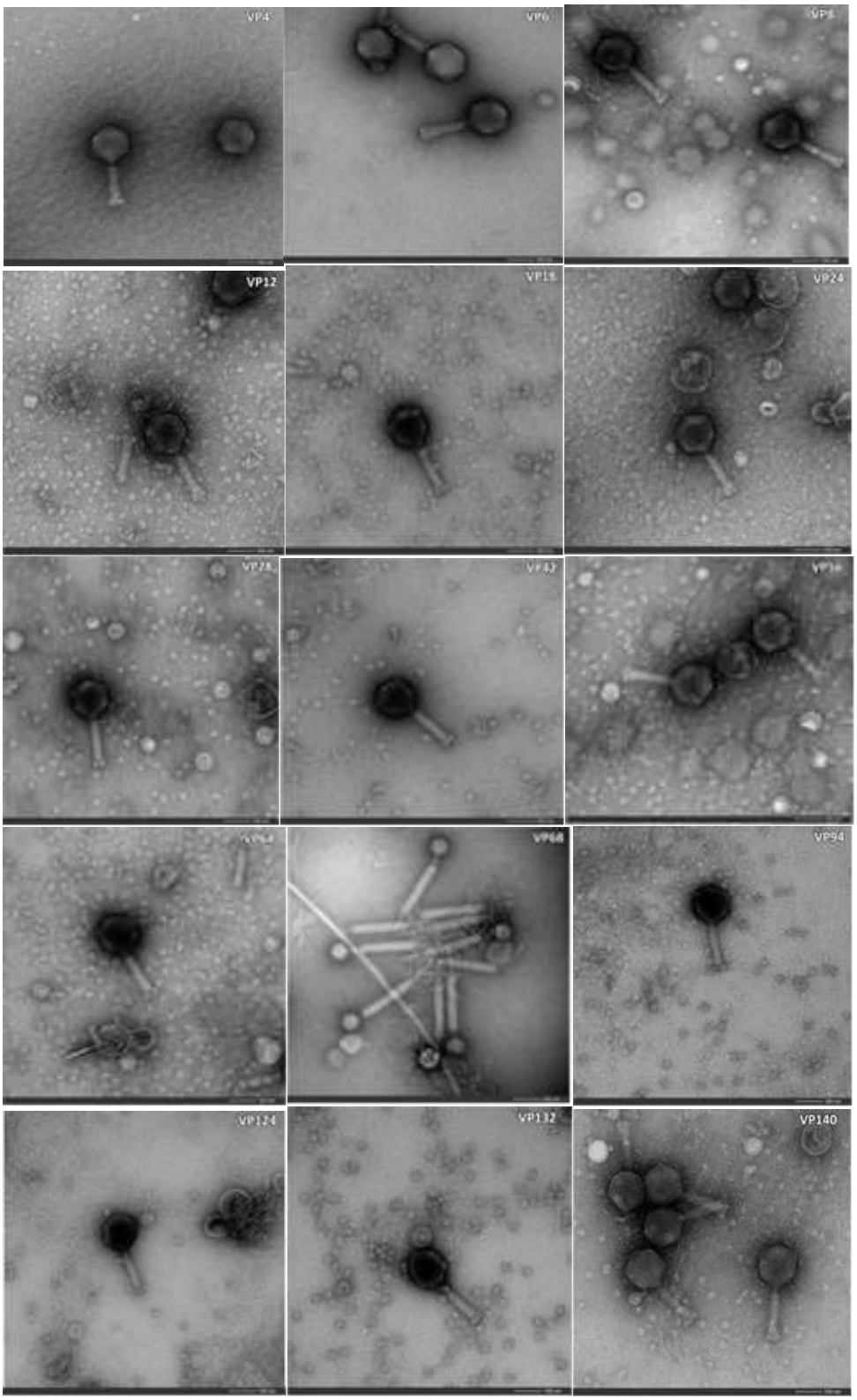
Table 4.4: Host range profiles of the *Vibrio cholerae* bacteriophages

Bacteriophages															
Host strain	**VP4**	**VP6**	**VP8**	**VP 12**	**VP 18**	**VP 24**	**VP 28**	**VP 42**	**VP 56**	**VP 64**	**VP 68**	**VP 94**	**VP 124**	**VP 132**	**VP 140**
***V. cholerae* (cl)**	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
***V. cholerae* (en)**	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
***P. mirabilis* (en)**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
***P. sneebia* (en)**	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
***E. coli:* 083(en)**	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-

Key: en-environmental, cl-clinical, ++ clear lysis, + opaque lysis, - no lysis

4.3.3: Morphological characterization of the bacteriophages

For determination of morphological features and classification according to International committee on taxonomy of viruses (ICTV), electron micrographs were obtained. Figure 4.8 displayed the morphological features of the fifteen bacteriophages isolated in this study. The 15 phages had icosahedral heads and contractile tails. Table 4.5 summarizes the structural features examined by electron microscope of the 15 phages isolated in this study. There were several features that were revealed by Transmission electron microscope. All the fifteen phages were assigned to the *Myoviridae* family of the order *Caudovirales*. In general, all the fifteen phages revealed some level of similarities except for phage VP68_Ke that revealed unique structural features. The head diameter ranged between 33 to 97nm with VP68_Ke having the smallest head diameter of 33nm and phages VP12_Ke had the largest diameter of 97nm. Neck diameter ranged between 8 and 16nm, VP8_Ke had the smallest neck diameter of 8nm while VP4_Ke and VP94_Ke had the highest neck diameter of 16nm. Tail diameter was between 14 and 21nm, VP8_Ke had the smallest while VP68_Ke the longest. VP68_Ke also displayed the longest diameter from tail to base plate of 130nm and also from base plate to the end it also had the longest, 47nm. Out of the fifteen phages only four were selected for further molecular characterization.



Bar =100nm

Figure 4.8: Transmission electron monographs of the 15 phages

Table 4.5: Classification and description of the phages

				Phage measurements (nm)				
Se. No.	***Vibrio cholerae* phage**	**Code**	**Classification (Family)**	**head diameter**	**Neck diameter**	**tail to bp**	**tail diameter**	**bp to end**
1	vB_VchM_Kuja	VP4_Ke	*Myoviridae*	85	16	95	16	13
2	vB_Vch6M _Ke	VP6_Ke	*Myoviridae*	85	14	100	16	13
3	vB_Vch8M _Ke	VP8_Ke	*Myoviridae*	82	14	97	14	14
4	vB_Vch12M _Ke	VP12_Ke	*Myoviridae*	85	15	101	16	12
5	vB_Vch18M _Ke	VP18_Ke	*Myoviridae*	79	15	103	16	12
6	vB_Vch24M _Ke	VP24_Ke	*Myoviridae*	78	13	99	16	12
7	vB_Vch28M _Ke	VP28_Ke	*Myoviridae*	85	15	95	17	14
8	vB _Vch42M _Ke	VP42_Ke	*Myoviridae*	80	15	96	16	12
9	vB _Vch56M _Ke	VP56_Ke	*Myoviridae*	87	15	98	17	14
10	vB _Vch64M _Ke	VP64_Ke	*Myoviridae*	85	15	100	15	14
11	vB _Vch68M _Ke	VP68_Ke	*Myoviridae*	33	8	130	21	47
12	vB _Vch94M _Ke	VP94_Ke	*Myoviridae*	79	16	78	17	13
13	vB_Vch124M _Ke	VP124_Ke	*Myoviridae*	80	15	85	16	12
14	vB_Vch132M _Ke	VP132_Ke	*Myoviridae*	75	15	102	16	13
15	vB _Vch140M _Ke	VP140_Ke	*Myoviridae*	86	14	100	17	13

KEY: bp-base plate

4.4: Genomic characterization

Agarose Gel electrophoresis was done for Purity and DNA integrity measurement. The results are shown in Figure 4.9. The four phages selected for further characterization in this study portrayed almost similar genome sizes: Vp4_Ke and Vp6_Ke had a genome size of each 148,180bp and 148,181 respectively while Vp18_Ke and Vp24_Ke had a size of 148,179bp. An assumption was that the bands shown in the Vp4 and Vp6 lanes might be a smear from DNA shearing.

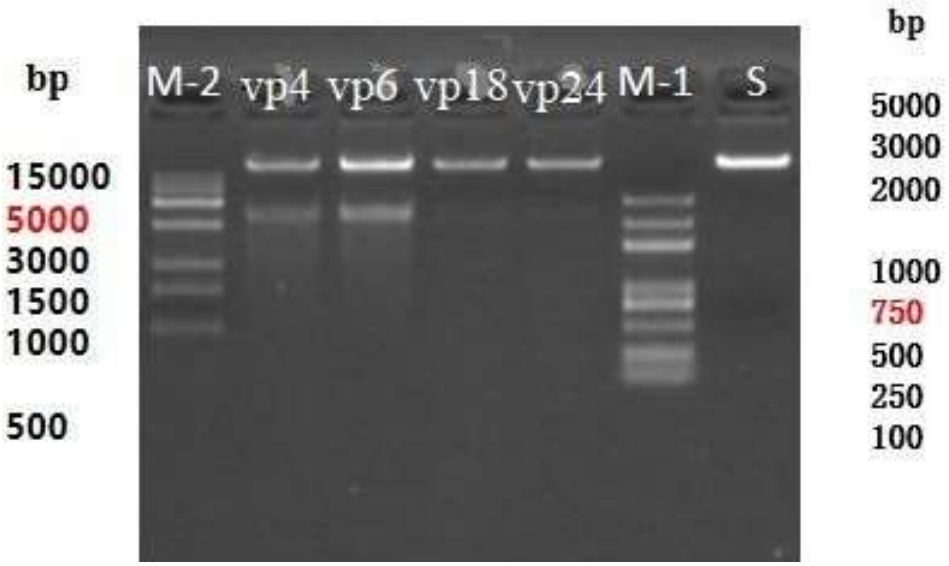


Figure 4.9: Agarose gel electrophoresis analysis of genomic DNA of phages

M-1 is Trans 2k plus with a loading volume of 2µl. M-2 is Trans 15k with a loading volume of 2µl. S is standard control with a loading volume of 5µl (10ng/µl). The four phage samples loading volume of 1µl.

4.4.1: Phage genome quality and concentration

The four phage DNA samples: VP4_Ke, VP6_Ke, VP18_Ke and VP24_Ke passed the quality control for library preparation and therefore small molecule library preparation was carried out. The concentration measurements of the four phage DNA samples are as depicted in Table 4.6

Table 4.6: Concentration of the phage DNA extracted from *V. cholerae* phages

Phage	Conc. in ng/µl	Volume in µL	Quantity in µg	Lib. prep type
VP4_Ke	25.20	38	0.958	Small molecule
VP6_Ke	46.80	38	1.778	Small molecule
VP18_Ke	21.20	39	0.827	Small molecule
VP24_Ke	21.20	39	0.842	Small molecule

4.4.2: Restriction patterns of phage DNA

As described in section 3.4.14 four restriction enzymes were used namely: *EcoRI*, *SalI*, *HindIII* and *XhoI*. These enzymes cut DNA at specific sites. Table 4.7 shows the endonucleases used in this study and their respective restriction sites. The results of restriciton digestion by the four enzymes are portrayed in Figure 4.10.

Table 4.7: Endonucleases and their restriction sites used in this study

Endonuclease	Restriction site (5' to 3')
EcoRI	G/AATTC
SalI	G/ TCGAC
HindIII	A/AGCTT
XhoI.	C/TCGAG

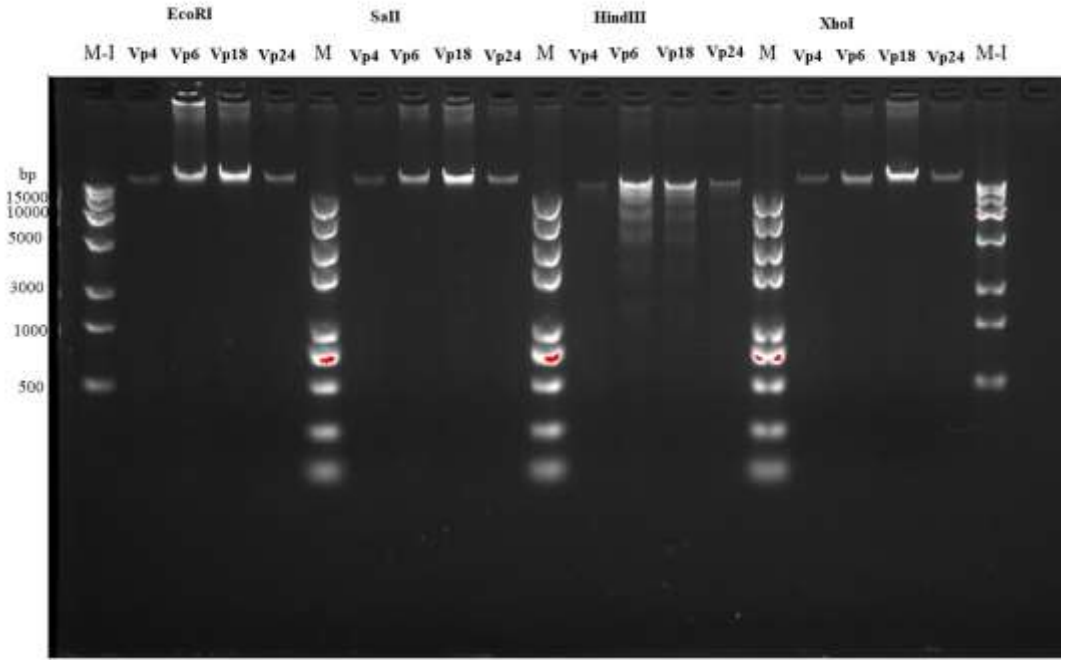


Figure 4.10: Restriction digests of the phage genomes

The restriction enzymes used were: *EcoRI*, *SalI*, *HindIII* and *XhoI*. M: 15k marker, M-1: 2k plus marker**.**

Table 4.8: Restriction enzymes with respective site numbers of the respective genomes

	VP4_Ke	VP6_Ke	VP18_Ke	VP24_Ke
BamHI	15	15	15	15
BglII	63	63	63	62
EcoRI	43	43	43	43
EcoRV	21	21	21	21
HindIII	57	57	57	57
PstI	10	10	10	10
SalI	3	3	3	3
SmaI	4	4	4	4
XbaI	33	33	33	33
XhoI	5	5	5	5

4.5: Phage genome assembly and annotation

The assembly and annotation of the phage genome sequences revealed the exact genome sizes of the four sequenced phages, total tRNAs in each sequence and number of genes among other details. The results are summarized in Table 4.9.

The results revealed that the genome sizes of each of the four sequences were almost similar: VP4_Ke: 148,180bp, VP6_Ke: 148,181bp with only a difference of one unit in each of these two. VP18_Ke and VP24_Ke had a genome length of the same size of 148,179bp. Each of the four phage sequences had a total of four tRNAs while the functional genes were 25 except for VP6_Ke that revealed 24 with only one-unit difference. The G+C content of 36.4% which was as well the same in all the four phage genome sequences. The above results revealed close relationship in the four genome sequences. The details of each of the four genome sequences are summarized in Tables 4.10 to 4.13.

Table 4.9: Post sequencing summary of the four phage genomes

V. cholerae **phage**	**Genome length(bp)**	**%G+C content**	**No. of tRNAs**	**Functional genes**
VP4_Ke	148,180	36.4	4	25
VP6_Ke	148,181	36.4	4	24
VP18_Ke	148,179	36.4	4	25
VP24_Ke	148,179	36.4	4	25

Table 4.10: Protein product identified of VP4_Ke, length, start, end and strand orientation

#	Name of the protein encoded by the gene	Start	End	Length	Strand
1	Phage baseplate wedge subunit (T4-like gp6)	6881	5169	1713	Negative
2	Gp44-sliding clamp holder	8927	9958	1032	Positive
3	DNA helicase, phage-associated #ATP-dependent	12527	14308	1782	Positive
4	Phage recombination-related endonuclease Gp47	15547	16674	1128	Positive
5	Ribonucleotide reductase of class Ia (aerobic), alpha subunit (EC 1.17.4.1)	24594	26900	2307	Positive
6	Ribonucleotide reductase of class Ia (aerobic), beta subunit (EC 1.17.4.1)	26945	28096	1152	Positive
7	Phage protein	29029	29424	396	Positive
8	Gp2 DNA end protector protein	33715	33017	699	Negative
9	Thymidylate synthase (EC 2.1.1.45)	39992	39138	855	Negative
10	tRNA-Met-CAT	41840	41767	74	Negative
11	tRNA-Arg-TCT	43219	43146	74	Negative
12	Phage protein (ACLAME 141)	44084	43455	630	Negative
13	tRNA-Arg-CCT	44721	44648	74	Negative
14	tRNA-Thr-CGT	44908	44822	87	Negative
15	Phage baseplate wedge subunit (T4-like gp25)	49125	49499	375	Positive
16	RND efflux system, inner membrane transporter CmeB	63095	63379	285	Positive
17	Phage head completion protein (gp4)	81713	82318	606	Positive
18	Gp61 DNA primase subunit	83360	82305	1056	Negative
19	*PhoH*-like protein	92575	91722	804	Negative
20	DNA polymerase III subunits gamma and tau (EC 2.7.7.7)	93525	92770	756	Negative
21	UDP-glucose 4-epimerase (EC 5.1.3.2)	94391	93537	855	Negative
22	UDP-glucose 6-dehydrogenase (EC 1.1.1.22)	95632	94391	1242	Negative
23	Gp41 DNA primase-helicase subunit	102938	101526	1413	Negative
24	conserved uncharacterised protein	117363	116641	723	Negative
25	Phage major capsid protein of Caudovirales (T4-like gp23)	124692	123334	1359	Negative

Table 4.11: Protein product of VP6_Ke, length, start, end and strand orientation

#	Name of the protein encoded by the gene	Start	End	Length	Strand
1	Phage baseplate wedge subunit (T4-like gp6)	6881	5169	1713	Negative
2	Gp44-sliding clamp holder	8927	9958	1032	Positive
3	DNA helicase, phage-associated #ATP-dependent	12527	14308	1782	Positive
4	Phage recombination-related endonuclease Gp47	15547	16674	1128	Positive
5	Ribonucleotide reductase of class Ia (aerobic), alpha subunit (EC 1.17.4.1)	24594	26900	2307	Positive
6	Ribonucleotide reductase of class Ia (aerobic), beta subunit (EC 1.17.4.1)	26945	28096	1152	Positive
7	Phage protein	29029	29424	396	Positive
8	Gp2 DNA end protector protein	33715	33017	699	Negative
9	Thymidylate synthase (EC 2.1.1.45)	39992	39138	855	Negative
10	tRNA-Met-CAT	41839	41766	74	Negative
11	tRNA-Arg-TCT	43218	43145	74	Negative
12	Phage protein (ACLAME 141)	44084	43455	630	Negative
13	tRNA-Arg-CCT	44720	44647	74	Negative
14	tRNA-Thr-CGT	44907	44821	87	Negative
15	Phage baseplate wedge subunit (T4-like gp25)	49121	49495	375	Positive
16	RND efflux system, inner membrane transporter CmeB	63091	63375	285	Positive
17	Gp61 DNA primase subunit	83361	82306	1056	Negative
18	*PhoH*-like protein	92576	91773	804	Negative
19	DNA polymerase III subunits gamma and tau (EC 2.7.7.7)	93526	92771	756	Negative
20	UDP-glucose 4-epimerase (EC 5.1.3.2)	94392	93538	855	Negative
21	UDP-glucose 6-dehydrogenase (EC 1.1.1.22)	95633	94392	1242	Negative
22	Gp41 DNA primase-helicase subunit	102939	101527	1413	Negative
23	conserved uncharacterised protein	117364	116642	723	Negative
24	Phage major capsid protein of Caudovirales (T4-like gp23)	124693	123335	1359	Negative

Table 4.12: Protein product of VP18_Ke, length, start, end and strand orientation

#	Name of the protein encoded by the gene	Start	End	Length	Strand
1	Phage baseplate wedge subunit (T4-like gp6)	6881	5169	1713	Negative
2	Gp44-sliding clamp holder	8927	9958	1032	Positive
3	DNA helicase, phage-associated #ATP-dependent	12527	14308	1782	Positive
4	Phage recombination-related endonuclease Gp47	15547	16674	1128	Positive
5	Ribonucleotide reductase of class Ia (aerobic), alpha subunit (EC 1.17.4.1)	24594	26900	2307	Positive
6	Ribonucleotide reductase of class Ia (aerobic), beta subunit (EC 1.17.4.1)	26945	28096	1152	Positive
7	Phage protein	29029	29424	396	Positive
8	Gp2 DNA end protector protein	33715	33017	699	Negative
9	Thymidylate synthase (EC 2.1.1.45)	39992	39138	855	Negative
10	tRNA-Met-CAT	41840	41767	74	Negative
11	tRNA-Arg-TCT	43219	43146	74	Negative
12	Phage protein (ACLAME 141)	44084	43455	630	Negative
13	tRNA-Arg-CCT	44721	44648	74	Negative
14	tRNA-Thr-CGT	44908	44822	87	Negative
15	Phage baseplate wedge subunit (T4-like gp25)	49124	49498	375	Positive
16	RND efflux system, inner membrane transporter CmeB	63094	63378	285	Positive
17	Phage head completion protein (gp4)	81712	82317	606	Positive
18	Gp61 DNA primase subunit	83359	82304	1056	Negative
19	*PhoH*-like protein	92574	91771	804	Negative
20	DNA polymerase III subunits gamma and tau (EC 2.7.7.7)	93524	92769	756	Negative
21	UDP-glucose 4-epimerase (EC 5.1.3.2)	94390	93536	855	Negative
22	UDP-glucose 6-dehydrogenase (EC 1.1.1.22)	95631	94390	1242	Negative
23	Gp41 DNA primase-helicase subunit	102937	101525	1413	Negative
24	conserved uncharacterised protein	117362	116640	723	Negative
25	Phage major capsid protein of Caudovirales (T4-like gp23)	124691	123333	1359	Negative

Table 4.13: Protein product of VP24_Ke, length, start, end and Strand orientation

#	Name of the protein encoded by the gene	Start	End	Length	Strand
1	Phage baseplate wedge subunit (T4-like gp6)	6881	5169	1713	Negative
2	Gp44-sliding clamp holder	8927	9958	1032	Positive
3	DNA helicase, phage-associated #ATP-dependent	12527	14308	1782	Positive
4	Phage recombination-related endonuclease Gp47	15547	16674	1128	Positive
5	Ribonucleotide reductase of class Ia (aerobic), alpha subunit (EC 1.17.4.1)	24594	26900	2307	Positive
6	Ribonucleotide reductase of class Ia (aerobic), beta subunit (EC 1.17.4.1)	26945	28096	1152	Positive
7	Phage protein	29029	29424	396	Positive
8	Gp2 DNA end protector protein	33715	33017	699	Negative
9	Thymidylate synthase (EC 2.1.1.45)	39992	39138	855	Negative
10	tRNA-Met-CAT	41840	41767	74	Negative
11	tRNA-Arg-TCT	43219	43146	74	Negative
12	Phage protein (ACLAME 141)	44084	43455	630	Negative
13	tRNA-Arg-CCT	44721	44648	74	Negative
14	tRNA-Thr-CGT	44908	44822	87	Negative
15	Phage baseplate wedge subunit (T4-like gp25)	49124	49498	375	Positive
16	RND efflux system, inner membrane transporter CmeB	63094	63378	285	Positive
17	Phage head completion protein (gp4)	81712	82317	606	Positive
18	Gp61 DNA primase subunit	83359	82304	1056	Negative
19	*PhoH*-like protein	92574	91771	804	Negative
20	DNA polymerase III subunits gamma and tau (EC 2.7.7.7)	93524	92769	756	Negative
21	UDP-glucose 4-epimerase (EC 5.1.3.2)	94390	93536	855	Negative
22	UDP-glucose 6-dehydrogenase (EC 1.1.1.22)	95631	94390	1242	Negative
23	Gp41 DNA primase-helicase subunit	102937	101525	1413	Negative
24	conserved uncharacterised protein	117362	116640	723	Negative
25	Phage major capsid protein of Caudovirales (T4-like gp23)	124691	123333	1359	Negative

For further confirmation of the results obtained after sequencing and annotation, nucleotide statistical analysis was done. The analysis revealed the details and also confirmed some results already obtained by annotation. Some of the results obtained from the annotated sequences after further nucleotide sequence statistical analysis included; count of atoms as

double or single stranded, weight, melting temperatures, total CDS, confirmed the percentage G+C content as well as total tRNAs and other details as summarized in the Tables (20.1 to 20.12) in Appendix 1.

The four phage genomes were closely related but novel as shown in Table 20.1 of Appendix 1. The phage is unknown as there is no other phage like the current one in this study that exists. The sizes of the four phage genomes were confirmed to be highly similar through the sequence statistical analysis that is: 148,180bp, 148,181bp, 148,179bp, for VP4_Ke, VP6_Ke, VP18_Ke and VP24_Ke respectively. Presence of both single and double DNA strand revealed, with respective weight of a single stranded sequence of 45MDa and double stranded 91MDa, hence portraying a high level of similarity in these four phage genome sequences.

In Table 20.2 of Appendix 1, the melting temperatures in degrees Celsius of the four phage genomes were also highly similar in salts of different concentrations (ranging from 0.1M to 0.5M) that ranged from 79.81°C to 91.41°C, respectively.

A lot of similarity in the four phage genomes was also revealed in Table 20.3 of Appendix 1. The tables confirmed the counts of annotations with 4 tRNAs and either 186 Coding sequences (CDSs) for VP4_Ke, VP18 _Ke or 185 for VP6_Ke, VP24_Ke in each of the four genome sequences.

The counts of atoms were also similar as shown in Table 20.4 of Appendix 1. Phosphorous lowest in all the phage genomes and hydrogen highest.

Table 20.6 of Appendix 1 showed also similarity in nucleotide sequences with A+T higher than G+C for all the phage genomes. The percent G+C content was 36.4 while the A+T content was 63.6 percent. The rest of the comprehensive statistical sequence nucleotide details are summarized in appendix one with detailed tables. A histogram of nucleotide distribution is shown in the Figure 4.11.

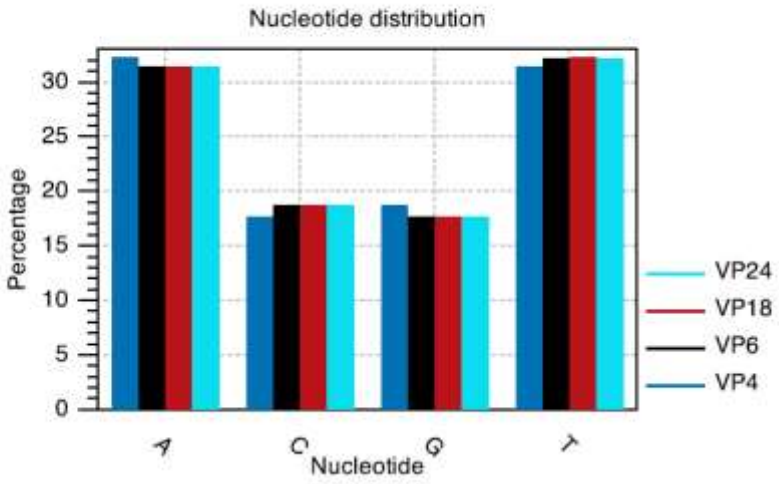


Figure 4.11: A histogram of nucleotides frequencies.

The complete genome sequences of the four phages according to the different bioinformatics' analysis used in this study, revealed the four phage genomes were double stranded and a smaller single stranded genome sequence. This is displayed in the genomic maps of VP4_Ke and VP24-2_Ke in Figures 4.12 and 4.13, respectively. Through the comparative analysis of the phage sequences it was revealed that VP18_Ke and VP24_Ke were similar since they shared 100% identity. Also as shown in Table 6.1 (20.1) the length of the two sequences was the same with 148179bp. VP4_Ke and VP6_Ke sequences shared the same identity of 99.99%. In these sequences, besides the *Myoviridae* phage there was a single stranded, circular *Inoviridae* DNA phage sequenced together with the *Myoviridae Vibrio cholerae* phages. The two different sequences, example found in phage VP24_Ke, of lengths 148179bp which was a *Myoviridae* while another smaller sequence had a length of 7180bp, was an *Inoviridae*. Since the genomic maps for all the *Myoviridae* phage genomes were almost similar, only the VP4_Ke genomic map was constructed (Figure 4.12). For genome of the filamentous phage, genomic map was constructed and named VP24-2_Ke as shown in Figure 4.13.

Figure 4.12 shows the Genomic map of VP4_Ke which was construction was done using Genome VX and the genome size was 148,180bp. The uncoded regions represent the hypothetical proteins

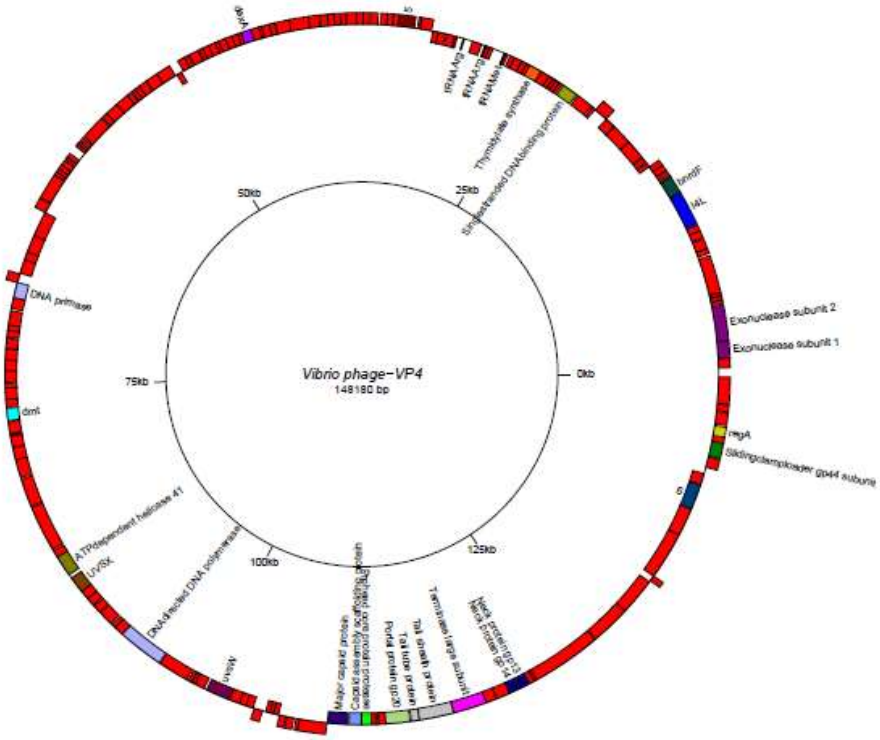


Figure 4.12: Genomic map of VP4_Ke.

Figure 4.13 is the genomic map of phage VP24-2 which is single strand sequence classified as Inoviridae. Genomic map construction was done using GenomeVX. The VP24-2_Ke phage was filamentous with genome size 7180bp.

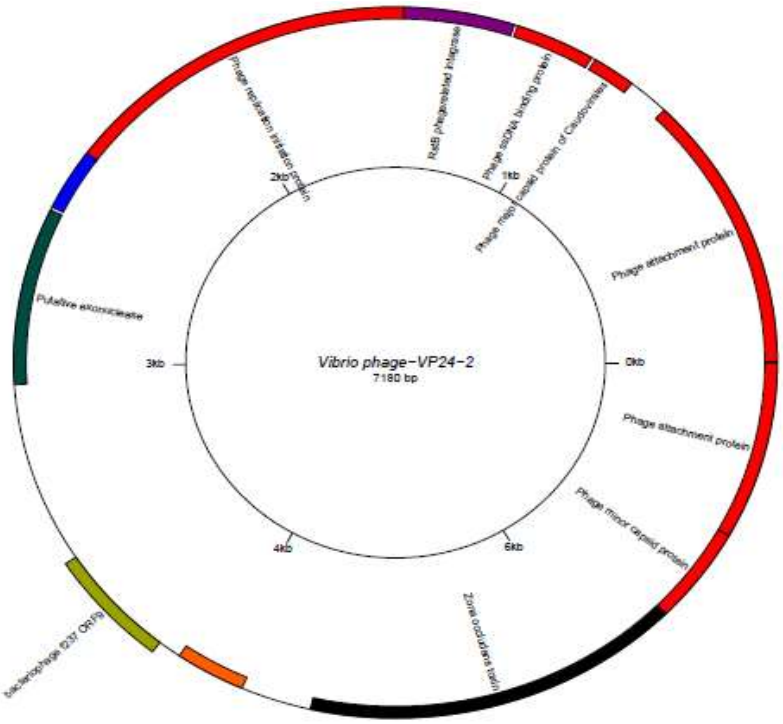


Figure 4.13: Genomic map of VP24-2_Ke.

With respect to the recent advances in bacteriophage research, more details of the phage genome in this study were revealed through Virfam webserver that uses annotations of a phage genome to predict structural proteins (Head-Neck- tail modules) then classify the phage. In this study the Virfam analysis was able to predict hit to: Major capsid protein, Large terminase and other features. The Virfam webserver is through which users can detect proteins involved in the neck structure of the phage of interest, identify the type of neck and deduce the likely morphological family of the phage, display the organisation of the genes encoding the major capsid protein, large terminase, portal, neck, major tail and sheath proteins and finally locate the given phage within classification. Results of the Virfam analysis on the neck module and part of the head and tail proteins are displayed in the Figures 4.14 and 4.15. The following deductions were made:

- The query phage was given the name Alice since it was a novel one.
- Category to which the Query Phage was assigned: ***Myoviridae* of Type 2**.
- Number of sequences analyzed: **186**.

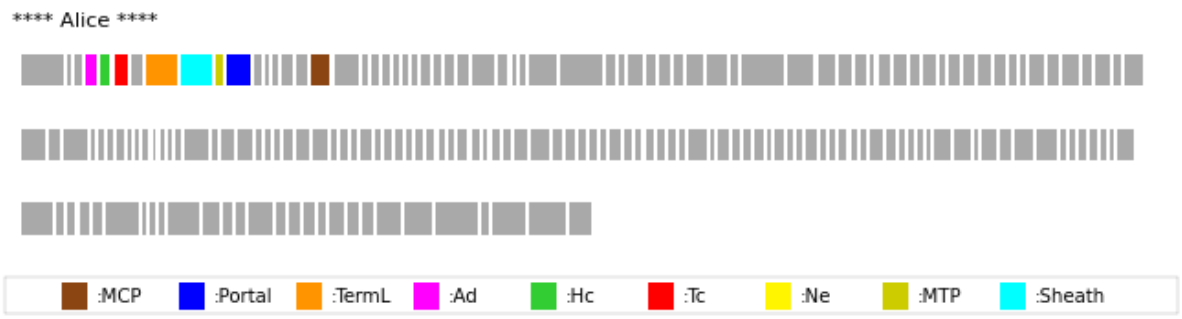


Figure 4.14: Proteins of the query phage detected.

Key: MCP-Major capsid protein, TermL/TerL-Large terminase, Ad- Adaptor, Hc-Head closure, Tc- Tail completion protein, Ne- Neck protein, MTP- Major tail protein.

These proteins can be classified as: Proteins from the head(Mcp, Portal and terminase, tail (major tail protein or MTP, sheath) of bacteriophages are generally well conserved and could be detected with standard bioinformatics strategies. Proteins lysing at the interface between the head and the tail components : Ad, Hc,Tc head to tail connection proteins can be much more difficult to detect due to drastic sequence divergence.

Figure 4.15 dispalyed the classification of the Query Phage with respect to other related Phages in Aclame. Query Phage in Green color: (VP4_Ke). The query phage was classified and placed in the above position indicated in green colour.

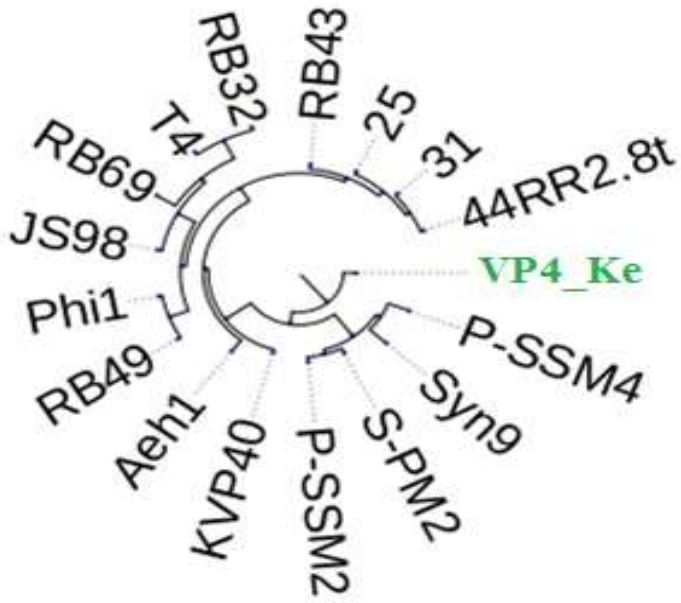


Figure 4.15: Comparison of the novel phage/query phage (VP4_Ke) with closely related studied phages/aclame phages

Table 4.14 shows the proteins of head-neck-tail detected by Virfam analysis and highest identities of protein superfamilies of the query phage with proteins of the acclame phages respectively.

Table 4.14: Head-Neck-Tail-Proteins of the phage genome

- Table of the Head-Neck-Tail Proteins Detected :

Protein Superfamily	Index in Query Genome	Sequence Header	Detection Method
MCP	17	S1_DMS33493-V-C_1.clean_(paired)_contig_2_selection_CDS_Phage_major_capsid_protein_of_Caudovirales_(	HHsearch (proba=100.00%)
Portal	11	S1_DMS33493-V-C_1.clean_(paired)_contig_2_selection_CDS_hypothetical_protein	HHsearch (proba=100.00%)
TermL	8	S1_DMS33493-V-C_1.clean_(paired)_contig_2_selection_CDS_hypothetical_protein	Blast (e-value=5e-93)
MTP	10	S1_DMS33493-V-C_1.clean_(paired)_contig_2_selection_CDS_hypothetical_protein	Blast (e-value=1e-12)
Sheath	9	S1_DMS33493-V-C_1.clean_(paired)_contig_2_selection_CDS_hypothetical_protein	HHsearch (proba=100.00%)
Ad1	NOT FOUND		
Hc1	NOT FOUND		
Ne1	NOT FOUND		
Tc1	NOT FOUND		
Ad2	4	S1_DMS33493-V-C_1.clean_(paired)_contig_2_selection_CDS_hypothetical_protein	HHsearch (proba=100.00%)
Hc2	5	S1_DMS33493-V-C_1.clean_(paired)_contig_2_selection_CDS_hypothetical_protein	HHsearch (proba=100.00%)
Tc2	6	S1_DMS33493-V-C_1.clean_(paired)_contig_2_selection_CDS_hypothetical_protein	HHsearch (proba=100.00%)
Ad3	NOT FOUND		
Hc3	NOT FOUND		
Ad4	NOT FOUND		

NB : Matches obtained with a HHsearch probability between 70 % and 90 % should be considered with caution. Check that the canonical intergene below is not violated in that case.

- This page has some unexpected intergene distance(s) with respect to those observed in Reference Phages from Aclame (Obs_InterGeneDist > Mean_InterGeneDist+2*Std_Dev) (Help):

No unsual intergene distance were detected.

- Highest Identities of Proteins of the Query Phage with at least 3 Proteins in Aclame Phages :

Protein Superfamily	Corresponding Protein in Aclame	Aclame Phage	Seq. Identity (%)
MCP	protein:vir:104915 protein:vir:103181 protein:vir:104549	P-SSM2 Syn9 P-SSM4	37 % 35 % 34 %
Portal	protein:vir:108049 protein:vir:103177 protein:vir:104500	3S98 Syn9 P-SSM4	32 % 32 % 31 %
TermL	protein:vir:104536 protein:vir:103169 protein:vir:104946	P-SSM4 Syn9 P-SSM2	38 % 37 % 36 %
MTP	protein:vir:104771 protein:vir:103176 protein:vir:106918	P-SSM2 Syn9 S-PM2	26 % 24 % 24 %
Sheath	protein:vir:80984 protein:vir:6594 protein:vir:101187	Phi1 RB49 44RR2.8t	27 % 27 % 26 %
Ad2	protein:vir:103451 protein:vir:7198 protein:vir:101153	RB32 T4 44RR2.8t	28 % 28 % 26 %
Hc2	protein:vir:5656 protein:vir:104739 protein:vir:103005	KVP40 P-SSM2 Syn9	27 % 26 % 26 %
Tc2	protein:vir:5659 protein:vir:98260 protein:vir:81023	KVP40 RB43 Phi1	23 % 23 % 22 %

4.6: Phylogenetic trees of phages VP4, VP6, VP18 and VP24 with reference viruses

In figure 4.16 the phylogenetic tree was constructed using protein sequences of the large terminase subunit. Phages VP4, VP6, VP18 and VP24 are shown in red. Phages used for genome alignments are highlighted with green dots. Bootstrap values greater than 70 are shown and trees are rooted by outlier.

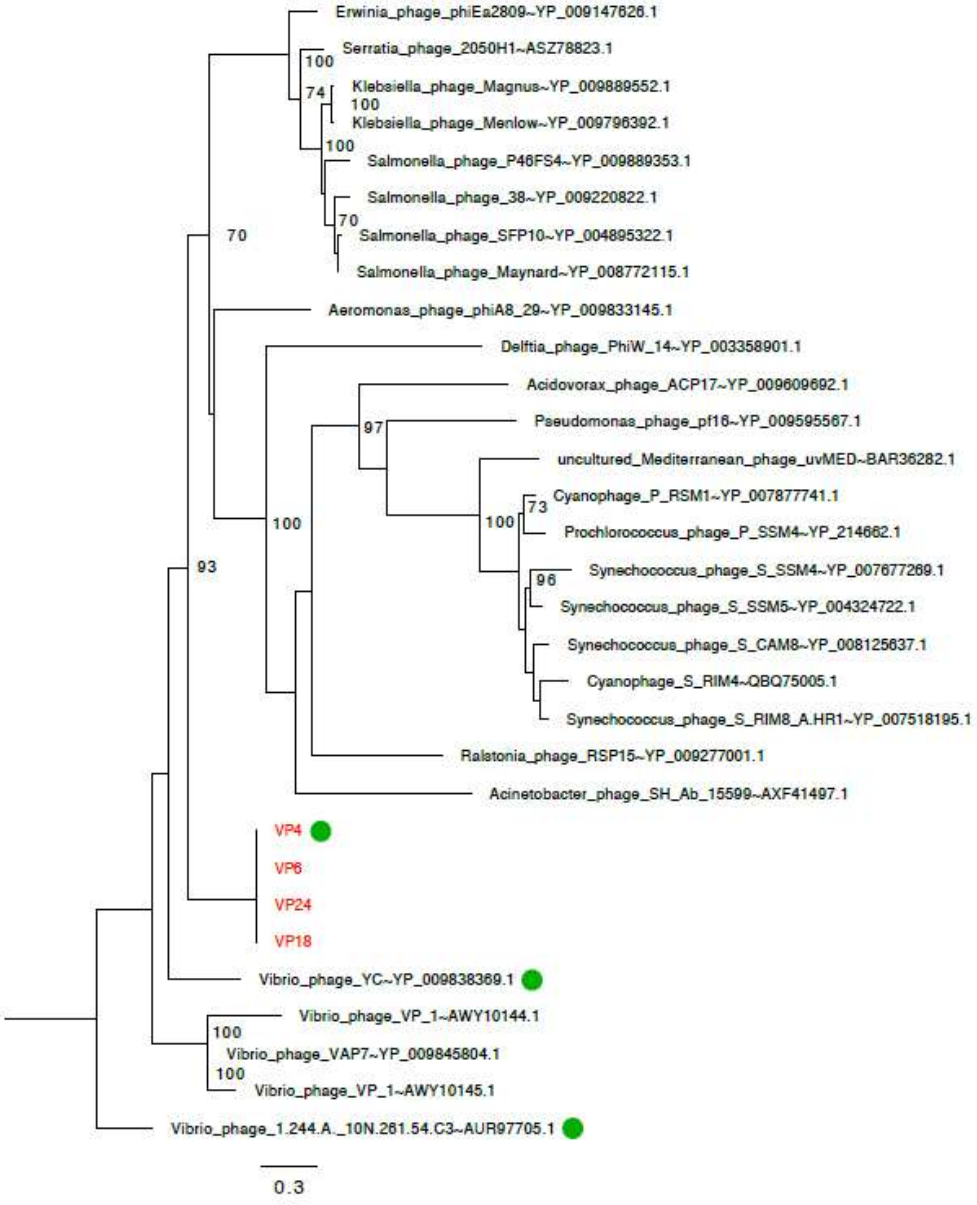


Figure 4.16: Phylogenetic tree of four phages based on large terminase subunit

The phylogenetic tree in Figure 4.17 was constructed using protein sequences of ribonucleotide reductase subunit alpha. Phages VP4, VP6, VP18 and VP24 are shown in red. Phages used for genome alignments are highlighted with green dots. Bootstrap values greater than 70 are shown and trees are rooted by outlier.

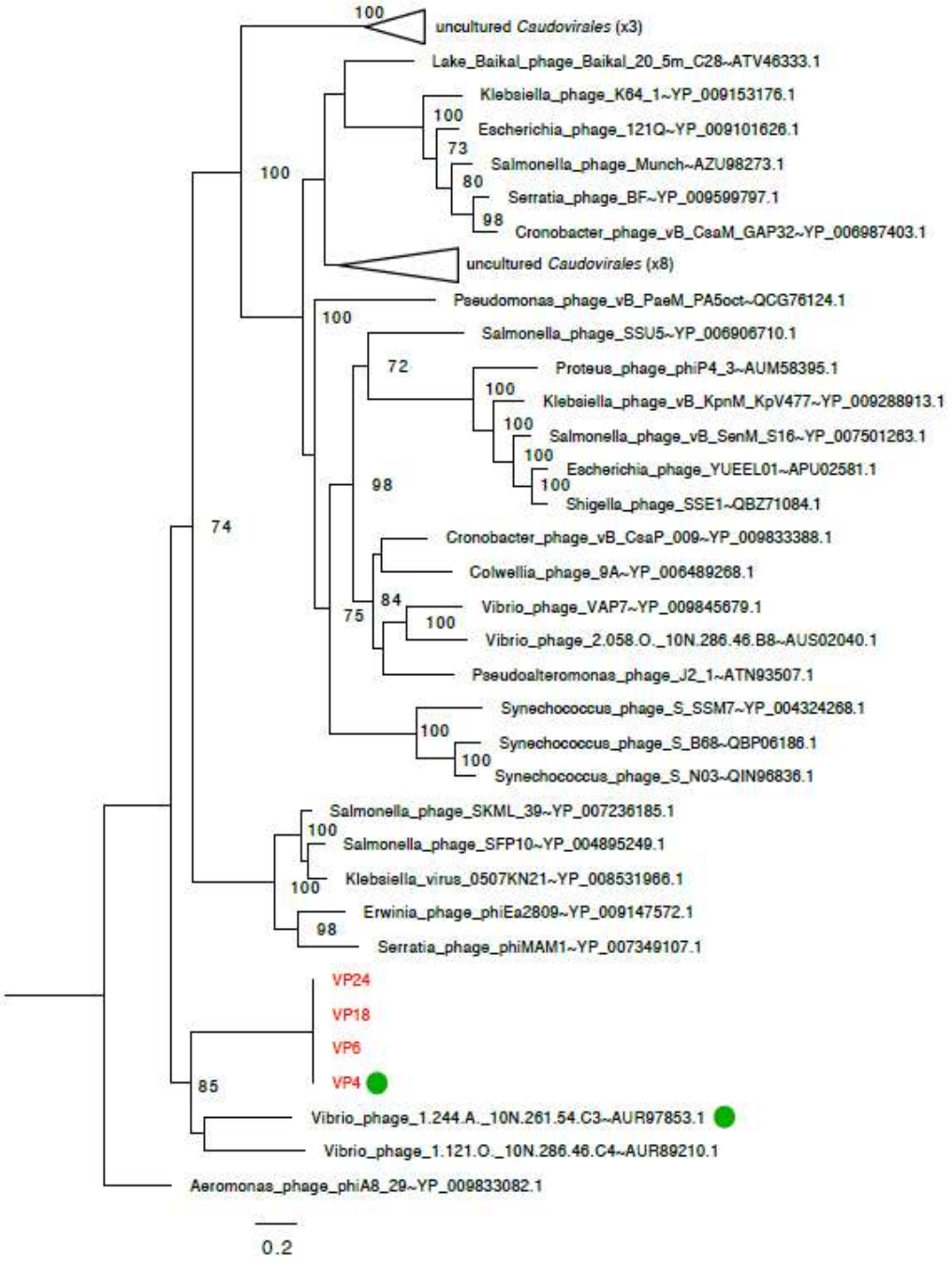


Figure 4.17: Phylogenetic tree of four phages based on ribonucleotide reductase subunit alpha

In Figure 4.18, phylogenetic tree was constructed using protein sequences based on ribonucleotide reductase subunit beta. Phages VP4, VP6, VP18 and VP24 are shown in red. Phages used for genome alignments are highlighted with green dots. Bootstrap values greater than 70 are shown and trees are rooted by outlier.

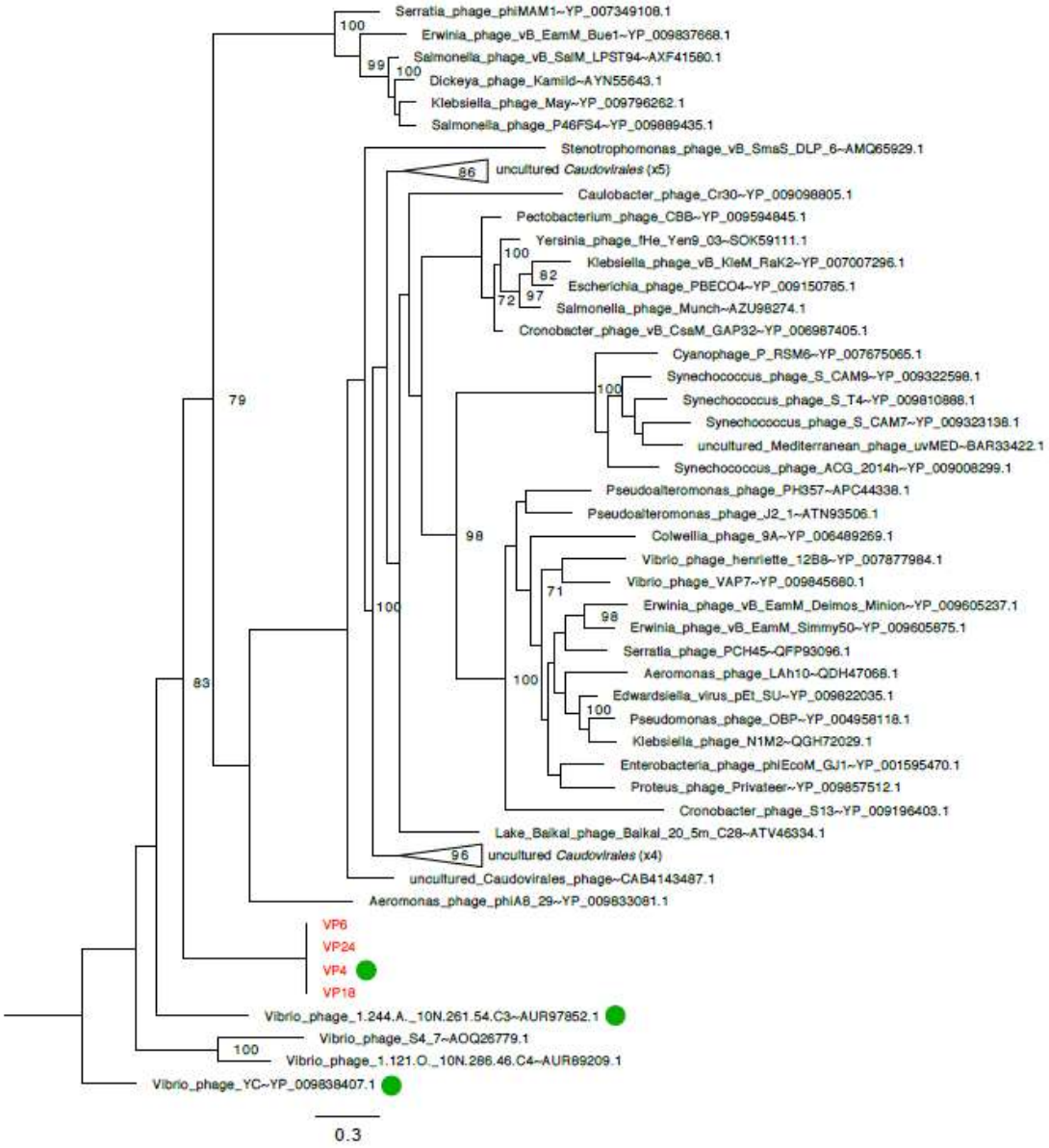


Figure 4.18: Phylogenetic tree of four phages based on ribonucleotide reductase subunit beta

Annotations from VIBRANT v1.2.1 are color-coded as follows: blue for proteins involved in nucleotide replication, repair, recombination, and metabolism; green for phage structural and hallmark proteins; and gray for proteins of unknown or uncategorized functions. *phoH* is shown in red (Figure 4.19). The dotted line indicates the site of circularization of the linear sequence.

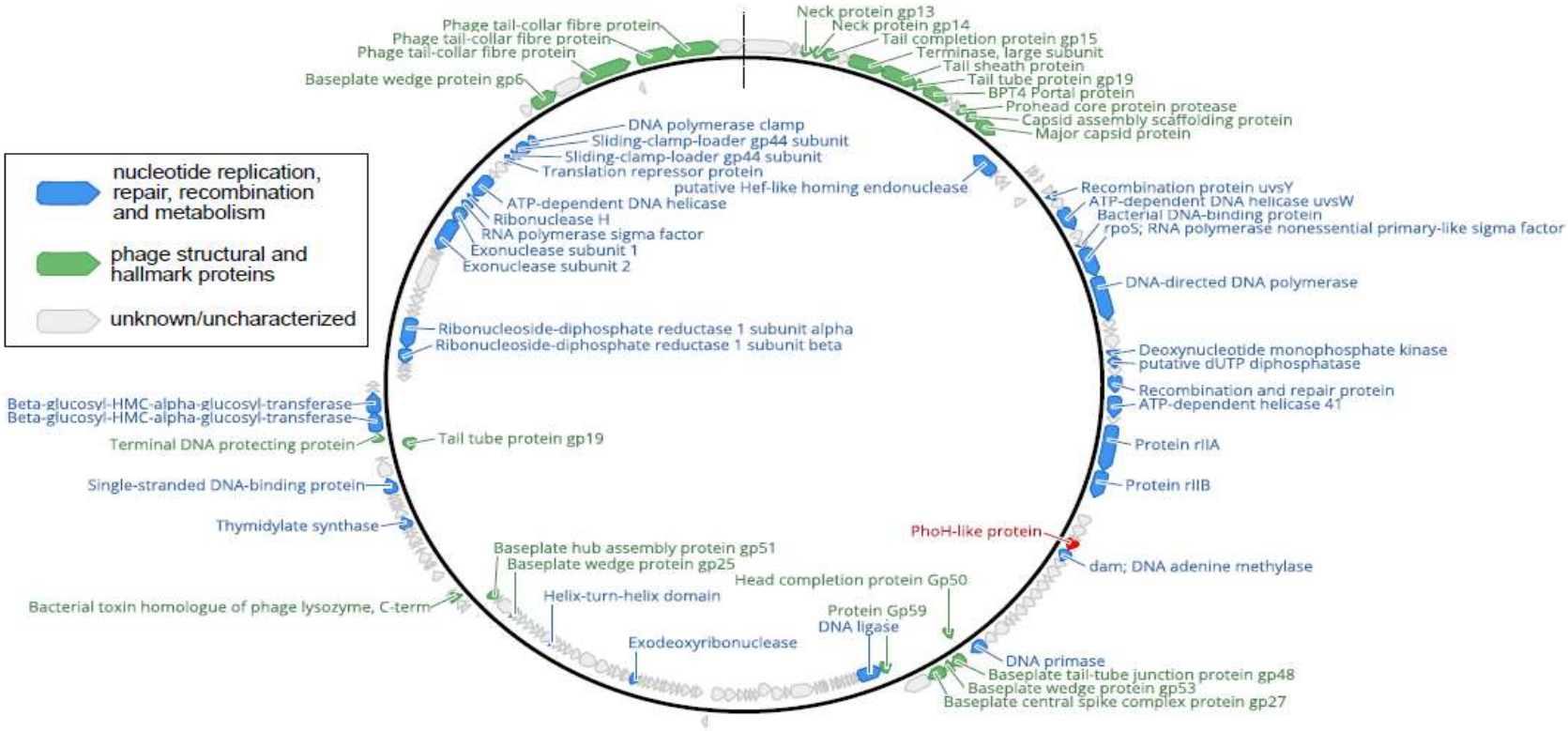


Figure 4.19: Circular diagram of annotated phage VP4 genome

Figure 4.20 shows the Genomic alignments of VP4 and reference phages. Genomes maps are connected by lines according to high (green) or low (red) tBLASTX identity. Open-reading frames are depicted by gray arrows, and genes with high identity between VP4 and the reference phages are annotated. Accession numbers for reference phages are MG592609.1 (*Vibrio* phage 1.244.A._10N.261.54.C3) and NC_048709.1 (*Vibrio* phage YC).

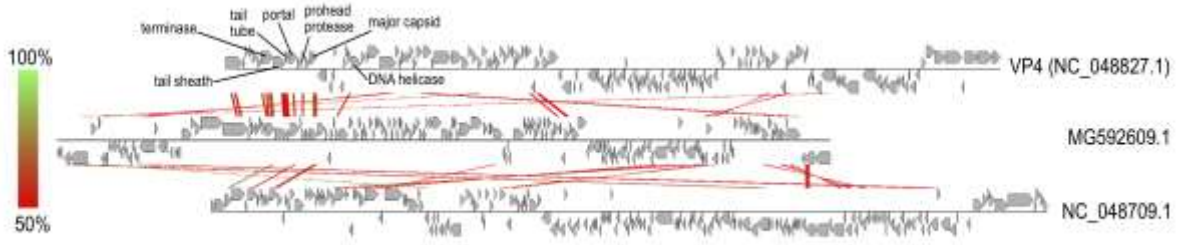


Figure 4.20: Genomic alignments of VP4 and reference phages

4.7: Genomic characteristics of filamentous phage VP24-2_Ke

Three putative proteins p111-like receptor binding, coat proteins and Zot like phage assembly proteins were used for the construction of the phylogenetic tree then compared with 9 reference genomes of filamentous phages searched on NCBI. Only values greater than 60 are shown, bootstrap based on 1000 computer generated trees indicated at the nodes. Names of the phages are shown on the right side. The filamentous Inoviridae phage VP24-2_Ke had a genome length of 7180bp, 11 functional genes with a single stranded circular DNA. The Phylogenetic tree of filamentous phage is displayed in Figure 4.21.

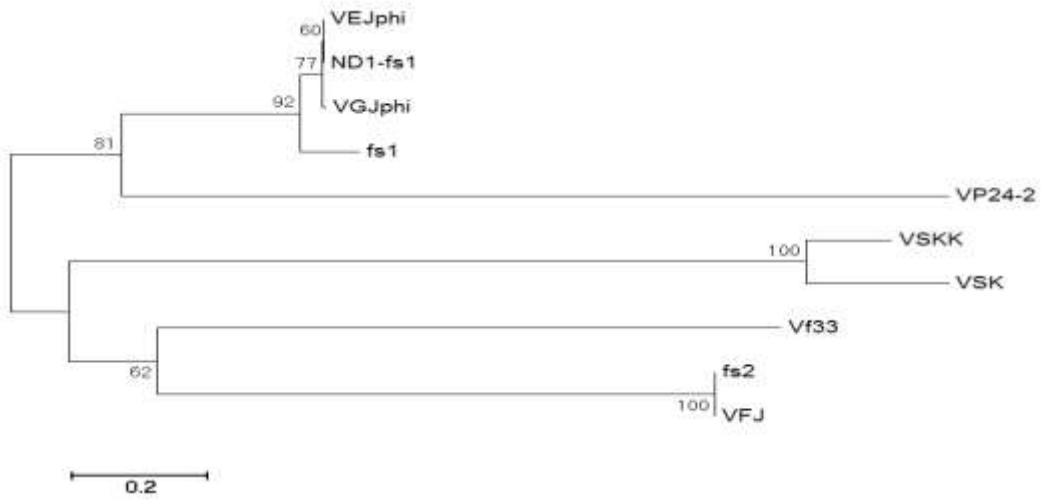


Figure 4.21: Phylogenetic tree of filamentous *Inoviridae* phage VP24-2_Ke

Proteins encoded by the genes in the filamentous phage VP24-2_Ke:

1. Phage replication initiation protein
2. rolling circle replication protein
3. RstB phage-related integrase
4. Phage ssDNA binding protein
5. Phage major capsid protein of Caudovirales
6. Putative minor capsid protein
7. Phage attachment protein
8. Phage minor capsid protein
9. RstR repressor
10. RstR repressor protein
11. Conserved hypothetical

CHAPTER FIVE: DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1: Discussion

Isolation of *Vibrio cholerae* El Tor O1 serotype was successful from the four study regions in Kenya. It was unsuccessful to isolate serotype O139 though it could have been more interesting to isolate both strains and consequently isolate phages from each of these strains. Strain collection was therefore dominated by *Vibrio cholerae* El Tor O1 serotype. It is worth noting here that all the bacteriophages, though isolated from different regions of Kenyan environmental waters, used the propagating *Vibrio cholerae* strain isolated from the respective regional source. This is envidence that *Vibrio cholerae* continues to persist in Kenyan environmental waters, hence the frequent outbreaks of cholera in the country.

Previous report by (Kahler et al., 2015) revealed that although *Vibrio cholerae* was present in the environment, detection could be difficult because of an abundance of non-toxigenic *Vibrio cholerae* in natural water systems. Furthermore, inability to isolate viable but nonculturable (VBNC) bacteria using traditional methods is another contributing factor to lack of detection (Kahler et al., 2015).

Vibrio cholerae bacterium is one of the oldest toxigenic bacteria known to cause infectious disease in human beings. It is therefore important to understand the transmission and evolution (Hounmanou et al., 2019) of the bacterium. Hounmanou et al. (2019) also reports that, even though most of cholera cases worldwide take place in Africa, there has been limited research on occurrence of *Vibrio cholerae* in aquatic environments in countries around African great lakes (Hounmanou et al., 2019). The knowledge on the occurance of *Vibrio cholerae* in aquatic environments is required. This is particularly when setting up control and preventive measures of cholera epidemics. Strategies to control cholera in endemic areas are required still if *Vibrio cholerae* is not detected in the environment. This is because the viable but non culturable type can go back to transmissable form under favourable conditions (Shishir et al., 2018). Studies done by Abana et al. (2019) in Accra, Ghana, confirmed presence of *Vibrio cholerae* in environmental waters (streams and wells). Similar studies in Haiti (Alam et al., 2014; Rahman et al., 2018) also confirmed that *Vibrio cholerae* thrived in environmental waters.

According to the results obtained in this current study, out of a total of 140 environmental water samples, toxigenic *Vibrio cholerae* was isolated from 15 samples. Consequently, it was from the same environmental water sources that the respective phages lytic to *Vibrio cholerae* were isolated.

Out of the 15 environmental water samples from which *Vibrio cholerae* was isolated, the distribution from various sources was as follows: 5 samples collected from river Kuja in Migori county, 2 samples were collected from Coastal region (river Kizulini and river Nsongoni), 2 samples were from Central region (river Kamiti and Riverside river) in Nairobi county. In summary, this contributed to a total of 9 environmental water sources (12%) collected from various regional rivers out of the total 75 samples that tested positive for *Vibrio cholerae*. *Vibrio cholerae* was also isolated from 3 beaches namely: Usenge, Launda and Osieko in Bondo translating to 14% of the total number of samples collected from beaches while 3 other *Vibrio cholerae* isolates were from three environmental water sources collected from each of the ponds; Koleche, Kotonde and Owira out of a total of eight. This translated to 37.5% of the total number of samples collected from ponds in Bondo/Rarienda . Conclusively, it was revealed that three different sources of environmental water tested positive for *Vibrio cholerae* ; rivers, ponds and beaches. *Vibrio cholerae* was neither isolated from boreholes, Lake Victoria, Indian Ocean, wells , nor springs.

In Kenya, outbreaks of cholera are experienced during the rainy season and the dry spell as well. The presence of this pathogen in rivers could have been attributed to run off into rivers especially in the areas that lack pit latrines. Rivers also become sites where human, animal and domestic waste is dumped. Rains lead to run off and flow of sewage causing contamination of the rivers by seepage. Ponds as well as beaches in Bondo and Rarienda were also open grounds for various human and animal activities. Some human activities like bathing in the river, washing clothes and swimming could have contributed to contamination of the surface waters with the pathogenic *Vibrio cholerae* bacterium. It should be noted here that in some of these sampling regions, a large percentage of the population lack basic ameneties like toilet facillites. The residential communities therefore, result to use of other means like bushes. The end results is the contamination of the surface water bodies during heavy rains. There are reports that around the great lakes the incidence of cholera varies by season, the level of fishing activities, rainful and plankton (Nkoko et al., 2011). A study by Hounmanou et al. (2019) provided evidence that *Vibrio cholerae* can remain in aquatic

environment during even when there is absence of cholera outbreak. The study also suggested that lake Victoria was a reservoir for *Vibrio cholerae* O1 with potential of causing cholera outbreak. Presence of *Vibrio cholerae* in environmental water sources may cause difficulties in eradication of cholera in the country. Cholera epidemics among humans are preceded by an environmental bloom of toxigenic *Vibrio cholerae* and spill over into human populace (Alam et al., 2014). The environmental reservoir and persons infected with the disease cholera can be considered as a potential source of outbreak but their respective relations to cholera transmission have been highily disputed (Rahman et al., 2018). *Vibrio cholerae* presence in ponds has also been reported (Murkherejee et al., 2011). Climate change, lack of disinfection of the surface waters, absence of piped clean water coupled with lack of resources for chlorination of the surface waters, were among other factors contributing to the outbreak of disease in these regions.

The absence of the pathogen from some of the environmental water sources in the current study could have been attributed to factors such as : low salinity, large organic matter, treatment of water before distribution and as said earlier, *Vibrio cholerae* may have existed in viable but non culturable state (VBNC) especially during unfavourable conditions (Pietras et al., 2016). This is a state whereby the pathogen loses its flagella and changes to a smaller spherical form in a sporelike stage. Such unfavourable conditions include : low nutrient levels, lower temperatures and higher osmolarities. These conditions could have contributed to the low number of isolates in the current study from the various environmental water sources. Alternatively, other advanced techniques on detection of toxigenic *Vibrio cholerae* in the surface environmental waters if employed would increase the chances of detecting even the VBNC forms.

The pathogenic *Vibrio cholerae* not only thrives well in saline environments but also in low saline environments as long as the environment is warm with adequate nutrients (Alam et al., 2014). In the environment, *Vibrio cholerae* can withstand a temperature of 30°C, pH 8.0, salinity of 600nM while in the gut it is able to survive at 37°C, pH 7.2 and saliniy of 150nM. The pH of the surface waters from where the pathogenic *Vibrio cholerae* was isolated in this study ranged from 7.6 to 8.1. The optimum pH for isolation of *Vibrio cholerae* is between 7 to 8.5 which is favourable for its growth.

In 2015, following an outbreak of cholera in Western Kenya, *Vibrio cholerae* was isolated from river Riana in Migori county. The same serotype of *Vibrio cholerae* confirmed to be the source of the outbreak was also isolated from a patients' rectal swab (Oyugi et al., 2017). The river was the source of water used for domestic purposes by the inhabitants of Migori county. According to World Health Organisation (WHO, 2018), Kenya experienced an outbreak of cholera that started in Tana river at the Coast and later reported in other counties: Garissa, Nakuru, Vihiga, Nairobi, Mombasa, Kiambu, Turkana, Kericho and Narok. The outbreak was reported both in general public and in refugee camps. A total of 1216 cases and 14 deaths was reported by July 2017. According to UNICEF bulletin on cholera and Acute waterly disease (AWD) outbreaks in Eastern and Southern Africa (ESAR), a total of 1198 cases including 4 deaths had been reported since the beginning of the year 2019 with cholera cases being confirmed in Nairobi and Machakos.

Gwenzi and Sanganyado (2019) reported that Kenya was among the seven countries in Subsaharan that experienced cholera outbreaks between 2017 to 2018. This is evidence that the presence of the pathogenic *Vibrio cholerae* found its route to a certain population of Kenyan people either through consumption of contaminated food or water with the pathogen. Ramamurthy et al. (2019) reported that only *Vibrio cholerae* serogroup O139 and O1 Classical or El Tor biotypes are known to cause cholera epidemics although there are more than 200 serogroups.

Vibrio cholerae proliferates and persists in aquatic systems that play a part as sinks after which it enters through consumption of contaminated food and water into the body of human beings (Gwenzi & Sanganyado, 2019). There is therefore a connection between the the environmental components and the human playing a vital part in promoting persistence of cholera and sustaining and that is drinking waters. Importantly therefore, efforts should be made to understand the continued persistence of *Vibrio cholerae* environmental reservoiurs in a bid to control cholera outbreaks. Other studies also showed presence of *Vibrio cholerae* in environmental waters of Kenya have been reported (Kiiru et al., 2013). Apart from *Vibrio cholerae*, in this study other bacteria from the various sources of Kenyan waters were isolated and characterised. These bacteria included: *Providencia sneebia*, *Proteus mirabilis*, *Escherichia coli:*O83 and *Pseudomonas solanacearum*. The partial 16S rRNA gene sequences of these isolated bacterial species were posted in GenBank and accession numbers assigned as follows: MN467398.1, MN467401.1, MN467400.1 and MN467399.1 for

Escherichia coli: O83 (isolated from Nairobi river), *Providencia sneebia*, *Proteus mirabilis* and *Vibrio cholerae,* respectively. Besides River Kuja, *Vibrio cholerae* was also isolated from Koleche pond in Bondo and river Nsongoni in Mombasa. The partial sequences of 16S rRNA gene of two *Vibrio cholerae* bacterial isolates were as well deposited in GenBank. The assigned accession numbers were: MN907464.1, MN907465.1 from Koleche pond and Nsongoni River, respectively. River Kuja in Migori was also a source of *Escherichia coli* isolate and the accession GenBank number of the 16S ribosomal RNA gene partial sequence of this isolate was MN907463. Alam et al. (2018) reported that *Proteus mirabilis* could be isolated from the environmental waters. Their presence in water and soil may indicate faecal contamination of the environment (Santo-Domingo & Edge, 2010). Besides river, well or lake waters, borehole waters have also been proven to harbour different bacteria. A study in Nigeria showed that borehole water was a source of *Vibrio cholerae*, *Escherichia coli* and *Providencia sneebia* during rainy and dry seasons (Onuorah et al., 2019). Faecal coliforms and *Escherichia coli* have been used for decades for identification of faecal matter contamination as an indicator for existence of pathogenic microorganisms in water (Payment & Locas, 2011). These indicator organisms demonstrate propable occurance of pathogenic bacteria in water but are not able speculate their occurance level (Payment & Locas, 2011). Therefore, the presence of *Escherichia coli*:O83 in surface waters of rivers: Kuja in Migori county, Kamiti river in Kiambu, Thika county and Nairobi river in Nairobi county was a clear indication that the water had been contaminated with pathogenic bacteria, hence not fit for human consumption. The possible contamination could have been attributed to heavy rains experienced in the country in the year 2018. This was the period during which sampling of environmental water took place in Nairobi and Thika counties. Faecal contamination of river Kuja, Migori county with *Escherichia coli* :O83 could have been attributed to various human activities mentioned earlier. Earlier studies on the bacteriological quality of water of river of metropolitan capital city of Kenya, Nairobi river are in agreement with the current study results where *E.coli* was also isolated (Musyoki et al., 2013). Other bacteria isolated from Nairobi river included *Vibrio cholerae*, *Proteus mirabilis* and *Pseudomonas aeruginosa* (Musyoki et al., 2013). *Proteus* species are human opportunistic pathogens and postulation is made that the pathogen's reservoir are the intestines. Their presence either in water or soils habitations is an indication of faecal contamination causing danger of intoxication after consumption of such infected water or sea food (Drzewiecka, 2016). In Netherlands *Pseudomonas solanacearum* was isolated from waterways as reported by (Bergsma-Vlami et

al., 2018; García et al., 2019). *Proteus* species bacteria were detected in two of five studied well waters treated as a source of drinking waters in the country of Nigeria (Aboh et al., 2015). Disturbing exsistence of resistant to multiple drugs in *Proteus vulgaris* and *Proteus mirabilis* strains among other bacteria in water used for consumption from the streams and springs in Sikkim rural area, was reported by (Drzewiecka, 2016). The absence of *Vibrio cholerae* from borehole water tested in this study could have been attributed to low salinity and low temperatures as compared to other surface environmental water sources. The current study has therefore proven that Kenyan surface environmental waters are contaminated with different bacteria that may contribute to diarrhoreal diseases. Majority of the rural communities in Kenya depend on surface water sources for all their water needs. The presence of these bacteria especially *Vibrio cholerae*, was an indication that these communities were at risk of cholera infection. This is because proliferation of the pathogen is favoured by the environmental conditions recorded during sampling. Environmental *Vibrio cholerae* strains are adapted well in the environs for they have potential to grow as biofilms on a scope of abiotic as well as biotic surfaces.

A selective culture media with a high pH (8.5-9.5) that supports growth of *Vibrio spp* is thiosulphate citrate bile salt sucrose (TCBS) that inhibits growth of intestinal flora, bile salts also prevents growth of Gram positive bacteria. In TCBS, *Vibrio cholerae* produced yellow colonies because it was able to ferment sucrose with production of small yellow colonies.

Colonies were also subjected to Triple Sugar Iron Agar test (TSI), Oxidase test as well as gram staining for further confirmation of *Vibrio cholerae*. On TSI, *Vibrio cholerae* produced yellow butt, yellow slant due to production of acid butt, acid slant without gas. These reactions of *Vibrio cholerae* in both TSI and TCBS were typical reactions of *Vibrio cholerae* as explained by (Dua et al., 2017). Triple sugar iron agar is a medium that is designed to differentiate among bacteria based on fermentation patterns of three sugars (Glucose, Lactose and Sucrose) and production of Hydrogen Sulfide gas. The sugars are in different percentages in the medium: Glucose 0.1%, Lactose and Sucrose 1%. TSI also contains Sodium thiosulfate and ferrous ammonium sulfate to detect presence of Hydrogen sulfide indicated by the black color in the butt of the tube. Hydrogen sulfide is produced when the sulfur compound is reduced by the bacterial isolate to sulfides. The Hydrogen sulfide gas produced reacts with the Iron compound to form black precipitate of ferric sulphide. The black color acts as an indicator for the presence of Hydrogen sulphide gas. When gas is produced there are cracks,

breaks or lifting of the agar in the tube. In the current study, the reaction of *Vibrio cholerae* on the TSI, there was no production of gas as depicted in figure 4.2. A pH indicator, phenol red is also present in the TSI which when pH is below 6.8 turns yellow and when pH is above 6.8, turns red. If any of the three sugars in TSI is fermented, the byproducts mostly acids are produced and the color of the medium changes because the phenol red which is sensitive to pH changes. When yellow color is produced in the butt, it indicates production of acid due to glucose fermentation. When the slant is yellow it indicates sucrose or lactose fermentation. In the TSI reaction of *Vibrio cholerae*, the butt was yellow and the slant was yellow (Acid/Acid). This was an indication that the isolate was able to ferment both glucose and sucrose. It also indicated both aerobic and anaerobic fermentation taking place as *Vibrio cholerae* is a facultative anaerobe.

A percentage of 60 of the phages were isolated from rivers, 20% from ponds and 20% from beaches in this study. This was also an indicator of the continued occurrence of the toxigenic *Vibrio cholerae* in the Kenyan environmental waters. Previous studies (Maina et al., 2014) isolated and characterized *Myoviridae* vibriophages from Lake Victoria region of Kenya. Isolation of bacteriophages from these areas also proved that these are cholera endemic zones hence the prevalence of cholera in these regions and the country at large. In Kenya, phage isolation lytic to other *Vibrio* spp than *Vibrio cholerae* is documented (Moulton et al., 2011; Akhwale et al., 2019). Vibriophages lytic to alkaliphilic *Vibrio metschnikovii* from Lake Magadi were isolated in these two studies.

Out of the 15 bacteriophages isolated from the Kenyan environmental water sources, nine of these phages were from different rivers. Three bacteriophages were isolated from ponds while three from beaches. Phages: VP4_Ke, VP6_Ke, VP8_Ke, VP12_Ke, VP18_Ke, VP94_Ke, VP124_Ke, VP132_Ke and VP140_Ke were isolated from nine different rivers in three different regions which accounted for 60% of all the phages isolated. Phages VP42_Ke, VP56_Ke and VP64_Ke were isolated from three ponds namely; Kotonde, Koleche and Owira which translated to 20% while phages VP24_Ke, VP28_Ke and VP68_Ke were isolated from three beaches accounting 20% of the total phages isolated. The results showed that rivers harbored the highest number of phages isolated in this study. Since the host had also been isolated from the respective rivers, pond or beaches, the results of isolation of these 15 bacteriophages lytic to environmental *Vibrio cholerae*, proved the coexistence of phage with the host. This is because bacteriophages are frequently based in huge populations where

the hosts thrive such as: hatchery, sewage, soils, thermal vents that are deep or in ordinary water bodies (Karunasagar et al., 2007). Vibriophages can also be isolated from sewage or fresh waters (Shrestha et al., 2018).

Plaque and spot assays are important during isolation of new bacteriophages. This is because complete clear zones in soft assay or clear plaques are indicators of lytic phages especially for DNA tailed phages (Hyman, 2019). In the current study, positive spot assays of phages VP4_Ke, VP6_ Ke and VP8_ Ke was an indication of cell lysis as well as a positive plaque assay. Some phages had plaques of large diameter while others had pinpoint plaques. Diameter of these plaques ranged between 0.5-3mm.

Related studies have been done indicating plaques of vibriophages isolated from environment waters with diameter 1mm (Naser et al., 2017). Research undertaken according to (Al-Fendi et al., 2014) in Kelantan, Malaysia showed that vibriophages lytic against *Vibrio cholerae* were isolated from ponds among other environmental sources. The plaques were small, round and clear with diameter 3-5mm (Al-Fendi et al., 2014). Studies done in Nepal, a total of sixty-seven diverse bacteriophages were isolated, up against eight differing organisms used as host from river Kathmandu. From the ten environmental water samples from the river, *Escherichia coli* phages were among those isolated. In the same study, 8 other water samples had phages against *Proteus* and *Pseudomonas* among other bacteria. The phages had clear and turbid plaques of different sizes (Bhetwal et al., 2017).

In the initial stages of characterizing El Tor, M4 which is a vibriophage, studies (Das & Ghosh, 2018) reported that the lytic phage showed clear zones or plaques with size of diameter about 3.6 ± 0.4 millimeter using Lauria Bertani (LB) as the propagating media after incubation overnight at 37 degrees centigrade.

Vibriophages, VP64_Ke and VP68_Ke formed turbid zones on a lawn of *Providencia sneebia* and *Escherichia coli:* 083. However, further purification on a lawn of each of these bacteria as propagating strains need to be carried out to confirm whether the two phages had a host range that was wide/broad. Wide/broad host range or polyvalent bacteriophages are more common than had been thought previously.

The stability of phages is very important and under any set of conditions it must be tested. After storage bacteriophage titre should be determined the day before a crucial analysis is

carried out for precise evaluation on number of infectious bacteriophage virions. Out of the 15 phages isolated in this study the titers ranged between 10^9- 10^{10} PFUs/mL. These figures were found within the required ranges because titers that are high may lead to aggregation or crystallisation while titers that are low give rise to stability decrease on long term basis. After phages storage in SM buffer (after one year storage) a spot assay was positive for phages VP4_Ke, VP6_Ke, VP8_Ke, VP12_Ke and VP18_Ke. The phages were also stocked in Trehalose and remained infective for 6 months. As a pilot study Trehalose did not affect the phages because each of the phages developed a zone of clearing on a lawn of the propagating strain of *Vibrio cholerae*. An important *in vitro* and *invivo* study showed that trehalose protects cells from heat by stabilising proteins at high temperatures hence a stabilizer of boilogics (Singer & Lindquist, 1998). It should be noted here that tailed phages can be fragile therefore vigourous vortexing, pipeting, unnecessary agitating or passing the phages through narrow apertures should be avoided. These practises create mechanical shear that harm aparatus used by bacteriophage for binding to the host. Some bacteriophages are not stable in chemically defined media or buffers and osmotic shock can cause harm to the phages. Mirzaei and Nilsson (2015) proved that phage titers were important during phage therapy for reaching a productive infection.

The shelf life of the 15 phages isolated in the study was found to be between one year in SM buffer and 6 months in Trehalose. Phages VP4_Ke, VP6_Ke, VP8_Ke, VP12_Ke and VP18_Ke had a shelf life of one year in SM buffer at 4°C. These vibriophages were isolated from River Kuja in Migori. All the 15 phages had a shelf life of 6 months in both SM buffer and Trehalose at 4°C. It is important to moniter the storage life of bacteriophage preparations becasue bacteriophages vary in their titer stability under conditions of storage, persistence depended on the different physical and external conditions as well as the suspended media (Weber-Dabrowska et al., 2016). The current study therefore proved that both Trehalose and SM buffer preserved the attachment appendices of the isolated phages. These two media preserved the capsid from any osmotic shock that would have made it burst thereby releasing the enclosed DNA, hence they could be recomended for vibriophage preservation.

Another factor that influence survival of bacteriophages is the pH. The 15 phages isolated in the current study were found to have an optimum pH between 6 to 8 within which phages infectivity remained stable. Extreme pH of 2 and pH above 12 showed that only afew phages remained viable. Similar studies conducted by Dąbrowska (2019) showed that vibriophages

had a maximum population at pH between 6 to 8. For therapautic purposes, pH is important in survival of phages especially where they are to be administered orally because of the gut acidic environment. In alkaline pH, vibriophages are usually found to be more stable than acidic pH (Das et al., 2019; Mitra & Ghosh et al., 2007). However, this report was disputed by Das and Ghosh (2018) who reported that phage M4 was interesting because inactivation effect was more in alkali than acid. The survival rate of vibriophage, M4 in pH 5 was found to be 40 while at pH 10 was 30. Sarkar et al. (2018) reported that the optimum stability of vibriophages was between pH 7-8.

Temperature is also an important component influencing survival of bacteriophages. Phages can survive a temperature range of between 40 to 90°C as well as in extreme thermal habitats Dąbrowska (2019). Some of the roles played by temperature include: multiplication viability, storage, attachment and penetration. All the 15 vibriophages isolated in this study were stable at 37°C but stability reduced with temperature rise. Inactivation of most of the vibriophages occured at temperatures above 50 degrees centigrade. Vibriophages isolated from Migori maintained their lytic activity against *Vibrio cholerae* at 4°C even after storage for one year as mentioned earlier in SM buffer. Phage M4 is a thermotolerant phage (Das & Ghosh, 2018) unlike other vibriophages. Highly thermo-stable bacteriophages have better chances of survival in different conditions where the temperature increases to 70 degrees centigrade (Das & Ghosh, 2018). Dąbrowska (2019) isolated vibriophages from sewage and fresh water that were stable at 45°C with gradual reduction in phages observed at 55°C and almost complete inactivation at 70°C. Entire 15 bacteriophages isolated in the current research were found to have 100% stability at 4°C and fairly stable between 20°C to 50°C and so it can be said that these vibriophages recorded a wide range of temperature stability. *In vitro* studies on 5 vibriophages showed that these phages that included: B5, B1, B3, B4 and B2 could withstand a wide range of pH between pH 2 and pH 12 and a temperature range of between 25°C to 60°C (Letchumanan et al., 2016). It is a prerequisite in study of newly isolated bacteriophages to have knowledge on the growth parameters of the host strain bacterium for the subsequent study of one step growth curve of respective bacteriophages. The growth curve of *Vibrio cholerae* host strain provided an estimate of the generation time and mid exponential phase (Martinez et al., 2010). The *Vibrio cholerae* generation time in the current study was approximately less than 50 min which agreed with the conclusions of the studies done before that suggested that generation time of *Vibrio cholerae* approximately ranged between 30 to 40 minutes (Beibei et al., 2010). The mid exponential phase was between 100 to 200 min. Figure

4.6 is a representative growth curve of the *Vibrio cholerae* strain isolated from river Kuja in Migori County.

Only five phages isolated in this study; VP4_Ke, VP6_Ke, VP18_Ke, VP24_Ke and VP68_Ke were selected for determination of one step growth curve. Latent period is defined as the time between the infection and the subsequent release of phage virions that is lysis (Bryan et al., 2016). Density of the host bacterium affects latent period whereby a lesser density of host strain results in a prolonged latent period (Abedon et al., 2001; 2003). The latent period of the phages in the current study varied from 30 - 50 minutes whilst the burst size was between 23 to 26 PFUs/10µl. Phage VP18_Ke showed the shortest latent period of 30min whilst VP4_Ke had the longest latent period of 50 minutes. Study done on specific bacteriophages of *Vibrio* spp, showed the shortest latent period of 10 min by P9C and 15 min by pVp-1 (Jun et al., 2014). Most of the vibriophages have latent periods above 30min and other vibriophages have latent periods that fall between 10 to 120min (Cohen et al., 2013). The latent period of the five phages in this study fell within the prescribed ranges.

Burst size of a phage is the number of progeny (phages) released by a single infected host cell bacterium. Bacteriophages with larger burst sizes are more desirable for treatment of infections because they may eradicate infections faster. Previous studies reported burst size of vibriophages ranging from 23 to 500 PFUs (Baudoux et al., 2012; Lee et al., 2014; Elbakidze et al., 2015; Thung et al., 2018). The burst sizes of the phages isolated in this study were therefore in agreement with previous studies as described.

One of the pivotal phage features that make them candidates for therapeutic studies is the host spectrum. In this study it was important to carry out the host range test in order to find out the bacteria that were able to support phage infections which produce new virions as described by Hyman (2019). Hyman (2019) also reported that the greater the breadth of host range within the target pathogen species the more likely a particular phage can be used for any infection by the target pathogen.

A survey was carried out by Hyman during the Evergreen Phage conference in 2017 on host range. Majority of the respondents recommended that both novel environmental or clinical isolated host strains, host range tests were critical Hyman (2019). It was therefore important to carry out host range analysis of the newly isolated vibriophages in the current study.

The 15 vibriophages isolated from different environmental water samples in this current study were tested for their host range against 16 *V. cholerae* bacterial isolates, one clinical, 15 *V. cholerae* environmental strains. The host range was also tested against three more bacterial strains isolated from the environmental water samples which were: *Escherichia coli*, *Providencia sneebia* and *Proteus mirabilis*. Phages VP64_Ke and VP68_Ke had turbid zones on each of bacterial lawns of *Escherichia coli* 083 and *Providencia sneebia*. Environmental conditions in cholera endemic areas prompt persistence of pathogenic *Vibrio cholerae* and enhances dramatic shifts in the strains that emerge to cause disease. Such a process might be driven by bacteriophages lytic to *Vibrio cholerae* or that can infect other aquatic bacterial species that act as alternative hosts (Faruque & Mekalanos, 2013). Certain vibriophages that infect *Vibrio cholerae* O1 and non O1 non-O139 strains as alternative hosts, were identified in Bangladesh. Some of these phages appear to exist as lysogens in *Vibrio cholerae* non-O1 non-O139 strains (Faruque & Mekalanos, 2013). It also seemed possible that *Vibrio cholerae* phages could also replicate in other common environmental species like *Aeromonas*, *Pleiseomonas* and *Pseudomonas*. This is important since identifying and characterising such non-cholera *Vibrios* spp may have predictive value in cholera epidemiology (Faruque & Mekalanos, 2013).

Further purification and analysis is required to verify that truly these two bacterophages,VP64_Ke and VP68_Ke can lyse more bacteria besides *Vibrio cholerae*. This was the intial stage of the spot assay test, hence it could not be used to draw conclusive remarks that truly these two phages had a broad host range. Also as recommended by Hyman and Abedon (2012) phages VP64_Ke and VP68_Ke that showed an intial positive spot assay on a lawn of each of the two bacteria, three rounds of plaque purification should be carried out. Further purification was not carried out on a lawn of each of these bacterium after the first intial positive spot assay hence, further analysis recommended. Broad host range bacteriopahges have been reported by Jensen et al. (1998). Some of the broad host range bacteriophages that infect a wide range of bacterial species are P1 and MU (Jensen et al., 1998). Host range is critical for deciding the usefulness of a particular bacteriophage for phage therapy (Hyman, 2019). Report by (Ross et al., 2016) showed host range as a key to property for phage therapy and biology of phages in general.

Vibrio cholerae has a duo lifecycle as it can exist naturally in the aquatic environment and can also colonise human beings. In both life cycles, *Vibrio cholerae* encounters a variety of

stressful conditions that may affect its cell envelop negatively (Saul-McBeth & Matson, 2019). It was therefore important to test the isolated phages for their lytic activity against both environmental and clinical isolates. This is because previous studies have reported resistance of environmental and clinical *Vibrio cholerae* to antibiotics. A study in Ghana (Abana et al., 2019), used eight antibiotics: (tetracycline, doxycycline, ciprofloxacin, erythromycin, chloramphenicol, azithromycin, trimethoprim/sulfamethoxazole, nalidixic acid against *Vibrio cholerae* and revealed a loss of sensitivity of many of them including erythromycin and nalidixic acid. Similar findings have been reported in the Democratic Republic of Congo (DRC) (Miwada et al., 2015. Thepa Shrestha et al. (2015) in Kathmandu City-Nepal reported 100% resistance of both clinical and environnmental *Vibrio cholerae* isolates to nalidixic acid.

The phages isolated in this study had therefore a narrow host range. Narrow host range phages complete their life cycles in only one host (Manohar et al., 2019). Host range modulation may depend on adaptations in the host receptor –binding proteins and on proteins that play a role in other life cycle stages. Monovalent phages that bind to a single receptor are more likely to have a narrow host range while polyvalent phages that bind to multiple different receptors may be able to infect more diverse hosts. Bhandare et al. (2019) isolated a vibriophage of the family *Podoviridae* . The study provided evidence that the phage could both reduce the severity of cholera and limit the spread of the organism in the environment. Narrow host range vibriophages against *Vibrio cholerae* have been reported by (Al-Fendi et al., 2014) with specificity to *Vibrio cholerae* either El Tor Inaba. Generally, bacteriophages infect a narrow range of bacteria closely related due to the specificity of the phages host binding proteins, biochemical interractions during infection, presence of related prophages or particular plasmids and bacterial phage –resistance mechanisms (Ross et al., 2016).

Lytic phages have a narrow host range but this limitation can be overcome by use of cocktails. Sarkar et al. (2011) reported that a total of five vibriophages with a broad host range which may be useful as cocktails for phage therapy to control the disease cholera, caused by *Vibrio cholerae* O1 bacterium.

A well-studied vibriophage in Japan, is KVP40 (Miller et al., 2003), that has a very broad host range. It was isolated using *Vibrio cholerae* O139 as the propagating strain. This vibriophage is lytic to several *Vibrio* spp including: *Vibrio cholerae, Vibrio anguillurum, Vibrio*

parahaemolyticus, non-pathogenic species: *Vibrio natriengens* and *Photobacterium leignathi* (Letchumanan et al., 2016). Similar reports by (Sarkar et al., 2018) showed broad host range of *Vibrio cholerae* bacteriophages. Previous report showed that CTXϕ of *Vibrio cholerae* O1 infected an enterotoxingenic *Escherichia coli* isolate. Four *Vibrio harveyi* isolates, one *Vibrio alginolyticus* and one *Escherichia coli* isolate, were shown to be infected by VHML bacteriophage, out of the 36 bacterial isolates (Oakey & Owens, 2000).

Interestingly, the *Myovirus*-like bacteriophages isolated from *Vibrio harveyi* was able to infect one out of 8 isolates of *Vibrio cholerae.* This could be explained by binding of phage to the host bacterium. The initial interaction between bacteriophages and their host strain is very important. Among the key factors that determine phage specificity for all phages upon interaction with their host are Receptor Binding Proteins (RBP), tail fiber protein (Dowah & Clokie, 2018). The Lipopolysaccharides are common phage receptors in Gram negative bacteria. The core oligosaccharide region of LPS was found to be necessary for binding of *Vibrio cholerae* typing phage VP4 (Wang et al., 2017). Other studies, (Seed et al., 2011; Fouts et al., 2013); have reported capsular O antigen identified as the receptor for Vibrio spp. The specificity of phage-host range is advantageous in phage therapy as non-pathogenic microbes are spared from being killed during the experiment (Ross et al., 2016).

Persistence of a resistant *Vibrio cholerae* O1 to commonly used antimicrobials such as Streptomycin, ammoxilin, clavulinic acid, trimethorpin and sulfamethoxazole has also been reported by Hounmanou et al. (2019). Occurrence of multidrug resistant *Vibrio cholerae* O1 in Lake Victoria that are genetically related to recent pandemic strains in Tanzania, Uganda and Kenya has also been reported (Hounmanou et al., 2019). There was close phylogenetic relationship between environmental and clinical *Vibrio cholerae* O1 strains in Haiti reported by Azarian et al. (2014). In Kenya *Vibrio cholerae* O1 resistant to common antimicrobials have been isolated in fish and water from Lake Victoria (Onyuka et al., 2011). Due to emergence of antibiotic-resistant bacterial strains there is an extensive search for an alternative treating method (Sen & Ghosh, 2018) and phage therapy is one of such potential alternative that is gaining a lot of attention throughout the world.

Use of bacteriophages to control pathogenic *Vibrio cholerae* was first done in India between 1927 and 1936 period of years. The early studies showed that the bacteriophages lytic to pathogenic *Vibrio cholerae* were applied in three ways: for treatment of active cases,

prophylactically during epidemics and for prevention in potentially epidemic areas (Nagel et al., 2016). In the current study, the same approach can be applied both prophylactically and by dissemination of the *Vibrio cholerae* phages in environmental water bodies. This can be done especially in specified areas of study where the pathogen has proved to be resistant to several antimicrobials. Since the newly isolated novel phages in the current study infected also the clinical *Vibrio cholerae*, they can be applied prophylactically to prevent the cholera outbreak.

In order to confirm that the environmental waters of Kenya were reservoirs of *Vibrio cholerae* bacteriophages, further characterization was done using the Transmission Electron Microscopy (Ntema et al., 2010). In accordance to International Committee on Taxonomy of Viruses (ICTV), electron micrographs of newly isolated bacteriophages were obtained in order to determine their morphological features and for purposes of classification. The 15 newly isolated bacteriophages had unique features displayed by the TEM images. The monographs revealed that these were truly different from others (Chakrabarti et al., 1993).

According to Ackermann (2007) description of phages, all the 15 bacteriophages isolated in this study belonged to the order *Caudovirales.* Owing to their icosahedral symmetric head, contractile tail defined by a base plate, though with slight differences except for phage VP68_Ke, all the 15 phages belonged to the *Myoviridae* family. The head diameter of the phages ranged between 85nm to 33nm. Five phages: VP4_Ke, VP6_Ke, VP12_Ke, VP28_Ke and VP64_Ke had similar heads, diameter of 85nm which constituted 33%. Two phages: VP42_Ke and VP124_Ke had same head diameter of the same size that is 80nm. Two phages: VP18_Ke and VP94_Ke equally had identical heads of 79nm. Phage VP68_Ke had the smallest head diameter of 33 nm while the remaining phages: VP56_Ke, VP140_Ke, VP24_Ke, VP8_Ke and VP132_Ke had head of diameters 87nm, 86nm, 78nm, 82nm and 75nm respectively. Two phages with identical head and tail of dimensions 85nm and 95nm respectively were VP4_Ke and VP28_Ke. The average head diameter of the 15 phages was 79 nm.

Previously, Maina et al. (2014) isolated 9 phages from the Lake Victoria region of Kenya. The phages had dimensions, 88.3nm and 84.9nm of head and tail respectively. The difference could have occurred due to the unique phage VP68_Ke that had the smallest head of 33nm. The tails' length of the 15 phages ranged between 78nm to 130nm with three phages (VP64_Ke, VP6_Ke and VP140_Ke) having the same tail length of 100nm. Two phages

(VP4_Ke and VP28_Ke) also had the same tail length of 95nm. Phage VP68_Ke had the longest tail length of 130nm which was very unique. Phage VP94_Ke had the shortest tail length of 78nm while the other phages: VP12_Ke, VP132_Ke, VP18_Ke, VP24_Ke, VP56_Ke, VP8_Ke, VP42_Ke, and VP124_Ke had tail length of diameter 101nm, 102nm, 103nm, 99nm, 98nm, 97nm, 96nm and 85nm, respectively. The average size of the tail length was 90nm.

A recent study by Sen and Ghosh (2018) on a comparison between *Myoviridae* phages revealed that phage M4 that had been propagated on El Tor *Vibrio cholerae* strain O1 MAK757, belonged to the family of *Myoviridae*. This phage had a head diameter of 85±3nm, a long contractile tail of length 98±2nm. In comparison was phage D10, of *Myoviridae* family which had a head diameter size of 52±2.3nm, a long contractile tail length of 101.4±0.3nm. Phage ICP1 isolated from rice water stools samples of a cholera patient in Bangladesh, belonged to *Myoviridae* family. The phage had a contractile tail 106nm long and 17nm wide. The host range was limited to *Vibrio cholerae* O1. The head was icosahedral of diameter 86nm long (Navarro-Ocaña et al., 2016). Another phage that was isolated from the environmental waters of Bangladesh also propagated on *Vibrio cholerae* El Tor O1 strain was JSF7. This was also a *Myoviridae* phage with a head diameter of 58.3±4nm and tail length of 55.9 ± 2.5nm (Naser et al., 2017). Phages AS1 and AS3 isolated from sewage and pond water collected from the outskirts of Kolkata, a high cholera endemic area had hexagonal heads and noncontractile tails. The propagating strain was *Vibrio cholerae* O1 El Tor (MAK 757) were placed in *Myoviridae* family. AS1 had a head diameter of 43.6± 2.34nm and a tail length of 85.21± 3.0nm, AS3 had a head diameter of 90.1±2.21nm, tail length 193.5± 14.5nm (Sen & Ghosh 2005a).

According to Ackermann (2007), typical dimensions of tailed phages are capsid of length between 20-160nm and tail length of 80-800nm. In the current study, the average length of head and tail dimensions were 79nm and 90nm respectively. In line with this the head and tail dimensions of *Myoviridae* family of phages infecting *Vibrio* spp are 43-107nm and 85-221nm respectively (Sen & Ghosh, 2005a; 2005b; Dutta & Ghosh 2007; Al-Fendi et al., 2014; De Sordi, Lourenço & Debarbieux, 2019). The only phage that did not fall under these dimensions was phage VP68_Ke. The measurement of the head was 33nm which was within the Ackermann's dimensions (30nm) of the head. The other 14 phages can be said to have relatively big heads and so they could be interesting subjects for further studies.

Phages with such long tails like VP68_Ke are rare in the environment and therefore it could have been interesting to further characterize the phage (Sen & Ghosh, 2005a). All the 15 phages even though they appeared similar in the TEM images except for VP68_Ke, portray a diversity of *Vibrio cholerae* phages in the Kenyan environmental waters. In addition to the names given to the 15 bacteriophages isolated in this study, they were further assigned names recommended by (Kroprinski et al., 2009). The presence of vibriophages in Kenyan environment has previously been described (Maina et al., 2014). However, the description was limited because the phages were only isolated from one region of Lake Victoria while the current study involved isolation from four regions of Kenyan environmental waters, hence more diversity. The limitation of the host strain could have been attributed to the fact that phages isolated belonging to only *Myoviridae* family. This study used strains of *Vibrio cholerae* El Tor each isolated from the respective region as the propagating strain to isolate phages.

Previous investigation of other lytic vibriophages in the environmental waters eg shrimp ponds, aquaculture system, sea water and aquatic product market from different regions of the world, has been reported (Luo et al., 2016; Stalin & Srinivasan, 2016*;* Katharios et al., 2017*;* Srinivasan & Ramasamy, 2017; Yang et al., 2019). From a total of 15 phages isolated from the different environmental waters of Kenya, only four were identified and selected for further molecular characterization and sequencing. The phage selection was based on: host range because they lysed both environmental and clinical *Vibrio cholerae* isolates, ability to produce large, clear regular plaques in plaque assays, shelf life in SM buffer and Trehalose which was one year, 6 months respectively for phages except for VP24_Ke and the high titer which was 10^{10}PFUs/ml for these four phages. Moreover, out of the four phages only VP24_Ke was isolated from Usenge beach. The other three phages (VP4_Ke, VP6_Ke and VP18_Ke) were isolated from river Kuja in Migori County.

A combination of analysis was used to determine the genome size and details of the genomes of the four phages. According to Strauch et al. (2001), use of restriction digestion of phage DNA has been for restriction mapping of the phage genome. Moreover, restriction digestion patterns have been used to differentiate between bacteriophages (Ogunseitan et al., 1992). The restriction digestion pattern using four enzymes showed that the four phage genomes could be cut by *HindIII* but could not be cut by the other three endonucleases used in this study: *ECOR1, Sal1* and *Xho1*. Bacteriophage resistance to restriction enzymes has been reported

previously. Sixteen *Campylobacter* bacteriophages were shown to be refractory to digestion by commonly used restriction enzymes. In relation to this *Lactococcus lactis* bacteriophages have been found to be highly refractory to digestion by several restriction enzymes (Barrangou et al., 2002). Phage DNA resistance to restriction enzymes is known as antirestriction mechanism and several explanations have been proposed. One such explanation is the adaptation of the bacteriophage genomes under selection pressure of widespread restriction-modified systems and lose restriction sites during evolution (Barrangou et al., 2002). Moreover, insensitivity of phage DNA can also be due to integration of unusual basis in the viral DNA such as hydroxymethyl uracil or hydroxymethyl cytosine that make the DNA refractory to endonuclease cleavage (Jensen et al., 1998). Alternatively, phage genomes may encode methyltransferases that modify specific nucleotides within the recognition site of one or more of the restriction endonucleases (Barrangou et al., 2002).

Previously, Al-Fendi et al. (2014) reported three bacteriophages (VPUSM 4, VPUSM 7, and VPUSM 8) isolated from environmental water samples of different areas in Kelantan, Malaysia using *Vibrio cholerae* El Tor as the propagating strain. The three bacteriophages were found to have a small genome size of 33.5kb. These phages belonged to *Myoviridae* family, had similar morphologies, indistinguishable genome characteristics, identical patterns of host range. These results suggested that the three phages represented one phage or several very closely related phages present in different waters (Al-Fendi et al., 2014). Eight restriction enzymes were used (*BglII, EcoRI, EcoRV, Hin1ll, HindIII, ScaI, SmaI*, and *XbaI*) only *HindIII* resulted in digestion of the nucleic acid of these three vibriophages and showed identical restriction digest profiles (Al-Fendi et al., 2014). The % G+C content was 48.9 of these three phages.

The visual classification of all tailed phages puts them in order *Caudovirales* but the classification does not take into account the life cycle of the phages, Genome replication and what takes place inside the host cell. Bacteriophages with contractile tails have larger genomes than others (Leiman & Shneider, 2012). The most abundant of tailed phages are the *Siphoviridae* with long non-contractile tails. Giant *Myoviridae* phages represent some of the most complex viruses with genomes as long as 700Kbp (Serwer et al., 2007). The results of post sequencing confirmed the size of the four phage genomes selected for further characterization. The percentage G+C content was also found to be the same in the four phage genomes i.e. 36.4 percent. Among the well-studied bacteriophages that infect *Vibrio cholerae*

O1 is the myophage ICP1, a double stranded DNA (previously known as JSF1) with a genome size of 125,956bp, G+C content of 37 percent and 230 coding sequences. A similar phage of the same series of phages JSF7 from propagating strain *Vibrio cholerae* O1, has a double stranded DNA genome with G+C content of 48.42 percent has a genome size of 46kb (Naser et al., 2017). The genome size of JSF7 is far much smaller than the four phages in this study. M4 is a double stranded myophage with a large genome, three times larger than other vibriophage D10 (family *Myoviridae*) that has a genome size of 32kb and can be cut by *Hind111* (Das & Ghosh, 2018). Therefore, in comparison with the most recent isolated *Myoviridae* phages of *Vibrio cholerae* O1, the four phages isolated in this current study have largest genomes among the recently isolated phages of *Vibrio cholerae* O1. Hence, they are novel vibriophages which can attract a lot of interest in the phage biology, in addition phages with big head required to accommodate large genomes are very interesting subjects for further study by Das and Ghosh (2018) The phage genomes are also important for further studies because inadequacy of information on vibriophages genome has been reported (Luo et al., 2015).

In each of the phage genome sequences there were four tRNAs present and the functional genes were 25 except for VP6_Ke that had 24 of them. This further confirmed the close similarity in these four phage genomes. Transfer RNA (tRNA) was present in each of the four phage genome sequences. Each of the four sequences had 4 tRNAs coding for amino acids. The role of tRNA is protein translation in living cells, replication among others.

The most characterized phage of the *Myoviridae* family is T4 phage. The T4 phage has a genome size of 168,903bp, elongated head (110x80nm), contractile tail that ends with a complex base plate with 6 long fibers radiating from it. It has a G+C content of 35 percent, 8 tRNAs with Accession number NC_000866. The propagating strain is *Escherichia coli*. More than 200 similar phages have been described that share common virion morphology and related features (Comeau & Krisch, 2008). The genomes of the four closely related phages isolated in the current study can therefore be placed in the T4 like phages. About 90% of the T4 like phages grow on *Escherichia coli* or other *Enterobacteria* but 10% grow on phylogenetically more distant bacteria such as *Aeromonas*, *Vibrio*, *Cyanobacteria* among others and they convey significancy in virion morphology (Comeau & Krisch, 2008). Phage JS98 propagated on *Escherichia coli* with a genome size of 170,523, 3 tRNAs, G+C content 39 percent and Accession number NC_ 010105 can be said to be closely related to the four

phage genomes in this study. The four phage genomes in the current study were also closely related to the well-studied vibriophage KVP40 that has a giant genome size of 244,834bp the host strain *Vibrio parahemolyticus*, G+C content of 42.6 percent, 30 tRNAs, Accession number NC_005083, isolated from polluted sea water in Japan belongs to the *Myoviridae* family and it is classified as a T4-like phage with abroad host range (Miller *et al.,* 2003). The four phages could also be closely related to two non-Vibrio phages; S-PM2 and S-RSM4 the host strain of these two was *Synechococcus,* % G+C contents of 37 and 41, genomes size 196,280bp and 194,454bp, Accession numbers NC_006820 and NC_013085 respectively (Clokie et al., 2010). Phages closely related in percent G+C content are P-SSM2 and P-SSM4 the host strain *Prochlorococcus* had G+C content of 35.5% and 36.7%, genome sizes 252,401 and 178,249, tRNAs 1 and 0 with accession numbers NC_ 006883 and NC_006884, respectively. These two bacteria, *Prochlorococcus* and *Synechococcus,* are marine cyanophages very closely related, highly abundant in world's oceans (Clokie *et al.,* 2010; Aziz *et al.,* 2015). Phages with contractile tails and large genomes are strictly lytic, that is the infection leads to host cell lysis and phage genome does not integrate into the host genome. All known phages with contractile tails have double stranded DNA genome (Orlova, 2012; Leiman & Shneider, 2012). A coliphage, RB69 has a genome size of 167,560, G+C content of 37.6 percent, 2 tRNAs and accession number NC_004928. The four phages isolated in this study fall in the category described of the T4-like phages because they can be considered to have a large genome, had 4 tRNAs, all of *Myoviridae* family of tailed phages and were lytic, dsDNA.

Detailed description of the proteins encoded by genes in each of the phage genomes is described in this study. The function of the proteins could be grouped into: Translation (eg tRNAs), nucleotide metabolism (Thymidylate synthase), Head functions (head completion protein) and tail and tail fiber (phage plate wedge subunit) as detected in the four genome sequences. Some of the genes detected have been described as genes of the core genome of T4-viruses. These genes could be grouped into two categories according to (Petrov et al., 2010):

i. DNA replication, Repair and recombination: In all the four genomes they were (gp 41, gp61, gp44 and gp47).
ii. Phage morphogenesis (Phage structure and assembly). These included in all the four phage genomes (gp4, gp6, gp2 and gp25).

As mentioned earlier, 186 CDSs were defined for phages VP4 and VP18, and 185 CDSs for phages VP6 and VP24. Out of these, 103 genes (55%) were annotated as 'hypothetical' or 'unknown' proteins. High frequency of proteins with unassigned functions is typical for phage genomes from previously unsampled geographical sites, and calls for further studies to identify the potential functions of those proteins. On the other hand, the annotation process identified 82 genes (45% of all CDSs) associated with two main categories: (1) functional subsystems/modules, including nucleotide replication, repair, recombination, and metabolism, and (2) phage structural and hallmark genes. The four phages had near identical set of predicted protein. Other than structural and functional phage domains, details for phage VP4 no unusual genes were detected. Specifically, both VIBRANT (Kieft et al., 2020) and the PATRIC (Davis et al., 2020) database indicated no known resistance or virulence genes, and no evidence of integrases was found.

A notable exception to the genome analysis was the *phoH* gene, the expression of which was linked to phosphate starvation conditions. The *phoH* gene is a host-derived auxiliary metabolic gene (AMG), sometimes known as moron (Hendrix et al., 2000; 2003), is commonly carried by some phages. It belongs to the phosphate regulon that regulates phosphate uptake and metabolism under conditions of low phosphate and phosphate limitation. The *phoH* gene homologs were detected in phages with various morphological types, e.g., siphophages, myophages, and podophages, and with a wide bacterial host range (including autotrophic and heterotrophic bacteria). They were even detected in viruses of autotrophic eukaryotes (Goldsmith et al., 2011). The *phoH* gene is not restricted to a certain morphological type of phage, which suggests that it could be a powerful biomarker gene for studying phage diversity. Goldsmith et al. (2011) found out that nearly 40% of marine phages contained *phoH*, compared to only 4% of nonmarine phages.

In a study by Wang et al. (2016), more than 400 phage-harbored *phoH* sequences were obtained from several paddy floodwaters in northeast China. Precisely, four specific groups and seven subgroups of this gene family were detected in phages from paddy waters (Wang et al., 2016). The study demonstrated that *phoH* was present in phage genomes of terrestrial environments and that this gene was useful for studying phage ecology in paddy ecosystems. These findings support the evidence that this biomarker gene can be used to investigate the diversity of phages in both marine and terrestrial environments (Li et al., 2019).

Phosphorus is a major element, necessary for nucleotide biosynthesis and DNA replication, but is extremely scarce in oligotrophic waters and is consequently thought to be one of the limiting factors for cyanobacterial growth (Martiny et al., 2006; Tetu et al., 2009; Kelly et al., 2013). Thus, it is not surprising that some phosphorus-acquisition genes, such as the phosphate-inducible genes, *pstS* and *phoH*, and the alkaline phosphatase gene *phoA*, which are regulated by the PhoR/PhoB two-component regulatory system to sense phosphorus availability, were found in the genomes of cyanophages infecting cyanobacteria (Martiny et al., 2006; Sullivan et al., 2010; Zeng & Chisholm, 2012). These genes could be upregulated in response to phosphate starvation in host cells, and their products could play an important role in regulating phosphorus absorption and transportation of host cells under low-phosphorus content or phosphorus-deprived conditions (Gao et al., 2016). It was also proposed that cyanophages maintain *phoH* to allow their host increased phosphate uptake during infection; however, the mechanism of how it occurs is not well known (Clokie et al., 2010), and *phoH* expression in phosphate-limited conditions appears to vary between hosts (Lindell et al., 2007; Tetu et al., 2009).

Based on these observations, *phoH* has been proposed as a novel signature gene to assess the genetic diversity of viruses in multiple families of double-stranded DNA tailed phages (Goldsmith et al., 2011). *phoH* was commonly discovered in cyanophages, such as marine cyanophages P-SSM2, P-SSM4, and Syn9 (Sullivan et al., 2005; Weigele et al., 2007), and freshwater cyanophages Ma-LMM01(Yoshida et al., 2008) and MaMV-DC (Ou et al., 2015). It was also found in non-marine phages, such as coliphage T5 (Wang et al., 2005) and Staphylococcus phages K, G1, and Twort (O'Flaherty et al., 2004; Kwan et al., 2005). The frequent detection of *phoH* genes in phage genomes suggests that their products play a role in the phosphate metabolism of the phage-infected cell (Sullivan et al., 2005). Based on bioinformatic analyses, *phoH* genes were suggested to be part of a multi-gene family with divergent functions from phospholipid metabolism and RNA modification to fatty acid beta-oxidation (Kazakov et al., 2003). *phoH* was also reported in vibriophages, represented by phage KVP40 (Miller et al., 2003) the well-studied T4-like phage isolated from polluted coastal sea water in Japan. The propagating host bacterium of phage KVP40 was *Vibrio parahaemolyticus*. Here, *phoH* was found in all the four phage genome sequences reported. *phoH* has already been reported in phages isolated from versatile geographic locations. Although most phages harboring the *phoH* gene originated from marine environments, some belonged to other habitats, such as soil, sewage and stool (Adriaenssens & Cowan, 2014).

Taken together, the presence of genes involved in phosphorous acquisition demonstrate how phages might have developed adaptation to life in oligotrophic environment (Baudoux et al., 2012).

The molecular weight of the four phage genomes in this study was also found to be similar that is 91.54MDa. Comparison among recently isolated phage M4 and other four phages of various families revealed that the genome size was 120kb and molecular weight 80MDa. The general summary was that the four genome sequences were almost similar but not identical. The similarity could have been attributed to the use of the propagating strain, *Vibrio cholerae* confirming that these two coexist in different environmental waters of Kenya

Alignment of phage VP4 with two closely related *Vibrio*-infecting phages shows that VP4 shares a few genes with known *Vibrio* phages. The genes with the highest similarity are those predicted to encode terminases and major capsid proteins. These similarities, along with the phylogenetic trees, confirm the phylogenetic relatedness, yet distinction, of the four novel phages relative to known *Vibrio* phages.

Phylogenetic analysis of RnR alpha, RnR beta and LT protein sequences of the four phages, compared to those from reference phages, displayed separate branching of the four phages. Although the four phages were phylogenetically distinct from reference phages, they were most closely related to other *Vibrio*-infecting phages. Based on the trees for RnR alpha and RnR beta, the four phages are most closely related to *Vibrio* phage 1.244.A._10N.261.54. C3 (NCBI accession number MG592609.1, 159 kb length), from seawater, which has a partial genome sequence and is estimated to belong to the family *Ackermannviridae* (Kauffman et al., 2018).

As for the LT-based phylogeny, LT from the four phages are most closely related to *Vibrio* phage YC (RefSeq NC_048709.1, 147 kb length), which belongs to *Ackermannviridae* and infects *Vibrio coralliilyticus* (Cohen et al., 2013). Other phages that are related based on the LT tree include *Vibrio* phage VP-1 (NCBI MH363700.1, 150 kb length) (Mateus et al., 2014) and *Vibrio* phage VAP7 (Ref- Seq NC_048765.1, 144 kb length), both of which belong to the family *Ackermannviridae* as well (Gao et al., 2020), but were found to infect *Vibrio parahaemolyticus* and *Vibrio alginolyticus*, respectively.

Although the four phages described here seem to belong to family *Ackermannviridae* and share a similar genome length (~ 150 kb) to those other phages, their separate phylogenetic branching and different host range suggest that they are evolutionarily distinct from other known phages.

According to current virus classification, phages that show some sequence similarity are members of the family, *Ackermannviridae*. The genomic classification of the four genome sequences in this study placed them further in the *Ackermannviridae* family. It should be well noted here that there has been continuous review of the ICTV by a committee of experts. After the July 2018 International Society on Viruses of Microbes, discussions by these experts at Wroclaw in Poland, more reviews have been made in the classification of viruses.

Virfam was proposal by (Lopes et al., 2014) as novel classification of phages dedicated to recognition of "head-neck-tail" modules and recombinase genes in phage genomes. The Virfam analysis was able to display the organization of the genes encoding the Major capsid protein (MCP), large terminase (TermL), Portal, neck, major tail and sheath proteins. The current study applied this novel classification and came up with various results. These include the MCP (major capsid protein), Portal, TermL (large terminase subunit), Ad (Adaptor protein), Hc (Head closure protein), Tc (Tail completion protein), Ne (Neck protein), MTP (major tail protein) and sheath. The MCP forms the icosahedral shell of the tailed phage head. The capsids of dsDNA phages have icosahedral symmetric with three functions namely: Protection of the nucleic acids from digestion by enzymes contains special sites on its structure that allow the virion to attach to the host cell and provision of proteins that enable the virion to penetrate the host membrane. The portal proteins form the hole at one vertex of the icosahedral shell through which the DNA is packaged and ejected. The TermL is the ATPase that pumps DNA into the capsid during packaging and after DNA is packaged tails attach to the portal vertex (Grose & Casjens, 2014). The head completion proteins (stopper and adaptor) are bound to the portal; complex preventing DNA leakage.

The assembly of capsid in tailed phages is initiated by construction of procapsid which is composed of MCP. When DNA packaging is complete the head-completion proteins prevent the leakage of the viral DNA and one protein binds to the portal called Adaptor protein (Ad) and can be supplemented with head closure protein (Hc). All together the head completion proteins provide platform for docking of pre-assembled long tails in *Myoviridae* and

Siphovidae families of *Caudovirales*. The tail completion proteins (Tc) form the head to tail connection and together with the portal proteins constitutes the virions neck. The major tail protein is the main component of the tail tube structure (Gontijo et al., 2017). The tail sheath protein in *Myoviridae* contracts upon infection initiating injection of viral DNA into the host cell (Lopes et al., 2014). The description of the phage genome structural proteins that were identified coding for the head, tail, base plate and also the proteins and protein superfamilies were carried out with the help of the various Bioinformatics. The description of the phage genome corresponds with the TEM images that classified the isolated phages into the family of tailed bacteriophages hence confirming their identity.

Since the Virfam analysis evaluates the sequence and composition diversity at head to tail connection then classifies the phages according to morphology, the highest identities of proteins of the query phage with at least three in the acclame phages. Among the three acclame phages, Syn9 compares closely with the sequences of the query phage in terms of MCP, portal, TermL, MTP and Hc2. Syn9 is a tailed dsDNA marine cyanophage, infecting both *Prochlorococcus* and *Syanechoccus* (Sullivan et al., 2010). Isolated from Woods Hole Harbour on *Synechoccus* strain WH8012, T4-like phage with genome size: 177,300bp, G+C content 40.5% distantly related to T4 coliphage together with S-PM2 and P-SSM4 placing SYn9 in the large group of T4 –type phages. In terms of protein superfamilies there seemed to be commonality with especially Syn9 and others in sharing genes that encode for functions required for any (Sullivan et al., 2010). The genes that adapt the four phage sequences to their specific niche are then unique to the four phages while genes allowing for other bacterial hosts are shared among the phages related to them depending on the conditions and environment.

In addition, Ad2, Hc2 and Tc2 were found to be present in the sequences of the four phage genomes in this study. According to Lopes et al. (2014), the type 2 necks T4-like are only found in *Myoviridae*. They comprise two head completion protein homologous to T4 gp13 (Ad2) and T4 gp14 (Hc2) and tail completion protein homologous (Tc2). The head –neck-tail proteins are important in defining the type of morphology a phage adapts. Neck proteins are important for capsid completion because they form the channel between the head and the tail that has to close immediately after DNA packaging to avoid leakage, reopen after recognition of the target host bacteria so that the infection process takes place. This process is important for the life cycle of a phage. This is the reason phage was classified in *Myopviridae* type 2.

The type three necks P22-like and Type 4 ϕ29-like were not found because they are usually associated with *Podoviridae*.

Among the Cyanophages clustered together were phages P-SSM2, P-SSM4, S-PM2 and Syn9. All the four cyanophages are common in that they were all isolated from the environmental sources of water just like the phages in this study. Phages P-SSM2 and P-SSM4 were isolated from Atlantic Ocean; the original host was *Prochlorococcus* NTL1A and NATL2A respectively. The G+C% content was closely related to the phage genome in the study i.e. 35.5, 36.7% respectively hence close relationship because the G+C in the phage genome in this study was 36.4%. Phages S-PM2 and Syn9 had been isolated from the original host S*ynechococcus* WH7803 and WH8109 from English Channel and woods hole harbor in Atlantic Ocean respectively. The four phage genomes in this study were also clustered along with non cyanophages of the T4-like phages all isolated from the environmental waters, *E. coli* as propagating host these included: T4, RB32, RB43, RB49, RB69, JS98 and Phi1. All these are *Myoviridae* phages with contractile long tails, prolate/icosahedral heads. Among the phages clustered together were phages that used a different host other than *Escherichia coli* for propagation that included: 44RR2.8t, PHG25, PHG31 the propagating host was *Aeromonas samonicida*, Phage 44RR2.8t was isolated from Ontario Canada trout pond while phages PHG25 and PHG31 were isolated from Europe fish hatchery and France fish hatchery respectively. Phage Aeh1 propagated on *Aeromonas hydrophila* was isolated from treated sewage also among the clustering. The only vibriophage that was clustered with vibriophage in this study was phage KVP40 the well-studied T4-like phage isolated from polluted coastal sea water in Japan. The propagating host bacterium of phage KVP40 was *Vibrio parahaemolyticus*.

Besides the lytic phages isolated in the current study, presence of a filamentous phage was also revealed after sequencing of the four genomes. The filamentous phage in the current study, given the code VP24-2_Ke had a genome size of 7180bp, classified in the *Inovirus* genera of the family *Inoviridae.* It was closely related to a *Vibrio cholerae* filamentous phage VEJphi, approximately 95 % identical except for one region (505-1916) that was divergent. The region encoded for three genes namely: phage related regulator and two hypothetical genes.

VEJphi phage was isolated from strain MO45 of *Vibrio cholerae* of the O139 serogroup. The genome nucleotide sequence of VEJphi, 6842 bp, was organized in modules of functionally related genes in an array characteristic of the genus *Inovirus* (filamentous phages). VEJphi was closely related to other previously described filamentous phages of *Vibrio cholerae*, including VGJphi, VSK and fs1 (Campos et al., 2010). Template vibriophages can grow lytically killing the host cell and producing numerous progeny phage particles or form lysogenic *Vibrio cholerae* cells (Faruque et al., 2005b). Filamentous phages of the family *Inoviridae* are ssDNA genomes packaged into filament like virions. They were initially identified in *Escherichia coli* and the best well known is M13. The key factor success of phage therapy of *Vibrio* spp is the selection of an appropriate bacteriophage (Mateus et al., 2014). Before using a bacteriophage for phage therapy, it is important to test for presence of virulence genes (Principi et al., 2019; Letchumanan et al., 2014). There are more than 200 serogroups of *Vibrio cholerae* but only two have been reported to cause the pandemic cholera disease these are the O1 and O139 serotypes (Harris et al., 2012). This is because of the production of two essential virulence factors that is the toxin co-regulated pilus (TCP) and the cholera toxin (CT). The genes ctxAB encoding the enterotoxin CT are not carried by core genome of the bacterium but can be acquired after infection by lysogenic phage CTXϕ. Once infected the bacterium produces CT and assembles new phage particles carrying the ctxAB genes that will be secreted in the environment and it may convert nonpathogenic to pathogenic *Vibrio cholerae* (Houot et al., 2017). The filamentous lysogenic CTXϕ vibriophage is one of the best characterized and described where horizontal gene transfers of the phage that encodes cholera toxin (CT) can convert nontoxigenic strains into highly virulence pathogens (Mai-Prochnow et al., 2015). Other filamentous phages described are: *Pseudomonas* Pf phage, *Xanthomonas* Cf phage, *Escherichia coli* Ike, IF1 and If2 phages, *Neisseria* Ngo and Nf phages, *Sawewanella* SW1phage and *Ralstonia* RSM phage. Despite their small size they have significant impacts on the evolution and behavior of their bacterial hosts. The size of a phage is a function of that genome that ranges from 4 to 12 Kbp and mostly carried by Gram negative bacteria but some Gram positive have filamentous phages as well e.g., *Propionibacterium freudenreichii* B5 phage and *Clostridium acetobutyricum* CAR1 phage. The ICTV has classified rod or filamentous –like phage into the family of I*noviridae* with two genera: *Inovirus* and *Plectrovirus* where the former appears as filamentous while the former as rods (Chibani et al., 2019). The novel filamentous phage VP24-2_Ke isolated in this study can either integrate into *Vibrio cholerae* phage or traduce the cholera toxin gene by

packing the CTXphi genome instead of its own. Figures 4.31 and 4.40 represent the genomic map and phylogenetic tree of VP24-2_Ke, the *Vibrio cholerae* filamentous phage respectively.

The persistence of two genomes sizes indicated that two viruses co-occurred after three rounds of plaque purification. This co-occurrence of viruses was a significant finding in the current study. Studies by (Comeau, Chan & Suttle, 2006) showed the presence of two different phages in four individual clones (LH1, 4a, 4c and 6a) first observed by PFGE and then confirmed via TEM. Previous studies by Kellogg and colleagues (1995) also isolated a VpV from the coast of Florida that, upon sequencing (Seguritan et al., 2003), revealed two closely related phages.

Multiple phage infection has been described where in some cases PFGE yielded double bands for a phage stock (Holmfeldt et al., 2007). Further genomic analysis was however recommended to elucidate the origin of multiple bands where vibriophages genomes of ϕH20, ϕ H8 and ϕH2 yielded several bands in PFGE analysis representing multiplications of its own genome sizes (Tan, Gram & Middelboe, 2014). Specific host strains may also be susceptible to more than one type of viruses (Middelboe, 2000). The first tailed vibriophage genome to be sequenced was that of κ139 which can be both lysogenic and lytic *(*Nelson et al., 2009).

Persistence of co-occurring phage morphotypes after multiple rounds of purification and amplification from single plaques is a mechanism which is unknown (Comeau & Krisch, 2008). In the current study, after sequencing, two phages were present that is a *Myoviridae* and a filamentous *Inoviridae* phage of genome sizes 148179bp and 7180bp respectively. The filamentous phage VP24-4_Ke was a single stranded circular DNA and contained 11 functional genes. The presence of lysogenic genes: integrase and repressor, was an indication that the filamentous phage was not a candidate for phage therapy or water decontamination.

To investigate phylogenetic relationship between common filamentous phages, trees are generated based on core and accessory genomes. Major coat protein that determines the structure of virion coat found in all filamentous phages was used to assemble the tree and is also the most abundant protein hence making it a good gene for comparison. Closely related to the VP24-2_Ke phage was phage VEJphi as has been discussed earlier in this text and as shown in figure 4.40 representing the phylogenetic tree of the filamentous phage.

Phage therapy is a potential life savior during cholera outbreaks for underprivileged countries owing to the relative ease and speed of phage preparation using basic inexpensive laboratory equipment (Bhandare et al., 2019). To further circumvent antibiotic resistance, more complex and stable phage preparatory methods are being explored (e.g., lyophilization, spray drying, emulsification, and microencapsulation (Malik et al., 2017).

Cholera epidemics are known to be self-limiting in nature since the epidemics subside after reaching a peak, even without any active human intervention (Hoque et al., 2016). Among other factors, lytic phages that kill *Vibrio cholerae* have been shown to play a significant role in modulating the course of epidemics, presumably through their inherent bactericidal activity. Studies suggested that seasonal cholera epidemics may end as a result of phage predation of the causative epidemic *Vibrio cholerae* strains (Faruque et al., 2005a; 2005b; Nelson et al., 2009).

One of the pivotal phage features, which affects their therapeutic value, is their host spectrum. The four phages isolated here were infective against the 15 different tested environmental *Vibrio cholerae* strains, in addition to a clinical strain. However, the phages were not infective against bacteria representing three other species: *E. coli*, *Proteus mirabilis* and *Providencia sneebia* so they remain of limited spectrum. A cocktail, composed of three different phages isolated from surface waters in Bangladesh and designated as JSF7, JSF4, and JSF3, could significantly influence the distribution and concentration of the active planktonic form and biofilm-associated form of toxigenic *Vibrio cholerae* in water (Naser et al., 2017). Therefore, the four phages in this study are potential candidates to be added to cocktails for water decontamination and control of *Vibrio cholerae* in environmental waters, used by poor communities for domestic purposes in Kenya.

5.2: Conclusion

i. Isolation of pathogenic *Vibrio cholerae* O1 El Tor from various environmental water sources
ii. Whole genome sequencing of four novel vibriophages that could be primarily classified in the subfamily *Ackermannviridae*
iii. The full genome sequence of phage Vp4 (systematically named vB_vcM_Kuja) is currently available in the NCBI nucleotide database under the accession number MN718199.1

iv. The wide host range indicated that the isolated and characterized phages could be applied as biocontrol agents against clinical and environmental *Vibrio cholerae*

5.3: Recommendations

i. Further studies to determine the gene sequence of the hypothetical protein portion of each of the four genome sequences
ii. Further characterization of the other 11 phages will broaden the knowledge of the Kenyan environmental vibriophages data
iii. Develop technology for biomass production of the vibriophage dry product for application in water decontamination and phage therapy
iv. Further characterization of the four presented vibriophages and aim to test their control over other pathogenic *Vibrio cholerae*

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APPENDICES

Appendix 1. Nucleotide sequence statistics of the phage genomes

Table 20.1: Sequence information				
Information	**VP4_Ke**	**VP6_Ke**	**VP18_Ke**	**VP24_Ke**
Sequence type	DNA	DNA	DNA	DNA
Length	148,180bp	148,181bp	148,179bp	148,179bp
Organism	Unknown sp.	Unknown sp.	Unknown sp.	Unknown sp.
Name	VP4_Ke	VP6_Ke	VP18_Ke	VP24_Ke
Description	Unknown sp.	Unknown sp.	Unknown sp.	Unknown sp.
Modification Date				
Weight (single-stranded)	45.806 MDa	45.734 MDa	45.733 MDa	45.734 MDa
Weight (double-stranded)	91.54 MDa	91.54 MDa	91.539 MDa	91.539 MDa
Table 20.2: Melting temperatures - degrees Celsius				
[salt]	VP4_Ke	VP6_Ke	VP18_Ke	VP24_Ke
0.1M	79.81	79.81	79.81	79.81
0.2M	84.81	84.81	84.81	84.81
0.3M	87.73	87.73	87.73	87.73
0.4M	89.81	89.81	89.81	89.81
0.5M	91.41	91.42	91.41	91.42

Table 20.3: Counts of annotations

Feature type	VP4_Ke	VP6_Ke	VP18_Ke	VP24_Ke
CDS	186	185	186	185
Source	1	1	1	1
tRNA	4	4	4	4

Table 20.4: Counts of atoms

As single-stranded

Atoms	VP4_Ke	VP6_Ke	VP18_Ke	VP24_Ke
hydrogen (H)	1824688	1825922	1825899	1825898
carbon (C)	1455611	1454090	1454074	1454075
nitrogen (N)	548944	542221	542216	542221
oxygen (O)	887858	890310	890298	890299
phosphorus (P)	148180	148181	148179	148179

Table 20.5: As double-stranded

Atoms	VP4_Ke	VP6_Ke	VP18_Ke	VP24_Ke
hydrogen (H)	3650599	3650621	3650575	3650572
carbon (C)	2909695	2909712	2909676	2909673
nitrogen (N)	1091165	1091175	1091157	1091160
oxygen (O)	1778162	1778174	1778150	1778150
phosphorus (P)	296360	296362	296358	296358

Table 20.4: Frequencies of atoms				
As single-stranded				
Atoms	**VP4_Ke**	**VP6_Ke**	**VP18_Ke**	**VP24_Ke**
hydrogen (H)	0.375	0.376	0.376	0.376
carbon (C)	0.299	0.299	0.299	0.299
nitrogen (N)	0.113	0.112	0.112	0.112
oxygen (O)	0.182	0.183	0.183	0.183
phosphorus (P)	0.03	0.03	0.03	0.03
Table 20.5: As double-stranded				
Atoms	**VP4_Ke**	**VP6_Ke**	**VP18_Ke**	**VP24_Ke**
hydrogen (H)	0.375	0.375	0.375	0.375
carbon (C)	0.299	0.299	0.299	0.299
nitrogen (N)	0.112	0.112	0.112	0.112
oxygen (O)	0.183	0.183	0.183	0.183
phosphorus (P)	0.03	0.03	0.03	0.03
Table 20.6: Counts of nucleotides				
Nucleotide	**VP4_Ke**	**VP6_Ke**	**VP18_Ke**	**VP24_Ke**
Adenine (A)	47749	46525	46526	46524
Cytosine (C)	26189	27720	27716	27715
Guanine (G)	27716	26188	26188	26192
Thymine (T)	46526	47748	47749	47748
C + G	53905	53908	53904	53907
A + T	94275	94273	94275	94272

Table 20.7: Frequencies of nucleotides

Nucleotide	VP4_Ke	VP6_Ke	VP18_Ke	VP24_Ke
Adenine (A)	0.322	0.314	0.314	0.314
Cytosine (C)	0.177	0.187	0.187	0.187
Guanine (G)	0.187	0.177	0.177	0.177
Thymine (T)	0.314	0.322	0.322	0.322
C + G	0.364	0.364	0.364	0.364
A + T	0.636	0.636	0.636	0.636

Table 20.8: Percentage Nucleotide frequencies

Nucleotide	Percentage: VP4_Ke	Percentage: VP6_Ke	Percentage: VP18_Ke	Percentage: VP24_Ke
A	32.22365	31.39741	31.39851	31.39716
C	17.67378	18.70685	18.7044	18.70373
G	18.70428	17.67298	17.67322	17.67592
T	31.3983	32.22275	32.22386	32.22319

Codon statistics from coding regions

Table 20.9: Number of codons

Codon	VP4_Ke	VP6_Ke	VP18_Ke	VP24_Ke
AAA	2501	2499	2500	2499
AAC	1220	1218	1217	1218
AAG	863	862	862	862
AAT	1653	1652	1653	1653
ACA	1069	1066	1068	1068
ACC	453	453	453	453

ACG	231	231	231	231
ACT	912	911	911	912
AGA	694	695	694	693
AGC	208	207	207	207
AGG	198	197	197	197
AGT	725	723	723	724
ATA	752	752	751	751
ATC	686	685	685	685
ATG	1204	1202	1202	1203
ATT	1802	1802	1802	1802
CAA	1288	1285	1285	1285
CAC	274	273	274	275
CAG	370	368	369	368
CAT	589	589	589	589
CCA	722	722	722	722
CCC	137	137	137	137
CCG	104	104	104	104
CCT	620	620	620	620
CGA	130	130	130	130
CGC	360	360	360	360
CGG	48	48	48	48
CGT	671	669	669	669
CTA	715	715	714	715
CTC	184	182	182	182

CTG	237	236	236	236
CTT	952	950	950	950
GAA	2622	2623	2623	2623
GAC	695	695	695	695
GAG	741	741	741	741
GAT	2108	2108	2108	2108
GCA	897	897	897	897
GCC	291	291	291	291
GCG	361	361	361	361
GCT	1065	1064	1065	1064
GGA	352	352	352	352
GGC	263	263	263	263
GGG	224	223	223	223
GGT	1900	1898	1899	1899
GTA	791	791	791	791
GTC	260	260	260	259
GTG	420	419	419	419
GTT	1853	1854	1853	1854
TAA	0	0	0	0
TAC	591	589	591	590
TAG	0	0	0	0
TAT	1258	1256	1258	1256
TCA	1022	1022	1021	1022
TCC	131	131	131	131

TCG	110	109	109	109
TCT	769	769	769	769
TGA	0	0	0	0
TGC	108	108	108	108
TGG	537	537	537	537
TGT	505	505	505	506
TTA	1088	1085	1086	1085
TTC	701	700	700	700
TTG	676	674	674	674
TTT	1472	1467	1468	1468

Table 20.10: Frequency of codons

Codon	VP4_Ke	VP6_Ke	VP18_Ke	VP24_Ke
AAA	0.05	0.05	0.05	0.05
AAC	0.03	0.03	0.03	0.03
AAG	0.02	0.02	0.02	0.02
AAT	0.04	0.04	0.04	0.04
ACA	0.02	0.02	0.02	0.02
ACC	0.01	0.01	0.01	0.01
ACG	0	0	0	0
ACT	0.02	0.02	0.02	0.02
AGA	0.01	0.01	0.01	0.01
AGC	0	0	0	0
AGG	0	0	0	0
AGT	0.02	0.02	0.02	0.02

ATA	0.02	0.02	0.02	0.02
ATC	0.01	0.01	0.01	0.01
ATG	0.03	0.03	0.03	0.03
ATT	0.04	0.04	0.04	0.04
CAA	0.03	0.03	0.03	0.03
CAC	0.01	0.01	0.01	0.01
CAG	0.01	0.01	0.01	0.01
CAT	0.01	0.01	0.01	0.01
CCA	0.02	0.02	0.02	0.02
CCC	0	0	0	0
CCG	0	0	0	0
CCT	0.01	0.01	0.01	0.01
CGA	0	0	0	0
CGC	0.01	0.01	0.01	0.01
CGG	0	0	0	0
CGT	0.01	0.01	0.01	0.01
CTA	0.02	0.02	0.02	0.02
CTC	0	0	0	0
CTG	0.01	0.01	0.01	0.01
CTT	0.02	0.02	0.02	0.02
GAA	0.06	0.06	0.06	0.06
GAC	0.01	0.01	0.01	0.01
GAG	0.02	0.02	0.02	0.02
GAT	0.05	0.05	0.05	0.05

GCA	0.02	0.02	0.02	0.02
GCC	0.01	0.01	0.01	0.01
GCG	0.01	0.01	0.01	0.01
GCT	0.02	0.02	0.02	0.02
GGA	0.01	0.01	0.01	0.01
GGC	0.01	0.01	0.01	0.01
GGG	0	0	0	0
GGT	0.04	0.04	0.04	0.04
GTA	0.02	0.02	0.02	0.02
GTC	0.01	0.01	0.01	0.01
GTG	0.01	0.01	0.01	0.01
GTT	0.04	0.04	0.04	0.04
TAA	0	0	0	0
TAC	0.01	0.01	0.01	0.01
TAG	0	0	0	0
TAT	0.03	0.03	0.03	0.03
TCA	0.02	0.02	0.02	0.02
TCC	0	0	0	0
TCG	0	0	0	0
TCT	0.02	0.02	0.02	0.02
TGA	0	0	0	0
TGC	0	0	0	0
TGG	0.01	0.01	0.01	0.01
TGT	0.01	0.01	0.01	0.01

TTA	0.02	0.02	0.02	0.02
TTC	0.02	0.02	0.02	0.02
TTG	0.01	0.01	0.01	0.01
TTT	0.03	0.03	0.03	0.03

Table 20.11: Nucleotide count in codon positions

Nucleotide per position	VP4_Ke	VP6_Ke	VP18_Ke	VP24_Ke
1. pos. A	15171	15155	15156	15158
1.pos. C	7401	7388	7389	7390
1.pos. G	14843	14840	14841	14840
1.pos. T	8968	8952	8957	8955
2.pos. A	16773	16758	16765	16762
2.pos. C	8894	8888	8890	8891
2.pos. G	6923	6915	6915	6916
2.pos. T	13793	13774	13773	13774
3.pos. A	14643	14634	14634	14633
3.pos. C	6562	6552	6554	6554
3.pos. G	6324	6312	6313	6313
3.pos. T	18854	18837	18842	18843

Table 20.12: Nucleotide frequency in codon positions

Nucleotide per position	VP4_Ke	VP6_Ke	VP18_Ke	VP24_Ke
1.pos. A	0.33	0.33	0.33	0.33
1.pos. C	0.16	0.16	0.16	0.16

1.pos. G	0.32	0.32	0.32	0.32
1.pos. T	0.19	0.19	0.19	0.19
2.pos. A	0.36	0.36	0.36	0.36
2.pos. C	0.19	0.19	0.19	0.19
2.pos. G	0.15	0.15	0.15	0.15
2.pos. T	0.3	0.3	0.3	0.3
3.pos. A	0.32	0.32	0.32	0.32
3.pos. C	0.14	0.14	0.14	0.14
3.pos. G	0.14	0.14	0.14	0.14
3.pos. T	0.41	0.41	0.41	0.41

Appendix 2. Partial sequences of bacteria isolated from environmental waters of Kenya

2.1. ***Escherichia coli.*** ***(***EC_ke) 16S ribosomal RNA gene, partial sequence (From Nairobi River, Nairobi, Kenya). 954 bp DNA linear

GTGTCCGCTTGGCCTTGTCTACTCTTAAAGCTTGCTGCTTTGCTGACGAG
TGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATA
ACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAG
GGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCT
AGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGA
GAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG
GAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCC
ATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGG
GGAGGAAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGA
AGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTG
CAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGT
TAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATAC
TGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGT
GAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTG
GACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCC
CTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGA
GTACGGCCGCAAGGTTATAACTCAAATGAATTGACGGGGGCCCGCCCAA
GCGGGGGAACATGTGGTTTATTCTATGCAACCCGATAACCTTACCTGTGC
TTGAACTCTCGGAAGTTT

2.2. ***Vibrio cholerae*** **(**Vc_ke) 16S ribosomal RNA gene, partial sequence (From River Kuja, Migori, Kenya). 1004 bp DNA linear

GGAGTAGCTACCTGCAAGTCGAGCGGCAGCACAGAGGAACTTGTTCCTT
GGGTGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGAAATTGCCCGGT
AGAGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAACCTCGCA
AGAGCAAAGCAGGGGACCTTCGGGCCTTGCGCTACCGGATATGCCCAG
GTGGGATTAGCTAGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATCCC
TAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAA

GCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAA
GTACTTTCAGTAGGGAGGAAGGTGGTTAAGCTAATACCTTAATCATTTG
ACGTTACCTACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGG
TAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCA
TGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCTGGGCTCAACCTAGG
AATCGCATTTGAAACTGACAAGCTAGAGTACTGTAGAGGGGGGTAGAAT
TTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGC
GAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGG
GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTC
TACTTGGAGGTTGTGCCCTAGAGGTGTGGCTTTCGGAGCTAACGCGTTA
AGTAGACCGCCTGGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAAA
TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTAATTCGATGCAA
CGCGAAGACCTTACCTACTCTTGACTCCAGAGATCTAGCGGAGACGTTG
GAGTGCTTCGGGAGCTCTGAAACAGGGG

2.3. ***Proteus mirabilis (***PREM_ke***)*** 16S ribosomal RNA gene, partial sequence (From River Kuja, Migori, Kenya). 986 bp DNA linear

GACGGATGCTACCTGCAGTCGAGCGGTAACAGGAGAAAGCTTGCTTTCT
TGCTGACGAGCGGCGGACGGGTGAGTAATGTATGGGGATCTGCCCGAT
AGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCATAATGTCTAC
GGACCAAAGCAGGGGCTCTTCGGACCTTGCACTATCGGATGAACCCATA
TGGGATTAGCTAGTAGGTGGGGTAAAGGCTCACCTAGGCGACGATCTCT
AGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCA
GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAG
CCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTAGGGTTGTAAAG
TACTTTCAGCGGGGAGGAAGGTGATAAGGTTAATACCCTTGTCAATTGA
CGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGT
AATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCAC
GCAGGCGGTCAATTAAGTCAGATGTGAAAGCCCCGAGCTTAACTTGGGA
ATTGCATCTGAAACTGGTTGGCTAGAGTCTTGTAGAGGGGGGTAGAATT
CCATGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGC
GAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGG
GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTC
GATTTAGAGGTTGTGGTCTTGAACCGTGGCTTCTGGAGCTAACGCGTTA

AATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATT
GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGGTTAATTCGATGCAA
CGCGAAGAACCTTACCTACTCTTGACATCCAGCGAATCCTTTAAAAGATA
GAGGAGTGCC

2.4. ***Providencia sneebia (***PROS_ke***)*** 16S ribosomal RNA gene, partial sequence (From River Kuja, Migori, Kenya). 986 bp DNA linear

GCGAGGCTACCTGCAGTCGGGCGGTAACAGGGAGAAGCTTGCTTCTCCG
CTGACGAGCGGCGGACGGGTGAGTAATGTATGGGGATCTGCCTGATGGC
GGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAATGTCTTCGGAC
CAAAGCGGGGGACCTCCGGGCCTCGCGCCATCAGATGAACCCATATGGG
ATTAGCTAGTAGGTGAGGTAACGGCTTACCTAGGCGACGATCCCTAGCTG
GTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGAT
GCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTC
AGTCGGGAGGAAGGTGTCAAGGTTAATAACCTTGTCAATTGACGTTACCG
ACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA
GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGG
TTGATTGAGTCAGATGTGAAATCCCCGGGCTTAACCCGGGAATTGCATCT
GATACTGGTCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCTGGTGTA
GCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGGCGAAGGCGGC
CCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACA
GGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGCGGACTTTGGAG
GTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCG
CCTGGGGGAGTACGGCCGCAAGGTAAAACTCAAATGAAATTGACGGGGG
CCCGCACAAGCGGTGGGAGCATGTGGTTTAATTCGATGCAACGCGAAGA
ACCTTACCTACTCTTGACTCCAGAAAACTTAGCAGAAGATGCTTGGGGGC

2.5. *Vibrio cholerae* (Vc_Koleche) 16S ribosomal RNA gene, partial sequence (From Koleche pond in Siaya) 1211bp DNA linear

ATGCCTTGCGGCAGCTACACATGCAGTCGAGCGGCAGCACAGAGGAACT
TGTTCCTTGGGTGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGAAATT
GCCCGGTAGAGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAA
CCTCGCAAGAGCAAAGCAGGGGACCTTCGGGCCTTGCGCTACCGGATAT

GCCCAGGTGGGATTAGCTAGTTGGTGAGGTAAGGGCTCACCAAGGCGAC
GATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACA
CGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG
CGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTT
GTAAAGTACTTTCAGTAGGGAGGAAGGTGGTTAAGCTAATACCTTAATCA
TTTGACGTTACCTACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCG
CGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGC
GCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCTGGGCTCAACCTA
GGAATCGCATTTGAAACTGACAAGCTAGAGTACTGTAGAGGGGGGTAGA
ATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTG
GCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTG
GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGT
CTACTTGGAGGTTGTGCCCTAGAGGTGTGGCTTTCGGAGCTAACGCGTTA
AGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATT
GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAAC
GCGAAGAACCTTACCTACTCTTGACATCCAGAGAATCTAGCGGAGACGCT
GGAGTGCCTTTCGGGAGCTCTGAGACAGGTGCTGCATGGCTGTCGTCAG
CTCGTGTTGTGAAATGTTGGGTAGTCCCGCACGAGCGCAAGCCTTATCTT
GTTTGCAGCACGTATTGTGGGACTCAGGATACTGCGTGATAATCGGAGAA
GCTGGGACGACGTCCAGTCATCATGACCTTACAGGTAGTCTACACGTGCT
ACATCCGTTATACAAGGGTCCGATCGT

2.6. *Vibrio cholerae* (Vc_Nsongoni) 16S ribosomal RNA gene, partial sequence (From river Nsongoni in Mombasa, Coast) 1226 bp DNA linear

GGCATGCGCAGCTACACATGCAGTCGAGCGGCAGCACAGAGGAACTT
GTTCCTTGGGTGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGAAAT
TGCCCGGTAGAGGGGGATAACCATTGGAAACGATGGCTAATACCGCA
TAACCTCGCAAGAGCAAAGCAGGGGACCTTCGGGCCTTGCGCTACCG
GATATGCCCAGGTGGGATTAGCTAGTTGGTGAGGTAAGGGCTCACCA
AGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGA
ACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT
TGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGA

AGGCCTTCGGGTTGTAAAGTACTTTCAGTAGGGAGGAAGGTGGTTAA
GCTAATACCTTAATCATTTGACGTTACCTACAGAAGAAGCACCGGCTA
ACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATC
GGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGAT
GTGAAAGCCCTGGGCTCAACCTAGGAATCGCATTTGAAACTGACAAG
CTAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAAT
GCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGAC
AGATACTGACACTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGC
CCTAGAGGTGTGGCTTTCGGAGCTAACGCGTTAAGTAGACCGCCTGG
GGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCG
CACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACC
TTACCTACTCTTGACATCCAGAGAATCTAGCGGAGACGCTGGGAGTGC
CTTCGGGAGCTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTG
TTGGTGAAATGGTTGGGTTAAGTCCCGCACGAGCGCACCCTTATCCTT
GTTTGCAAGCACGTATGGTGGGTACTCCAGGCAGACTGCGTGATAAC
CGGAGGAAGTGGGACGACGTCCAGTCATCATGGACTTACGAGTAGGC
TTAACACGTGCTACATTGGCCGTTATACAGAGGGCAGCCGAATTACG

2.7. *Escherichia coli* (Ec_Kuja) 16S ribosomal gene partial sequence (from river Kuja, Migori). 1055bp DNA linear

GTAGAGCGCGGCCTACCATGCAGTCGAACGGTAACAGGAAGCAGCTTGC
TGCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCC
TGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGT
CGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCC
CAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGAT
CCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGG
TCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGC
AAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTA
AAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATACCTTTGCTCATT
GACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCG

GTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC
ACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGG
GAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAA
TTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGG
CGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGG
GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTC
GACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTA
AGTCGACCGCCTGGGGAGTACGGCCGCAAGGTAAAACTCAAATGAATTG
ACGGGGGCCGCACAGCGGGGAGCTGTGGTTATTCGATGCACGCGAAAAC
CTACCTGGTCTGACTTCACGGAAGTTTCCGAGATGAAATGGGCCTTCGGA
ACCGGGAAACGGGGCTGCTTGGCTGCGCAACTCCGGTTGGAAATGTTGG
GTTAATCCCGCAAAGAGGGAACCC

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