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# NEWLY ISOLATED *VIBRIO CHOLERAE* BACTERIOPHAGES FROM ENVIRONMENTAL WATERS OF KENYA

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***Abstract:*** **Viruses that infect bacteria are known as bacteriophages and can be used as biocontrol agents to complement antibiotics. The aim of the study was to isolate *Vibrio cholerae* lytic bacteriophages from environmental waters of different regions in Kenya that included: Lake Victoria, Coast, Nairobi and Central. A total of 140 environmental water samples were collected from ponds, rivers, lake, beaches, springs, boreholes, wells and Indian Ocean. Pathogenic *Vibrio cholerae* strains isolated from some these sources were used as respective propagating strains for isolation of vibriophages. Identification of the *Vibrio cholerae* bacterial strains by Polymerase Chain reaction was through amplification and sequencing of partial 16S ribosomal RNA gene. In total, 15 *Vibrio cholerae* bacteriophages were isolated; nine from rivers, three from beaches and three from ponds using the double layer method of purification. Lytic spectrum confirmed that all the 15 bacteriophages were infective to both environmental and clinical *Vibrio cholerae* isolates. Further characterization by Transmission Electron Microscope assigned the vibriophages to order *Caudovirales* of *Myoviridae* family owing to their icosahedral capsid and contractile tails. The average tail length, head diameter were 90nm and 79nm respectively. The current study has proved that vibriophages have established a niche in Kenyan environmental waters. The bacteriophages may have the potential for biocontrol of *Vibrio cholerae* bacterium.**

***Keywords: Vibrio cholerae*, vibriophages, cholera, biocontrol, *Myoviridae*.**

## I. INTRODUCTION

Kenyan republic has suffered from cholera outbreaks for more than a decade due to heavy rains, poor sanitation and lack of access to clean, potable drinking water especially the low-income poor communities. Since December 2014, a cumulative total of 10,568 cases were reported in 2015 and 6,448 in 2016 [1]. In 2017, Kenya experienced a surge in cholera cases across the country, including urban outbreaks in the capital city, Nairobi. A total of 3,967 cases including 76 deaths were reported across 20 of 47 counties (43%) in the country. By the end of the year, seven counties continued to have active cholera outbreaks. There have also been cholera outbreaks in refugee camps in Kenya like Dadaab, Kakuma and Kalobeyei camps. In 2018, March to May long rains in Kenya were the heaviest in the past 55 years. A total of 5,756 cholera cases and 78 deaths were reported in 2018 [1]. Out of 47 counties, 20 were affected including: Mombasa, Kirinyaga, Garissa, Siaya, Tharaka Nithi, Meru, Tana River, and Turkana. There has been occurrence and persistence of *V. cholerae* in various aquatic systems like lakes and rivers. *V.cholerae* is discharged directly into the environment during an outbreak through the human excretions and waste water effluent from municipal councils [2]. Considering that cholera

is a waterborne disease, aquatic systems act as a sink of bacterium as well as the source [2]. Since the bacterium persists and proliferates in these environmental waters through consumption of contaminated food and water, the bacterium gets into the body of the human beings. In this regard, drinking water is the ecotone between aquatic systems and the human body [3] and this forms the linkage between the human and environment that plays a major role in sustaining and promoting the persistence of cholera outbreaks [2]. Bacteriophages have a huge influence on the environment because they play a vital role in maintaining microbial balance [4]. Antibiotic resistance from clinical and environmental origin in strains of the genus Vibrios has been reported. Therefore, coupled with the occurrence of multidrug resistance strains of *V.cholerae* in the environment, there has been a renewed search for an alternative source of treatment such as the lytic bacteriophages [6].

## II. MATERIALS AND METHODS

### *2.1 Propagating Vibrio cholerae strain*

For isolation of bacteriophages, *Vibrio cholerae* strains isolated were used for propagation. Water samples were collected from several sources that included: wells, rivers, boreholes, beaches, springs, ponds, Lake Victoria and Indian Ocean. The regions where the samples were collected were: Lake Victoria, Nairobi, Central, and Coast. The strains were isolated from respective sources of environmental waters that included; five strains of *V. cholerae* were isolated from from river Kuja in Migori, three strains from each of the ponds (Koleche, Owira and Kitonde) and three strains from each of the beaches (Usenge, Leuda and Osieko) in Siaya. From the coastal region two *V. cholerae* strains were isolated from each of the two rivers: Nsongoni and Kizulini in Mombasa. Two more strains were isolated from each of the rivers; Kamiti and Riverside for respective phage propagation. Alkaline peptone water (APW) (HiMedia, Mumbai, India) broth was used for enrichment of *V. cholerae* isolated from the different environmental water sources. For screening and identification, a selective media, Thiosulfate Citrate Bile Salt Sucrose (TCBS) media was used (HiMedia, Mumbai, India). Identification of the bacterial strains was by Polymerase Chain reaction (PCR) amplification of 16S rRNA partial gene with universal primers as described by [7]. 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.

### *2.2 Enrichment of the environmental water samples for bacteriophages isolation.*

Enrichment procedures for phage isolation were performed as described by Kroprinski [8], with slight modifications. This was done by adding 10ml of *V. cholerae* overnight culture (12hour) grown in single strength Tryptose Soy Broth (TSB) (Oxoid, Basingstoke, Hampshire, England) sample into 10ml of the water sample in a 250ml Erlenmeyer flask. This was mixed with 20ml double strength TSB that had been supplemented with 2mM $CaCl_2$. The reaction mixture was incubated at 37°C for 48h in a water bath shaker at 100 revolutions per minute (RPM). After 48h, approximately 30ml of the reaction mixture was transferred onto a 50ml falcon tube, centrifuged at 3400 × g for 15 minutes. The supernatant was filtered through a sterile syringe mounted 0.45µm pore sized filter to remove any contamination by bacterial cells onto small screw capped bottles that were labeled “crude lysate”. This was suspected to contain bacteriophages lytic to *V. cholerae* and was stored at 4°C for further testing.

### *2.3 Assays and purification of bacteriophages*

Purification of bacteriophages was done according to method described by Kroprinski [8]. A spot assay was carried out by spotting an aliquot of 10µl of the crude lysate in triplicates on a lawn of *V. cholerae* that had been isolated from the respective regional environmental water samples used for enrichment. After incubation at 37℃ for 12h the bacterial lawns were inspected for a plaque, that is a clear zone on the lawn of the host bacterium caused by successive infection and lytic phage burst cycles. A clear zone indicated presence of lytic bacteriophages in the environmental water samples. The clear zones were later suspended in SM buffer and three rounds of purification carried out by plaque assay method for further characterisation. A control was set where sterile SM buffer instead of the crude lysate was spotted on the lawn of the bacterial host cells. If no clear zone was observed the sample was considered negative for bacteriophages against the host bacterium.

### *2.4 Host range profiles of the isolated vibriophages*

Host range examination was carried out as described by Kropinski [8]. Bacteriophages host range was determined based on the ability to form plaques on a lawn of all the 15 *V. cholerae* strains isolated from the different environmental waters,

a clinical strain and three other bacteria isolated from some of the environmental waters collected from the same regions. The clinical strain, previously isolated from a patient suffering from cholera infection, was kindly provided by the Department of Medical Microbiology, The University of Nairobi. Three other bacteria were; *Escherichia coli*, *Proteus mirabilis* and *Providencia sneebia*. Lawns were prepared from each bacterium by mixing 500µl (12hr culture) of the host strain with 4ml of soft agar then poured on the surface of the Tryptose Soy Agar (TSA) plates (Oxoid, Basingstoke, Hampshire, England). Spot assay was done where 10µl of the purified phage lysate was spotted on each of the lawns of the propagating host bacterial strain. After the spots were allowed to set, plates were incubated for 12h at 37℃, examined for zones of clearing where the phage lysate had been spotted [9]. If a clear zone was observed, the sample was declared positive for bacteriophage that is the bacteriophage was lytic against that bacterium and negative where no clear zone was observed. A control was set where instead of phage lysate; a sterile SM buffer was spotted on the lawn of the propagating bacterial strain.

### *2.5 Transmission Electron Microscopy*

Processing of samples for TEM was done at The Wellcome Sanger Institute operated by Genome Research Limited, UK. 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 [3]. 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.

## III. RESULTS AND DISCUSSION

### *Bacteriophage isolation*

Out of the 15 bacteriophages isolated from the Kenyan environmental water sources, nine were from different rivers, three from ponds and three from beaches. Phages: VP4, VP6, VP8, VP12, VP18, VP94, VP124, VP132 and VP140 were isolated from different rivers in three different regions which accounted for 60% of all the phages isolated. Phages VP42, VP56 and VP64 were isolated from three ponds namely; Kitonde, Koleche and Owira which translated to 20% while phages VP24, VP28 and VP68 were isolated from three beaches (Osieko, Lauda and Usenge) accounting 20% of the total phages. The results showed that highest number of phages was isolated from rivers. Five phages out of the 15 phages were isolated from river Kuja (55.6%), one phage from each of the rivers Kamiti, Nsongoni, Kizulini and Riverside. The total number of samples collected from river Kuja was 9, Kamiti I, Nsongoni 2, Kizulini 2 and I sample from Riverside River.

Since the host had also been isolated from the respective rivers, pond or beaches, the results proved the coexistence of phage with the host. This is because bacteriophages are commonly found in large numbers wherever their hosts live namely: sewage, soils, hatchery, thermal vents that are deep or in natural bodies of water [10]. Vibriophages can also be isolated from sewage or fresh waters [11].

In Kenya, *V. cholerae* O1 resistant to common antimicrobials have been isolated in fish and water from Lake Victoria [12]. Due to emergence of antibiotic-resistant bacterial strains there is an extensive search for an alternative treating method [13] and phage therapy is one of such potential alternatives that is gaining a lot of attention throughout the world.

### *Morphology of bacteriophages by TEM*

In order to confirm the morphology of the *Vibrio cholerae* bacteriophages, further characterization as required by Transmission Electron Microscopy was carried out [3]. In accordance to International Classification on Taxonomy of Viruses (ICTV), electron micrographs of newly isolated bacteriophages were obtained to determine their morphological features and for purposes of classification. The results of the classification, sources and description of the 15 phages were summarized in Table 1. From the table it was evident that out of the 15 newly isolated bacteriophages there were unique features displayed by the TEM images that revealed that these were truly different from others [14].

According to Ackermann 2007 [15] 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, all the 15 phages belonged to the *Myoviridae* family. Figure 1 showed plates 1-15 that displayed the TEM morphological characteristics of the 15 phages isolated in this study. The head diameter of the phages ranged between 85nm to 33nm. Five phages: VP4, VP6, VP12, VP28 and VP64 had a similar head diameter of 85nm which constituted 33%. Two phages: VP42 and VP124 had same head diameter of the same size that is 80nm. Two

phages: VP18 and VP94 equally had identical heads of 79nm. Phage VP68 had the smallest diameter of 33 nm while the remaining phages: VP56, VP140, VP24, VP8 and VP132 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 and VP28. The average head diameter of the 15 phages was 79nm.

Previous studies by Maina et al [16] reported isolation of 9 vibriophages from the Lake Victoria region that had dimensions 88.3nm and 84.9nm of head and tail respectively. The tail length of the 15 phages ranged between 78nm to 130nm with three phages (VP64, VP6 and VP140) having the same tail length of 100nm. Two phages (VP4 and VP28) also had the same tail length of 95nm. Phage VP68 had the longest tail length of 130nm which was very unique. Phage VP94 had the shortest tail length of 78nm while the other phages: VP12, VP132, VP18, VP24, VP56, VP8, VP42, and VP124 had tail length of diameter 101, 102, 103, 99, 98, 97, 96 and 85nm respectively. The average size of the tail length was 90nm.

A recent study [17] on a comparison between *Myoviridae* phages revealed that phage M4 that had been propagated on El Tor *V. cholerae* strain O1 MAK757, belonged to the family of *Myoviridae* had a head diameter of 85±3 nm, 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 *V. cholerae* O1. The head was icosahedral of diameter 86nm long [18]. Another phage that was isolated from the environmental waters of Bangladesh also propagated on *V. 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 [19]. 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 tail. The propagating strain was *V. 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±2.21 nm, tail length 193.5± 14.5nm [13].

According to Ackermann [15], 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 [13], [20], [21],[22], [23]. The only phage that did not fall under these dimensions was phage VP68 that had a head of 33nm but was within the Ackermann's dimensions of the head which is 30nm. The other 14 phages can be said to have relatively bigger heads and so they could be interesting subjects for further studies.

Phages with such long tails like VP68 are rare in the environment and therefore it can be interesting to further characterize such a phage [13]. All the 15 phages even though they appeared similar in the TEM images except for VP68, portray a diversity of *V. 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 [8] as depicted in Table 1.

***Host range determination***

The host range profiles as presented in Table 2 of the 15 vibriophages isolated showed that they were lytic to both environmental and clinical *Vibrio cholerae* isolates. The phages were not infective to *Escherichia coli*, *Proteus mirabilis* and *Providencia sneebia*. Phages VP64_Ke and VP68_Ke formed turbid zones on each of bacterial lawns of *Escherichia coli* 083 and *Providencia sneebia* however, this was not an indication of complete lysis for these two phages. The phages isolated in this study had therefore a narrow host range. Narrow host range vibriophages against *Vibrio cholerae* have been reported by Ali [22] with specificity to *Vibrio cholerae* either El Tor Inaba. Lytic phages have a narrow host range but this limitation can be overcome by use of cocktails. Sarkar *et al*, [24] 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.

## IV. CONCLUSION AND RECOMMENDATION

In conclusion, bacteriophages lytic to environmental *Vibrio cholerae* strains were isolated from environmental waters of different regions in Kenya. The current study showed that vibriophages have established a niche in Kenyan environmental waters. The host range profiles of the vibriophages isolated against three other gram-negative bacteria isolated in this

study gave an indication of narrow spectrum. The bacteriophages were however able to lyse a *V. cholerae* clinical isolate thus offering a potential application as biocontrol agents against the pathogenic *Vibrio cholerae.* Such application gives hope to the communities in cholera endemic areas in Kenya. Application of Phage will be environmentally safe and cheap. In the current study, even though both the host strain and the phage infectious to the respective host strains were isolated from sites several kilometers apart there were no major differences between both the host and the phages. It will therefore be interesting to have these phages further characterized to establish their comparative genomics.

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## APPENDIX-A

### TABLES AND FIGURES

**Table 1: Sources, Classification and description of the bacteriophages**

| | | | | | Phage measurements (nm) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **#** | **Name of the phage** | **Source of the phage** | **Code** | **Classification (Family)** | **Head diameter** | **Neck diameter** | **tail to bp** | **tail diameter** | **bp to end** |
| 1 | vB_Vch4M_Kuja | River Kuja | VP4 | *Myoviridae* | 85 | 16 | 95 | 16 | 13 |
| 2 | vB_Vch6M _Ke | River Kuja | VP6 | *Myoviridae* | 85 | 14 | 100 | 16 | 13 |
| 3 | vB_Vch8M _Ke | River Kuja | VP8 | *Myoviridae* | 82 | 14 | 97 | 14 | 14 |
| 4 | vB_Vch12M _Ke | River Kuja | VP12 | *Myoviridae* | 85 | 15 | 101 | 16 | 12 |
| 5 | vB_Vch18M _Ke | River Kuja | VP18 | *Myoviridae* | 79 | 15 | 103 | 16 | 12 |
| 6 | vB_Vch24M _Ke | Usenge beach | VP24 | *Myoviridae* | 78 | 13 | 99 | 16 | 12 |
| 7 | vB_Vch28M _Ke | Leuda beach | VP28 | *Myoviridae* | 85 | 15 | 95 | 17 | 14 |
| 8 | vB _Vch42M _Ke | Owira pond | VP42 | *Myoviridae* | 80 | 15 | 96 | 16 | 12 |
| 9 | vB _Vch56M _Ke | Koleche pond | VP56 | *Myoviridae* | 87 | 15 | 98 | 17 | 14 |
| 10 | vB _Vch64M _Ke | Kotonde pond | VP64 | *Myoviridae* | 85 | 15 | 100 | 15 | 14 |
| 11 | vB _Vch68M _Ke | Osieko beach | VP68 | *Myoviridae* | 33 | 8 | 130 | 21 | 47 |
| 12 | vB _Vch94M _Ke | Nsongoni river | VP94 | *Myoviridae* | 79 | 16 | 78 | 17 | 13 |
| 13 | vB_Vch124M _Ke | Kizulini river | VP124 | *Myoviridae* | 80 | 15 | 85 | 16 | 12 |
| 14 | vB_Vch132M _Ke | River Kamiti | VP132 | *Myoviridae* | 75 | 15 | 102 | 16 | 13 |
| 15 | vB _Vch140M _Ke | Riverside river | VP140 | *Myoviridae* | 86 | 14 | 100 | 17 | 13 |

***KEY:*** *bp-base plate*

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**Table 2: Host Range Profiles of the *V. cholerae* Bacteriophages**

| | Bacteriophages | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Host strain | VP4 | VP6 | VP8 | VP12 | VP18 | VP24 | VP 28 | VP42 | VP 56 | VP64 | VP 68 | VP94 | VP124 | 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/complete, + opaque lysis, - no lysis*

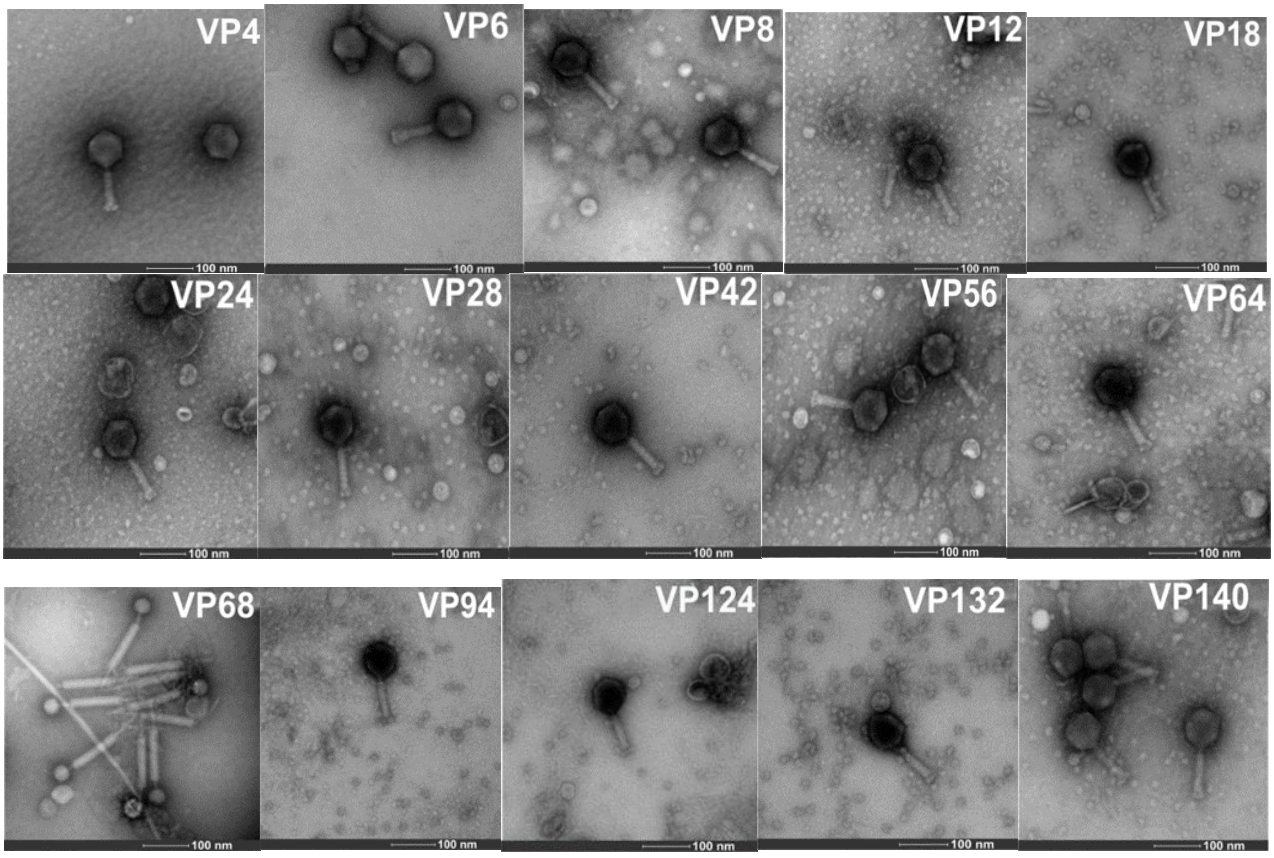


**Fig 1**: **Transmission electron micrographs of 15 newly vibriophages isolated from Kenyan environmental waters**