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RESEARCH PAPER

Journal of Basic Microbiology

Phenotypic characterization of phage vB_vcM_Kuja

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Abstract

Bacteriophage therapy targeting the increasingly resistant *Vibrio cholerae* is highly needed. Hence, studying the phenotypic behavior of potential phages under different conditions is a prerequisite to delivering the phage in an active infective form. The objective of this study was to characterize phage VP4 (vB_vcM_Kuja), an environmental vibriophage isolated from River Kuja in Migori County, Kenya in 2015. The phenotypic characteristics of the phage were determined using a one-step growth curve, restriction digestion profile, pH, and temperature stability tests. The results revealed that the phage is stable through a wide range of temperatures (20−50°C) and maintains its plaque-forming ability at pH ranging from 6 to 12. The one-step growth curve showed a latent period falling between 40 and 60 min, while burst size ranged from 23 to 30 plaque-forming units/10 μl at the same host strain. The restriction digestion pattern using *EcoRI*, *SalI*, *HindIII*, and *XhoI* enzymes showed that *HindIII* could cut the phage genome. The phage DNA could not be restricted by the other three enzymes. The findings of this study can be used in future studies to determine phage−host interactions.

KEYWORDS
endonucleases, plaque forming units, restriction digestion, *Vibrio cholera*

1 | INTRODUCTION

Cholera is a diarrheal disease that has afflicted mankind across ages and generations, especially in developing countries. It is mainly caused by toxigenic O1 and O139 *Vibrio cholerae* serotypes. The disease is spread through faecal-oral route; hence, it is strongly associated with poverty, poor hygiene, clean water shortage, and lack of adequate sanitation [1].

V. cholerae is known to exist and persist in various aquatic and maritime systems, such as lakes and rivers. Being a waterborne pathogen that causes the infectious diarrheal disease cholera, *V. cholerae* must persist in aquatic reservoirs and flourish in the human gut, in either of which it is attacked by predatory phages [2]. The bacterium is released into the environment directly during an outbreak via human excreta and wastewater discharge from municipal sources. Aquatic and maritime establishments provide both a sink and a source for the bacterium [3]: the pathogen persists and proliferates in these environmental waters and infects humans through the intake of contaminated water and food. Water used for human consumption becomes a transitional zone between humans and maritime systems [4], forming a

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linkage between humans and the environment that plays a major role in sustaining and promoting the persistence of cholera outbreaks.

Bacteriophages, also known as phages, are precise bacterial obligate parasites which do not affect other organisms [5]. They are bountiful, diverse, and coexist with the host bacteria influencing their evolution [6]. Even under adverse conditions, bacteriophages can retain their ability to infect bacteria [7]. Bacteriophages have an enormous influence on the environment largely by maintaining microbial balance [8]. In aquatic reservoirs, evolution of resistance to phage, as well as counter-resistance, gives rise to cyclic patterns of increasing *V. cholerae* populations, followed by phage amplification and coincident bacterial decline, leading to phage decline and bacterial blooms once again [2]. Besides being distinctively host-specific by targeting only specific species or even strains [9], bacteriophages at high specificity levels could minimize the interference of commensal bacteria [10]. Use of phages to identify types of *V. cholerae* or address the clonality of cholera outbreaks has fostered a long-established interest in cholera-associated phages.

Temperature and pH are important factors that influence the survival of bacteriophages during infection of host bacteria or administration in humans and animals. Phages can survive at extreme thermal habitats, at temperatures ranging between 40°C and 90°C. During phage propagation, temperature affects viability, multiplication, storage, attachment, and penetration [7].

Burst size of a phage is the number of progeny (phages) released by a single infected host bacterial cell. Bacteriophages with larger burst sizes are more desirable for the treatment of infections because they may eradicate bacteria faster [11]. Restriction digestion of phage DNA has been used for restriction mapping of phage genomes and to differentiate between bacteriophages [12]. Besides the application of bacteriophages in clinical trials to fight against bacterial infections, they can also be used in livestock protection to lower the mortality rate of animals and intensify food safety levels by controlling the growth of foodborne pathogens [13].

Genomic characterization of four environmental bacteriophages from Kenyan waters revealed that VP4 (vB_vcM_Kuja) is a PhoH-encoding vibriophage that has potential biocontrol properties for water decontamination against pathogenic *V. cholerae* [14]. The study also described the host range, genome size, G + C content of the four phages, and their morphology as displayed by transmission electron microscopy.

Bacteriophages success as a complement for antibiotics depends on their stability in different environmental conditions. It is against this background that this study investigated the restriction digestion profile, pH, and temperature stability of the environmental bacteriophage, VP4 (vB_vcM_Kuja), isolated from River Kuja in Migori County, Kenya. In addition, a one-step growth analysis of the phage was performed.

2 | MATERIALS AND METHODS

2.1 | Growth kinetics of *V. cholerae* strain

Growth kinetics was determined as previously described [15], except for using trypticase soy agar (TSA) as the culture media of choice. The growth kinetics involved the establishment of *V. cholerae* growth curve with lag, exponential, and death phases. A single colony of the host strain was picked, subcultured on thiosulfate-citrate-bile salts-sucrose (TCBS) agar and incubated for 12 h at 37°C. From the TCBS, a single yellow colony of *V. cholerae* was transferred to 10 ml trypticase soy broth (TSB). This was considered the zero time and 10 μl was immediately transferred to a 96-well plate containing 90 μl TSB to make the first serial dilution before transferring the host strain culture to a shaker water bath (Bibby Sterilin Ltd) at 100 rpm. After every 10 min, aliquots of 10 μl of the host strain were removed from the 10 ml culture, serially diluted, plated on TSA plates, and incubated for 12 h at 37°C. This was done for 8 h. Colonies were counted and only plates that contained 3−30 colonies were considered.

2.2 | Host strain and isolation of phage vB_vcM_Kuja

Steps of phage isolation and purification by the double agar layer method have been previously described [16]. Trypticase soy agar was the molten soft top layer (0.6% agar) media of choice as an alternative for Luria−Bertani agar. Vibriophage vB_vcM_Kuja was isolated as previously described [14]. Environmental toxigenic El Tor strain of *V. cholerae*, Vc_ke (MN467399.1), isolated from river Kuja, Migori County in western Kenya in 2015, was used for propagation and isolation of bacteriophage vB_vcM_Kuja.

2.3 | One-step growth curve of phage vB_vcM_Kuja

For determination of the burst size and latent period, a one-step growth curve was used as previously described,

except for the use of TSB instead of Luria−Bertani agar [17]. A colony from an overnight culture of *V. cholerae* was infused in 100 ml TSB and incubated for approximately 3 h at 37°C at 200 rpm. After 3 h, the culture was mixed with 1 ml of phage suspension and incubated at 37°C for 15 min to enable the adsorption of phages to the propagating bacterial strain. The suspension was thereafter centrifuged for 5 min at 14000*g*, the supernatant was removed and the pellet was resuspended in 15 ml TSB broth media and incubated at 37°C at 200 rpm. Approximately 100 μl aliquots of the sample were withdrawn every 15 min for a period of 3 h and the titer of the bacteriophage determined by the double agar overlay plaque method. The latent period and burst size of the bacteriophage were evaluated from the curve.

2.4 | Thermal stability of phage vB_vcM_Kuja

The thermal stability of vibriophage vB_vcM_Kuja was investigated as previously described [18], with slight modifications. The initial phage stock titer was determined 1 day ahead of the experiment. Bacteriophage suspensions were then dispensed into 1.5 ml Eppendorf tubes and exposed to six different temperature regimes 20°C, 30°C, 40°C, 50°C, 60°C and 70°C for 2 h, and a control was set at 37°C. After incubation, the surviving phages were titrated by the double-layer method as previously described [19].

2.5 | pH stability of phage vB_vcM_Kuja

pH stability of the phage was carried out as previously described [18], with slight modifications. The pH of TSB media was adjusted with either 1 M HCl or 1 M NaOH to obtain pH ranges of 2−12. Phage suspensions of 1 ml were added onto 9 ml TSB having pH levels of 2, 4, 6, 8, 10, 12, and incubated overnight (12 h) at 37°C. After 12 h, the phage titer was determined by the double-layer method, on a lawn of *V. cholerae* host propagating strain [19].

2.6 | Phage DNA restriction of vB_vcM_Kuja and three other phage genomes

Four restriction endonucleases were used to digest the phage genomic DNA for comparison of the DNA fragment patterns. The three other phage genomes were VP6 and VP18 isolated in 2015 from river Kuja in Migori county while VP24 had been isolated from Usenge beach in Siaya

county, Kenya in 2016 [14]. Restriction enzymes (Promega) used were: *HindIII*, *EcoRI*, *SalI*, and *XhoI*. The following components were mixed at room temperature (23 ± 2°C): 30 μl nuclease-free water, 5 μl of 10x fast digest buffer, 10 μl DNA, and 5 μl fast digest enzyme. The contents were thoroughly mixed and kept at 37°C for 10 min, after which the restriction fragments were separated by gel electrophoresis (1% agarose) as previously described [20] and visualized by an Agilent Bioanalyzer 2100 system (Agilent Technologies). Before phage restriction digestion, phage DNA had been extracted by a standard phenol-chloroform protocol [21]. The accession number for bacteriophage VP4 is vB_vcM_Kuja is MN718199 while that for *V. cholerae* Vc_ke is MN467399.1 [14].

3 | RESULTS

3.1 | Growth kinetics of the *V. cholerae* strain

A growth curve was generated for *V. cholerae* (MN467399.1), the host strain used for the isolation of the phage (Figure 1). The curve showed a lag phase between 0 and 40, while the mid-exponential time was between 140 and 160 min. The stationary phase was between 260 and 440 min.

3.2 | One-step growth curve of phage vB_vcM_Kuja

From the one-step growth curve of phageVP4 (Figure 2), the latent period, defined as the time between infection and subsequent release of phage virions [22], ranged from 40 to 60 min, while the burst size was in the range of 23−30 PFU/10 μl.

3.3 | pH stability of phage vB_vcM_Kuja

According to pH stability analysis (Figure 3), VP4 phage was most stable at a pH 10 and relatively stable between pH 6 and 8. The highest phage titer (10^7 PFUs/ml) was recorded at a pH of 10. However, no plaques were observed at pH between 2 and 4, indicating phage inactivation at low pH.

3.4 | Thermal stability of phage vB_vcM_Kuja

In the study of VP4 phage thermal stability (Figure 4), a titer between 10^5 and 10^9 PFUs/ml was observed between
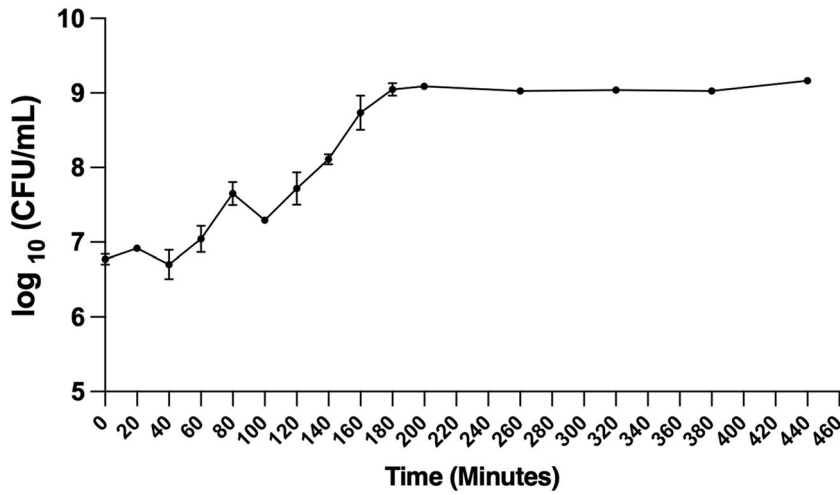
FIGURE 1 Growth curve of *Vibrio cholerae* strain Vc_ke isolated from river Kuja in Migori county, Kenya. The viable counts were calculated as CFU/ml and Log_{10} data were plotted against time. The error bars represent the standard error of the means.
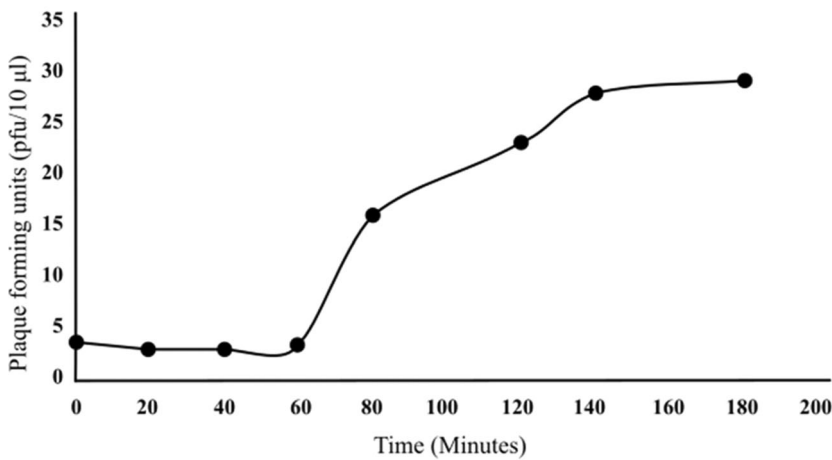

FIGURE 2 One-step growth curve of *Vibrio cholerae* bacteriophage vB_vcM_Kuja isolated from river Kuja in Migori county, Kenya.
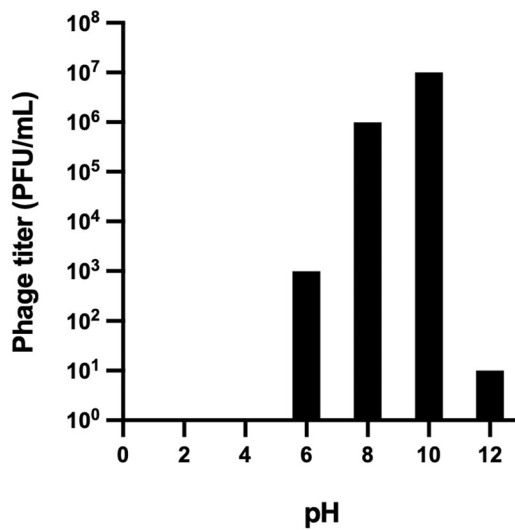

FIGURE 3 A bar chart showing Phage vB_vcM_Kuja titer at different pH. The phage stability was tested in acidic and alkaline adjusted trypticase soya broth, then incubated for 12 h at 37°C. At the end of the experiment, phage titers were enumerated by double agar overlay plaque assay.
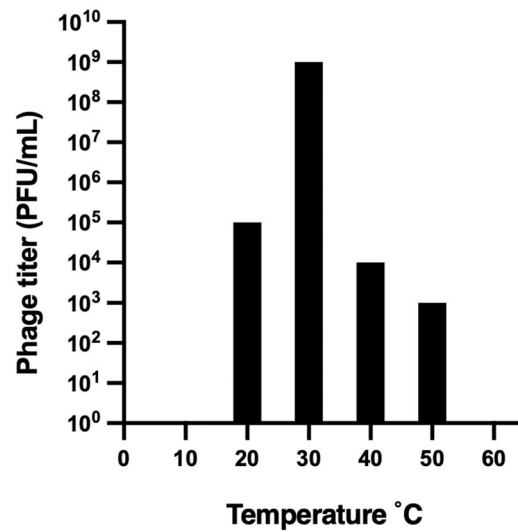

FIGURE 4 A bar chart showing Phage vB_vcM_Kuja titer at different tested temperatures. The phage stability was monitored at six different temperatures ranging from 10°C to 60°C for 2 h. At the end of the experiment, the surviving phages were measured using the double-layer method.
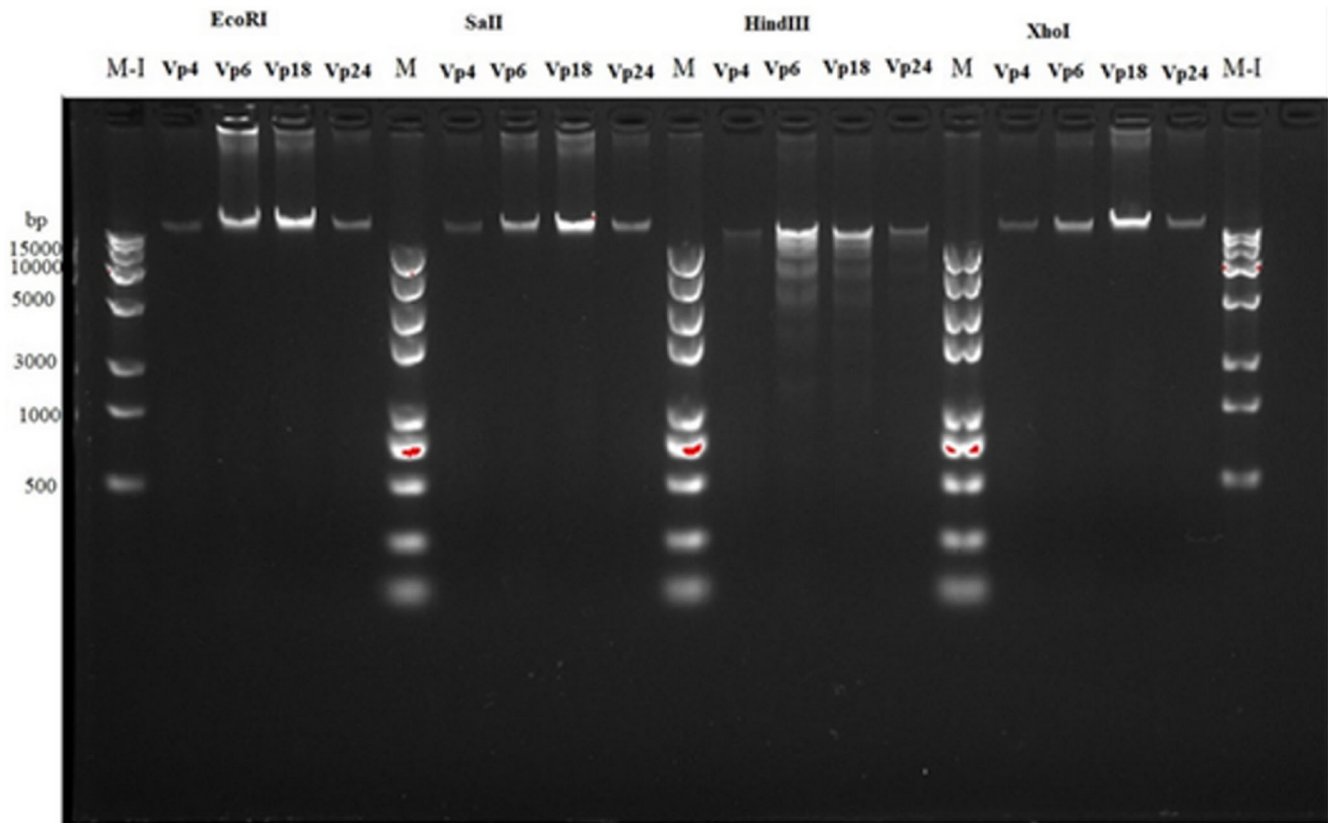
FIGURE 5 Restriction digestion profiles of the genomic DNA of four bacteriophages (VP4, VP6, VP18, and VP24) using the enzymes *HindIII*, *EcoRI*, *SalI*, and *XhoI*.

20°C and 40°C, with a maximum titer of 10^9 PFUs/ml at 30°C. After 12 h of incubation on a lawn of the host bacterium, there was a drastic reduction in titer at 60°C, with no plaques observed.

3.5 | The restriction endonuclease digestion patterns

Restriction digestion profiles of bacteriophage vB_vcM_Kuja (VP4) and three other phages are displayed in Figure 5.

4 | DISCUSSION

Understanding the host bacterium is of paramount importance before isolating potentially therapeutic bacteriophages, as abundance and distribution of phages are linked to their hosts [23]. Some of the host key aspects that influence the abundance and phage distribution include nutrient availability, environmental conditions, and control of population dynamics between bacteriophage and the host [24]. The growth

curve of *V. cholerae* was therefore put into consideration to help in the establishment of pH, temperature, and the one-step growth curve of bacteriophage VP4. The growth curve of *V. cholerae* showed a lag phase between minutes 0 and 40, while the mid-exponential time was between 140 and 160 min. The generation time was estimated at 60 min while stationary phase was between 260 and 440 min with the growth curve of the bacterium showing a sigmoid curve. The current results fit well in the Buchanan model stipulating that *V. cholerae* used in this study was dominated by a single phenotype of bacteria [25]. Additionally, bacterial hosts indirectly control phage production by their physiology, like growth rate, which is in turn often linked to the availability of resources, including nutrients [26].

Density of the host bacterium affects the latent period whereby a lesser density of host strain results in a prolonged latent period [27, 28]. From the one-step growth curve of phageVP4, the latent period ranged from 40 to 60 min, while the burst size was in the range of 23−30 PFU/10 μl. Most vibriophages have latent periods above 30 min while vibriophages have latent periods that fall between 10 and 120 min [29]. Previous studies

reported burst sizes of vibriophages ranging from 23 to 500 PFUs [30–32].

Based on the initial growth kinetics of the host bacterium and the one-step growth curve of phage VP4, it was of interest to further study their population dynamics. More comprehensive studies on VP4 predacity potential on *V. cholerae* in small-scale versions imitating fresh and estuarine aquatic environments can be considered. Such studies may lead to the future application of phage VP4 as a candidate for water decontamination or in phage cocktails. Applying phages in nonclinical fields, such as environments with great variability in parameters throughout the year, necessitates the study of the phage application impact on the environment and the effect of abiotic factors like temperature, pH, and UV radiation on the viability of phages [33].

The effectiveness of bacteriophage applications against pathogenic bacteria is affected by physicochemical conditions such as temperature and pH levels [34]. According to pH stability analysis, the highest phage titer (10^7 PFUs/ml) was recorded at a pH of 10. Phage VP4 was most stable at a pH 10 and relatively stable between pH 6 and 8. However, no plaques were observed at pH between 2 and 4, indicating phage inactivation at low pH. The results imply that bacteriophage VP4 is more stable in alkaline pH, concurring with other studies on vibriophages [35].

A previous study on three vibriophages ICP1, ICP2, and ICP3 to understand their equilibrium with *V. cholerae* in nutrient-limiting aquatic microcosms showed that each of the phages could infect and kill *V. cholerae* at 30°C. Phage ICP1 could kill *V. cholerae* and replicate in a higher salinity condition than the one found in freshwater [36]. Concentration of hydrogen ions in an acidic solution can cause a decline in phage concentration owing to the aggregation process [7, 37]. Moreover, *V. cholerae* is a halophile; hence, alkaline pH favors the coexistence of both the phage and the host. Previous, in vitro studies on five vibriophages showed that they could withstand a wide range of pH (between 2 and 12) and a temperature range of 25°C−60°C [38]. Survival of bacteriophages in divergent environments is governed by conditions, such as the level of pH, ionic strength, temperature, moisture, and host/bacteria concentration. Nevertheless, bacteriophages are abundant entities from a variety of environmental sources [7]. Therefore, determination of physicochemical parameters, such as temperature and pH are indispensable data supporting efforts toward utilization of the environmental phage VP4 in phage therapy, water decontamination, or inclusion in phage cocktails.

In the study of VP4 phage thermal stability, a titer between 10^5 and 10^9 PFUs/ml was observed between 20°C and 40°C, with a maximum titer of 10^9 PFUs/ml at 30°C. After 12 h incubation on a lawn of the host bacterium, there was a drastic reduction in titer at 60°C, with no plaques observed. These findings lead to the conclusion that phage VP4 has a relatively wide range of temperature stability. Such wide range is a positive attribute, especially for downstream biocontrol or therapeutic applications. A wide temperature range provides flexibility in preparing the phage in different pharmaceutical formulations, in administering it to infected patients in different conditions, and in the stability of the phage during storage—if it is to be used alone or as a part of a therapeutic cocktail. In a previous study on phage M4, a thermostable myoviral vibriophage, inactivation was reported when the phage was exposed to temperatures above 60°C [35]. Higher temperatures may result in permanent damage or denaturation of the virion [39].

As exhibited in the one-step growth curve, phage VP4 showed interesting traits, such as burst size and latent periods within ranges of other studied vibriophages. These traits make it environmentally appealing for application in water treatment to control pathogenic *V. cholerae* [14]. Previous studies have demonstrated that the thermal and pH stability of phages varies depending on the strain [40–42]. In this study, phage VP4 was thermally stable and active at 50°C, yet, it was completely inactivated at 60°C. The phage was also tolerant to a broad range of pH (6−10) making it suitable for application in different climatic zones as well as in alkaline foods.

Several studies supported the role of *V. cholerae* phages in modulating cholera outbreaks, leading to proposals on the use of phage cocktails prophylactically to curb cholera transmission [43].

VP4 phage had a maximum titer of 10^9 PFUs/ml at 30°C, which is supported by other studies [36]. This temperature, 30°C is that of optimum *V. cholerae* growth, demonstrating that the phage can replicate and ultimately cause lysis of the host. In Kenya, where periodic cholera outbreaks have occurred in recent years, detailed studies on the ecology of *V. cholerae* and its phages in aquatic reservoirs could contribute to understanding the impact of virulent phages on *V. cholerae* during cholera outbreaks.

Deriving out of the restriction endonuclease digestion, the genomic DNA of VP4 and the other three bacteriophages remained intact and could not be digested by the *EcoRI, SalI*, and *XhoI* restriction enzymes but could be cut by *HindIII*.

The restriction endonuclease digestion patterns showed that the VP4 phage genome could be digested

by the *HindIII* restriction enzyme. Phage DNA resistance to restriction enzymes is known as the anti-restriction mechanism and one explanation for this is adaptation of the bacteriophage genomes under the selection pressure of widespread restriction-modified systems and loss of restriction sites during evolution [44]. Insensitivity of phage DNA can also be attributed to the integration of unusual bases in the viral DNA such as hydroxymethyl uracil or hydroxymethyl cytosine that make the DNA refractory to endonuclease cleavage [45]. In addition, phage genomes may encode methyltransferases that modify specific nucleotides within the recognition site of one or more of the restriction endonucleases.

V. cholerae bacteriophages naturally reside in the environmental waters. In conclusion, according to the results obtained in this study, bacteriophage VP4 (vB_vcM_Kuja) was stable at a temperature range of between 20°C and 50°C and a pH range of between 6 and 12. One-step growth curve revealed that the latent period fell in the range of 40– 60 min while burst size ranged between 23 and 30 plaque-forming units/10 μl. The restriction digestion pattern using *EcoRI*, *SalI*, *HindIII*, and *XhoI* enzymes showed that the phage genome could be cut by *HindIII*.

The postantibiotic era we are living in stirred our attention to revitalize phage application as a potential antibacterial agent. However, it is of crucial importance to well characterize phages to maximize their usage benefits and improve phage therapy, either as an adjuvant or an alternative for available antibiotics.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available from the corresponding author.

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