Pak. J. Biotechnol. Vol. 21(2),528-533, ***2024.*** http://doi.org/10.34016/pjbt.2024.21.02.961

Research Article

OPEN ACCESS

Available on http://www.pjbt.org
Pakistan Journal of Biotechnology
(PJBT)
(P-ISSN: 1812-1837 and E-ISSN: 2312-7791)

ANTIBACTERIAL EFFICACY OF BACTERIOPHAGES AGAINST *VIBRIO CHOLERAE*

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Article Received 25-07-2024, Article Revised 28-08-2024, Article Accepted 01-09-2024.

ABSTRACT

This study investigates the use of bacteriophages as an alternative antibacterial approach to combat antibiotic-resistant *Vibrio cholerae*, a major public health concern. *Vibrio cholerae* strains were identified through culture on TCBS agar, microscopy, and biochemical tests (catalase, oxidase, indole, and citrate). Antibiotic susceptibility was assessed using the Kirby-Bauer disk diffusion method across five antibiotics: tetracycline, norfloxacin, ampicillin, ciprofloxacin, and erythromycin. Results showed resistance to ampicillin, tetracycline, and erythromycin, while strains remained susceptible to norfloxacin and ciprofloxacin Five bacteriophages against *Vibrio cholerae* were isolated from 50 wastewater samples, and only two (F1 and F2) showed lytic activity, forming clear plaques. These two lytic phages were further purified and characterized for stability across different temperatures, pH levels, and host range. The isolated phages were tested for host range against six *Vibrio cholerae* strains, with F1 lysing five strains and F2 lysing three, and demonstrated stability across temperatures (20°C to 55°C) and pH levels (4 to 9). They remained active across a wider temperature and pH range demonstrating their potential for therapeutic use. The study shows that these phages are highly specific, targeting only *Vibrio cholerae* strains from various sources without affecting other bacterial species. This specificity makes them safer for therapeutic use, as they are less likely to disrupt the body's natural bacterial flora. Further genomic studies are recommended to characterize the genetic makeup of these phages, optimizing their application in cholera treatment. This study provides critical insights into the potential of bacteriophages as an alternative treatment for antibiotic-resistant *Vibrio cholerae*.

KEYWORDS: *Vibrio cholerae*, bacteriophage, Phage therapy, Antibiotic resistance

INTRODUCTION

Vibrio cholerae is the causative agent of cholera, a diarrheal disease. *Vibrio* is naturally present in the aquatic and marine ecosystems. O139 and O serogroups of *Vibrio cholerae* are involved in causing diarrheal disease throughout the world (Fleischmann *et al.,* 2022; Hu *et al.,* 2016). Cholera is more prevalent in those countries that do not have facility of clean drinking water and have unhygienic conditions (Zahid *et al.,* 2008; Shaw *et al.,* 2022). In the 19th and 20th centuries, cholera spread globally which was considered cholera pandemic. In 1817 the first pandemic of cholera was started and later, further pandemics occurred in 1829, 1852, 1863, 1881, 1889, and 1961, and the last pandemic is continuing. Asia, Europe, America, and Africa (Deen *et al.,* 2020). Various drugs are recommended to cure the patients suffering from cholera. Antibiotics such as Tetracycline, Azithromycin, and Fluoroquinolone. In the last decade, a state of antibiotic resistance has been seen against *Vibrio cholerae* worldwide (Abed *et al.,* 2022). The World Health Organization reported that resistance against antibiotics is a main problem to public health worldwide. So, there is need to reassess the use of phage to cure bacterial diseases (Shrivastava *et al.,* 2018). In the last decade, a state of antibiotic resistance has been seen against *Vibrio cholerae* worldwide (Shah *et al.,* 2023; Das *et al.,* 2020). As multidrug resistance is increasing by *Vibrio cholerae* to antibiotics, there is a need to reconsider the usage of phage to treat diseases caused by bacteria. It is observed that the use of phage therapy is a good approach to cure diseases caused by *Vibrio cholerae* (Jaiswal *et al.,* 2013). Bacteriophage can kill even antibiotic-resistant bacteria which makes the phage an attractive approach to be used to treat bacterial diseases. The phage count may increase depending on the number of bacteria and phage also may not exert an impact on the resident microbiome (Hsueh *et al.,* 2019). Cholera caused by *Vibrio cholerae* remains a significant global health concern, particularly in regions with limited access to clean water and sanitation. In Pakistan, the rise in cholera cases has been observed since 2022 due to flooding and poor sanitation. *Vibrio cholerae* resistance to antibiotics has been increasing so to cure patients

suffering from cholera, alternate therapy is required. Therefore, there is a need to explore new alternative approaches

MATERIALS AND METHODS

Culturing of Bacteria and Identification: In the current investigation, TCBS Agar was used to cultivate preserved clinical samples of *Vibrio cholerae* strains. Using conventional methods such as colony morphology, Gram staining, and biochemical testing including the Catalase, Oxidase, Indole, and Simmons Citrate tests, *Vibrio cholerae* was detected.

Antibiotic Susceptibility Test: Kirby Bauer's disc diffusion method was employed to assess *Vibrio cholerae*'s susceptibility to antibiotics. On Mueller Hinton Agar, every verified *Vibrio cholerae* isolate was investigated. Five distinct antibiotic discs were used: Ciprofloxacin, Erythromycin, Tetracycline, Ampicillin, and Norfloxacin.

Bacteriophage Isolation and purification: To isolate the bacteriophage against *Vibrio cholerae*, 50 wastewater samples were gathered in a sterile falcon tube. The bacteria enriched in the LB broth were incubated for 24 hours at 37°C to isolate the phage. Next, fill a sterile falcon tube with 40 ml of wastewater sample, 20 ml of LB broth, and 5 ml of enriched bacteria. Incubate at 37°C.Following incubation, the sample was centrifuged for 20 minutes at 8000 rpm. A 0.22µm membrane filter was used to membrane-filter the supernatant. For the spot test, 100µl of enriched bacteria were added to the LB agar plate, soft agar was placed on top, and the bacteria were allowed to dry. Add 10µl of the supernatant filtrate to the area, let it dry, and then incubated it at 37°C (Jaiswal *et al.*, 2013).

To purify the phage, a single plaque was taken from the isolated phage and purified using a plaque assay. To make the phages pure, a single plaque was purified three or four times.

Bacteriophage Characterization

Temperature Effect: The purified phage was incubated for 1h at various temperatures and the phage titer was confirmed using the double-layer plate method.

pH Effect: At different pH values purified phage was incubated at 37°C for 18 h. After that, the phage titer was determined based on double-layer agar method.

Host Range: Host range of phage was determined by spotting the phage on lawns of *Vibrio cholerae.*

RESULTS

Clinical bacterial isolates were first cultured on TCBS agar. Gram staining, microscopy, and several biochemical assays, including catalase, oxidase, indole, and citrate tests, were used to further identify *Vibrio cholerae*. Antibiotic susceptibility tests revealed that *Vibrio cholerae* was sensitive to Ciprofloxacin and Norfloxacin but resistant to Ampicillin, Tetracycline, and Erythromycin.

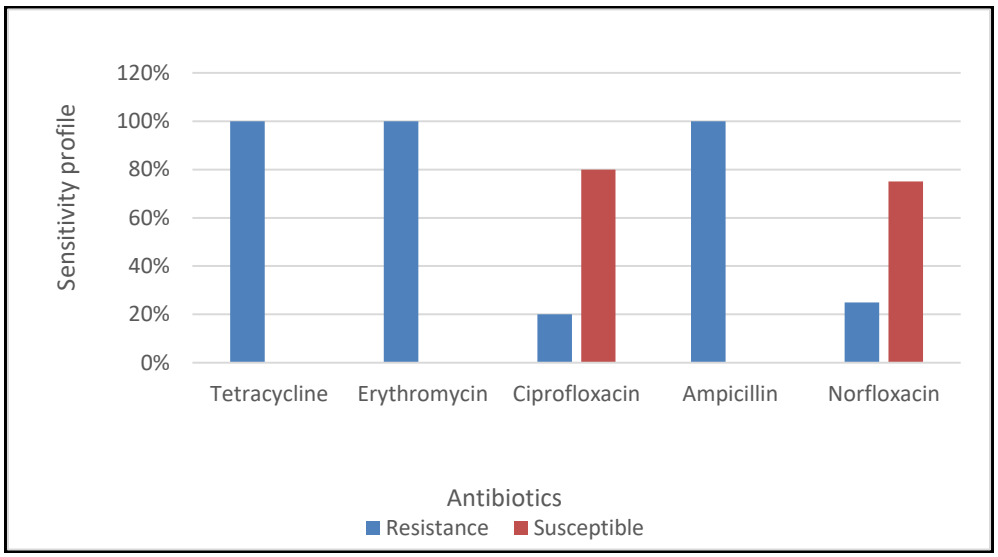

Figure 1: Antibiotic Resistance Profile of Various Antibiotics on *Vibrio cholerae* Isolates

Fifty wastewater samples in all were collected from various wastewater locations. Five bacteriophage isolates produced positive spot test findings. Only two isolated phages, F1 and F2, displayed lytic plaque formation. The phages were isolated and then purified using the plaque assay.

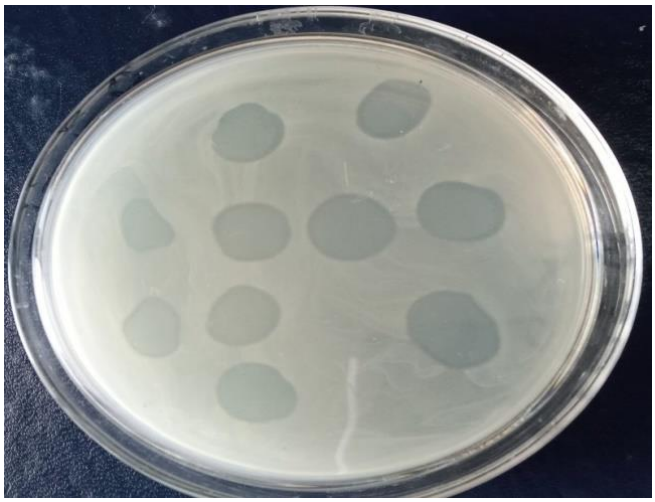

Figure:2 Spot test from initially collected water samples

The temperature, pH, and host range of two bacteriophages, designated F1 and F2, were described. Significant growth rates were demonstrated by the separated phages at 37°C, 45°C, and 55°C; however, no plaque-forming units were seen at 65°C.

Table 1: Growth of bacteriophages at different temperatures

Temperature	20°C	37°C	45°C	55°C	65°C
F1 Phage	105 pfu	277 pfu	230 pfu	160 pfu	0 pfu
F2 Phage	80 pfu	265 pfu	218 pfu	105 pfu	0 pfu

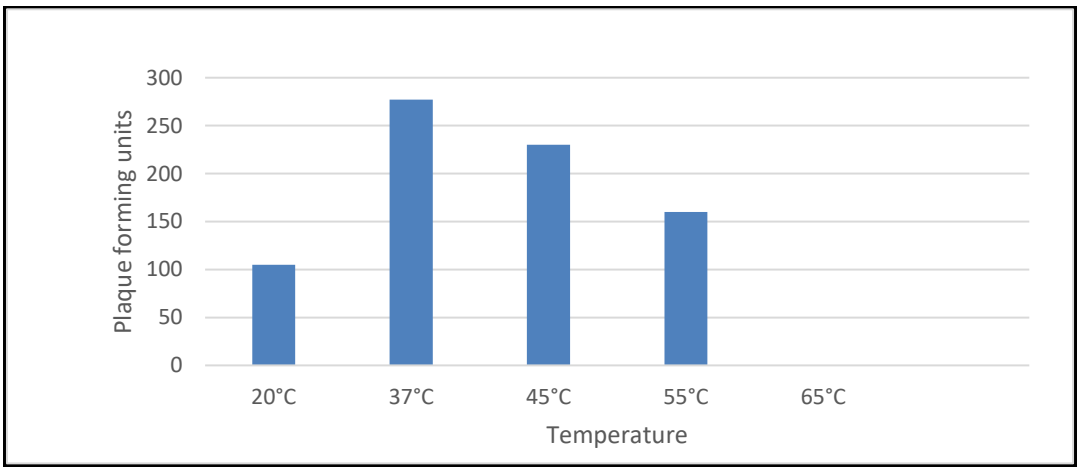

Figure 3: Bacteriophage F1 grew with maximum plaque forming unit at 37°C.

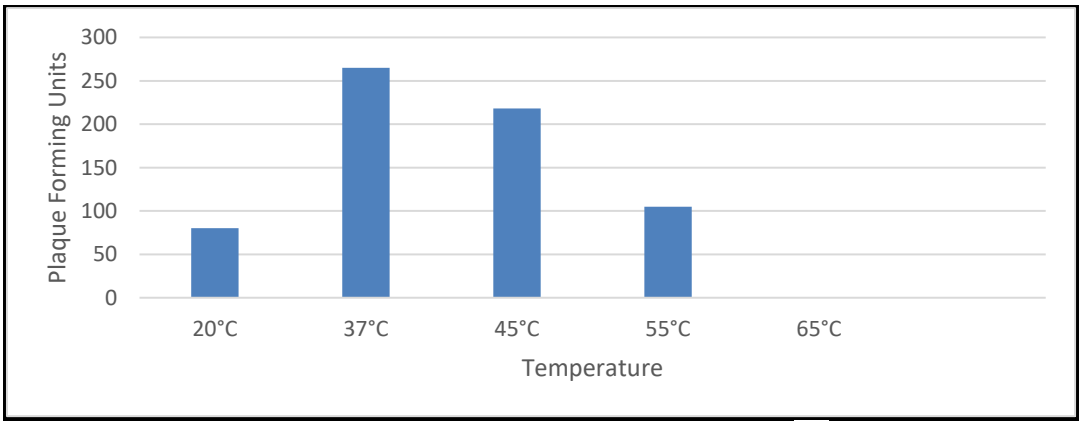

Figure 4: Bacteriophage F2 grew with maximum plaque forming unit at 37°C.

Bacteriophage growth at varying pH values revealed that they proliferated at a wider pH between 2, 4, 6, 7, 8, and 9, but at pH 12, no plaque-forming units were visible.

Table 2 Growth of bacteriophages at different pH

pH	2	4	6	7	8	9	12
F1 Phage	30 pfu	120 pfu	220 pfu	276 pfu	270 pfu	145 pfu	0 pfu
F2 Phage	25 pfu	96 pfu	205 pfu	291 pfu	283 pfu	142 pfu	0 pfu

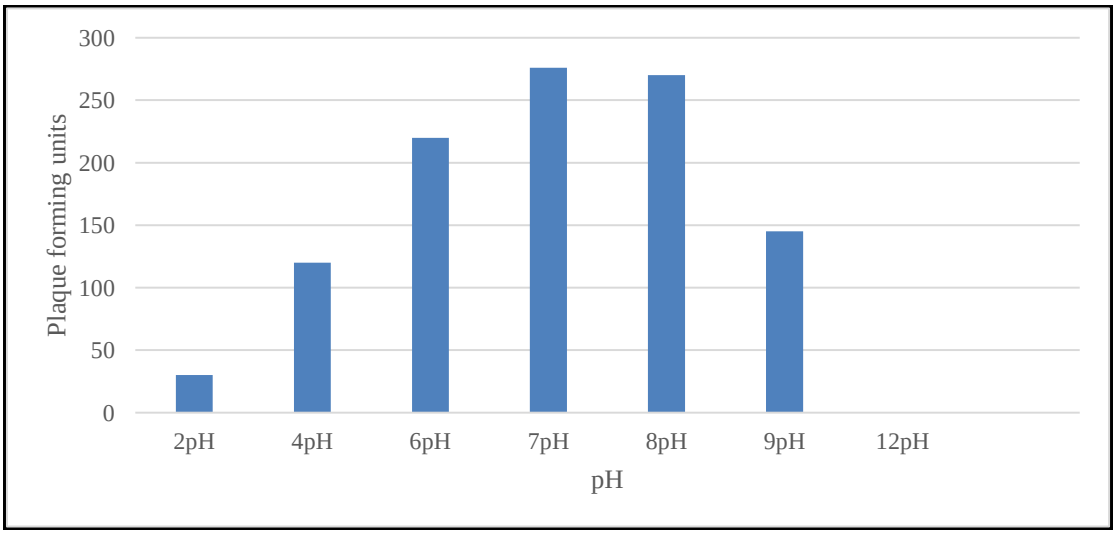

Figure 5: Maximum Plaque-Forming Units of Bacteriophage F1 at pH 7

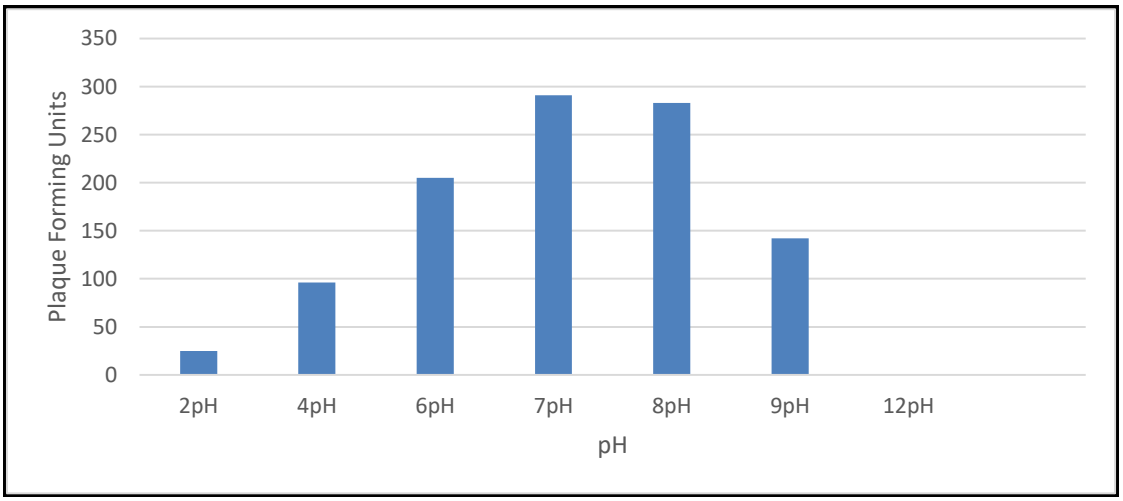

Figure 6: Bacteriophage F2 grew with maximum plaque forming unit at 7 pH. The host range of bacteriophages against different bacterial strains was done by spot test. Bacteriophages showed a wider host range against different bacterial strains. They showed a host range of 50-83%.

Table 3: Assessment of the host specificity range of isolated phages against various bacteria

Bacterial Strain	Phage F1	Phage F2
L14A *Vibrio cholerae*	+	-
9A *Vibrio cholerae*	+	+
4A *Vibrio cholerae*	+	-
4B *Vibrio cholerae*	+	+
8a *Vibrio cholerae*	+	-
15A *Vibrio cholerae*	-	+

DISCUSSION

Phage therapy offers a promising solution for bacterial infections amid rising antibiotic resistance (Mittal *et al.,* 2023). Antibiotic susceptibility pattern of the *Vibrio cholerae* isolates in this study are like another recent study conducted in 2024 where *Vibrio cholerae* were found resistant to Tetracycline, Ampicillin, and Erythromycin, but susceptible to Ciprofloxacin and Norfloxacin (Shah *et al.,* 2024).

To isolate bacteriophages against *Vibrio cholerae*, wastewater samples were collected from wastewater locations for the investigation. Five bacteriophages against *Vibrio cholerae* were identified from the wastewater samples. Another similar study also isolated eleven bacteriophages from 75 environmental water samples, including rivers, ponds, lakes, and sewage, with three of them being lytic and further characterized (Al-Fendi *et al*., 2014). Plaque test was used to isolate the bacteriophage against *Vibrio cholerae*. Only two of the five bacteriophage isolates underwent additional purification and characterization. Significant growth was shown by the phages recovered in our investigation at 20°C, 37°C, 45°C, and 55°C. At 65°C, no plaque-forming units (PFUs) were detected. Their wider host range (50–83%) suggested that they were more effective in killing *Vibrio cholerae.* Current study aligns with Cai *et al.,* (2023), where a lytic phage from China showed growth across 4°C–50°C and pH 6–11. Similarly, our study tested phage infectivity and persistence at temperatures 20°C, 37°C, 45°C, 55°C, and 65°C. In another study in Bangladesh phages isolated from Bangladeshi waters were tested for host specificity against *V. cholerae* O1 and O139 and their ability to disrupt lab-formed biofilms. A phage cocktail (JSF7, JSF4, JSF3) effectively reduced both planktonic and biofilm-associated forms of toxigenic *V. cholerae* (Naser *et al.,* 2017).

Our study's results demonstrate that bacteriophages have the potential to be useful biocontrol agents against *Vibrio cholerae*. The robust growth features and wider host range of the phages obtained in this work indicate that they are promising candidates for additional development and use in phage therapy. A recent study isolated a phage from cholera patient samples and found that VSP-I and VSP-II inhibit phage propagation, with these defenses regulated by quorum sensing. Additionally, a naturally occurring phage variant was identified that resists these defences, showcasing phage-counter strategies (O' Hara *et al.,* 2022).

Because of the inappropriate and excessive use of antibiotics, microorganisms are becoming more resistant to many drugs. The increase in resistance necessitates more research into the interactions between bacterial strains and their related bacteriophages. Because *Vibrio cholerae* strains vary widely, it's necessary to create phage cocktails with a broad range of efficiency that can target several strains. However, further research is required on genomic characterization of the phages to know their genetic components.

This study's limitations include the specificity of bacteriophage isolation to wastewater samples, which may not represent other environments, and variations in phage infectivity across *Vibrio cholerae* strains. Future research should explore phage isolation from diverse sources, assess phage stability under environmental conditions, and investigate genetic characterization to improve potential applications in cholera control.

CONCLUSION

The study demonstrates the promising potential of bacteriophages isolated from wastewater as effective agents against *Vibrio cholerae*, showing strong stability across varied conditions and a broad host range. This highlights phage therapy as a viable alternative to combat antibiotic-resistant *Vibrio cholerae*, paving the way for safer, sustainable treatment options. The next step is to evaluate the efficacy of these phages in various environmental conditions and assess their impact on Vibrio cholerae populations in real-world settings.

AUTHORS' CONTRIBUTION

Sidra Fareed: Executed study experiments and contributed to the manuscript write-up. Afshan Saleem: Conceptualized the study, provided materials and resources, supervised overall research progress. Rafiq Ahmad: Provided flood wastewater samples, supervised the isolation and identification of *Vibrio cholerae* Muhammad Fayaz Khan: Provided technical assistance in bacteriophage optimization experiments and contributed to study design. Hifza Rehman and Samia Gul: Conducted preliminary identification of *Vibrio cholerae* and sample processing from floodwater. Daniyal Akram: Contributed to the manuscript write-up.

CONFLICT OF AUTHORS

The authors declare no conflicts of interest.

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