Original Article

Isolation and Characterization of Novel Broad Host Range Bacteriophages of *Vibrio cholerae* O1 from Bengal

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Abstract

Objectives: We have isolated a total of five newer cholera phages which are novel broad host range to incorporate with the existing phage typing schemes for an extended typing scheme. **Materials and Methods:** These newly isolated phages were well characterized including the electron micrograph. A total of 300 *Vibrio cholerae* strains were isolated from the different endemic region in India were included in phage typing study. **Results:** These phages were found different from the existing phages. Electron microscopic results showed that the phages belonged to myophage and podophage group. Characterization of the phages based on pH, temperature, and organic solvent sensitivity showed differences among the phages used in this study. All the strains of *Vibrio* O1 were typeable (100%) with the five set of cholera phages. Of these, 40% strains were clustered under Type-1. **Conclusion:** The newer *Vibrio* phages are novel and broad host range and will be useful to incorporate with the existing phage typing system for more precisely discriminate the strains of *Vibrio cholerae*.

Keywords: Bacteriophages, isolation, morphology, phage typing, plaque, *Vibrio cholerae*

Introduction

Cholera is a severe dehydrating never-ending problem of human civilization. *Vibrio cholerae*, a Gram negative comma-shaped bacteria, is the causative agents of the disease cholera, and is one of the most life-threatening pathogens, we encounter in our daily activities. Cholera is an infectious disease that causes severe watery diarrhea leading to dehydration and even death. Worldwide cholera cases each year indicates It is estimated that 1.3–4.0 million cholera cases with 21,000–143,000 deaths occur each year worldwide. Today, the global burden of cholera is high, and Africa seems to be the major locus for this disease burden. An analysis suggests that approximately 29 million cases and 95,000 deaths occur annually in countries with endemic cholera, with 60% of cases and 68% of deaths recorded in Africa.[1] Based on their lipopolysaccharide composition and antigenic properties, more than 200 serogroups of *Vibrio* were invented, but only O1 and O139 are mostly causative agent. The genus *Vibrio* includes more than 70 species of which at least 12 *Vibrio* species are pathogenic for humans and important to public health.[2] The O1 serogroup is further classified into two biotypes, namely, classical and El Tor and each biotype into two major serotypes, i.e., Ogawa and Inaba. In India, the city of Kolkata is known as one of the major infected regions in Asia affected by cholera disease. National Institute of Cholera and Enteric Diseases (NICED) engaged in-depth research and study of cholera. Ensuring access to adequate supplies of safe water has traditionally been the primary response to cholera. Cholera is still a public health threat in all developing countries where clean drinking water is not available to the local populations, particularly in remote areas.

Bacteriophages (phages) or viruses attack bacteria; the killing ability of "phages" makes them the "natural enemies" of bacteria. Use of phage typing method expands a wide region of classifying *V. cholerae* strains to understand the epidemiology of cholera.

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NICED is a national reference laboratory for phage typing of *V. cholerae*. This institute receives an average 1000 strains of *V. cholerae* per year from different parts of India and abroad for conformation, biotyping, serotyping, and phage typing at the WHO Phage Reference Laboratory. Two phage typing schemes specific for *V. cholerae* O1 and O139 are being routinely used for the classification and discrimination of strains of *V. cholerae*.

Vibrio species are ubiquitous and abundant in aquatic environments. It has been reported that *V. cholerae* O1 and O139 survive in the environment in the viable but nonculturable (VBNC) state during inter-epidemic periods. Cholera epidemics, caused by toxigenic *V. cholerae* O1 or O139, are a major public health problem in many developing countries.[3] Earlier studies showed a correlation between *V. cholerae* O1 and its phages (vibriophages) in different environmental water samples of natural water bodies.[4,5] Because of its stability and less sensitivity vibriophages appear to be better indicators and suggested to be used as a biomonitoring agent in different aquatic ecosystems. Although *V. cholerae* is a human pathogen, they constitute part of the normal aquatic flora also in water environments,[6] and water is established a transmission vehicle. Perpetually changing environmental conditions (e.g., increasing surface water temperatures) can significantly elevate the risk of infections related to potentially human-pathogenic *Vibrio* species.

In our laboratory, a surveillance study is ongoing to isolate the vibriophages from the environmental reservoir on a seasonal basis in and around Kolkata to assess the scenario of phages. Besides, it has been shown that the presence of vibriophages in sewage water is a potential tool for the prediction of cholera outbreaks.[7] Earlier, we reported that vibriophages exist in the natural aquatic environment between epidemic periods as a surrogate marker, leading to the conclusion that vibriophages may play a significant role in the annual cycle of *V. cholerae* O1 and O139 in the environment and notably in annual epidemics of cholera in Kolkata.[4]

In light of these factors, the aim of this study was to conduct the environmental surveillance to assess the abundance and diversity of clinically important vibriophages in Kolkata freshwater reservoirs to explicitly address the seasonality of vibriophages. The study also aimed to evaluate the efficacy of phage typing methods with these newer phages to incorporate in our existing phage typing system. The new typing scheme will be used to discriminate strains of *V. cholerae* O1 for more efficiently and effectively and is an eloquent testimony of its important in the area of *V. cholerae* epidemiology.

Materials and Methods

Bacterial strains

All bacterial strains used in this study are listed in Table 1. A total of 300 isolates of *V. cholerae* O1 biotype El Tor from different parts of India were received by NICED during 2011–2015 for biotyping, serotyping, and phage typing were included in this study. In addition, *V. cholerae* O1 biotype E1 Tor strain MAK 757 (ATCC 51352) and O139 strain NPR-4 was also used in this study.[8,9]

Table 1: *Vibrio cholerae* isolates used in study

State	Number of isolates	Biotype		Serotype	
		El Tor	Classical	Ogawa	Inaba
Assam	12	12		12	
Andhra Pradesh	31	31	-	31	
Gujarat	29	29	-	29	
Karnataka	27	27		24	3
Madhya Pradesh	14	14	-	14	
Maharashtra	101	101	-	101	
Punjab	16	16	-	16	
Rajasthan	22	22	-	20	2
Tamilnadu	23	23		20	3
West Bengal	25	25	-	25	
Grand total	300	300	-	292	8
Total (%)	100	100	-	97.0	2.6

Bacteriology

V. cholerae O1 isolates were confirmed using the standard techniques.[10] Serological identification was performed using polyvalent O1 and subsequently mono-specific Inaba and Ogawa antisera (Difco, USA).

Isolation and purification of phages

Phages were isolated from water samples and purified using the standard techniques[5] as published earlier. Samples were collected at 30 days periodic intervals from January 2014 to December 2015 from five sites [Figure 1], four located in various parts of Eastern Metropolitan Bypass and one located in Subhas Sarobar lake, at Kolkata (latitude 22°33'N and longitude 88°20'E). Sampling sites on Eastern Metropolitan (EM) Bypass represented sewage canals receive both domestic and industrial effluents, and site of Subhas Sarobar were artificial freshwater reservoir.

Characterization of phages

Phage characterization was performed by the standard methods which include electron microscopy, pH sensitivity, organic solvent sensitivity, influence of temperature, and host range.[11]

Transmission electron microscopy

Electron microscopic study was performed to determine the morphologies of the phages. The phage lysates were purified using a CsCl gradient before electron microscopy. The phage samples (~1 × 10^8 PFU/ml) were placed on a glow-discharged carbon-coated Pioloform grid and stained with 1% uranyl acetate. Phages were imaged using a JEM-1400 EM transmission electron microscope.

pH sensitivity

Phage stability was determined at different pH values. pH of the nutrient broth (Himedia, Mumbai, India) was adjusted with either 1M HCl or 1M NaOH to obtain a pH range 2.0–12.0. 100 µl of phage suspension at a titer of 1 × 10^9 PFU/ml was mixed with 900 µl of pH adjusted medium to obtain a final concentration of 1 × 10^8 PFU/ml. The preparation was then incubated at 37°C for

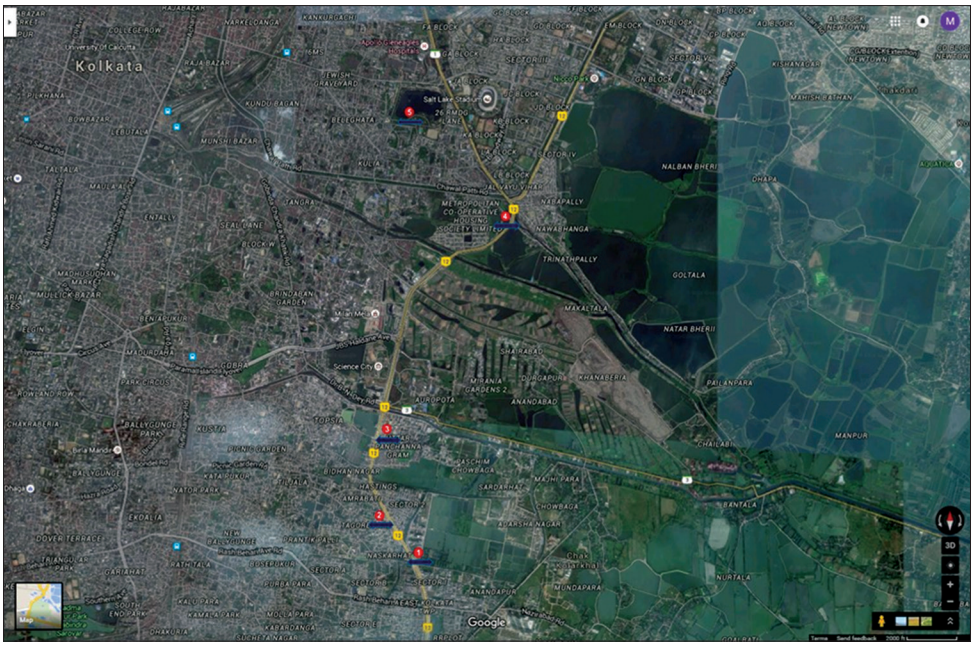

Figure 1: Map of Kolkata showing sampling sites. Sites are marked with red dot with number. Site 1: Tagore Park; Site 2: Mano Vikas, Site 3: Aykar Residency, Site 4: Chingrighata, Site 5: Subhas Sarobar. Map is obtained from Google Earth

1 h. Phage suspension at pH 7.0 used as control. After incubation, phage titer was estimated by soft agar overlay method against *V. cholerae* MAK 757 as described by Sarkar *et al*.

Organic solvent sensitivity

Phages (1×10^8 PFU/ml) were mixed with equal volume of organic solvent (chloroform, diethyl ether or ethanol) as appropriate and incubated at room temperature with recurrent shaking. After 1 h, mixture was centrifuged at 10,000 rpm for 10 min, and phage concentration in the supernatant was estimated by soft-agar overlay method against *V. cholerae* MAK 757 as propagating strain.

Temperature stability

Stability of phages in different temperatures (4, 25, 37, 45, 55, and 60, 65, 70°C) was determined by incubating the phages (1×10^8 PFU/ml) at respective temperatures for 1 h. After incubation, phage samples were centrifuged at 12000 rpm for 10 min, and phage titer in the supernatant was estimated by softagar overlay method as described.

Phage typing procedure

Phage typing was performed on the basis of standard methodology adopted in our laboratory.[8,9,12] A single colony of *V. cholerae* strain MAK 757 from nutrient agar was inoculated into nutrient broth and incubated for 4 h at 37°C. Stationary culture (0.1 ml) was mixed with molten (42°C) 0.8% soft agar (3 ml) and poured onto nutrient agar plate. The aim was to produce a uniform lawn of the growth of *V. choleare* on the surface of the agar to supply an adequate substrate for phage action but not so heavy as to obscure the plaque. The plates were then allowed to dry at room temperature (20–30 min). Small drops of (0.05 ml) phage lysates (1×10^4 PFU/ml) from the routine test dilution (RTD) were then applied onto the plates. The plates were kept at room temperature for at least 10 min to dry and incubated at 37°C for 16–18 h. For O1 and O139 strains, MAK 757 (ATCC 51352) and NPR-4 respectively, were used as controls. After overnight incubation, each reaction was recorded as positive if the number of plaques was five or more.

Results

Phage isolation

Periodic sampling from five different sites [Figure 1] was performed to isolate phages that can form plaques on lawns of *V. cholerae* strain MAK 757. Both sewage and fresh water yielded phages form plaques on lawns of *V. cholerae*. Of the phages isolated, A2, A4, A10, A12, and A13 are representative. These phages were different from each other and also from the existing O1 phages of the typing scheme. All the five phages were isolated from sewage water sites on EM Bypass.

Phage characterization

Bacteriophages used in this study were characterized by physicochemical and morphological parameters. Vibriophages A2, A10, A12, and A13 [Figure 2a and c-e] exhibit podophage morphology, with isometric heads (~48–67 nm) and short stubby tails (~12–17 nm), whereas A4 [Figure 2b] exhibits myophage morphology with isometric head (~80 nm) and contractile tail (~108 nm). The Podophages formed large clear plaques, whereas myophage formed small clear plaques.

All five vibriophages were stable at a range of pH 6–8 with optimum stability observed at pH 7.0 and pH 8.0. In organic solvent sensitivity experiments, no effect was observed on survival of vibriophages after incubation with chloroform. Diethyl ether does not affect the viability of vibriophages A12 and A13, but it results in negligible reduction of population of vibriophages A2,

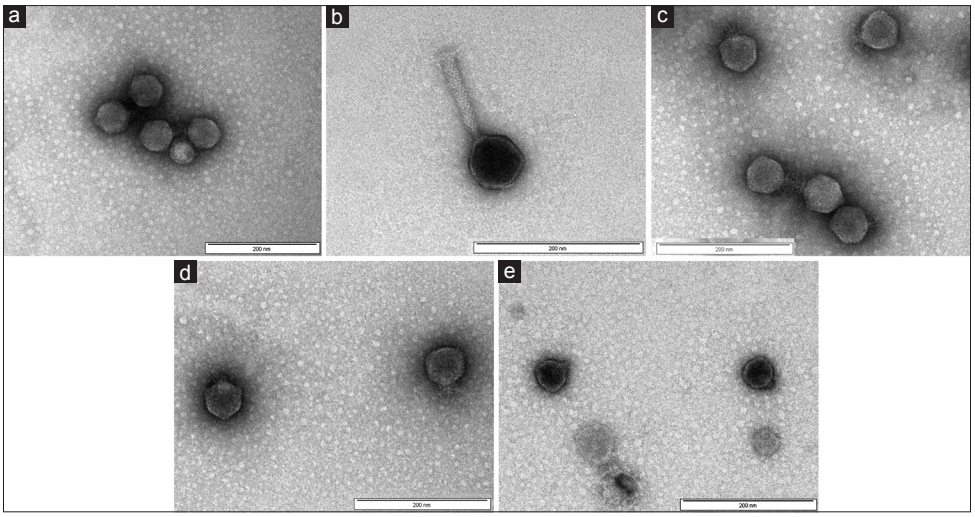

Figure 2: Morphology of vibriophages. Transmission electron micrographs show A2 (a), A4 (b), A10 (c), A12, (d) and A13 (e) CsCl purified phage particles. Bars, 200 nm

A4, and A10 after 1 h incubation (data not shown). In contrast, ethanol treatment showed complete loss of phage activity for all five vibriophages. Temperature sensitivity experiments indicated vibriophages were very stable in 4 ºC, 37 ºC and 45°C, whereas gradual reduction in vibriophage population observed when temperature increases to 55°C and above. Vibriophages were found almost completely inactivated at 70°C.

The host range of five newly isolated vibriophages was evaluated using phage typing of 300 *V. cholerae* O1 biotype El Tor isolates from different geographical regions of India [Tables 1]. All isolates were sensitive to the vibriophages [Table 2]. However, Phage Types T-1, T-7, and T-2 were predominant type. Phage types are the possible combinations of all five vibriophages with overlaps. The observed overlaps indicated that the vibriophages had broad host range activity individually and collectively. In addition, vibriophages A2, A4, A10, A12, and A13 do not form plaques on *V. cholerae* O139 strain NPR-4, indicated phage specificity to O1 biotype El Tor.

Table 2: Phage typing of Vibrio cholerae O1 isolates

Phage type	Vibriophages					Sensitive isolates (%)
	A2	A4	A10	A12	A13	
T-1	-	-	-	-	+	122 (40)
T-2	-	-	+	+	+	51 (17)
T-3	-	-	+	-	-	11 (0.3)
T-4	-	-	+	+	-	15 (5)
T-5	-	+	-	+	+	12 (4)
T-6	+	+	-	+	-	17 (5)
T-7	+	+	+	+	-	59 (19)
T-8	+	-	+	+	+	4 (1)
T-9	+	+	-	-	+	3 (1)
T-10	+	-	-	+	+	6 (2)

+= Sensitive, - = Resistance

Table 3: Morphological characterization of vibriophages

Properties	Vibriophages				
	A2	A4	A10	A12	A13
Mean capsid width (nm)±SD[a]	55.9±4.7	79.8±6.0	55.7±3.8	66.7±7.7	48.5±2.5
Mean tail length (nm)±SD[a]	15.0±3.1	108.1±8.1	15.7±1.7	17.2±3.2	12.5±2.3

[a]Phage physical dimensions are the means of measurements of ten virions, and values in parentheses indicate SDs. SDs: Standard deviations

Discussion

Cholera is a water-borne disease and still a serious threat. Cholera, though rare in the industrialized nations, has increased globally and still occurs in many places including Africa, Southeast Asia and Haiti. In India, 4031 cholera cases including 21 deaths Case Fatality Rrate (CFR 0.52%) were reported from 12 states (out of 29 states and 7 Union Territories). The majority of cases (49%) were from West Bengal on the border with Bangladesh. It has been estimated that an average of 22,000 cases occurs in India each year.[13]

Our analysis showed that the annual number of cholera cases reported to the WHO by the government was several times lower than the numbers; we obtained through culture positive strains received at the Phage Typing Unit, NICED.[14] This may be due to lack of surveillance and proper laboratory support. Therefore, the burden of cholera in India is clearly underestimated. The persistence of this bacterium in aquatic environments is a key epidemiological concern, as cholera is transmitted through contaminated water.[15] We have previously reported that phages act as a biomonitoring agent in the environment, provides important insights in *V. cholerae* surveillance.[4] Predation on pathogenic *V. cholerae* may be an important factor influencing the epidemic cycle on short-time scales and may act to modify the duration and severity of cholera outbreaks.[16]

During a span of 2 years (2014–2015) sampling in Kolkata, a total of 28 vibriophages isolated from the sewage and freshwater

samples (data not shown). Five viobrophages showing a broad host range were selected for this study which showed differences based on the characterization performed in this study. Capsid sizes of all the five vibriophages are different from each other with a range of 48–80 nm [Table 3]. A proportional allometric relationship between virion size and genome length of viruses was well documented.[17] In addition, it was reported that capsid size of virus particle can make quantitative prediction of its genome length.[18] Therefore, the above morphological analysis indicated that all the five vibriophages have different genome lengths which support diversity among the phages.

The survival and persistence of phages are affected by different physicochemical factors (pH, temperature, ions, etc.).[19] pH is a crucial factor for survival of bacteriophages. Regarding the therapeutic application of phages as antimicrobials for enteric pathogenic bacteria, always a major concern has been the survival of phages in acidic gut environment during oral administration. Oral administration worked better as documented, when antacid was used, which increased the number of phages surviving while passing through the acidic pH of stomach.[20,21] pH sensitivity results showed that vibriophages were stable at a range of pH 6–8. We found that at low (pH 2.0) and high (pH 12.0) pH a small amount of vibriophages retained viability. This property of vibriophages to resist extreme pH probably enabled them to overcome high and low pH in the gastrointestinal tract when they are used in therapeutic application. Other important factors influencing phage stability are solvent and temperature. Organic solvent sensitivity showed that chloroform and diethyl ether does not have significant effect on vibriophages. Temperature plays a fundamental role in attachment, penetration, multiplication, viability, and storage of phages.[22] Phage can survive at high temperatures (40°C–90°C) and could also be found in other environments beyond extreme thermal habitats.[23] Our observation was indicative of almost 100% stability of vibriophages at 4, 37 and 45°C and around 70% stability at 55°C suggesting a very long range (4°C–70°C) of temperature stability of five vibriophages.

In addition, 100% typeability was observed with newly isolated vibriophages for *V. cholerae* O1 isolates from different parts of India [Tables 1 and 2], confirmed that vibriophages are with broad host range. This indicated that these newly isolated phages are suitable to incorporate with the existing typing scheme for more precisely discriminate the strains of *V. cholerae*.

Conclusion

The reported vibriophages with diverse morphology, low and high pH stability, long-range temperature stability, broad host range, and 100% typeability are attractive candidates which may be used in an efficient phage typing scheme. Moreover, these phages may be useful as cocktails for phage therapy to control the disease cholera caused by *V. cholerae* O1.

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Conflicts of interest

There are no conflicts of interest.

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