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Bacteriophage JSF4 can be a Potential Prophylaxis Therapy for Cholera: An Alternative Approach to Antibiotics

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ABSTRACT

Cholera remains a major risk in developing countries like Bangladesh, particularly after natural or man-made disasters and becoming increasingly resistant to antibiotics. Effective prevention strategies will be essential in reducing the disease burden of these bacterial infections. Here, we used the specificity and rapid-acting properties of bacteriophages as a potential prophylaxis therapy for cholera, a severely dehydrating disease caused by *Vibrio cholerae 01* or 0139 serogroup. In this study, a single bacteriophage, JSF4 specific for *V. cholerae 01* serogroup, was used to reduce the severity of cholera therapeutically in the infant mice model. Bacterial counts were decreased up to 106 times in the intestines of bacteriophage-treated animals and increased up to 24 times in the untreated control mice intestines. This is the first report that a single bacteriophage JSF4 might be useful to treat cholera caused by *V. cholerae 01* serogroup strains and could be an alternative to antibiotics. In the future, JSF4 bacteriophages may also have profound implications in phage therapy for controlling cholera caused by pathogenic *V. cholerae 01* serogroup strains.

Keywords: Cholera, Dehydrating disease, *Vibrio cholerae*, Prophylaxis therapy, Bacteriophage, and Therapeutic.

INTRODUCTION:

The causative organism *Vibrio cholerae* is a facultative anaerobe, gram-negative, non-spore forming curved rod, about 1.04-1.06 µm long flagellated bacterium (Lekshmi *et al*., 2018; Maheshwari *et al*., 2011). Cholera (meaning being 'a gutter') is the correct name for the disease, caused by the entry of contaminated water (Lekshmi *et al*., 2018). Due to poor infra-structure, sanitation, and access to clean drinking water in developing countries like Bangladesh cholera has become an emerging disease for centuries. It also flourishes at the time of societal disruption, such as natural calamities like the 2010 earthquake in Haiti or the current refugee crisis in Yemen (Orata *et al*., 2014; Rabaan *et al*., 2019).

V. cholerae is classified based on its somatic antigens (O antigens) into serovers or serogroups. Out of 200 serogroups of *V. cholerae*, only the serogroups O1 and O139 were the causative agent of current epidemics, and *V. cholerae 01* is the major infectious agent (Orata

et al., 2014; Rabaan *et al*., 2019). The non-O1/non-O139 biotypes do not cause cholera, because they do not contain TCP/CT genes, but in some cases where they may show diarrheal symptoms (Hasan *et al*., 2015). The O1 and O139 strains are prevalent in several endemic regions, including Yemen, parts of Africa, Southeast Asia, and Haiti (Orata *et al*.,2014; Kühn *et al*., 2014; Hsueh *et al*., 2019; Kirigia *et al*., 2009; Hossain *et al*., 2018; Khan *et al*., 2018; Leitner *et al*., 2015).

The first 6 pandemics (1816-1923) were caused by the Classical O1 biotype, whereas the seventh (1961 to the present) was caused by the El Tor biotype (Hu *et al*., 2016). The current pandemic affects 3-5 million persons per annum, causing 21000-143000 deaths (Hu *et al*., 2016; Ali *et al*., 2015). A healthy person within an hour of the onset symptoms may become hypotensive and may die between 2-3 hours if proper treatment is not provided (Todar *et al*., 2004). However, the World Health Organization gave the advice that only the severe cases of cholera should be treated with antibiotics due to the spread of antimicrobial resistance. Alternative approaches to control cholera are emergency needed. Biological control using bacteriophages is a good alternative, particularly where antibiotic resistance may be a problem (Czaplewski *et al*., 2016).

In this study, a novel *V. cholerae* 01 serogroup-specific bacteriophage JSF4 is used. This bacteriophage is very efficient to lyse both the clinical and environmental pathogenic *V. cholerae* 01 serogroup strains, and one of our previous reports showed that the seasonal epidemics of cholera caused by *V. cholerae* 01 serogroup strains in Bangladesh is mostly regulated by this bacteriophage (Faruque *et al*., 2005). So, we assumed that these bacteriophages could be an effective therapeutic agent for controlling cholera caused by *V.cholerae* 01 serogroup strains. In the current study, we showed that the oral administration of JSF4 bacteriophages could reduce *V. cholerae* 01 concentration in infant mice. Given the well-documented challenges associated with the emergence of antibiotic-resistant bacteria, bacteriophages might yet provide a viable alternative to antibiotics. So, in future, we can use JSF4 bacteriophages for the development of a new type of therapeutic agent against cholera.

MATERIALS AND METHODS:

Media preparation: Taurocholate tellurite gelatin agar (TTGA), a highly selective medium for the isolation of *V. cholerae* was used (Monsur *et al*.,1961). To prepare TTGA plates, 14 gm of TTGA powder (Hi-Media, India) was added in 1 litre of distilled water and autoclaved at 121°C for 15 minutes and pour 25 mL of media into each 10-cm Petri plate. To prepare Luria Agar (LA) plates, 14 gm of Luria Agar powder (Biomark Laboratories, India) was added in 500 mL of distilled water and sterilization was done by autoclaving at 121°C for 15 minutes.

To prepare liquid media, Nutrient Broth (NB), 13 gms of Nutrient Broth powder (Hi-Media, India) and 10 gms of NaCl were added in 1 litre of distilled water and autoclaved at 121°C for 15 minutes. To prepare semi-solid media (0.8% soft-agar for bacteriophage growth), 0.8 gm agar was added in 100 mL of NB and autoclaved it.

Growth of bacterial culture: Mainly *V. cholerae* 01 serogroup strain named N-16961 was used in this study. A single colony of *V. cholerae* 01 strain was inoculated into the nutrient broth and incubated at 37°C for overnight. To get a single colony of *V. cholerae* 01 strain, *V. cholerae* 01 cultured was streaked onto TTGA plate and incubated at 37°C for overnight.

Antibiotic sensitivity test (AST) through the disc diffusion method: Bacterial growth inhibition was determined by standard disc diffusion method (Bauer *et al*., 1966). Briefly, Petri plates were prepared by pouring 25 mL of Nutrient Agar (Biomark Laboratories, India) and allowed to solidify. Plates were dried and 100 µL of bacterial suspension containing $1x10^6$ cells/mL was poured in each plate and spread uniformly. The plates were allowed to dry for 5 minutes. The antibiotic discs (Oxoid Ltd., UK) were then placed on the surface of the plates (Bauer *et al*.,1966). After that, the plates were incubated at 37°C for 24 h and the zone of inhibition was observed and measured in millimeters.

Collection, cultivation and harvest of JSF4 bacteriophages: We have collected *V. cholerae* 01 serogroup-specific bacteriophage JSF4 from Molecular Genetics Laboratory, International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B) (as a kind gift from Dr Shah Mohammad Faruque, a former director of the Centre for Food and Water Borne Diseases in ICDDR, B). *Faruque et al.* isolated and purified this bacteriophage from environmental waters in Bangladesh (Faruque *et al*., 2005). After collection, we cultivated the phage on its host strain by the method described previously (Faruque *et al*., 2005). Briefly, the phage was inoculated in nutrient broth containing the host strain and incubated at 37°C for overnight with shake, then filtered the broth with 0.22µ pore size of filters (Millipore Corporation, Bedford, Mass.) to remove the bacteria and bacterial debris. The filtrate contained the phages and maintained the phage stock in normal saline at 4°C. We then detected and quantified the bacteriophages by the standard double-layer plaque assay method as described previously (Faruque *et al*., 2005) and below.

Laboratory animals: Swiss albino mice (5 days old) of either sex obtained from the Department of Pharmacy, Jahangirnagar University, Savar, Dhaka, Bangladesh were used in this study. The animals were kept in well-ventilated and hygienic compartments, maintained under standard environmental conditions, fed with standard rodent pellet and water.

Quantification of host bacterial strain at the feeding time and after incubation in mice intestines: The ability of JSF4 bacteriophages to reduce the severity of cholera was determined by using batches of ten 5-day old mice with modifications previously described method (Nishibuchi *et al*.,1983). We divided twenty 5-day-old infant mice into two batches (control batch and experimental batch). Each batch contained 10 mice. In control batch, each mouse was given 50 µL of overnight cultured bacterial suspension containing $3.94\pm0.54\times10^8$ cfu/mL orally. In mice of the experimental batch, we tried to feed the equal number of bacteria to each mouse like the control batch, but in practice, each mouse of the experimental batch was inoculated $3.96\pm0.42\times10^8$ cfu/mL orally. After 1 hour incubation, each mouse of the experimental batch was given 50 µL of JSF4 phage from the stock containing $2.04\pm0.31\times10^9$ pfu/mL orally. All mice were kept at RT for 18 hrs from initial inoculation time. After 18 hrs incubation, the mice were sacrificed, and the small intestines were removed and homogenized. The homogenates were serial diluted, plated on TTGA plates and incubated at 37°C for overnight. The colonies were counted and compared with the controls and initial input.

Quantification of JSF4 bacteriophages at the feeding time and after incubation in mice intestines: To determine how much JSF4 bacteriophages inoculated and how much increase after incubation in mice intestine, we performed it by plaque assay method described previously (Faruque *et al*., 2005). Briefly, logarithmic-phase cells (500µL) of host *V. cholerae* 01 strain (N-16961) in nutrient broth were mixed with 3.5-ml aliquots of soft agar (nutrient broth containing 0.8% agar), and the mixtures were overlayed on LA plates. Dilutions of bacteriophages were inoculated on the plates, and the plates were incubated for 16 h at 37°C. A sample was scored positive for bacteriophages when a plaque was observed on the bacterial lawn. To estimate the concentration of bacteriophage particles the plaques were counted in the sample and expressed as pfu/mL.

RESULTS:

Antibiogram profile of *V.cholerae* 01 serogroup strain N-16961: This study needed a multi-drug resistant, pathogenic *V.cholerae* 01 serogroup strain as a host for JSF4 bacteriophages. The strain N-16961 was highly pathogenic and belonged to *V.cholerae* 01 serogroup (Islam *et al*., 2013), so we choose this strain as a host and determined its antibiogram profile through the disc diffusion method. From antibiotic sensitivity test, we have found that this strain is resistant to Ampicillin, Ceftriaxone, Methicilline and Trimethoprim. So, it is a multiple drug-resistant strain (**Table 1**). These types of multi-drug resistant strains are frequently emerged and make the treatment predicament through antibiotics. So, it is imperative to develop alternative prophylaxis therapy against such kinds of multiple drug-resistant pathogenic strains and phage therapy can be a suitable alternative.

Table 1: Antibiogram profile of *V.cholerae* 01 strain N-16961.

Antibiotic name	Code	Disc concentration	Zone of inhibition (mm)
Ampicillin	AMP	10µg	**00 (R)**
Ciprofloxacin	CIP	5µg	30 (S)
Chloramphenicol	C	30µg	30 (S)
Ceftriaxone	CFO	30µg	**00 (R)**
Kanamycin	K	30µg	20 (S)
Methicilline	MET	5µg	**00 (R)**
Nalidixic acid	NA	30µg	10 (I)
Norfloxacin	NOR	10µg	26 (S)
Trimethoprim	W	5µg	**00 (R)**

Sensitive (S), Resistant (R), Intermediate (I)

Host specificity of JSF4 bacteriophages: Before inoculation of JSF4 bacteriophages into experimental mice, we determined its host specificity through the standard double layer plaque assay method (Faruque *et al*., 2005). From this assay, we have found that the bacteriophage JSF4 was 100% host specific and very efficient to lyse pathogenic *V.cholerae* 01 serogroup strain N-16961 (**Fig 1A**). Through its efficient lysis activity, we used this bacteriophage as a therapeutic agent in our current *in vivo* experiment.

Phage therapeutic activity of JSF4 bacteriophages inside mice intestines: To determine whether bacteriophage JSF4 acts as a therapeutic agent, we inoculated the pathogenic host *V. cholerae* 01 strain and its specific bacteriophage JSF4 in the experimental batch of 5 days old mice. In control mice, we fed only the bacteria. After 18h incubation, the mice were sacrificed, and the small intestines were removed and homogenized. From the homogenates, the bacteria were enumerated through the standard serial dilution plate count method. From this *in vivo* experiment, we have found that the bacterial counts in the intestines of phage-treated animals were reduced by up to 106 times (**Fig 3**) and increased up to 24 times in the untreated control mice intestines (**Fig 2**). This result indicated that JSF4 bacteriophages reduce the severity of cholera caused by pathogenic *V. cholerae* 01 strains. At the same time, we have enumerated JSF4 phages in the intestines of phage-treated animals through the plaque assay method (**Fig 1B**) and found that the number of phages increased up to 45 times (**Fig 4**) compared to the initial input. The increasing number of phage indicated that it reduces the severity of disease through its efficient lysing activity the host.

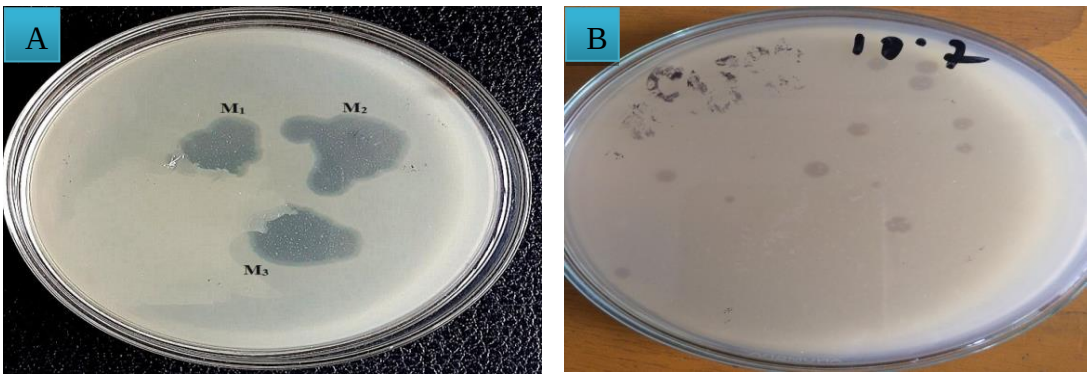

Fig 1: (A) Host specificity of JSF4 bacteriophages; (B) Plaques of JSF4 bacteriophages on the lawn of host strain N-16961.

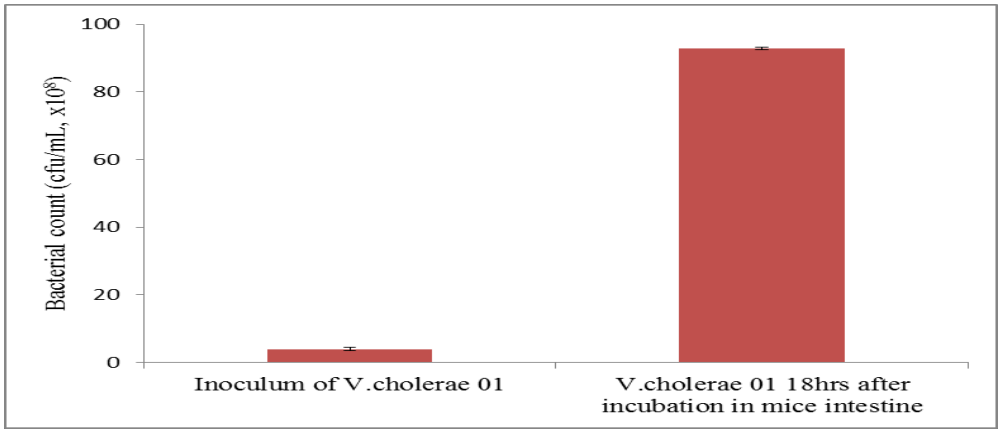

Fig 2: Bacterial count (cfu/mL) at the feeding time and 18 hrs after incubation in the intestines of control batch mice. Data shown were mean±SD of ten individual mice of each group.

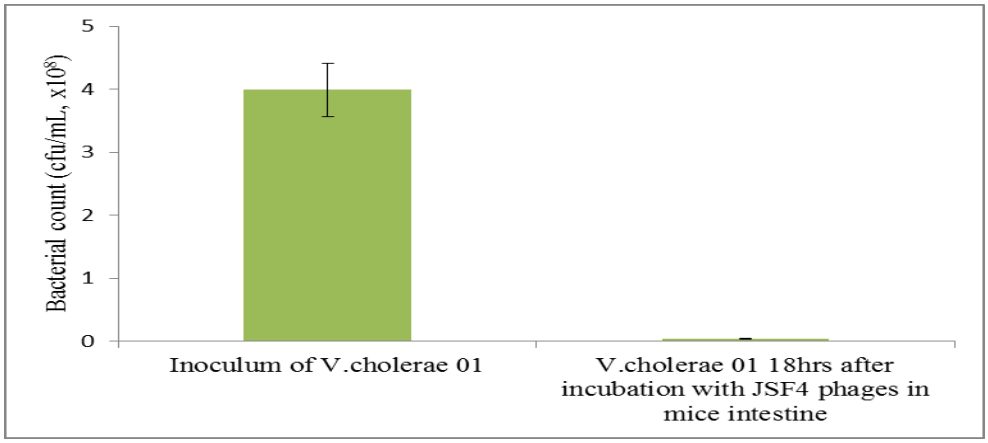

Fig 3: Bacterial count (cfu/mL) at the feeding time and 18 hrs after incubation with JSF4 bacteriophages in the intestines of experimental batch mice. Data shown were mean±SD of ten individual mice of each group.

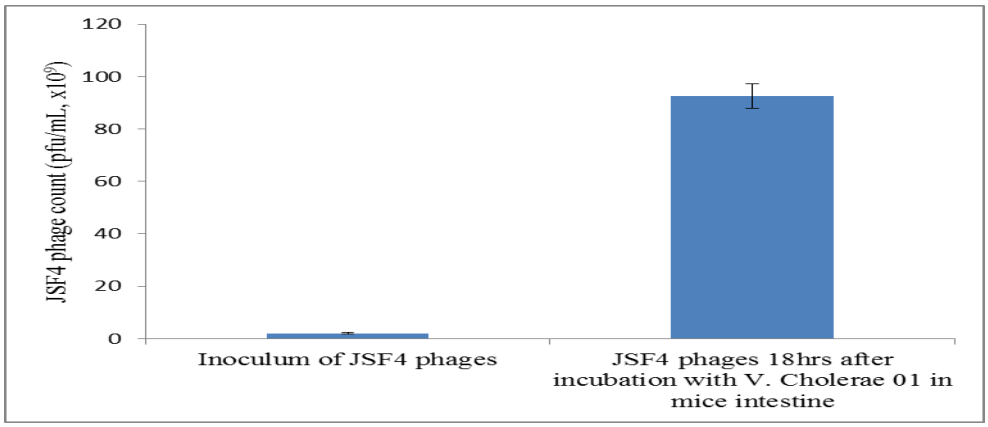

Fig 4: Count of JSF4 bacteriophage (pfu/mL) at the feeding time and 18 hrs after incubation with its host strain in the intestines of experimental batch mice. Data shown were mean±SD of ten individual mice of each group.

DISCUSSION:

V. cholerae belonging to the O1 or O139 serogroups causes severe dehydrating diarrheal disease. In Asia, Africa and many developing countries cholera has become endemic that causes a substantial global health burden (Faruque *et al.*, 2005). Every year more than 95000 deaths occur worldwide for the outbreaks of cholera, among them the majority are of children

(Islam *et al*., 2020). The people lived in the delta region are at high risk because of the frequent emergence of multiple drug-resistant *V. cholerae* serogroup strain (Islam *et al*., 2020).

The currently recommended preventatives include mass vaccinations with the World Health Organization-prequalified oral cholera vaccine and increased awareness of sanitation and hygiene practice (Qadri *et al*., 2016 ; Taylor *et al*., 2015). However, it is very difficult to clean water and vaccination campaigns are very time consuming for efficacy. So in the event of an outbreak, both methods may not be logistically feasible for immediate protection. One of the major contributors to the rapid spread of *V. cholerae* within communities is household trans-mission. Household contacts patients become sick before 2-3 days of the initial symptoms of cholera (Harris *et al*., 2008). Chemoprophylaxis with antibiotics might effectively reduce cholera burden (Reveiz *et al*., 2011), but due to the development and spread of drug-resistant bacteria, the World Health Organization does not advise this practice.

Lytic bacteriophages generally disrupt bacterial metabolism and lyse the bacterial host, indicating bactericidal activity. Besides, human phage therapy trials have shown a high level of safety without any side effects indicating safety for human applications. For many years in Eastern Europe and Russia, phage therapy has been used to treat infections caused by *V. cholerae* (Cisek *et al*., 2017).

CONCLUSION:

A novel treatment for cholera involves the therapeutic use of lytic bacteriophages. Phages are able to kill antibiotic-resistant bacteria and amount of phages increases proportionally to the number of infecting bacteria. This treatment strategy is inspired by the natural life cycle of *V. cholerae* in which blooms of the bacteria during outbreaks are followed by the expansion of lytic bacteriophage (Reyes-Robles *et al*., 2018; Silva-Valenzuela *et al*., 2019), The micro-organism we examined in this study was Gram-negative bacteria. It was suggested that the lipo-polysaccharides present in the outer membrane of Gram-negative bacteria gave them more resistance towards antibacterial agents than Gram-positive bacteria. Nevertheless, we demonstrated here potent antibacterial activity of JSF4 phages against Gram-negative enteric pathogen *V. Cholerae* 01 serogroup. In the present study, we show that the oral administration of JSF4 bacteriophages could reduce *V. cholerae* concentration in infant mice models. Given the well-documented challenges associated with the emergence of antibiotic-resistant bacteria, bacteriophages can yet provide a viable alternative to antibiotics. Moreover, this bacteriophage JSF4 can not only able to reduce the population of viable *V. Cholerae* 01 strain but also can survive a wide range of temperature, pH and salinity (Islam *et al*., 2020).

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ETHICAL STATEMENT:

All animal **e**xperimental procedures were conducted according to the NIH Guide for the Care and Use of Laboratory Animals.

CONFLICTS OF INTEREST:

All authors of this manuscript declare no conflict of interest to publish it.

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