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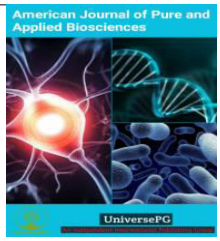

Determination of Optimum Survivability Factors of Highly Pathogenic *Vibrio cholerae* 01 Serogroup-Specific Bacteriophage JSF4ϕ

Mohammod Johirul Islam[1]*, Titash Chandra Sarker[1], Ruksana Akter Jebin[1], Farjana Yasmin[1], Mousumi Khatun[1], Md. Ashraful Islam[1], Sadia Binte Ramzan[1], Fahim Alam Nobel[1], Sharmin Akter[1], Monira Islam[1], Mohammad Mehedi Hasan[1], Mst. Mahmuda Khatun[1], Shahnaz Yesmin[1], Saima Sabrina[1], Marina Khatun[1], Abdullah Al Mamun[1], Md. Khairul Islam[1], Mohammad Asaduzzaman[2], Keshob Chandra Das[3], Mohammed Badrul Amin[4], and Mohammad Khaja Mafij Uddin[5]

[1]Dept. of Biochemistry and Molecular Biology, Mawlana Bhashani Science and Technology University, Santosh, Tangail, Bangladesh; [2]Dept. of Biochemistry, Primeasia University, Banani, Dhaka, Bangladesh; [3]Dept. of Molecular Biotechnology, National Institute of Biotechnology, Savar, Bangladesh; [4]Food Microbiology Laboratory, Laboratory Sciences and Services Division, ICDDR,B, Mohakhali, Dhaka, Bangladesh; and [5]Mycobacteriology Laboratory, Infectious Disease Division, ICDDR,B, Mohakhali, Dhaka, Bangladesh

*Correspondence: johir75@yahoo.com

ABSTRACT

Cholera is severe watery diarrhea caused by pathogenic *V. cholerae* 01 or 0139 serogroups. In each year, 2.9 millions of people are affected with cholera worldwide and 95000 deaths occur from the disease annually. In Bangladesh, around 100000 people are affected by this disease and approximately 4500 deaths occur each year. In this study, a novel *V. cholerae* 01 serogroup-specific bacteriophage JSF4ϕ was used. This phage was able to lyse both the clinical and environmental pathogenic *V. cholerae* 01 serogroup strains and one of our previous studies demonstrated that the seasonal outbreaks of cholera caused by *V. cholerae* 01 serogroup strains in Bangladesh are mostly regulated by this bacteriophage. In this current study, we determined the optimum survivability factors of JSF4ϕ bacteriophages. This study showed that the temperature 25^{0}C, pH 7 and normal saline are the optimal survivability factors for JSF4ϕ bacteriophages because, at these conditions, we have got the maximum number of plaque-forming unit (PFU/mL) of these bacteriophages. This study also showed that the JSF4ϕ bacteriophages can survive at a wide range of temperature, pH and salinity. So, the study presented here may have an impact on the controlling of cholera epidemics caused by environmental and clinical pathogenic *V. cholerae* 01 serogroup strains if we can use JSF4ϕ bacteriophages as a biocontrol agent. This study may also have profound implications for future studies of JSF4ϕ bacteriophages as a good food additive or in phage therapy for its efficient lysing capacity against the pathogenic *V. cholerae* 01 serogroup strains.

Keywords: Cholera, Diarrhea, *Vibrio cholerae*, Optimum survivability, Pathogenic, and Bacteriophages.

INTRODUCTION

Vibrio cholerae is a facultative anaerobe, gram-negative, non-spore forming curved rod, about 1.04-1.06 µm long. It is classified based on its somatic - antigens (O antigens) into serovers or serogroups, and there are at least 155 known serogroups (Maheshwari *et al*., 2011). Serogroup 01 was supposed to include all the strains responsible for epidemic and endemic

cholera. There are 154 known serogroups of non-01 vibrios. In 1992, a new serogroup, 0139 appeared in areas surrounding the Bay of Bengal produced major epidemics in India and Bangladesh, spread to neighboring countries, and continues to cause epidemic cholera in many of these areas (Shimada *et al*., 1995). *V. cholerae* 0139 was first identified in Bangladesh in 1992. This organism did not belong to any of the 138 known O serogroups of *V. cholerae* but to a new serogroup, which was later designated 0139. Hence, there are now two serogroups of *V. cholerae*, 01 and 0139 that have been associated with epidemic disease. Vibrios are the most common organisms in surface waters of the world (Jensen *et al*., 2006). They occur in both marine and freshwater habitats and associations with aquatic animals (Jensen *et al*., 2006, Zo *et al*., 2002, Sack *et al*., 2003). Cholera, whose "traditional home" has been the Ganges Delta and Southeast Asia, is one of man's oldest scourges. Cholera is characterized by severe watery diarrhea caused by pathogenic *V. cholerae*. A healthy person may become hypotensive within an hour of the onset of symptoms and may die within 2-3 hours if no treatment is provided (Todar *et al*., 2006; and Islam *et al*., 2020).

The meaning of bacteriophage is bacteria eater. Bacteriophages are approximately 50 times smaller than bacteria and ubiquitous in the soil, water, and several food products. They have also been isolated from humans and animals, for example from feces, urine, saliva, spit and serum (Jończyk *et al*., 2011). About 96% of them are tailed, but there are filamentous and pleomorphic ones as well (Jończyk *et al*., 2011, Ackermann *et al*., 2007, Hendrix *et al*., 2002). Generally, the phage virion consists of two basic components: nucleic acid (double- or single-stranded RNA or DNA) and a protein envelope (Jończyk *et al*., 2011; and Ackermann *et al*., 2003).

In this study, a novel *V. cholerae* 01 serogroup-specific bacteriophage JSF4ϕ, that infects both clinical and environmental pathogenic *V. cholerae* 01 strains were used. The outbreaks of cholera in Bangladesh are usually caused by pathogenic *V. cholerae* 01 or 0139 strains (Faruque *et al*., 2005). Since the JSF4ϕ bacteriophages are very efficient to lyse both the clinical and environmental pathogenic *V. cholerae* 01 serogroup strains and one of our previous studies showed that the seasonal outbreaks of cholera caused by *V. cholerae* 01 serogroup in Bangladesh are mostly regulated by this bacteriophage (Faruque *et al*., 2005). So, it is important to know what the optimum survivability factors of these bacteriophages are. In this current study, we tried to determine the survivability factors of this bacteriophage and we have found that the temperature 25°C, pH 7 and normal saline are the optimal survivability factors for JSF4ϕ bacteriophages because, at these conditions, we have got the maximum number of plaque-forming unit (PFU/mL) of these bacteriophages. This study also showed that the JSF4ϕ bacteriophages can survive at a wide range of temperature, pH and salinity (Uddin *et al*., 2014).

So, the study presented here may have an impact on the controlling of cholera epidemics caused by the environmental and clinical pathogenic *V. cholerae* 01 strains if we can use JSF4ϕ bacteriophages as a biocontrol agent in future. Since *V. cholerae* 01 serogroup strains are found in different types of food samples categorically including meat, fish, vegetables, fruits, street food, bakery shop food, fast food, sweets and dairy products (Mrityunjoy *et al*., 2013, . Lee *et al*., 2014), in future, we can also use JSF4ϕ bacteriophages as a good food additive or in phage therapy with its efficient lysing capacity against pathogenic *V. cholerae* 01 strains.

MATERIALS & METHODS

Media preparation: To prepare a nutrient broth, add 13g of nutrient broth powder in 1L of distilled water. Mix and dissolve them completely. Pour them into the conical flask. Sterilize by autoclaving at 121°C for 20 minutes. To prepare nutrient agar plat, dissolve the dehydrated medium in the appropriate volume of distilled water i.e., 28 gm dehydrated nutrient agar in 1000 mL of distilled water. Sterilize by autoclaving at 121°C for 20 minutes and pour the sterilized media into 10 cm Petri dishes.

Growth of bacterial culture: Mainly two types of *V. cholerae* 01 serogroup strains were used in this experiment, one is clinical named N-16961 and

another is environmental named Env-201. 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 a nutrient agar plate and incubated at 37°C for overnight.

Collection and detection of *V. cholerae* 01 serogroup-specific bacteriophage JSF4ϕ: We have collected *V. cholerae* 01 serogroup specific bacteriophage JSF4ϕ from Molecular Genetics Laboratory, International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). Faruque *et.al* isolated and purified this bacteriophage from environmental waters in Bangladesh (Faruque *et al*., 2005). After collection, we enriched the phage in *V. cholerae* 01 hosts strain named N-16961, then filtered with 0.22 µm pore-sizes of filters (Millipore Corporation, Bedford, Mass.) to exclude bacteria and maintained the phage stock in normal saline at room temperature. We then detected and quantified JSF4 bacteriophages by the standard double layer plaque assay method as described previously (Faruque *et al*., 2005) and below.

Quantification of JSF4 bacteriophages: In order to enumerate *V. cholerae* 01 serogroup specific bacteriophages JSF4 by plaque assay method, logarithmic-phase cells (500µL) of host *V. cholerae* 01 strain in nutrient broth were mixed with 3.5 ml aliquots of soft agar (nutrient broth containing 0.8% Agar), and the mixtures were over layed on nutrient agar plates. Dilutions of JSF4ϕ bacteriophages were inoculated on the plates, and the plates were incubated for 16 h at 37°C. A sample was considered positive for bacteriophages when a plaque was observed on the bacterial lawn. Plaques were counted to estimate the concentration of bacteriophage particles in the sample and expressed as PFU/mL.

Effect of temperature on the survivability of JSF4 bacteriophages: Temperature is a crucial factor for bacteriophage survivability. It plays a fundamental role in attachment, penetration, multiplication. At lower than optimal temperatures, fewer phages genetic material penetrates bacterial host cells; therefore, fewer of them can be involved in the multiplication phase. Higher temperatures can prolong the length of the latent stage. Moreover, temperature determines the occurrence, viability, and storage of bacteriophages. The impact of temperature on the JSF4ϕ bacteriophages was examined at distinctive temperatures i.e. 15°C, 20°C, 25°C, 30°C, 40°C, 50°C.

To the determination of the optimum phage survivability at different temperatures, an equal number of JSF4ϕ bacteriophages from the stock was added to normal saline, and the mixtures were incubated in preheated water at 15°C, 20°C, 25°C, 30°C, 40°C, and 50°C for 24 hours. After 24 hours incubation, the phage titers were estimated by the double-layer plaque assay method.

Effect of pH on the survivability of JSF4ϕ bacteriophages: Development and survivability of microorganisms are fundamentally affected by nature's pH. Bacteriophages can grow and develop at a particular pH range. This experiment was performed to determine the impact of pH on the survivability of JSF4ϕ bacteriophages. To perform this experiment, an equal number of JSF4ϕ bacteriophages from the stock were added to normal saline and the pH of saline was adjusted from 5 to 9 by using HCl or NaOH solution. The mixtures were then incubated for 24 hours at room temperature. After 24 hours incubation, the phage titers were quantified by the double-layer plaque assay method.

Effect of salinity on the survivability of JSF4ϕ bacteriophages: Osmotic shock has been shown to inactivate bacteriophages (Jończyk *et al*., 2011) Bacteriophages can survive and show infectivity inside the cell at a particular saline concentration. The impact of salinity on JSF4ϕ bacteriophages was examined at different saline concentrations i.e 1.2N, 0.9N, 0.7N, 0.3N.

To the determination of the optimum phage survivability at different saline concentrations, an equal number of JSF4ϕ bacteriophages from the phage stock were added to the aforementioned saline concentrations and the mixtures were then incubated at room temperature for 24 hours. After 24 hours incubation, the phage titers were counted by the double-layer plaque assay method.

RESULTS & OBSERVATION

Effect of temperature on the lytic activity of JSF4ϕ bacteriophages: Temperature is one of the most important environmental factors that strongly affect many aspects of the biological systems (Taj *et al.*, 2014, Young *et al.*, 2000, Wang *et al.*, 2000). Temperature is a crucial factor for bacteriophage survivability (Jończyk *et al.*, 2011, Olson *et al.*, 2004, Srinivasan *et al.*, 2007). Thermal survivability of *V. cholerae* 01 serogroup-specific bacteriophage JSF4ϕ was determined after calculating the PFU in per millilitre of incubated samples through the appeared plaque count on the host bacterial strain (**Fig 1A** & **Fig 1B**).

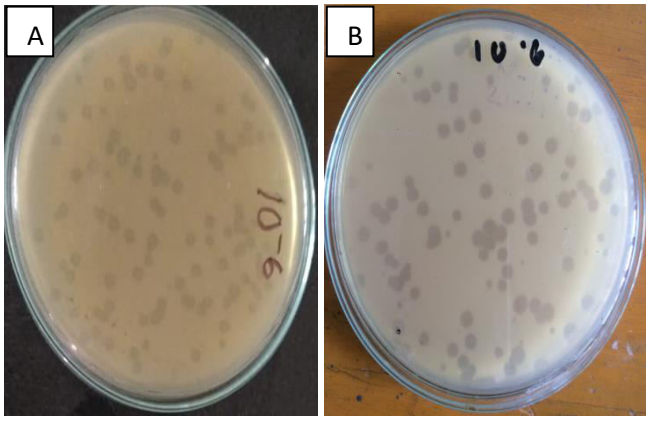

Fig 1: (A) Plaques of JSF4ϕ bacteriophages on the lawn of host clinical *V. cholerae* 01 strain N-16961; (B) Plaques of JSF4ϕ bacteriophages on the lawn of host environmental *V. cholerae* 01 strain Env-201.

The impact of temperature on JSF4ϕ bacteriophages was examined at distinctive temperatures i.e., 15°C, 20°C, 25°C, 30°C, 40°C, 50°C. It was found that the temperature 25°C is the optimal temperature for the survivability of JSF4ϕ bacteriophages because at this temperature we have got the maximum number of phage particles ($1.62x10^{11}$ PFU/mL) in the sample (**Fig 2**).

The lytic activity gradually declined when the bacteriophages were treated at less than or greater than 25°C. No plaques were observed at 50°C (**Fig 2**). It might be due to the protein coat denaturation of bacteriophages. So, at this temperature, no phage particles were able to infect bacteria.

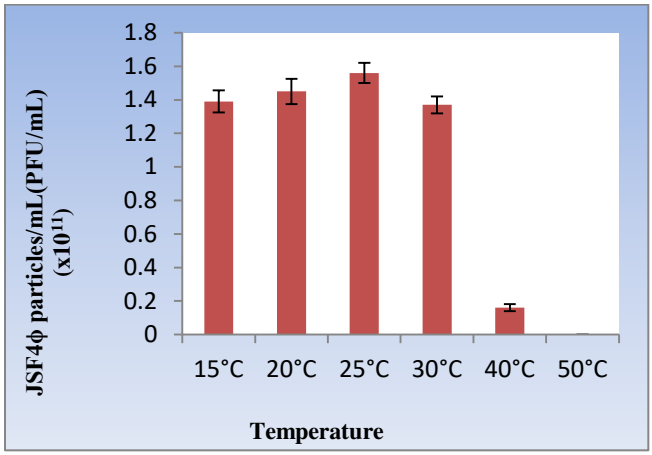

Fig 2: Effect of temperature on the lytic activity of JSF4ϕ bacteriophages. Data shown are mean ± S.D. of three individual experiments of each group of temperature.

Effect of pH on the lytic activity of JSF4ϕ bacteriophages: pH is an important control factor for influencing attachment, infectivity, intracellular replication and multiplication of bacteriophages (Chatain-ly *et al.*, 2014). Generally, studies on the lytic activity of bacteriophages have shown them to be most sensitive to pH values less than 5 and over 10.

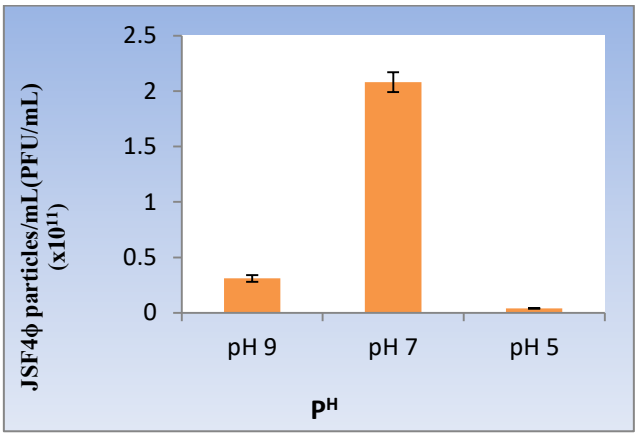

Fig 3: Effect of pH on the lytic activity of JSF4ϕ bacteriophages. Data shown are mean ± S.D. of three individual experiments of each group of pH.

In the current study, we have found that the lytic activity of the JSF4ϕ bacteriophages varied in the range of pH 5 to 9 and the lysis activity showed highest at pH 7 ($2.08x10^{11}$ PFU/mL) (**Fig 3**). The lysis activity of JSF4ϕ bacteriophages was gradually decreased when the bacteriophages were treated at less than or greater than of pH 7 (**Fig 3**). This result

indicates that pH 7 is the optimal survival factor for JSF4ϕ bacteriophages. At less than or greater than of pH 7, the PFU of JSF4ϕ bacteriophages decreased, it might be due to the alteration of the protein coat of bacteriophages, so the infectious capacity of phages becomes reduced.

Effect of salinity on the lytic activity of JSF4ϕ bacteriophages: Osmotic shock has been shown to inactivate bacteriophages (Jończyk *et al*., 2011). The impact of salinity on JSF4ϕ bacteriophages was examined at different saline concentrations i.e., 1.2N, 0.9N, 0.7N, 0.3N. From this experiment, we have found that the normal saline concentration (0.9N NaCl solution) is the optimal survivability factor for JSF4ϕ bacteriophages because at this concentration the bacteriophage JSF4ϕ showed the highest lytic activity ($1.39x10^{11}$ PFU/mL) (**Fig 4**). The lytic activity was gradually declined when the bacteriophages were treated at less than or greater than of 0.9N NaCl solution (**Fig 4**). This indicates that at less than or greater than of 0.9N NaCl solution, the PFU of JSF4ϕ bacteriophages gradually reduced, it might be due to the osmotic shock of bacteriophages.

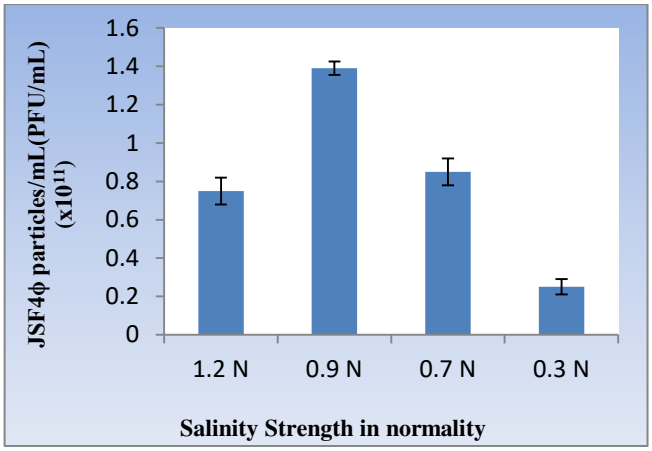

Fig 4: Effect of salinity on the lytic activity of JSF4ϕ bacteriophages. Data shown are mean ± S.D. of three individual experiments of each group of sodium chloride concentration.

DISCUSSION

Epidemics of cholera caused by pathogenic *V. cholerae* belonging to the 01 or 0139 serogroups are a major public health problem in many developing countries of Asia, Africa, and Latin America. In Bangladesh, all the cholera pandemics except the seventh started because the people of this region come into direct contact with the surface water for drinking, bathing, cooking, and irrigation. Outbreaks of cholera caused deaths estimated at 95000 annually worldwide and many more cases each year, of which the vast majority occurs in children. It is threatening for people lived in the delta region for frequent emergence of multiple drug resistance *V. cholerae* 01 serogroup strains (Das *et al*., 2011; and Faruque *et al*., 2007).

The antibiotic resistance of *V. cholerae* 01 serogroup suggested that the development of alternative therapy is immediately needed. 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 (Bruttin *et al*., 2005, Sarker *et al*., 2016). Phages can be applied by oral, intraperitoneal, intravenous, or intranasal administration (Chatain-ly *et al*., 2014). *V. cholerae* isolated from 40 types of food samples categorically including meat, fish, vegetables, fruits, street food, bakery shop food, fast food, sweets, dairy products and seafood (Mrityunjoy *et al*., 2013). In 2006, the FDA approved the use and the preparation of bacteriophages generally recognized as safe (GRAS) as food additives for the control of the pathogenic bacterium *L. monocytogenes* in meat and poultry products (Chatain-ly *et al*., 2014).

Our current phage survivability study showed that the bacteriophage JSF4ϕ can survive under a wide range of temperatures, pH and salinity. Moreover, these bacteriophages JSF4ϕ are strongly able to lyse both the clinical and environmental pathogenic *V. cholerae* 01 serogroup strains suggesting that the bactericidal activity of these bacteriophages should be retained during food processing and preservation. So, we can use these bacteriophages as a good food additive in future. This bacteriophage can also be used as a good biocontrol agent in controlling the epidemics of cholera caused by pathogenic *V. cholerae* 01 serogroup strains. Furthermore, these bacteriophages JSF4ϕ can be used in phage therapy with its efficient lysing capacity against *V. cholerae* 01 serogroup strains.

CONCLUSION

In conclusion, our study demonstrated the survival parameters of *V. cholerae* 01 serogroup-specific bacteriophages JSF4ϕ in laboratory condition. Even though this study did not simulate the survivability factors of the actual aquatic environment where these phages were habituated and first isolated from. As per as we know this study is the first of its kind for *V. cholerae* 01 serogroup-specific bacteriophages JSF4ϕ. We hope this study may have an impact on the controlling of cholera epidemics caused by environmental and clinical pathogenic *V. cholerae* 01 strains if we can use JSF4ϕ bacteriophages as a biocontrol agent. This study will also have profound implications for future studies of JSF4ϕ bacteriophages as a good food additive or in phage therapy for its efficient lysing activity.

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CONFLICT OF INTEREST

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

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