RESEARCH ARTICLE

Detection of Bacteriophages against *ESKAPE* Group of Nosocomial Pathogens from Ganga River Water During Community Bath at Various Rituals: Since 2013–2019

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Abstract

Introduction: Several species of bacterial contaminants are at a high level in river Ganga water, but the question arises: Why Ganga water has not been spoiled? Even left for a long time and answer is a presence of biological components, including bacteriophage and bioactive components such as nanoparticles.

Objective: In the present study, we aimed to detect bacteriophages of resistant microbes such as *the ESKAPE* group of nosocomial and *S. Typhi* from different Ganga water samples collected on different rituals.

Material and Methods: This study started in 2013 and completed in 2020. As per the study design, water sample from different places (Prayagraj, Mirzapur, and Varanasi) and sites were collected. A total 210 strains (30 each) of *Enterococcus faecium (E. faecium), Staphylococcus aureus* (*S. aureus*), *Klebsiella pneumoniae (K. pneumoniae), Acinetobacter baumanii (A. baumannii), Pseudomonas aeruginosa (P. aeruginosa), Escherichia coli* (*E. coli*) (Called as *ESKAPE* group) and additionally *S. Typhi* were identified from the in 500 clinical samples. These identified strains were processed for their biochemical test microscopy and antibiotic sensitivity for its conformation. Confirmed *ESKAPE* and *S. Typhi* strains were used for lawn culture. The bacteriophages were isolated from the collected Ganga water samples by using the double layer agar assay method.

Results and Discussion: Bacteriophages were observed in the form of plaques on the bacterial lawn culture. Among 210 strains (30 each) of *E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa, E. coli* and *S. Typhi* total 52 phages were detected in the form of plaques on the bacterial lawn culture. Maximum no of phage sensitivity were identified with *E. coli* (13) then in *S. aureus* (11). Eight phages of ware specific to *S. Typhi* and seven were specific to *P. aeruginosa* and how ever in six phages are specific to *K. pneumoniae* and *E. faecium.* Minimum no of phage sensitivity were identified with *A. baumanii* (1).

Conclusion: Our study concludes that Ganga water is a huge source of above-detected bacteriophages among all possible natural sources with full of diversity. This is the development of a phage bank, which will be useful for bacteriophage therapy in the near future.

Keywords: Biochemical test, Double-layer agar assay, *ESKAPE* group of nosocomial, Microscopy, River Ganga Bacteriophage.

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Introduction

The great river Ganga is a divine and longest river of India arising in the Himalaya Mountains and flowing about 2,601 km (1,616 mi) generally eastward through a vast plain to the Bay of Bengal. It flows southeast through the Indian states of Uttar Pradesh, Bihar, and West Bengal and millions of people depend on the water such as for: drinking, bathing, agriculture, industry, and other household chores. [1] In between every 6 and 12 year, millions of people come and take a bath in the Ganga water for their mythological regions known as Kumbh and Maha Kumbh. Mahakumbh (at every 12th year) is the largest community bath of world and important Hindu ritual held on the banks of Sangam (Ganga, Yamuna, and Saraswati) in Prayagraj. Mahakumbh is a combination of various rituals that starts from Makar Sankranti in December/January and ends at Maha Shivratri in February/March.[2] Few workers had been repotted faecal bacterial contaminants such as *Actinomyces* sp., *Aerobacter aerogenes (A. aerogenes), Aerobacter cloacae (A. cloacae), Micrococcus* sp., Salmonella sp., *Staphylococcus aureus (S. aureus), Bacillus* sp. *Escherichia coli (E. coli), Enterococcus faecium (E. faecium), Pseudomonas aeruginosa (P. aeruginosa), Acinetobacter baumanii (A. baumanii), Mycobacterium tuberculosis (M. tuberculosis)* and *Shigella* sp. in Ganga water.[3] A group of such bacterium commonly pronounced as *ESKAPE* stands; *E. faecium, S. aureus, Klebsiella pneumoniae (K. pneumoniae), A. baumannii, P. aeruginosa,* and *E. coli* like enterobacter species. [4] These are basically nosocomial be indebted as *superbug* status not to enhanced pathogenicity or virulence but to their resistance to multiple antimicrobial agents. *S. aureus* is a frequent component of the human microbial pathogenic flora capable of causing a wide range of human diseases. Most of strains do not respond to treatments with almost all known and powerful antibiotics; therefore, it has also been termed as "golden *superbug*".[5] In our country recently reported that *superbugs* recognized as New Delhi metallo-beta-lactamase-1 (NDM-1). In NDM-1 transmissible genetic element encoding, multiple

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Conflict of interest: None

resistance genes were initially isolated from a strain of *Klebsiella* obtained from a patient who acquired the organism in New Delhi, India. The present study designed and framed with the objective of the discovery of *bacteriophage* from Ganga water samples against antibiotic-resistant isolates of phatogenic bacteria and its quantitative load evaluation of *bacteriophages* on a different ritual date between MahaKumbh. The above facts are keeping in mind and design this study to explore how many types of bacteriophages in Ganga water. We had collected water sample during Mahakumbh Period (14th January – 10th March 2013) because at this time population density of Ganga bath is very high, and comparison makes between Preretual bath (05/12/2012) and post ritual bath (05/05/2013 and 30/06/2013) with the Ardh Kumbh that was held in 2019 (15th January– 4th March 2019) and comparison made between Preretual bath (25/12/2018) and post ritual bath (05/05/2019 and 30/6/19). It is assumed that so many people hold so many types of pathogenic infection. So far bacteriophage identification against *various* infections was our main objective.

Materials and Methods

Culture collection

This study conducted in between 2013-2020 at the Medical Laboratory Technology, DDU Kaushal Kendra, RGSC and Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi with the initial grant of Council of Scientific and Industrial Research, New Delhi (No.: 9/13(306)/2010-EMR-I).

A total 500 strains of pathogen were collected from the bacteriology section. These strains were isolated from various clinical specimens, including pus, wound swab aspirates, etc. *ESKAPE* group member and *S. Typhi* strains were confirmed as using microscopic[6] and biochemical observation[7] and antibiotic sensitivity.

Collection of water samples

To isolate bacteriophages, water samples were collected from different sources; these were mainly from river Ganga at different places, including Prayagraj, Mirzapur, and Varanasi and site at different rituals (Table 1). From a site, nine samples were collected: three form corners, three from middle, and three from main river flow.

Removal of water contamination and bacteriophages sensitization

Previously developed Kisselgurh G filtration technique (2.5% Kisselgurh G with 15% $CaSO_4$) with our lab modifications[8] was applied for the removal of contaminants from the water sample. Briefly, *culture* strain of each was inoculated in 5.0 mL of Luria burtini (LB) broth and incubated at 37°C overnight. The second day 5.0 mL of water sample was added with 5.0 mL of 2XLB broth, and 10 µL of log phage culture of *bacterium* was added. It was incubated overnight at 37°C in a water bath shaker. Third day it was centrifuged, and supernatant was collected. 1.0 mL of supernatant was taken in a microcentrifuge tube and 1% of chloroform was added and then centrifuged at 10,000 rpm for 10 minutes. The resulting supernatant was sensitized, and it was collected for the phage generation.

Table 1: Proposed natural place/site of water to use for isolation of bacteriophages.

No.	*Prayagraj*	*Mirzapur*	*Varanasi*
1.	Shringaverpur ghat	Vindhyachal ghat	Ramnagar Ghat
2.	Phaphamau ghat	Sastripul ghat	Assi ghat
3.	Nagvasuki ghat		Harichandra ghat
4.	Sangam ghat		Dasaswmegh ghat
5.	Kila ghat		Manikarnika ghat
6.	Arail ghat		Varuna ghat

Isolation and harvesting of bacteriophage

The overlay method for plaques formation was used with our lab modifications.[9] In the overlay method, 100 µL of the above supernatant was added with 890 µL TMG (Tris $MgSO_4$ and Gelatin) buffer and 10 µL of log-phase *bacterial* culture. The total liquid content in the test tube was mixed and incubated at 37°C at least for 20 min in water bath shaker. After incubation 4.0 mL of soft agar (cooled to 50°C) added to the test tube and immediately pour on the respective Mueller Hinton (MH) agar plates. Plates were swirled gently to spread the liquid contents on the whole plate. It was allowed to solidify, and after solidification, the plates were incubated at 37°C for overnight. The surface of the lawn culture containing plaques on the plates was washed with TMG (Tris $MgSO_4$ and Gelatin) buffer with the help of a cotton swab. Washed out were collected in centrifuge tubes, and 1% of chloroform was added mixed by repeated inversion. The centrifuge tubes were centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was collected and allowed it for re-centrifugation.

Bulk production and concentration of bacteriophages

The plaque assay technique processed harvested phages. At which the confluent plaques were obtained, used for bulk production. The supernatant was treated with polyethylene glycol (PEG)-6,000/2.5M NaCl solution. Then it was incubated overnight at 4°C. Next day supernatants were centrifuged at 15,000 rpm for 30 minutes, and milky pellets were collected. The centrifugation processes were continued 2-3 times to remove all the PEG solutions with STE buffer. The milky pellets were dissolved in 80 µL STE buffer solution and then stored for further use.

Sensitivity and specificity analysis of bacteriophages

Total isolated phages were used to determine their activity with their specific host strain including *E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa, E. coli* and *S. Typhi* (0.5 OD on McFarland represented 1.5 X 10^8 CFU per ml) were swabbed over the plates and incubated for 3.0 hour at 37°C to maintain the bacteria in the form of log phase. Subsequent to incubation, 5 µL of respective bacteriophage with 10^{12}pfu were dropped on swabbed MH agar plates according to the bacteriophage naming. Phage lytic zone was examined the next day to analyze bacteriophage sensitivity. On the basis of their sensitivity pattern NTYS dendrogram tree plot was plotted for it and analysys was made.

Results

Host identification for isolation of bacteriophage from different water sources

Among 500 isolates 30 isolates of each *E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa, E. coli* and *S. Typhi* were identified without any other contamination grouped as Gram +ve (*E. faecium, S. aureus*) and -ve (*K. pneumoniae, A. baumannii P. aeruginosa, E. coli*, and *S. Typhi*) (Figure 1) were used for the isolation of bacteriophage from the water.

Identification and confirmation of bacteriophages

The identification was done by the microscopic observation and by biochemical tests of the bacterial culture growing around the plaques (Table 2). After the observation of the incubated plates, the number of plaques on the bacterial lawn in four respective plates from 10^1 to 10^4 was seen. It means that, the harvested phage supernatant contained 10^4 PFU/mL. By the plaque assay it also confirmed the presence of phage for the respective bacteria (Figure 2).

Sensitivity Analysis of Bacteriophages on Host

Isolated 52 bacteriophages were checked for the sensitivity analysis over *ESKAPE* and *S. typhi* strains. 30 MH agar media plates of each *ESKAPE* and *S. typhi* were prepared and marked 1 to 52 and all the strains were swabbed over these plates by using sterile cotton swabs (Figure 3). Maximum no of phage sensitivity (Figure 4) were identified with *E. coli* (13) then in *S. aureus* (11). Eight phages of ware specific to *S. Typhi* and seven were specific to *P. aeruginosa* and how ever in six phages are specific to *K. pneumoniae* and *E. faecium*. Minimum no of phage sensitivity were identified with *A. baumanii (1).*

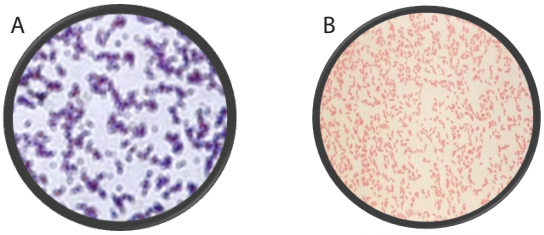

Figure 1: Slide processed for Gram-staining to observe under a microscope. A Gram-positive B Gram-negative

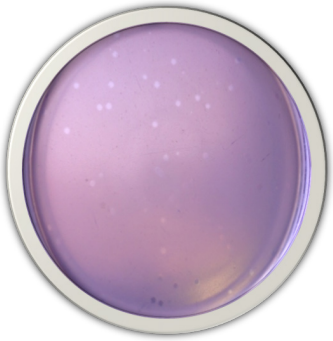

Figure 2: Representative culture plate for the isolation and propagation of bacteriophages against *ESKAPE* and *S. typhi* pathogen from different sources of water samples.

Table 2: Water samples that form plaques on lawn culture and their sensitivity to strain

No.	*Water sample*	*Plaque formation*	*Sensitivity observed with the ESKAPE and S. typhi Strain*
1	Shringaverpur ghat	No	None
2	Phaphamau ghat	No	*E. coli*
3	Nagvasuki ghat	Yes	*E. coli*
4	Sangam ghat	Yes	*S. aureus*, *E. coli*
5	Kila Ghat	Yes	*P. aeruginosa*
6	Arail Ghat	No	None
7	Vindhyachal ghat	No	None
8	Sastripul ghat	Yes	*K. pneumoniae*
9	Ramnagar ghat	No	None
10	Assi ghat	Yes	*S. Typhi*
11	Harichandra ghat	Yes	*A. baumannii*
12	Dasaswmegh ghat	Yes	*E. coli*
13	Manikarnika ghat	Yes	*P. aeruginosa*
14	Varuna ghat	Yes	*E. faecium*

Discussion

In 2010 reported the *emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK* hospitals. The report was with modest convincing scientific evidence. This report makes comprehensive conclusions against surgical treatment opting in India. In this regard, the government of India framed a Task Force to review the current situation regarding the manufacture, use, and misuse of antibiotics in the country and recommend the design for the creation of a National Surveillance System for Antibiotic Resistance (NSSAR).[10] In this situation, we are compelled to explain possibilities for cure of such type of MDR pathogens. Among many options bacteriophages may be new hope for their treatment.

Bacteriophage was discovered in 1915 by British microbiologist Felix Twort and French-Canadian microbiologist Felix d'Hérelle in 1917. These are viruses that infect and can kill bacteria.[11] d'Hérelle systematically described the nature of bacteriophages and explored their ability to function as therapeutic agents.[12-15] Bacteriophages are very specific; they attack only host bacterial cells without any effect on normal microflora. In the environment, bacteriophages

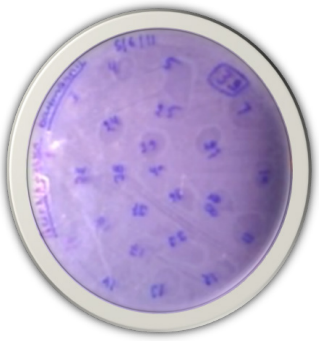

Figure 3: Sensitivity of 5 µL phage ingestible was performing on to the host swabbed plate.

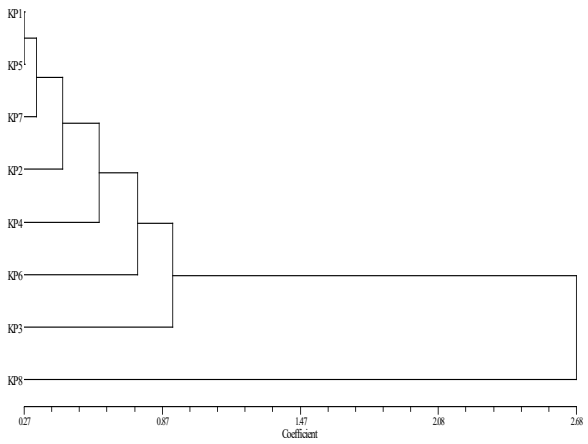

Figure 4: Dendogram representation of phage sensitivity of *ESKAPE* group member and *S. typhi.*

are commensal with animal or plants cells.[16] Bacteriophages appear too evolved with bacteria as they are ubiquitous in nature. Increasing antibiotics-resistance is one of the reasons for the growing interest in the therapeutic use of bacteriophages. Phages continue to be used for bacterial infections, in place of antibiotics, in Soviet Union and Eastern Europe.[17,18] Since more than eighty years, phage therapy was used as bio-agents for the treatment of bacterial infections.[19] The therapeutic efficacy of phage therapy proved in cases of *P. aeruginosa,*[20] *S. aureus* (including Methicillin-resistant *S. aureus:* MRSA),[21] *E. coli,*[22] *E. faecium* (including Vancomycin-resistant *Enterococcus:* VRE),[23] and *Streptococcus pneumoniae* (*S. pneumoniae*)[24] associated experimental animal models. However, the models used in these studies were simple models of infection that did not closely resemble the pathophysiology of human diseases.[25] To date, the increasing problems related to the worldwide emergence of antibiotic resistance in common pathogenic bacteria. We had discovered bacteriophages represents a potentially viable alternative to antibiotics and to other antibacterial compounds to inactivate indigenous and non-indigenous pathogenic bacteria.[26-27]

Our experiment was performed for the isolation of bacteriophages of *seven strains* from the different sources of aquatic environments, where it was observed the presence vast population of bacteriophages. For the isolation of bacteriophages, we collected the three places of water samples, including river Ganga water at Prayagraj, Mirzapur, and Varanasi from at least six different locations. Recombinant protein StaphTAME from the bacteriophages, isolated from Ganges water[28] that proved active against the antibiotic-resistant *S. aureus*. Our study also proves Ganga river water may be good source of *S. aureus* bacteriophage that is successfully used as therapy in an animal model.[26] The bacteriophages were isolated by using the double layer agar assay method. As a result the plaques were observed on the bacterial lawn, which confirms the presence of bacteriophages in the water samples that had been collected. The plaques are the cleared zone on the bacterial lawn that has been raised due to killing of bacteria by the bacteriophages present in the water samples. The killing efficiency of bacteriophage is due to the presence of two enzymes, endolysin and holin.[29] Endolysins are the phage-encoded peptidoglycan hydrolyzes, which produced in phage infected bacterial cells towards the end of the lytic cycle of phage. They reach the peptidoglycan through the membrane lesions formed by holin and cleave it, inducing the lysis of the bacterial cell and enabling the progeny phages to be released.[30-31] As per our aim, bacteriophages of *ESKAPE* group member and *S. typhi*. from different sources of water samples have been identified, and this discovery very useful in therapeutics.[32-33]

Conclusion

Phages are very specific; hence do not harm the commensal microflora, thus no side effects, is an advantage of phage therapy. Phages multiply at the site of infection till the host is eliminated, then they are excreted, and specific phage resistant bacteria can be destroyed by other phages. Phages are easily isolated from the source because their evolution occurs along with the evolution of bacteria. The emergence of antibiotic and multiple drug-resistant bacteria such as *ESKAPE* group member *S. typhi* and *Superbugs* are a major concern which emphasizes the need for alternate means of antibacterial therapy renewing the interest in phage mediated control of pathogenic bacteria. With the above advantageous properties, the phage treatment has extended from the medical field to others such as agriculture, fisheries, food industry, and wastewater treatments. Our studies conclude various natural interactions among microbes boost phage generation as we see in the case of community bath in Ganga River induced high number of diversified bacteriophages.

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