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Comparative study of Different Water Sources for Presence of Bacteriophages against Bacterial strains

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ABSTRACT

There are number of antibiotics or drugs which are found non-effective against various diseases caused by specific pathogens. The current challenges of multi-drug resistance (MDR), bacteria have compelled to find alternative antibacterial agents. Bacteriophages as lytic virus for bacteria seem a potential candidate to develop alternative antimicrobial agents. Bacteriophage specificity towards bacteria make it unique tool to counter bacterial infection and pathogens. In current comparative study, an attempt was made to isolate and screen bacteriophage against bacterial strains from different water sources as sewage, rain and pond against *Pseudomonas strain (Gram-negative)* and *Staphylococcus strain (Gram-positive)*. Water samples were filtered by using of 0.45µM and 0.2µM syringe filters. Double layer agar method was used to isolate specific bacteriophage. Number of plaques/concentration of Bacteriophage is observed better with sewage water sample filtered through 0.45µM pore size syringe filters. For further characterization, isolated bacteriophage was enriched in liquid culture and further concentrated through using filter membrane of 0.45µM. Further studies will be carried out to standardize the optimal viral dose to control growth of its respective host rapidly and to check its cross activity.

Keywords: Bacteriophage, Comparative study, double layer agar method, liquid method, *Pseudomonas strain*, *Staphylococcus strain*.

INTRODUCTION

The pathogenic bacteria are mainly resistant to different types of antibiotics nowadays.[1] This uncontrolled resistive nature of microorganisms emerging novel challenges worldwide[2]. Some modern medicines are also ineffective against a variety of microorganisms[3,4]. Bacteriophage can be a sustainable solution of these novel challenges[5]. It can be used as a modern weapon against the resistive nature of pathogenic bacteria[6,7]. Bacterial infection can be easily treated by using Bacteriophage due to its specificity and various applications[8,9,10].

Bacteriophage is a diverse entity that belongs to the prokaryotic microbial virus family[11,12]. It can digest different types of bacterial strains easily due to its infective mechanism against pathogens[13]. In India, Bacteriophage was first reported in 1896 by Ernes Hankin during experiments with river water

sample[14]. The bacteriophage mechanism depends on the availability of a specific host in the form of bacteria[15]. Due to its specificity, Bacteriophage has been used for many years for different types of applications[16]. There are a few examples like drug-resistant bacterial infections[17,18,19], pathogens in food items[20], water infection[21,22], bacterial diseases[23], and evaluation of bacteria[24]. Bacteriophages can survive at every environment in parallel with bacteria strains[25]. Bacteriophages are acting as divine in today's health sector[26] as they act specifically against bacteria[27] without affecting humans because bacteriophages become inactive after complete digestion of bacteria. Composition of the bacteriophages depends upon the nucleic entity[28,29] that can be RNA or DNA, but never exists together. The nucleic entity of phages generally has modified bases. It protects the phages from its lysis which ultimately avoids phage infection[30]. In normal phages, the number of genes is very less while more than 100 genes exist with complex phages[31]. There are some specific phages called single-stranded DNA due to the design of their nucleic entity[32]. The infection process of phages initiates from its adsorption to the bacterial cells[33]. Phages attract towards available receptors on the bacterial cell. Bacterial fluids and its structural entities in the form of protein help in this specific attachment. The hollow tail helps nucleic acid to pass through and to enter inside bacterial cell[34]. The remaining part of the phage outside can be called as "ghost". Some bacterial entities do not adopt this infection process. For such cases, phage is injected artificially which is known as transfection. Based on their life cycle, Phages are of two types as lytic and lysogenic[35]. Lytic phages ensure the killing of bacteria after infection while lysogenic phages are responsible for infection only. Phages have a sequential growth process in lytic cycle. It includes, the growth of phage components, required assembly, development of matured cells and finally the release of fully developed cells. The bacterial cell wall initiates to penetrate due to continuous accumulation of phage lysis protein which helps to release the intracellular phage into the medium. Phages have specific enzymes[36] which are responsible for weakening the bacterial cell wall. Phages releases more than 1000 of particles per infected bacterial cell, which is calculated in terms of burst size[34]. Burst size is considered as yield average of phages for a specific bacterial cell.

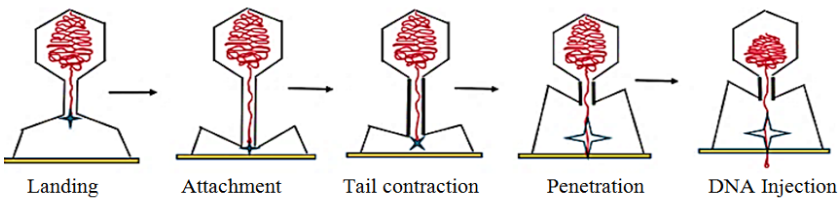


Fig. 1. Infection mechanism of Bacteriophage

These viral forms (phages) can be isolated from different types of sewage water samples[37]. There are the number of techniques to isolate it followed by purification and characterization[38,39]. In this study, samples were collected from different water sources such as rain, pond, and sewage for potential presence of the bacteriophages. Two types of bacterial strains i.e. *Pseudomonas* and *Staphylococcus strains* were isolated from household wastewater. These bacterial species were isolated and differentiated by using selective media. Some specific tests like serial dilution, gram staining, streaking and spreading on selective media, culture purification, and filtration with different pore size filters were performed for isolation, identification, and characterization of these bacterial strains. Bacteriophages were isolated by using two different methods viz. "double layer agar method" and "liquid method". Agar plates having plaques and filtrate of liquid technique were used for screening and further characterization of bacteriophages.

MATERIALS AND METHODS

Nutrient Agar, Nutrient Broth, Agar Powder, Mannitol Salt Agar and Cetrimide Agar of Himedia were used for media preparation. Gram staining kit of Himedia was used to evaluate the type of bacterial strains. Qualified and calibrated equipment like Autoclave, Laminar Air Flow, Incubators, Microscope and Cooling incubator etc. were used for test performance, incubation and storage of required solvents and microorganisms.

Broth preparation: 13 g of nutrient broth was mixed per 1000 mL of the demineralized water and sterilized for 15 min at 121°C. After sterilization, it was cooled inside Laminar Air Flow and stored at Cooling incubator for further usage.

Agar preparation: 13 g of nutrient broth was mixed per 1000 mL of the demineralized water. Additionally, 15 g of agar powder added in the solution and mixed uniformly. It was sterilized for 15 min at 121°C. After sterilization, agar was poured into 90 mm sterile petri-plates after reaching temperature up to 40°C. Agar plates were labeled and stored at Cooling incubator for further usage.

Preparation of dilution test tubes: Test tubes were filled by 9 mL of demineralized water. All test tubes were sterilized for 15 min at 121°C. After sterilization, it was cooled inside Laminar Air flow Unit and stored at Cooling incubator for further usage.

Collection of household waste water sample: Samples were collected in sterilized conical flasks/ bottles. These collected samples then filtered through 0.45µM polypropylene filter to separate unwanted bacterial strains/contaminants. After filtration, labeling was done, and filtered samples were stored at appropriate storage condition.

Development of bacterial strains: 1 mL of household waste sample was mixed per 100 mL of sterilized nutrient broth and incubated for not more than 3 days at 30°C to 35°C. Growth pattern were observed regularly for each incubation day. After completion of incubation period, grown culture was store at 2°C to 8°C.

Isolation and analysis of prepared bacterial culture: For testing purpose, bacterial culture was taken out from the Cooling incubator and kept inside Laminar Air Flow to attain the room temperature. Full loop of grown culture was streaked on Mannitol salt agar plate and another full loop was streaked on Cetrimide agar plate. It was incubated for not more than 3 days at 30°C to 35°C. After completion of incubation period, morphological analysis for shape, color, surface and structure were performed and recorded. Gram staining was also performed to confirm type of bacteria (either *Gram-Positive* or *Gram-Negative*).

Sub-culturing of purified bacterial strain: Based on seed lot technique, a single colony from each of streaked plate was mixed in sterile nutrient broth. It was incubated for not more than 3 days at 30°C to 35°C. After incubation, these sub cultures were compared with mother culture based on their morphological characteristics.

Concentration checks by serial dilution method: 1 mL of stock culture was transferred to the labeled test tube of 9 mL sterile water and swirled well for its uniform suspension. It was considered first dilution. 1 mL of this dilution is now mixed to next volume of 9 mL sterile water testube, likewise dilutions were prepared upto 8th dilution. 1 mL suspension of each dilution is poured on the agar plate. Also, 1 mL of each dilution were filtered through membrane filtration method and membrane was transferred to the agar surface. All agar petriplate was incubated for not more than 3 days at 30°C to 35°C. After incubation, colony forming units were observed and recorded for its numbers and morphological characteristics.

Collection of water samples: For isolation of bacteriophages, three types of water sources were used to collect the test samples as Rain water, Pond water and Sewage water. All water samples were filtered through 0.45µM and 0.2µM size polypropylene syringe filters. All filtered samples were labeled and stored at appropriate storage condition.

Development of Bacteriophages by Double layer agar method/ agar overlay method: In this method, known bacterial strains were mixed with filtered water samples and molten nutrient agar as defined as per volume defined in below table.

Table 1: Development of Bacteriophages by Double layer agar method

Name of Culture	Rain water (1 mL)	Pond water (1 mL)	Sewage water (1 mL)
Pseudomonas (mL)	1	1	1
Staphylococcus (mL)	1	1	1
Molten agar (mL)	5	5	5

This mixed suspension then loaded to the semisolid nutrient agar petri plate. These double layered plates were incubated for not more than 3 days at 30°C to 35°C. This method has one practical limitation as separation of plaque from agar was a tough and challenging task. Advantage of this method was that bacteriophage colonies were seen by naked eyes. Sewage water with *Pseudomonas* species shown the expected better results.

Development of Bacteriophages by Liquid method: In this method, liquid media (nutrient broth) was used for Bacteriophage development. It

is for omission of agar to overcome the challenge of bacteriophage separation. Refer below table for prepared suspension for liquid method. Nutrient broth (20 mL) and bacterial strains (1 mL) were mixed uniformly with 1 mL of each water sample. Prepared suspension was incubated at 32.5°C for not more than 3 days. After completion of incubation period, suspension was filtered through 0.45µM syringe filter and collected in sterile bottles for future characterization testing by using SEM/TEM.

Table 2: Development of Bacteriophages by Liquid method

Name of Culture/media	Rain water (1 mL)	Pond water (1 mL)	Sewage water (1 mL)
Pseudomonas species (mL)	1 mL	1 mL	1 mL
Staphylococcus (mL)	1 mL	1 mL	1 mL
Nutrient broth (mL)	20 mL	20 mL	20 mL

After completion of incubation period, this incubated suspension was filtered by 0.45µM syringe filter and collected in sterile bottles for future characterization testing by using SEM/ TEM.

Development of test set-up: All test samples, culture flasks, agar plates and liquid media etc. were wiped by 70% IPA and aseptically shifted inside Laminar air flow unit. Required PPEs were used while handled the specific specimens and samples. Refer below figure for test set-up.

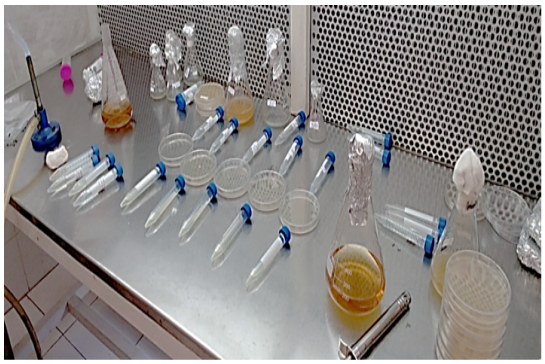


Fig. 2. Development of test set-up

Plaques assessment and result recording: After incubation, transparent round shape plaques/ colonies were observed on agar plates. It was evaluated and recorded for transparent area of plaques, color and shape.

RESULTS AND DISCUSSION

As shown in Table 3, all type of water samples (rain water, pond water and sewage water) showing good water quality and acceptable appearances after filtration. These water samples were filtered by two types of syringe filters i.e. with 0.45µM and 0.2µM pore size. Household waste water was used for isolation of bacterial strains. Incubated media with household waste water shown enriched turbid growth.

Table 3: Water sample quality and appearance

Water Samples	Appearance before filtration	Appearance after filtration (0.45µM)	Appearance after filtration (0.2µM)	pH
Rain	Clear	Transparent	Transparent	8.1
Pond	Turbid	Transparent	Transparent	9.2
Sewage	Yellow	Transparent	Transparent	6.1

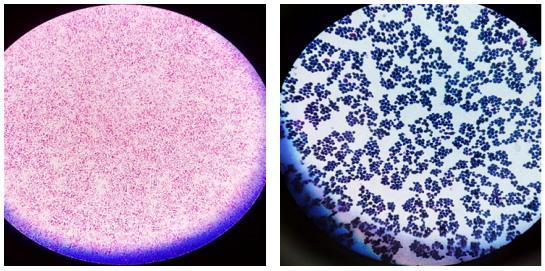
Mannitol salt agar was used for presence *Gram-positive* strains and Cetrimide agar was used for resence of *Gram-negative* strains. As per characterization result shown in Table 4 and 5, these isolated strains on specified agar media, expected strains were observed with specified color, shape, elevation and surface morphology. Based on outcome of strain characterization and microscopic identification, pinkish colored rod shape strain was observed on Cetrimide agar and bluish purple cocci shape strain was observed with Mannitol salt agar. As per this observation the isolated strain with Cetrimide agar was considered *Gram-negative* i.e. *Pseudomonas* species and the strain with Mannitol salt agar was considered *Gram-positive Staphylococcus* species. These isolated strains were also diluted, plated and incubated to know the microbial concentration.

Table 4: Culture characterization

Culture identification parameters on agar plate	Cfus on Cetrimide Agar	Cfus on Mannitol Salt Agar
Color (On Agar)	Green	Yellowish
Shape	Circular	Circular
Elevation	Convex	Low Convex
Surface	Smooth	Smooth
Expected Strain Name	*Pseudomonas*	*Staphylococcus*

Table 5: Microscopically identification

Parameters	Cfus on Cetrimide Agar	Cfus on Mannitol Salt Agar
Color	Pinkish red	Bluish purple
Shape	Rod	Cocci
Arrangement	Mixed rods	Round cocci of Grape structure
Type	*Gram-negative*	*Gram-positive*



Gram-negative *Gram-positive*

Fig. 3. Microscopic view of bacterial strains

Visible CFUS (colony forming units) (Table 6) were observed in 10^{-7} dilution with both type of strains. Below 10^{-7} dilution concentration was TNTC (too numerous to count). These isolated strains were tested against all type of water samples by mixing the defined volume of strain and water. This mixed suspension then poured on agar surface through two layer agar method.

AS shown in Table 7, after incubation of these plates, visible plaques were observed only with combination of sewage water sample tested with pseudomonas strain. Number of visible plaques were seen with the sewage water sample filtered through 0.45µM syringe filters. Plaques were also present with sewage water sample filtered through 0.2µM syringe filters but the plaque number was very low. Plaque is the technical form of Bacteriophage.

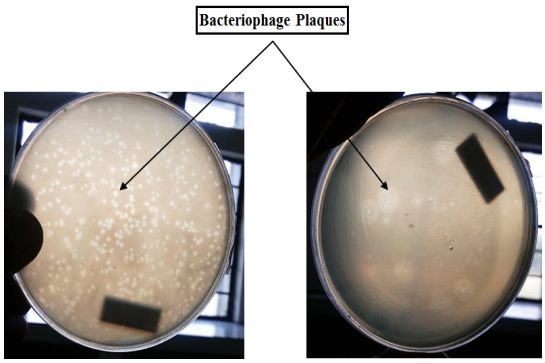
Plaque obtained from different water samples against *Pseudomonas* and *Staphylococcus* species:

Table 6: Microbial concentration

Culture Name	Cfus in dilution 10^{-5}	Cfus in dilution 10^{-6}	Cfus in dilution 10^{-7}	Cfus in dilution 10^{-8}
Pseudomonas	TNTC	TNTC	17, 22	2, 1
Staphylococcus	TNTC	TNTC	19, 25	1, 2

Table 7: Plaque observation

Water Sample Source	Plaque Obtained Against Pseudomonas species		Plaque Obtained Against Staphylococcus species	
Syringe filters pore size	0.2µM	0.45µM	0.2µM	0.45µM
Rain	Not present	Not present	Not present	Not present
Pond	Not present	Not present	Not present	Not present
Sewage	Present	Present	Not present	Not present



0.45µM filtered sewage water 0.2µM filtered sewage water

Fig. 4. Plaque obtained against *Pseudomonas* by double layer agar method

So, it can be concluded that sewage water sample tested with *Pseudomonas* strain have good capability of Bacteriophage presence.

CONCLUSION

This study concluded that bacteriophages are present in Sewage water sample which are effective against *Pseudomonas* strain. Number of optimization studies were performed to select the suitable methods as well as the quantity/ concentration of used media and solvents. The validated methods for isolation and characterization of phages were double layer agar method and liquid method. In double layer agar method visible transparent plaques were observed on agar plates, which is planned for further analysis with SEM/ TEM for morphological characteristics. In same manner, samples from liquid method are also planned for morphological characteristics.

Based on specific properties, its applications

are diverse in water treatment, therapeutic usage and gene therapy etc. Bacteriophages are the novel solution against various type of microbial infections and much promising agents for the cases of antibiotic resistance. Based on these classified applications and properties, usage of Bacteriophages can save time, cost and efforts for livelihood. Novel field of Bacteriophages can act as a strong backbone for future therapeutic and industrial challenges.

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Conflict of interest

The authors disclose no competing interests. The content and writing of the research paper are the sole responsibility of the authors.

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