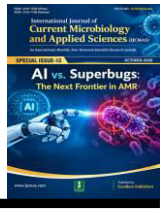
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Isolation and Characterization of Bacteriophages from Sewage Water and Evaluation of their Lytic Activity against Selected Pathogenic Bacteria

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A B S T R A C T

Bacteriophages are viruses that specifically infect bacterial cells and have gained increasing attention as potential alternatives or complementary agents for controlling bacterial pathogens. Sewage and wastewater represent important environmental reservoirs of bacteriophages due to their diverse bacterial populations. The present study was undertaken to isolate bacteriophages from sewage water and preliminarily characterize their plaque-forming activity against selected bacterial hosts. Sewage samples were processed by clarification and membrane filtration, followed by enrichment using *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Vibrio cholerae*. Bacteriophages were detected and isolated using the double agar overlay plaque assay, and plaque characteristics were evaluated based on density, size, clarity, shape and distribution. Distinct plaques were observed with *E. coli*, *S. aureus*, *K. pneumoniae* and *P. aeruginosa*, whereas no detectable plaques were observed with *V. cholerae* under the conditions tested. *S. aureus* showed a high density of predominantly small, circular plaques, while *E. coli* exhibited prominent, circular and well-defined plaques. *K. pneumoniae* showed numerous predominantly circular plaques, whereas *P. aeruginosa* exhibited fewer and relatively diffuse plaques. The findings demonstrate host-dependent variation in plaque formation and indicate sewage as a potential source of bacteriophages for further investigation in phage therapy, food safety, aquaculture and wastewater biocontrol.

Introduction

Bacteriophages, commonly referred to as phages, are viruses that specifically infect bacterial cells and are widely distributed in natural environments, including soil, freshwater, marine environments, sewage and wastewater. Their distribution is closely associated with the occurrence of suitable bacterial hosts. Through

infection and lysis of susceptible bacteria, bacteriophages influence bacterial population dynamics and microbial community structure. Their highly specific interactions with bacterial hosts have generated considerable interest in microbiology, biotechnology, environmental applications and antibacterial research (Hyman, 2019).

The emergence and increasing dissemination of antimicrobial-resistant bacterial pathogens have created major challenges for conventional antimicrobial therapy. The reduced effectiveness of several antibiotics has encouraged the investigation of alternative or complementary approaches for bacterial control. Bacteriophages are of particular interest because of their ability to infect susceptible bacterial cells and replicate within their hosts. Their host specificity provides the possibility of targeted bacterial control, although the biological characteristics, host range and safety of individual phages need to be established before considering practical applications (Hyman, 2019; Pires *et al.,* 2017).

Sewage and wastewater are important environmental reservoirs of bacteriophages because they receive bacterial populations from diverse sources and contain high concentrations of microbial biomass. Consequently, sewage has frequently been used as a source for isolation of bacteriophages against clinically and environmentally important bacterial hosts. The general strategy involves enrichment of environmental samples with a susceptible bacterial host, removal of bacterial cells and debris, and subsequent detection of phages by plaque formation (Hyman, 2019).

Bacteriophage isolation generally involves clarification of environmental samples, membrane filtration, enrichment with an appropriate bacterial host and detection by plaque assay. The double agar overlay plaque assay is a standard method for detecting and enumerating infectious bacteriophages. In this technique, phage particles are mixed with susceptible bacterial cells in molten soft agar and overlaid onto a solid agar surface. Following incubation, localized bacterial lysis results in the formation of visible plaques. Plaque assays can therefore be used for detection, isolation, purification and enumeration of infectious phage particles (Kropinski *et al.,* 2009).

Plaque morphology may differ with respect to size, clarity, shape and edge characteristics. These features can be influenced by phage adsorption, replication, latent period, burst size, diffusion through the agar and bacterial growth characteristics. Clear plaques generally indicate efficient bacterial lysis, whereas turbid plaques may occur because of incomplete lysis or other phage–host interactions. However, plaque morphology alone is insufficient to definitively establish whether a phage is strictly lytic or temperate, and additional biological or genomic characterization is required for confirmation (Hyman, 2019).

Host range is an important biological characteristic of bacteriophages and describes the bacterial hosts that a particular phage can infect. Host-range assessment may initially be performed by spot testing as a qualitative screening procedure, followed by plaque assays to determine productive infection.

Quantitative comparison of phage infectivity among different bacterial hosts can be performed using efficiency of plating (EOP), which compares the phage titre obtained on a test host with that obtained on the reference or propagation host (Kutter, 2009; Letarov and Kulikov, 2018).

Several bacterial pathogens, including *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Vibrio cholerae*, are important in clinical and environmental microbiology. Bacteriophages infecting these bacterial species have been recovered from sewage and other environmental sources, demonstrating the usefulness of wastewater-associated environments for phage discovery (Hyman, 2019).

The isolation and preliminary characterization of bacteriophages from sewage may therefore provide useful information regarding their plaque-forming activity and interactions with selected bacterial hosts. The present study was undertaken to isolate bacteriophages from sewage water and to preliminarily characterize their activity based on plaque formation, plaque morphology and apparent susceptibility of selected bacterial hosts.

Materials and Methods

Biological Materials

Sewage water samples were used as the environmental source for bacteriophage isolation. Sewage-associated environments were selected because they contain diverse

bacterial populations and consequently provide a suitable ecological environment for the occurrence and recovery of bacteriophages. The bacterial hosts used for phage isolation and plaque formation were *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Vibrio cholerae*. These bacterial species were selected because they represent important bacterial hosts of microbiological, clinical and environmental interest. The bacterial cultures were maintained under appropriate laboratory conditions and freshly prepared cultures were used for enrichment and plaque assays. Environmental sewage is frequently used as a source for bacteriophage isolation because of the abundance and diversity of bacterial hosts present in wastewater-associated environments (Hyman, 2019).

Culture Media and Reagents

Nutrient agar and nutrient broth were used for routine cultivation of the bacterial host cultures and for bacteriophage assays. Nutrient agar provided a solid growth surface for the development of bacterial lawns and plaque visualization, whereas nutrient broth was used for propagation of the bacterial host cultures and enrichment of bacteriophages from sewage samples. Soft agar containing 0.7% agar was used for the double agar overlay plaque assay. The relatively low agar concentration allowed bacterial cells and bacteriophage particles to remain distributed within the overlay while permitting diffusion of newly released phage particles and localized development of plaques. Sterile distilled water and phosphate-buffered saline were used as required during the experimental procedures. SM buffer was used for serial dilution and recovery of phage particles from isolated plaques. The use of SM buffer for phage handling and plaque recovery is consistent with established bacteriophage isolation procedures (Kropinski *et al.,* 2009).

Sample Collection

Sewage water samples were collected aseptically from selected sewage-associated locations using sterile polypropylene containers. The containers were appropriately labelled with the sampling location, date and time of collection to facilitate sample identification and traceability. Care was taken during collection to minimize external contamination of the samples. Following collection, the sewage samples were transported to the laboratory under cooled conditions and processed as soon as possible. When immediate processing was not possible, the samples were stored at 4°C and processed within 24 h of collection. Proper handling and relatively rapid processing of environmental samples were adopted to minimize changes in the microbial population and preserve the detectable phage population in the collected sewage samples.

Processing of Sewage Samples

The collected sewage samples were initially allowed to stand for approximately 10–15 min to facilitate the settling of larger suspended particles and coarse particulate matter. Following settling, the supernatant was carefully transferred without disturbing the settled material. The recovered supernatant was centrifuged at 4000 × *g* for 10–15 min at 4°C to remove suspended material and bacterial cells. This clarification step reduced the particulate and cellular content of the sewage sample before membrane filtration. The clarified supernatant was subsequently subjected to sequential membrane filtration using 0.45 µm and 0.22 µm membrane filters. Filtration was performed to reduce bacterial contamination and particulate matter while allowing bacteriophage particles to remain in the filtrate. The resulting filtrate was used for subsequent enrichment procedures with the selected bacterial hosts. Clarification followed by membrane filtration is commonly employed during environmental bacteriophage isolation to obtain a phage-containing preparation with reduced bacterial contamination (Hyman, 2019).

Preparation of Host Bacterial Cultures

The selected bacterial cultures were inoculated into nutrient broth and incubated at 37°C for 18–24 h. Fresh bacterial cultures were subsequently prepared and used during the exponential growth phase for bacteriophage enrichment and plaque assays. Actively growing bacterial cells are preferred for phage isolation because bacterial physiological state can influence phage adsorption and productive infection. The use of actively growing host cells also facilitates the formation of a confluent bacterial lawn required for visualization of plaques during the double agar overlay assay. The bacterial cultures were handled aseptically throughout the experimental procedures to minimize contamination and ensure reliable plaque formation (Hyman, 2019).

Enrichment of Bacteriophages

The sewage filtrate was mixed with nutrient broth and an actively growing culture of the selected bacterial host and incubated at 37°C for 12–18 h with gentle shaking. Separate enrichment procedures were performed using the selected bacterial hosts. During enrichment, bacteriophages capable of infecting a susceptible bacterial host could undergo successive rounds of infection and multiplication, followed by release of newly formed phage particles from lysed bacterial cells. This enrichment procedure was intended to increase the detectable population of host-associated bacteriophages present in the original sewage sample. Following the enrichment period, the enriched culture was centrifuged at 5000 × *g* for 10 min to remove bacterial cells and cellular debris. The resulting supernatant was carefully collected and passed through a 0.22 µm membrane filter to obtain a clarified phage-containing lysate. The filtered preparation was subsequently used for detection and isolation of bacteriophages by the double agar overlay plaque assay. Enrichment using a susceptible bacterial host followed by removal of bacterial cells and plaque-based detection is a commonly used approach for the isolation of environmental bacteriophages (Hyman, 2019).

Isolation of Bacteriophages by Double Agar Overlay Plaque Assay

The enriched phage preparation was serially diluted ten-fold using sterile SM buffer. An aliquot of the appropriate dilution was mixed with an actively growing culture of the corresponding bacterial host and allowed to stand briefly to facilitate interaction and adsorption between the phage particles and bacterial cells. Approximately 3 mL of molten soft agar containing 0.7% agar and maintained at approximately 45°C was added to the phage–bacterial mixture. The contents were mixed gently to ensure uniform distribution of bacterial cells and phage particles throughout the soft agar.

The mixture was immediately overlaid onto a pre-solidified nutrient agar plate and allowed to solidify. Following solidification of the soft agar layer, the plates were incubated in an inverted position at 37°C for 18–24 h. After incubation, the bacterial lawns were examined for the development of discrete, localized zones of bacterial lysis, referred to as plaques. Plaques were identified based on their visible appearance within the bacterial lawn. Well-separated plaques were selected for subsequent purification and characterization.

The double agar overlay technique allows infectious bacteriophages to produce localized zones of bacterial lysis and is widely used for bacteriophage detection, isolation and enumeration. The method also permits individual plaques to be selected for purification and subsequent propagation (Kropinski *et al.,* 2009). In the present study, plaque formation was used as the primary criterion for detecting bacteriophages capable of producing visible lysis under the experimental conditions.

Purification of Bacteriophages

Well-isolated plaques were selected aseptically from the bacterial lawn and transferred into sterile SM buffer. The suspension was gently mixed and allowed to stand under refrigerated conditions to facilitate the elution of bacteriophage particles from the agar. The recovered phage suspension was subsequently subjected to another round of double agar overlay plaque assay using the appropriate bacterial host. Individual well-separated plaques obtained during this assay were again selected and transferred into fresh SM buffer. The plaque-picking and re-plating procedure was repeated at least three times to obtain a purified phage preparation. Repeated plaque purification is commonly performed to reduce the possibility of mixed phage populations and to obtain a more homogeneous phage preparation for subsequent characterization. Following purification, the recovered phage preparations were used for preparation of phage lysates and preliminary characterization based on plaque morphology (Hyman, 2019).

Preparation of Phage Lysate

A fresh, actively growing culture of the susceptible bacterial host was infected with the purified phage preparation and incubated at 37°C with gentle shaking until visible bacterial lysis occurred. The appearance of bacterial lysis indicated the production and release of phage particles during the propagation process. Following visible lysis, the culture was centrifuged at 5000 × *g* for 10 min to remove bacterial cells and cellular debris. The resulting supernatant was carefully collected and passed through a 0.22 µm membrane filter to remove residual bacterial cells. The filtrate obtained after filtration was considered the phage lysate and was stored at 4°C until further analysis. The prepared lysates were subsequently used for plaque-forming assays and

preliminary assessment of plaque characteristics.

Determination of Plaque Morphology

Plaques produced on the bacterial lawns were examined for their visible morphological characteristics. The parameters assessed included plaque size, clarity, shape, edge characteristics and distribution across the bacterial lawn. The observed plaque characteristics were recorded and compared among the bacterial hosts used in the study. Plaques were qualitatively categorized according to their apparent size and visual characteristics rather than by precise numerical measurement.

Plaque morphology can provide useful preliminary information regarding phage–host interactions. However, plaque appearance can be influenced by several biological and physical factors, including bacterial growth rate, phage adsorption, phage replication, diffusion through the agar, incubation conditions and the physiological characteristics of the bacterial host. Therefore, differences in plaque size or clarity were interpreted as preliminary morphological observations rather than definitive evidence of differences in phage replication efficiency or life cycle.

Clear, turbid or diffuse plaque appearances were recorded according to their visual characteristics on the bacterial lawn. The presence of plaques was considered evidence of detectable plaque-forming activity under the experimental conditions, whereas the absence of plaques was recorded as no detectable plaque formation under the conditions tested. Plaque morphology was therefore used as a preliminary characterization parameter, and definitive determination of phage life cycle, infectivity, host range or replication efficiency was not based solely on plaque appearance (Hyman, 2019).

Results and Discussion

The plaque morphology varied considerably among the bacterial hosts tested. In the *Staphylococcus aureus* lawn (Fig. 1), numerous small plaques were observed throughout the bacterial lawn. The plaques were predominantly circular and relatively distinct, although several plaques were closely spaced and difficult to distinguish individually. The high visible plaque density indicated substantial plaque-forming activity against *S. aureus* under the conditions tested.

The *Escherichia coli* lawn (Fig. 2) showed numerous relatively large plaques distributed across the plate. The plaques were predominantly circular with relatively distinct margins, although some plaques were closely adjacent or partially merged.

Compared with the other bacterial hosts, the plaques observed with *E. coli* were more prominent in size and showed extensive areas of visible lysis. The plaque morphology therefore demonstrated clear plaque-forming activity against *E. coli*.

In the *Pseudomonas aeruginosa* lawn (Fig. 3), a comparatively smaller number of plaques were observed. The plaques were medium-sized and predominantly circular, but their margins were less distinct and the zones of lysis appeared slightly turbid or diffuse. The lower visible plaque density and reduced clarity indicated comparatively lower plaque-forming activity under the experimental conditions used.

The *Klebsiella pneumoniae* lawn (Fig. 4) showed numerous medium-sized plaques distributed across the bacterial lawn. Most plaques were circular and relatively well defined, although several closely situated plaques appeared to overlap. The observed plaque morphology indicated clear plaque-forming activity against *K. pneumoniae*, with a greater visible plaque density than that observed for *P. aeruginosa*.

The plate containing *Vibrio cholera* showed no visible plaques, and the bacterial lawn remained intact under the experimental conditions. No detectable plaque-forming activity was observed against *V. cholerae* in the tested sewage-derived phage preparation. The absence of plaques may indicate that the phage preparation did not produce a productive infection in *V. cholerae* under the conditions used. However, the lack of plaque formation alone does not establish phage resistance or confirm the absence of specific receptors required for phage adsorption. Therefore, *V. cholerae* was considered non-susceptible to detectable plaque formation under the experimental conditions tested. Further studies, including host-range analysis and efficiency-of-plating assays, would be required to determine whether the observed lack of plaque formation reflects host resistance, inefficient adsorption, or other phage–host interactions.

Fig.1 Isolation of bacteriophage showing plaques on *Staphylococcus aureus*



Fig.2 Isolation of bacteriophage showing plaques on *Escherichia coli*

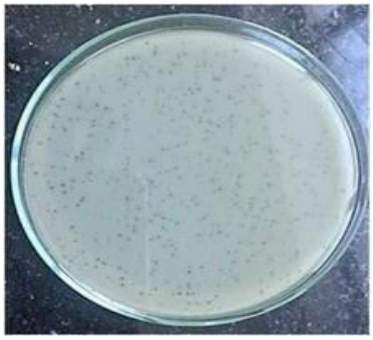


Fig.3 Isolation of bacteriophage showing plaques on *Pseudomonas aeruginosa*

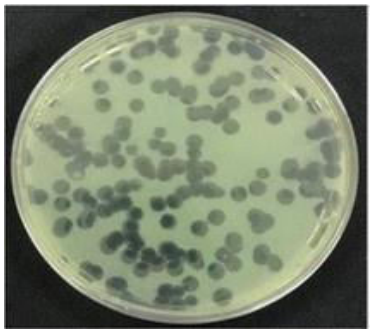


Fig.4 Isolation of bacteriophage showing plaques on *Klebsiella Pneumoniae*

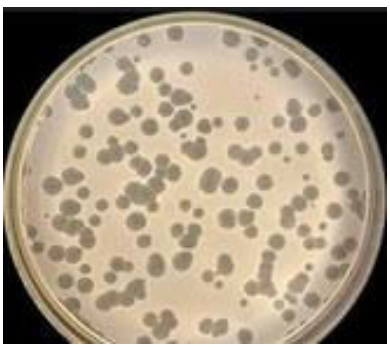


Table.1 Plaque characteristics against different bacterial hosts

S. No.	Bacterial Host	Plaque density	Plaque Size
1	Escherichia coli	Moderate to High	Small
2	Staphylococcus aureus	High	Medium to large
3	Klebsiella pneumoniae	High	Medium to large
4	Pseudomonas aeruginosa	Low	Medium to large
5	Vibrio cholerae	No detectable plaques	No Plaques

The present study demonstrated the occurrence of bacteriophages in sewage water capable of producing plaques against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Differences in plaque density, size, clarity and morphology were observed among the bacterial hosts, indicating variation in phage–host interactions under the experimental conditions. Sewage and wastewater contain diverse bacterial populations and are important environmental sources of bacteriophages. Ballesté *et al.,* (2022) highlighted sewage as an important reservoir of bacteriophages, while Runa *et al.,* (2021) reported their occurrence and ecological significance in wastewater treatment systems. These observations support the suitability of sewage for preliminary bacteriophage isolation.

The plaque-forming activity observed in the present study agrees with previous investigations of sewage-derived phages. Jurczak-Kurek *et al.,* (2016) reported considerable morphological and biological diversity among bacteriophages isolated from urban sewage. Similarly, Topka *et al.,* (2019) characterized an *E. coli* bacteriophage isolated from urban sewage with distinct lytic development. These studies indicate that sewage can harbour bacteriophages with different plaque-forming characteristics and host interactions.

In the present study, *S. aureus* showed a high density of predominantly small, circular and relatively distinct plaques. Synnott *et al.,* (2009) reported sewage-derived *S. aureus* bacteriophages exhibiting variation in plaque morphology and lytic characteristics. Fanaei *et al.,* (2021) also reported bacteriophages from wastewater capable of infecting methicillin-resistant *S. aureus*, *E. coli* and *K. pneumoniae*. The *E. coli* lawn in the present study showed numerous relatively large, circular and well-defined plaques, with some closely adjacent or partially merged. Similar variation in plaque morphology among sewage-derived coliphages was reported by Jurczak-Kurek *et al.,* (2016), while Topka *et al.,* (2019) demonstrated lytic activity of an urban sewage-derived *E. coli* phage. Fanaei *et al.,* (2021) also reported phage activity against an ESBL-producing *E. coli* strain. However, plaque size alone should not be interpreted as an indicator of phage replication efficiency because plaque development is influenced by several biological and physical factors (Abedon and Yin, 2009; Hyman, 2019).

In *P. aeruginosa*, comparatively fewer medium-sized, slightly diffuse or turbid plaques were observed. Aghaee *et al.,* (2021) identified sewage and sewage-contaminated environments as important sources of *P. aeruginosa* phages. Menon *et al.,* (2021) also characterized a wastewater-derived *P. aeruginosa* phage from South India. The lower plaque density observed in the present study indicates comparatively lower visible plaque-forming activity under the conditions tested. However, the turbid appearance should not be considered definitive evidence of a temperate phage without further characterization.

The *K. pneumoniae* lawn showed numerous predominantly circular and relatively well-defined plaques. Liang *et al.,* (2022) reported bacteriophages isolated from sewage using multidrug-resistant and non-multidrug-resistant *K. pneumoniae* strains. Townsend *et al.,* (2021) also demonstrated diversity among *Klebsiella* phages, while Fanaei *et al.,* (2021) reported phage activity against an ESBL-producing *K. pneumoniae* strain. These findings support the present observation that wastewater can serve as a source of *K. pneumoniae*-infecting phages.

Overall, the observed differences in plaque density and morphology may reflect variations in phage adsorption, bacterial susceptibility, replication and diffusion through agar. Abedon and Yin (2009) emphasized that plaque formation is influenced by multiple factors, and Hyman

(2019) recommended interpreting plaque morphology as a preliminary characterization parameter. Therefore, the present findings demonstrate host-associated differences in plaque formation but do not establish differences in phage infectivity or replication efficiency. Further determination of phage titre, efficiency of plating, host range, stability and genomic characteristics would provide quantitative and molecular evidence for comprehensive characterization of the isolated bacteriophages.

In conclusion, the present study demonstrated that sewage water is a potential source of bacteriophages capable of producing detectable plaques against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Differences in plaque density, size, clarity and morphology indicated variation in plaque-forming characteristics among the bacterial hosts under the tested conditions. The findings highlight the potential of sewage as a source of bacteriophages of possible relevance to phage therapy and phage-based control of bacterial pathogens in food, aquaculture and wastewater environments. However, the present study represents preliminary characterization, and plaque morphology alone cannot establish phage titre, infectivity, host range, replication efficiency or life cycle. Further studies involving phage titre, efficiency of plating, host-range determination, stability testing and genomic characterization are required to establish the biological properties and safety of the isolated phages and to evaluate their suitability for potential applications, including phage therapy.

Author Contributions

Gomathi N: Conceived the original idea and designed the model and wrote the manuscript. Mounika M: Contribued in the Experimental worksand documentation. Keerthana G: Documentation of results. Mageshwari S: Effectively participated in the sample collection and processing. Nandini J: Contributed in the experimental work. Lavanya G: Helped in Reviewing the research work.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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