EAS Journal of Biotechnology and Genetics
Abbreviated Key Title: EAS J Biotechnol Genet
ISSN: 2663-189X (Print) & ISSN: 2663-7286 (Online)
Published By East African Scholars Publisher, Kenya

OPEN ACCESS

Volume-8 | Issue-3 | Jul-Sep-2026 | DOI: https://doi.org/10.36349/easjbg.2026.v08i03.001

Original Research Article

Isolation of Podoviridae Bacteriophages of Pseudomonas Aeruginosa from Hospital Sewage Water and the River Ganges

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Article History
Received: 28.05.2026
Accepted: 22.07.2026
Published: 24.07.2026

Journal homepage:
https://www.easpublisher.com

Quick Response Code

Abstract: The effectiveness of conventional antibiotic regimens has been severely compromised by the rapid dissemination of the multidrug-resistant (MDR) *Pseudomonas aeruginosa,* a critical ESKAPE pathogen characterized by impermeable outer membranes, active efflux mechanism, and robust biofilm matrix production. Lytic bacteriophages therapy represents a highly promising alternative treatment approach. In this study, we isolated and characterized virulent bacteriophages targeting a MDR *P.aeruginosa*host from hospital sewage water and the River Ganges, and for this we adapted the host-mediated selective enrichment and double layer agar typing methodologies that was first developed by Dr. Sachindranath Mukherjee. The isolated phages generated homogenous, transparent plaques ranging from 2.5 to 4.5 mm in a diameter. The absence of central turbidity or peripheral halo zones confirms a lytic, obligate lifecycle. These findings demonstrates that bioprospecting geographically diverse environmental reservoirs to produce lytic phages for downstream lyophilization, encapsulation against chronic nosocomial infections.

Keywords: *Pseudomonas Aeruginosa,* Bacteriophage Therapy, Pbunavirus, Multidrug Resistance (MDR), Plaque Morphology, Environmental Wastewater, Lytic Cycle.

1. INTRODUCTION

Multidrug-resistant (MDR) bacteria are rising worldwide and creating a global crisis. As a result, certain superbug infections are becoming exceptionally difficult and, sometimes, impossible to treat using traditional antibiotics [1]. Phage therapy, a treatment strategy that predated antibiotics but has attracted increased attention in light of the current resistance challenge, is one of the most promising and potential alternatives to conventional antibiotics. Phages, also known as bacteriophages, are viruses that only infect and target bacteria [2]. Unlike broad-spectrum antibiotics, which can affect a large range of bacterial species, phages are quite selective, usually targeting a specific strain of bacteria. This specificity results from the phage's ability to recognize and attach to particular receptors on the surface of bacterial cells, starting a process that destroys the bacterium. Once a phage adheres to its bacterial target, it inserts its genetic material into the bacterium, using the bacterial machinery to produce new phage particles [3]. One of the most essential characteristics of phage action is its life cycle, of which there are two primary types, the lytic and lysogenic cycles [4]. The lytic cycle is particularly relevant for therapeutic applications since it includes the direct killing of bacterial cells. The phage binds to the bacterial cell during the lytic cycle, injecting its DNA, which then circularizes. To create new phage DNA and proteins and assemble them into new virions, the phage DNA hijacks the machinery of the host cell. As a result, additional phage particles are released, and cells lyse (Fig. 1) [5]. During the lysogenic cycle, the phage combines with the bacterial chromosome to form a prophage. The prophage and the bacterial DNA replicate during normal cell division. On rare occasions, the prophage may be extracted from the bacterial chromosome and re-enter the lytic cycle [6].

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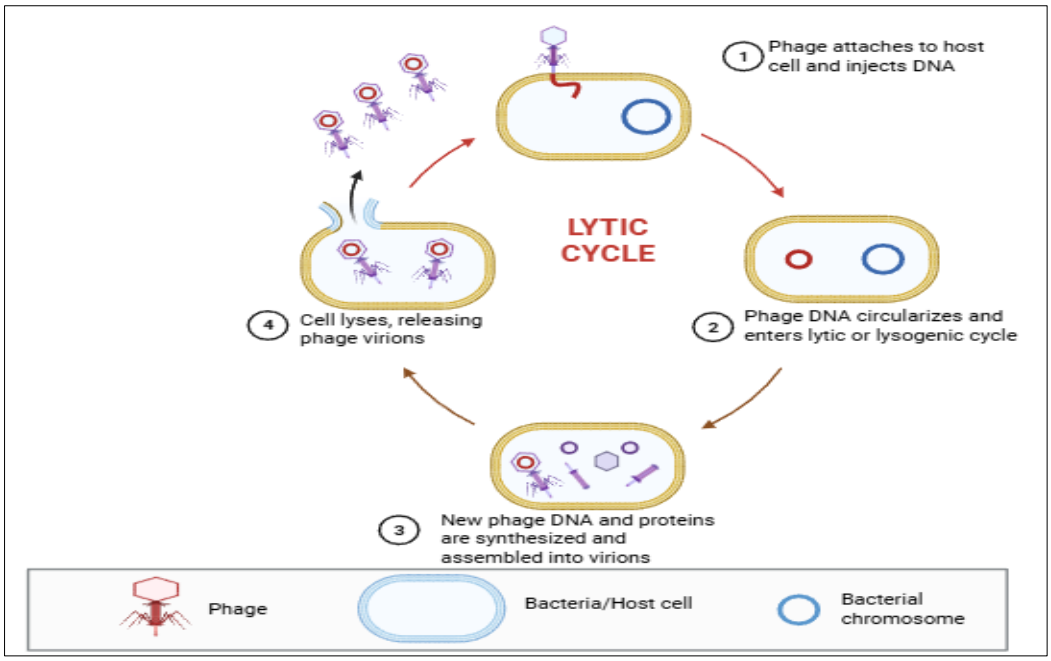

Figure 1: Mechanism of phage action in the lytic cycle

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen, predominantly found in hospitals, sewage water, etc. In most cases, treatment of *P. aeruginosa* is very challenging owing to its multiple mechanisms to resist antibiotics. Multidrug-resistant (MDR) *Pseudomonas aeruginosa*is a major ESKAPE pathogen, causing severe nosocomial infections and high mortality, largely because of its intrinsic efflux pumps, low outer-membrane permeability, and robust biofilm-forming capacity [7]. *P. aeruginosa* carries multiple RND family efflux systems encoded in its core genome, giving multidrug efflux capacity. These tripartite pumps span the inner and outer membranes and extrude many classes of antibiotics, directly supporting intrinsic and acquired multidrug resistance (Fig. 2). The outer membrane is a primary barrier that restricts antibiotic influx. Altered permeability via porins and LPS limits penetration and reduced membrane permeability, which acts in synergy with efflux systems to significantly restrict the intracellular accumulation of the drug [8]. *P. aeruginosa* establishes robust biofilms structurally mediated by exopolysaccharides, including alginate, Psl, and Pel, which facilitate cellular adhesion, immune evasion, and profound antimicrobial resistance. These biofilm matrices function as physical and physiological barriers that harbor tolerant, metabolically dormant subpopulations, thereby enabling survival against lethal antibiotic concentrations and driving multidrug resistance in chronic infections [9]. The presence of flagella and type IV pili allows motility on solid or semi-solid surfaces, which can lead to cross-contamination of surfaces and tools, particularly in clinical settings [7].

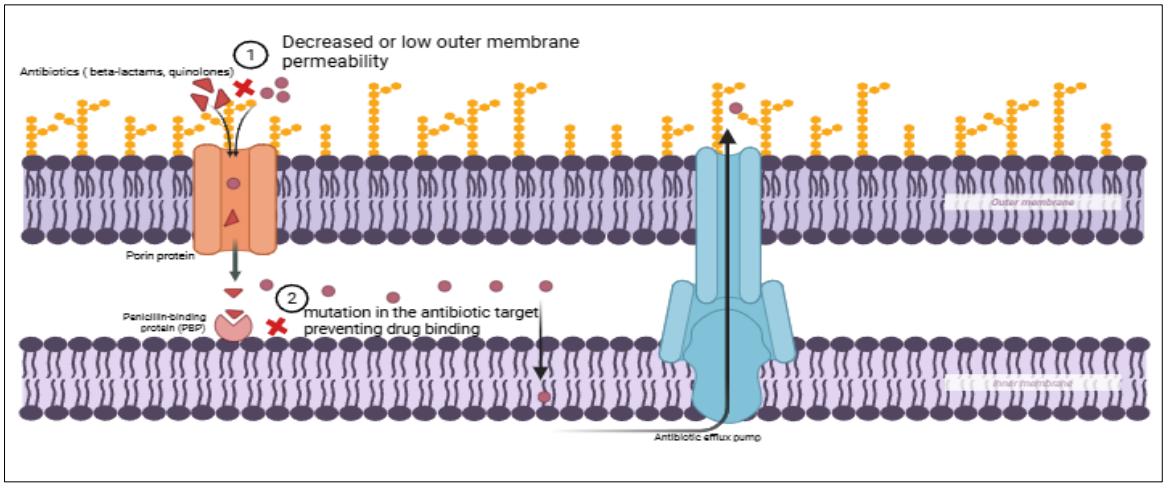

Figure 2: Multidrug resistance mechanism by *Pseudomonas aeruginosa*

The resurgence of phage therapy has offered optimism and a paradigm shift in the development of a new class of antibacterial agents in the face of growing antibiotic resistance among severe infections due to *P.*

aeruginosa. According to transmission electron microscopy (TEM) and kinetic assays, the isolated phages have distinct biophysical profiles, positioning them within the class Caudoviricetes as tailed double-stranded DNA (dsDNA) viruses [10]. The morphotypes vary across taxonomic boundaries; for example, phage PaCC1 represents the septimatrevirus genus, whereas PaCC2 and DRL-P1 are categorized under the Pbunavirus genus [11]. Members of the Pbunavirus genus typically manifest a classic contractile-tailed morphotype characterized by an icosahedral head with a diameter of about 60.89 ± 2.37 nm, a strong neck, and a contractile tail structure. Purified suspensions display rapid adsorption and short latent periods, giving rise to highly productive burst sizes of approximately 100 plaque-forming units (PFU) per infected cell. The purified phages exhibit outstanding physicochemical stability over a wide range of temperatures (4 degrees Celsius to 40 degrees Celsius or sometimes up to 50 degrees Celsius) and preserve a high viability of approximately 70% within a wide pH threshold of 5.0 to 10.0, remarkably resistant to subsequent processing such as lyophilization and encapsulation [12].

Sewage water that receives human feces is thought to be an effective reservoir of phages against various harmful bacteria, abundant in a given population, despite the fact that bacteriophages are ubiquitous in our environment. Isolating virulent bacteriophages targeting multidrug-resistant *Pseudomonas aeruginosa* from a sewage water sample involves sequential stages of sample clarification, host-mediated enrichment, and clonal purification via the conventional double-layer agar method. Clonal virulent lines, such as the isolates PaCCP1, PaCCP2, and DRL-P1, characteristically form distinct, circular, and completely transparent zones of clearance (plaques) on a dense host bacterial lawn. The primary phenotypic indicator that verifies a strictly lytic lifestyle and excludes temperate or lysogenic activity is absolute clarity and the lack of central bacterial growth. The plaque dimensions are highly uniform for each specific line, typically exhibiting diameters ranging from small to moderate sizes (approximately 2.0 ± 0.23 mm for DRL-P1) [12].

2. MATERIALS AND METHODS

2.1. Location of the Study

Sewage water sample required for the study was collected on July 3,2026, from a tertiary care hospital located in Kolkata, India (22.4761° N, 88.3965° E). Meteorological conditions on the day of sampling were characteristic of the regional southwest monsoon, with a mean day temperature of 30°C, high relative humidity, and heavy cloud accompanied by intense precipitation and thunderstorm activity.

The water from river Ganges was collected from the Hoogly River at Bhatpara near Naihati, West Bengal, India (22.874702° N, 88.405127° E) on July 5, 2026. This was performed under typical monsoon conditions, with an ambient temperature of 29.3°C, a relative humidity of 89%, and a wind speed of 23.4 km/h.

2.2. Collection of *Pseudomonas Aeruginosa*

The *Pseudomonas aeruginosa* strain used in this study was sourced from hospital stock culture. Antimicrobial susceptibility test (AST) and strain identification was performed using the VITEK 2 Compact automated system (bioMérieux). The isolate was verified as multidrug-resistant (MDR) based on its resistance profile against categories of standard antimicrobial agents. The phenotypic antibiogram of the multidrug-resistant *Pseudomonas aeruginosa* strain determined by the VITEK 2 Compact is given below in the table 1.

Table 1: Antibiogram of Multidrug resistant pseudomonas. MIC is Minimum Inhibitory Concentration (µg/ml) and R is Resistant.

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Piperacillin	>=128	R	Gentamicin	>=16	R
Ceftazidime	>=64	R	Ciprofloxacin	>=4	R
Cefoperazone /Sulbactam	>=64	R	Levofloxacin	>=8	R
Cefepime	>=32	R	Minocycline		
Aztreonam	>=64	R	Tigecycline	>=8	R
+Doripenem		R	Fosfomycin		
Imipenem	>=16	R	Colistin	>=16	R
Meropenem	>=16	R	+Polymyxin B		R
Amikacin	>=64	R	Trimethoprim / Sulfamethoxazole		

The isolation and enrichment strategy employed in this study is adapted from the classical technique originally developed by Dr. Sachindranath Mukherjee for the isolation *Vibrio* bacteriophages. Historically, this method served as the foundational protocol for the phage typing of *Vibrio cholerae,* utilizing a distinct group of viral agents widely referred to in epidemiological literature as the “Mukherjee phages”. In the present investigation, the core principles of Mukherjee’s selective enrichment and filtration methodology were modified and adapted to isolate bacteriophages specific to *Pseudomonas aeruginosa* from environmental wastewater matrices [13].

2.3. Enrichment of Bacteriophages

Bacteriophages isolation from wastewater was performed using an enrichment protocol with *Pseudomonas aeruginosa* as the host organism. Briefly, a 190 ml sample of raw sewage water was initially passed through Whatman filter paper to eliminate coarse suspended solids. The clarified filtrate was supplemented with 10ml of 20%(w/v) peptone water to facilitate the ensuing bacterial growth and phage propagation. The hydrogen ion concentration of the supplemented sample was then adjusted to a physiological range of pH 7.4-7.8. The prepared mixture was inoculated with 1ml of a log-phase (2-hour) broth culture of the propagating *P. aeruginosa* strain for the enrichment phase. The inoculated sample was incubated overnight at 37^0C under aerobic conditions to enable host bacterial replication and concurrent phage lysis [14, 15]. After the culture was incubated, the culture was subjected to membrane filtration using 0.22µm pore size membrane to separate the bacterial debris and intact host cells from the viral particles. The resulting cell- free filtrate, containing the isolated bacteriophages, which was collected and stored at 4^0C [16].

2.4. Phage Enumeration and Isolation via Double-Agar Layer Plaque Assay

The double-agar layer method was used to quantify bacteriophages and isolate clones. The cell-free filtrate was assessed both undiluted and through a series of tenfold serial dilutions (10^{-2}, 10^{-4}, 10^{-6}), 5ml aliquots of 0.7% (w/v) soft nutrient agar kept in a molten state within a 42°C water bath prior to inoculation were used to prepare the overlay architecture. A 0.1 ml volume of the corresponding phage preparation was thoroughly mixed with 0.1ml of mid-log phase (4 hour) nutrient broth culture of the indicator *Pseudomonas aeruginosa* strain within the molten agar matrix for each assay plate. This mixture was then immediately decanted on to a pre-dried, solid nutrient agar base plate. The top agar overlays were allowed to solidify undisturbed at room temperature for 15 minutes before the plates were inverted and incubated at 37°C for 16-18 hours. Viable viral particles were identified and quantified by observing distinct, transparent zones of lysis, i.e., plaques disrupting the otherwise confluent, opaque background of bacterial growth [14]. The plates were gently rotated by hand to achieve uniform distribution of both viral and host components across the basal layer [14].

2.5 Purification and Clonal Selection of Bacteriophages

Based on phenotypic traits, individual plaque purification was carried out to produce genetically homogenous viral lineages. In cases where uniform plaque morphology was observed, one or two well-resolved, isolated plaques were aseptically harvested by excising the surrounding top agar matrix using a sterile inoculation loop. The excised agar plugs were transferred into fresh, actively growing broth cultures of *Pseudomonas aeruginosa* to initiate host lysis and viral propagation. The lysates were processed through membrane filtration following overnight incubation. The cycle of single-plaque isolation, propagation, and microfiltration was repeated for three consecutive rounds to ensure absolute clonal purity [10]. When heterogeneous plaque morphologies were exhibited, each morphologically distinct plaque type was categorized, individually harvested, and propagated independently. The purified clonal phage stocks were transferred into sterile, hermetically sealed vials and kept at 4°C for long-term preservation [17].

3. RESULT AND DISCUSSION

Phage plaque morphology on double-layer agar plates serves as a primary phenotypic indicator of viral fitness, host-interaction dynamics, and structural classification. The isolated MDR *Pseudomonas aeruginosa* bacteriophages exhibited distinct, completely translucent plaques with sizes ranging from 2.5 mm to 4.5 mm. The burst size, latent period duration, and passive diffusion rate of the virion particles through the porous agar matrix are the main factors that determine the plaque macroscopic dimensions, which are essentially a direct reflection of underlying viral life-cycle kinetics and physical parameters [18]. A more comprehensive examination of *Pseudomonas* phages reveals a varied morphological spectrum, usually divided into three different size cohorts based on diameter. Small plaques (≤1 mm to 2 mm) are characteristically observed in standard *Podoviridae* morphotypes. Such restricted clearing zones are commonly associated with either prolonged replication cycles that constrain spatial propagation before host bacterial confluence is reached or compromised diffusion rates, which are frequently caused by structural dimensions or surface charges. Medium plaques (2 mm to 5 mm), which include several members of the Myoviridae and Podoviridae families, are the most common morphological template found for wild-type lytic *Pseudomonas*phages. The 2.5-4.5 mm plaques isolated, indicating a balanced equilibrium between the viral latent period and the rate of radial macromolecular diffusion. Large plaques (5 mm to 15 mm), exceptionally massive clear zones are hallmarks of highly virulent genera, such as phikmvvirus. Because of their fast replication kinetics and high diffusion efficiency, certain ultra-virulent or depolymerase-producing phages in this category have the ability to sustain radial growth, widening the zones of lysis up to 2.0 cm over a prolonged 24-hour incubation [19].

Figure 3: Plaque morphology of *Pseudomonas* bacteriophages isolated from hospital sewage water (A) and the river Ganges water (B)

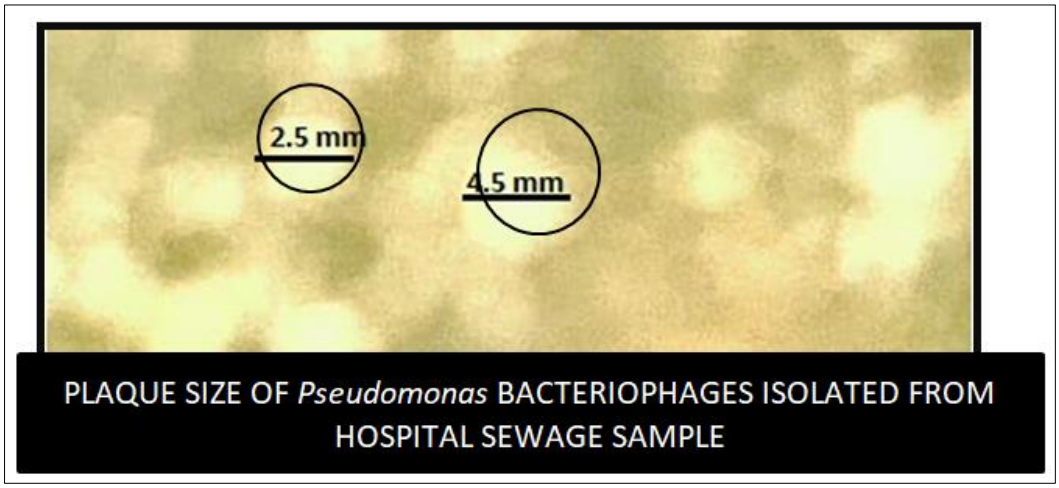

Figure 4: Plaque size of *Pseudomonas* Bacteriophages isolated from hospital sewage water

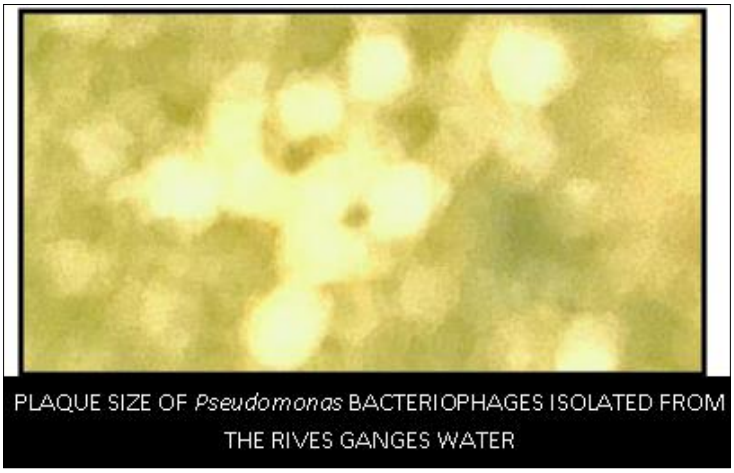

Figure 5: Plaque size of *Pseudomonas* Bacteriophages isolated from the river Ganges water

The physical characteristics of the newly isolated bacteriophage's plaque were methodically assessed in order to determine its virulence and possible taxonomic classification. The isolate produced extremely clear, distinct plaques with no detectable peripheral changes and sharp, well-defined boundaries. This physical hallmark which consists of small to medium clear zones with sharp borders that align with

the typical lytic behaviors reported for the *Podoviridae* family [19, 20]. In contrast to the distinct plaque phenotypes of rapid-lysis mutants of phages, which typically yield substantially larger, hazy, or irregular, rough-bordered lysis zones due to altered structural dimensions, accelerated replication kinetics, or erratic host-lysis pathways [21].

The lack of peripheral halo development around the primary zones of clearing was a crucial diagnostic characteristic of the isolated phage. The expression and extracellular secretion of phage-encoded depolymerase in numerous *Pseudomonas* bacteriophage, particularly those belongs to the *Phikzvirus* genus, plays major role in structure of plaque [18-22]. These enzymes degrade the bacterial extracellular polymeric substances (EPS) and capsular polysaccharides, resulting in a distinctive plaque with a fully cleared central lytic core encircled by a gradually growing, translucent outer halo of decapsulated host cells [23]. The isolated *Podovirus* does not express active, diffusible polysaccharide depolymerases that target the host's outer capsule under the investigated conditions, as shown by the strict absence of halo zones in our experimental assays. This lack of halo formation further supports its classification as a very highly localised lytic *Podovirus* [18].

The zones of clearance generated by the isolate were uniformly transparent, showing no turbidity or residual bacterial growth within the lysed areas. This absence of opacity indicates rapid cell destruction, a characteristic phenotypic trait of lytic obligate lytic phages [24]. On the other hand, temperate or lysogenic phages, such as specific species within the *Casadabanvirus*genus, often produce turbid plaques with cloudy centres due to the survival and subsequent replication of lysogenized, phage-resistant immune cells within the zone of infection [25].

Complex, circular plaque structures, which are characterised by a translucent core surrounded by a massive, gradually expanding peripheral halo that can exceed 2.0 cm within 24 hours, were not present in the isolate.

The absence of these highly dynamic circular structures indicates that, viral propagation is restricted to standard lytic diffusion without secondary, enzyme-mediated degradation of the surrounding bacterial matrix [26]. The spatial stability and clear delineation of the plaques over a 24-hour, confirm a lytic cycle and uniform and homogenous viral distribution within the agar layer [18].

4. CONCLUSION

The findings of this study highlight the potential of river systems and environmental wastewater, particularly hospital sewage and the Ganges River, as rich reservoir for isolating highly potent, obligate, lytic phages capable of targeting pathogen like MDR *Pseudomonas aeruginosa.* Using plaque assay and structured enrichment methodologies, we isolated and characterized different viral lineages. The physical and biological characteristics of the isolates includes clear, well-defined plaques that range in size from 2.5-4.5 mm and lack peripheral halos, confirms lytic characteristics devoid of lysogenic tendencies or diffusible extracellular depolymerase activity.

Although the sampling sites are separated by a considerable geographical distance of approximately 45 to 50 kilometers, structurally and behaviourally identical *Pseudomonas aeruginosa* phages were isolated from both distinct environments. This consistency over a wide geographic range indicates widespread ecological prevalence of highly specialized *Myoviridae* and *Podoviridae* lineages that have adapted to exploit ubiquitous clinical and municipal wastewater niches. This study thus demonstrates that targeted environmental biodiscovery yields highly stable lytic phages that effectively evade the complex resistance mechanism of ESKAPE pathogens. The characterised phage isolate provide a strong basis of developing targeted adjuvants or alternative conventional antibiotics.

Conflict of Interest: The authors declare no conflict of interest.

Author's Contribution: Dr. Bhasker Narayan Chaudhuri, Dr. Partha Guchhait and Dr. Satadal Das designed the study procedure, helped in carrying out the experiment properly and corrected the manuscript. Koena Barik carried out the experiment and prepared the manuscript.

Funding Source: This study was not supported by any funding.

ACKNOWLEDGEMENT

We hereby acknowledge the Dr Sudipta Mitra of Peerless Hospitex Hospital & Research Centre Limited, Kolkata, India for the administrative support.

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Cite This Article: Koena Barik, Bhaskar Narayan Chaudhuri, Partha Guchhait, Shreya Bera, Arup Kumar Dawn, Satadal Das (2026). Isolation of Podoviridae Bacteriophages of Pseudomonas Aeruginosa from Hospital Sewage Water and the River Ganges. *EAS J Biotechnol Genet, 8*(3), 42-49.