

Phage based control of clinical MDR strain of *Pseudomonas aeruginosa*

NIHALANI Yukta S

Department of Microbiology

C.B.Patel computer college and

J.N.M.Patel Science College,

New City Light Road, Bharthana (Vesu) Surat 395017

(affiliated to VNSGU)

Email:- yuktanihalani7998@gmail.com

JAIN Mannu R

Department of Microbiology

Surat Municipal Institute of Medical Education and

Research, Bombay Market Rd, Sahara Darwaja,

Umarwada, Surat, Gujarat 395010

(affiliated to VNSGU)

Email:- jainmannu01@gmail.com

GHEVARIYA Kajal K

Department of Microbiology

C.B.Patel computer college and

J.N.M.Patel Science College,

New City Light Road, Bharthana (Vesu) Surat 395017

(affiliated to VNSGU)

Email:- kaju239@gmail.com

Abstract:

Pseudomonas aeruginosa, an opportunistic gram-negative lactose non fermenting pathogen is often associated with high level of drug resistance to multiple classes and chronic biofilm mediated infections, especially in urinary tract cases. Where biofilms complicate the situation of drug resistance, bacteriophage therapy offers a promising alternative.

A clinical isolate of *P. aeruginosa*(YU27) was isolated from urine sample, identified via biochemical profiling and MALDI-TOF (score: 1.72; match to *P. aeruginosa* ATCC 27853) and checked for its MDR status using Kirby-Bauer antibiotic susceptibility testing. Against this isolate, phage YSNP7 was isolated using standard enrichment and standard double layer agar assay from nanpura sewage waste water. After plaque purification, host range was analysed by spot assay against 63 MDR isolates and it showed lytic activity against 47.83 %, indicating a moderate but clinically relevant range. Further biofilm degradation was assessed by 96 well ethanol-crystal violet assay showing a biomass reduction of 87.9 % and 76.7 % for 24 hrs and 48 hrs old mature biofilm, showing its power even against mature biofilm supporting its candidacy for therapeutic applications.

Introduction:

Over 5 million deaths globally in 2019 were directly caused by antimicrobial resistance (AMR), a developing health epidemic [1]. *Pseudomonas aeruginosa* because of its inherent resistance mechanism, exceptional flexibility, and ability to quickly acquire multidrug resistance (MDR), is one of the most worrisome organisms and a major contributor to hospital-acquired infections[2, 3].

In its 2024 revised list, the World Health Organization (WHO) named carbapenem-resistant *P.aeruginosa* a “critical priority pathogen,” highlighting the pressing need for innovative therapeutic strategies [4]. Alarming resistance trends are highlighted by surveillance data: In many critical care units, the prevalence of MDR *P.aeruginosa* surpasses 30%; in Iran, it has been reported to reach 87.5%, whereas in the US, it is 19.7% [5, 6].High morbidity, mortality, extended hospital admissions, and substantial medical expenses are linked to these infections, especially ventilator-associated pneumonia, urinary tract infections, and bacteraemia [7].

Capacity of *P. aeruginosa* to produce biofilms, which are organised bacterial colonies covered in an extracellular polymeric matrix, is a key factor in the persistence of its infections. Biofilms make illnesses resistant and recurring by shielding germs from immune clearance and medications [8, 9]. Alternative approaches are desperately needed because conventional antibiotic therapy frequently fails to treat infections linked to biofilms.

Bacteriophages (phage), viruses which specifically infect and lyse bacteria, have made a comeback as possible medicinal agents. Phage exhibit potent antibiofilm activity in addition to direct bacteriolysis[10]. Their potential against MDR bacteria, such as *P. aeruginosa*, have been demonstrated by several preclinical and clinical investigations [11, 12]. Targeting an MDR isolate of *P. aeruginosa* (YU27), the current study focuses on the identification and characterisation of novel lytic phage, YSNP7. We investigated its antibiofilm efficacy against both early and mature biofilms and examined its host range across a variety of MDR strains. Our results are intended to show therapeutic value of YSNP7 as a potential treatment for MDR *P. aeruginosa*, specifically in infections linked to biofilms.

Methodology: -

1. Bacterial isolation[13, 14] and characterisation

1.1 Clinical sample collection and its processing to get isolate

Midstream urine samples were collected aseptically from the patient and transported immediately to microbiology lab, where they were streaked onto sterile nutrient agar and MacConkey agar plates and incubated at 37°C for 24 h. Distinct colonies were picked and purified by repeated streaking and added to sterile peptone water for further analysis.

1.2 Gram staining

Bacterial sample prepared in peptone water was smeared on slide, heat fixed, stained with crystal violet and iodine, decolourised and counter stained using safranin.

1.3 Biochemical analysis of the obtained isolate

1.3.1 Catalase test:

Prepared bacterial sample was added to 3% hydrogen peroxide and observed for bubble formation

1.3.2 Oxidase test:

Prepared bacterial sample was applied to oxidase strip

1.3.3 Indole test:

Inoculate the bacterium into sterile tryptone broth, incubate at 37°C for 24h, then add Kovac's reagent

1.3.4 Citrate test:

Inoculate the organism into citrate agar incubate at 37°C for 24h

1.3.5 Triple Sugar Iron (TSI):

Stab the inoculum into sterile TSI agar, streak the slant, and incubate at 37°C for 24h

1.3.6 Methyl Red (MR) test:

Inoculate sterile MR-VP broth with organism, incubate at 37°C for 24h then add a few drops of MR indicator.

1.3.7 Voges-Proskauer (VP) test:

Inoculate sterile MR-VP broth with organism, incubate at 37°C for 24h then add Barritt's A and B reagents to detect acetoin.

1.3.8 Motility test:

Inoculate motile bacteria into a semi-soft sterile agar medium

1.3.9 Phenylalanine test:

Inoculate the bacteria by streaking phenylalanine slant, incubate at 37 for 24 hrs, then directly add several drops of an acidified 10% ferric chloride solution

1.4 Antibiotic susceptibility testing (AST) [15, 16]

The Kirby-Bauer method for antibiotic susceptibility testing involves inoculating a Mueller-Hinton agar plate with a standardized bacterial suspension and placing antibiotic-impregnated disks on the surface. After incubation, antibiotics diffuse, creating a concentration gradient, and the resulting zone of inhibition (clear area where growth is prevented) is measured. The size of this zone determines if the bacteria are susceptible, intermediate, or resistant to the antibiotic, with interpretation based on annually updated guidelines from the Clinical and Laboratory Standards Institute (CLSI), such as the 2024 edition (M100-Ed34), which incorporates revised breakpoints and a tiered reporting system to enhance accuracy and combat antimicrobial resistance

1.5 MALDI-TOF analysis[17, 18]

MALDI-TOF is a soft ionization mass spectrometry technique that identifies molecules by their mass to charge ratio (m/z). The process involves mixing a sample with an energy-absorbing matrix, which co-crystallizes on a target plate. A pulsed laser is used to desorb and ionize the sample and matrix molecules, transferring a charged to the analyte without causing fragmentation. The resulting ions are then accelerated by an electric field into a

vacuum-filled flight tube, where they separate based on their m/z. Since lighter ions travel faster than heavier ions, their arrival time at a detector is used to determine their mass. The final data produces a mass spectrum; a unique protein fingerprint used for identification by comparing it to a reference database.

1.6 Biofilm formation by 96 well crystal violet ethanol standard assay[19]

Biofilm formation and reduction were quantified using the standard 96-well crystal violet assay. Bacterial suspensions were allowed to adhere and form biofilms in sterile polystyrene 96-well plates for 24 h and 48 h at 37 °C. Following incubation, non-adherent cells were removed by washing with sterile PBS, air-dried, and stained with 0.1% crystal violet for 15 min. Excess stain was removed, and the bound dye was solubilized with ethanol. Absorbance was measured at 570 nm using a microplate reader to quantify biofilm biomass.

2. Phage isolation[20-24] and characterization

2.1. Collection and processing of water sample for usage

The environmental water sample was collected from nanpura sewage station in sterile container and transported immediately to lab, where they were allowed for gravitational settlement of large debris. A 25-50ml of water sample from the top was collected and used for enrichment.

2.2. Enrichment of the water sample

For phage enrichment, mid-log phase bacterial cultures were added to the sewage sample along with sterile nutrient broth, followed by incubation at 37 °C for 24 h with shaking at 100 rpm.

2.3. Spot assay of enriched sample to check presence of phage

The enrichment mixture was centrifuged at 10,000 rpm for 10 min, and the supernatant was passed through a 0.22 µm sterile syringe filter to remove bacterial cells. Then the 5ul filtrate was spotted onto bacterial lawn prepared by swabbing bacteria onto sterile agar plates and incubated overnight at 37 °C.

2.4. Standard double layer agar assay

The filtrate of enriched mixture showing positive spot was double dilute in normal saline and used for standard double layer agar assay where the dilutions were mixed

with equal amount of overnight bacteria into sterile top agar and poured onto pre-prepared sterile nutrient agar plates. The obtained plaques were purified by repeating the double layer agar assay till uniform plaque morphologies were obtained or at least thrice (whichever more).

2.5. Bulk production of bacteriophage

The plaques were stabbed using sterile needle and added to overnight bacteria containing nutrient broth, incubated overnight at 37°C, centrifuged at 10,000 rpm for 10 min, and the supernatant was passed through a 0.22 µm sterile syringe filter to remove bacterial cells. The filtrate was used as lysate.

2.6. Biofilm reduction by 96 well crystal violet ethanol assay [19]

Biofilm formation and reduction were quantified using the standard 96-well crystal violet assay. Bacterial suspensions were allowed to adhere and form biofilms in sterile polystyrene 96-well plates for 24 h and 48 h at 37 °C. Following incubation, non-adherent cells were removed by washing with sterile PBS, and phage lysates were added to the wells. After an additional 24 h incubation, wells were washed, air-dried, and stained with 0.1% crystal violet for 15 min. Excess stain was removed, and the bound dye was solubilized with ethanol. Absorbance was measured at 570 nm using a microplate reader to quantify biofilm biomass.

2.7. Transmission electron microscopy (TEM) for visual confirmation of virus[20]

Phage morphology was examined using transmission electron microscopy (TEM). Purified high-titer phage suspensions (~10⁹ PFU/mL) were applied to glow-discharged holey carbon grids, blotted to form a thin film, and were negatively stained by 2% uranyl acetate. Images were recorded digitally to analyse phage morphology and structural characteristics.

Results: -

1. For bacterial isolate:

1.1 Isolation of bacteria:

Midstream urine samples streaked on nutrient agar and MacConkey agar yielded distinct non-lactose fermenting colonies with characteristic greenish pigmentation, figure 1.



Figure 1: The small plates show isolation while the large shows AST

1.2 Gramstaining:

On gram staining, the isolate appeared as gram-negative rods, figure 2.



Figure 2: Gram negative bacilli

1.3 Biochemical characterization:

The isolate was catalase positive (figure 3), oxidase positive (figure 4), citrate positive (figure 5; tube no.4), motile (figure 5; tube no.6), indole negative (figure 5; tube no.1), MR negative (figure 5; tube no.2), VP negative (figure 5; tube no.3), phenylalanine negative (figure 5; tube no.7) and showed alkaline slant and butt without H_2S or gas in TSI (figure 5; tube no.5). The biochemical profile confirmed the isolate as *Pseudomonas aeruginosa*.



Negative Positive

Figure 3: Catalase test

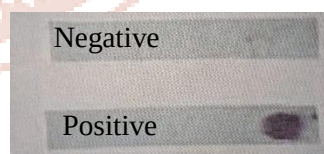


Figure 4: Oxidase test



1 2 3 4 5 6 7

1.4 Antibiotic susceptibility testing (AST):

AST was determined by kirby-Bauer disc diffusion method on Muller-Hinton agar and interpreted according to CLSI (2024) guidelines. The isolate displayed resistance to multiple antibiotics but retained susceptibility to selected antipseudomonal agents as shown in table 1 and figure 1.

Table 1: AST of the obtained clinical isolate

Sr.no	Antibiotic class	Antibiotic name	Antibiotic code	Resistant (R) / Sensitive (S)
1.	β - lactam	Cefoparazone	CPZ	R
2.	β - lactam	Cetazidime	CAZ	S
3.	β - lactam	Cefepime	CPM	S
4.	β - lactam	Amoxycilling clavulanic acid	AMC	R
5.	β - lactam	Piperacillin tazobactam	PIT	S
6.	β - lactam	Ticarcillin clavulanic acid	TCC	R
7.	β - lactam	Cefoparazone sulbactam	CFS	S
8.	Aminoglycoside	Amikacin	AK	S
9.	Aminoglycoside	Gentamicin	GEN	S
10.	Fluoroquinolone	Levofloxacin	LE	R
11.	Fluoroquinolone	Ofloxacin	OF	R
12.	Phenicol	Chloramphenicol	C	R
13.	Tetracycline	Minocycline	MI	R
14.	Carbapenem	Ertapenem	ETP	R
15.	Carbapenem	Meropenem	MRP	MS
16.	Carbapenem	Imipenem	IPM	R
17.	Carbapenem	Doripenem	DOR	S
18.	Monobactam	Aztreonam	AT	S
19.	Polypeptide	Polymyxin b	PB	S
20.	Polypeptide	Colistin	CL	S

1.5 MALDI-TOF analysis:

The MALDI-TOF mass spectrometry generated a unique protein fingerprint that matched *Pseudomonas aeruginosa* ATCC 27853 THL with confidence score of 1.72 and NCBI Identifier 287.

1.6 Biofilm formation assay:

Quantification of biofilm biomass by 96-well crystal violet assay showed significant biofilm production by the isolate. At 24 h of incubation, the OD₅₇₀ was 1.292, while at 48 h the OD₅₇₀ increased to 2.583, indicating strong biofilm forming ability with time-dependent enhancement.

2. For phage isolation and characterization:

2.1 Collection and processing of water sample

Water collected from Nanpura sewage station was clarified by gravitational settlement, and the supernatant was used for further processing. (figure 6)



Figure 6: water sample collection at Nanpura sewage station

2.2 Enrichment of water sample

The clarified sample, when incubated with bacterial host culture under enrichment conditions, was processed for further phage detection assay. (figure 7)

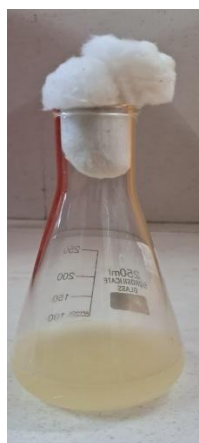


Figure 7: Enrichment flask after 24 h

2.3 Spot assay for phage detection

Filtrates from the enrichment step produced distinct clear zone on bacterial lawn confirming the presence of bacteriophage infecting the clinical isolate. (figure 8)

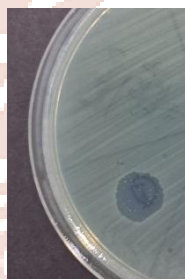


Figure 8: Spot assay for checking phage presence in the enrichment broth

2.4 Standard double layer agar assay

Serial dilutions of the filtrate generated well-defined plaques against bacteria. Repeated purifications resulted in uniform pin-point plaque morphology, validating successful isolation of bacteriophage. (figure 9)

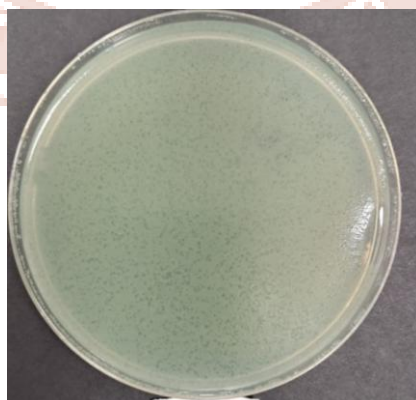


Figure 9: Uniform morphologies of the obtained phage

2.5 Bulk production of bacteriophages

High-titre lysate was obtained by amplification in liquid culture. After centrifugation and filtration, the lysate was free of bacterial contamination and suitable for downstream analysis.

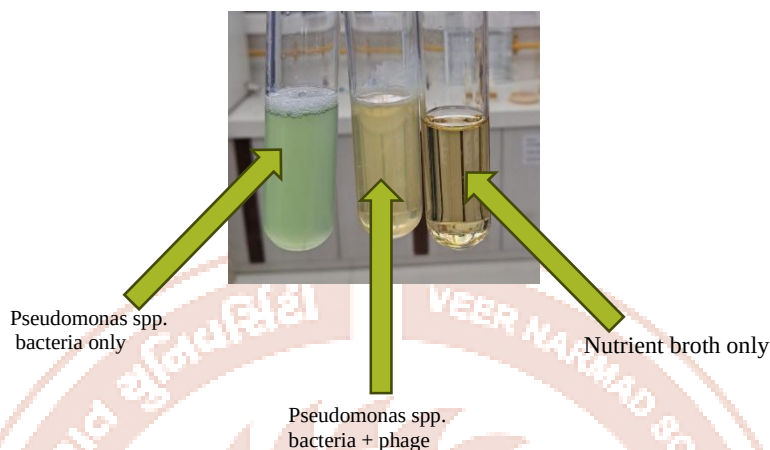


Figure 10: Bulk production of bacteriophages

2.6 Storage of bacteriophages

The phage preparation preserved in all the formats provided stable lysates for subsequent experiments.

2.7 Host range analysis

Spot assay performed on different clinical isolates showed lytic activity against 47.83 % of the checked MDR strains, with variation in the clearing across isolates (only fully clear were considered positive). When checked with other bacterial genera namely *K. pneumonia*, *V. cholera*, *P. mirabilis*, *E. coli*, *Citrobacter* spp., *Streptococcus* spp., *Enterococcus* spp., *Staphylococcus* spp and *Acinetobacter* spp. it did not cause any lyses. (figure 11)



Figure 11: Spot assay of multiple phage's against various bacterial strains

2.8 Biofilm reduction assay

Phage treatment led to measurable and visually noticeable biofilm reduction in both early and mature biofilms to almost of 87.9 % and 76.7 % for 24 hrs and 48 hrs old biofilms respectively. (figure 12)

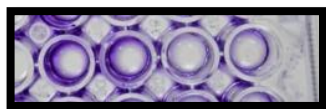


Figure 12: Biofilm formation and reduction

(Well 1 and 2 biofilm formation at 48 h, 24 h; well 3 and 4 biofilm reduction after phage treatment of 24 h biofilm and 48 h biofilm.)

2.9 Transmission electron microscopy (TEM)

The imaging showed viral particle with distinct head (70nm) and contractile tail structure (108 nm) on a scale bar of 200nm. The classification based on structure under TEM places it under Myoviridae like structure, but being replaced by genomics for classification it is not used here classification. (figure 13).



Figure 13: TEM confirming the phage presence

Discussion: -

Because of its antibiotic resistance and capacity to form biofilms, *Pseudomonas aeruginosa* presents a significant clinical problem, especially in nosocomial and urinary tract infections. The YU27 showed resistance to several antibiotics, highlighting the need for alternative treatment approaches. Biochemical profiling and MALDI-TOF validated the isolate's identity as *P. aeruginosa*.

One of the promising strategies for treating MDR infections is bacteriophage therapy. The sewage-derived phage YSNP7 showed lytic activity against 47.83 % of the studied MDR *P. aeruginosa*, suggesting a moderate host range that is clinically relevant. Crucially, biofilms were successfully broken up by YSNP7, which decreased biomass by 87.9% in 24 h biofilm and 76.7 % in 48 h mature biofilm. This demonstrates its capacity to attack biofilms that are

usually resistant to traditional antibiotics, both early and established, which is in correlation with Shafique et.al., 2017 studies.

The somewhat decreased activity against mature biofilms is consistent with the extracellular matrix's protective properties. All things considered, the findings provide credence to phage YSNP7's potential as a treatment option for MDR *P. aeruginosa*, especially for infections linked to biofilms. To properly evaluate its clinical application, future research should concentrate on resistance monitoring, in vivo assessment, and genetic characterisation.

Conclusion: -

Phage as therapeutic and as biocontrol agents may provide an alternative method in near future taking us to pre-antibiotic era where modern therapeutic agents are failing. Even though YSNP7 exhibited moderate host range it was clinically significant while considering personalised therapy. It also showed pronounced biomass reduction in both early and mature biofilms proving and underscoring the relevance of YSNP7 as an alternative or adjunctive strategy to combat biofilm associated infections where conventional antibiotics fail. Further in vivo and genomic characterizations are warranted to establish its safety and optimize its therapeutic applicability.

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