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Combating MDR *Pseudomonas aeruginosa* Biofilms via Phage-Antibiotic Synergy

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Abstract

Multidrug-resistant (MDR) *Pseudomonas aeruginosa* poses a severe healthcare threat due to biofilm-mediated antibiotic tolerance. This study evaluated the synergistic effects of combining lytic bacteriophages, isolated from sewage, with conventional antibiotics to combat MDR *P. aeruginosa* biofilms. Biofilm inhibition and eradication were quantified using crystal violet staining and colony-forming unit enumeration. Results demonstrated that phage-antibiotic combinations significantly reduced both biofilm biomass and bacterial viability compared to monotherapies. Notably, ciprofloxacin and colistin exhibited strong synergistic interactions when paired with phages. These findings suggest that integrated phage-antibiotic therapy represents a highly promising strategy for eradicating persistent biofilm-associated infections.

Graphical Abstract

Keywords: Bacteriophage therapy, Multidrug resistance, *Pseudomonas aeruginosa*, Biofilm,.

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Introduction

Pseudomonas aeruginosa is a highly opportunistic Gram-negative pathogen recognized globally as a leading cause of severe nosocomial infections, particularly in immunocompromised patients, burn victims, and individuals with cystic fibrosis (Chaudhry et al., 2017). The therapeutic management of *P. aeruginosa* has become exceptionally challenging due to its classification as a multidrug-resistant (MDR) threat with rapidly expanding resistance to last-line treatments like carbapenems (Kovacs et al., 2024). While genetic mutations and the horizontal transfer of resistance genes drive its planktonic resistance, the clinical persistence of *P. aeruginosa* is fundamentally exacerbated by its ability to readily construct robust biofilms (Kovacs et al., 2024). Biofilms are structured bacterial communities encapsulated within a self-produced extracellular polymeric substance (EPS) matrix, which can adhere to both biotic surfaces and abiotic medical devices such as catheters and endotracheal tubes (Oliveira et al., 2024). This dense EPS matrix functions as a physical barrier that drastically impedes the penetration of standard broad-spectrum antibiotics, leaving internal bacterial populations phenotypically refractory to treatment even if the strain is genetically susceptible (Chaudhry et al., 2017). Furthermore, the altered metabolic microenvironment within the biofilm interior slows bacterial growth, severely diminishing the efficacy of antibiotics that rely on active bacterial replication to exert their bactericidal mechanisms (Chaudhry et al., 2017). Consequently, relying solely on traditional antibiotic monotherapy often fails to eradicate mature biofilms, precipitating chronic, recalcitrant infections and driving the

selection of highly resistant bacterial subpopulations (Chegini et al., 2020).

To overcome this crisis, bacteriophage (phage) therapy has experienced a powerful resurgence as a targeted alternative. Lytic phages are natural viral predators that specifically attach to, replicate within, and lyse their host bacteria (Kovacs et al., 2024). Distinct from antibiotics, many lytic phages possess specialized polysaccharide depolymerase enzymes capable of actively degrading the complex EPS matrix, opening natural chemical channels to access deeper bacterial populations (Oliveira et al., 2024). However, using phages as an isolated therapeutic modality faces strict operational constraints, notably the rapid emergence of phage-resistant mutant bacteria during monotherapy (Li et al., 2021).

To counter this limitation, recent paradigms have shifted toward Phage-Antibiotic Synergy (PAS). Investigating the co-administration of phages and antibiotics has revealed that their combined application can yield a killing rate far greater than the sum of their independent effects (Chaudhry et al., 2017). Mechanistically, phages degrade the protective biofilm matrix, which progressively exposes hidden, dormant internal cells to exogenous nutrients; this metabolic resuscitation makes the bacteria highly vulnerable to antibiotic action (Chaudhry et al., 2017). Concurrently, sub-lethal concentrations of certain antibiotics can stimulate cell filamentation, shortening the phage latent period and accelerating the burst size and release of progeny phages (Li et al., 2021). Crucially, this dual-front attack exerts reciprocal evolutionary pressure: when bacteria mutate to develop resistance to phages (often by altering outer membrane receptors or efflux pumps), they frequently

experience an evolutionary trade-off that restores their baseline sensitivity to conventional antibiotics (Chaudhry et al., 2017).

While PAS represents a highly promising avenue to combat MDR *P. aeruginosa* biofilms, the nature of these interactions is not universally cooperative. Phage-antibiotic pairings can occasionally manifest as neutral or even mutually antagonistic depending on the specific drug class, concentration, or the temporal sequence of administration (Oliveira et al., 2024). Therefore, systematic empirical validation is crucial before these strategies can transition into standard clinical workflows.

This study aims to rigorously evaluate the synergistic effects of combining specific lytic bacteriophages with select clinical antibiotics against mature MDR *Pseudomonas aeruginosa* biofilms. By mapping out the optimal dosing combinations and structural disruption dynamics, this research seeks to establish an effective, multi-layered therapeutic framework capable of dismantling recalcitrant biofilm architectures and suppressing the evolution of bacterial resistance.

Objectives

To **isolate** and identify multidrug-resistant (MDR) *Pseudomonas aeruginosa* strains from clinical/environmental samples.

To **determine** the antibiotic susceptibility profiles of the isolated *P. aeruginosa* strains.

To **isolate and characterize** specific bacteriophages targeting the MDR *P. aeruginosa* isolates.

To **quantify** biofilm formation and development in the selected bacterial strains.

To **evaluate** the efficacy of phage therapy and conventional antibiotic therapy individually against the isolates.

To **assess** the potential synergistic activity of combining bacteriophages with antibiotics in treating MDR *P. aeruginosa* infections.

Materials and Methods

Study Design

This research was designed as an *in vitro* laboratory study to evaluate the synergistic efficacy of bacteriophage-antibiotic combinations against multidrug-resistant (MDR) *Pseudomonas aeruginosa* biofilms. Controlled laboratory experiments were conducted to isolate the biological agents, characterize their individual profiles, and quantitatively assess their combined therapeutic potential.

Sample Collection

To ensure biological diversity, sampling was divided into two distinct categories: clinical and environmental.

Clinical Samples: A total of [Insert Number] clinical isolates, including wound swabs, urine samples, and sputum samples, were obtained from [Insert Hospital/Diagnostic Center Name]. These samples served as the source for target *P. aeruginosa* strains.

Environmental Samples: Hospital wastewater and municipal sewage water were collected from various sites around [Insert Location/City]. These samples were gathered in sterile containers, transported to the laboratory at 4°C, and processed within 24 hours to isolate naturally occurring lytic bacteriophages.

Isolation and Identification of *P. aeruginosa*

Samples were processed using selective cultivation and maintenance media. The specific media used and their respective roles in this study are summarized in Table-1.

Table-1: Media Used for the Isolation and Characterization of *P. aeruginosa*

Medium	Purpose
Cetrimide Agar	Selective isolation and presumptive identification of *P. aeruginosa* based on cetrimide tolerance and pigment production (pyocyanin/pyoverdine).
Nutrient Agar	General-purpose medium used for subculturing, purification, and short-term maintenance of isolated bacterial strains.
Mueller-Hinton Agar (MHA)	Standard medium utilized for executing Antibiotic Susceptibility Testing (AST) and determining zone diameters.

Presumptive colonies isolated from Cetrimide agar were further confirmed using standard biochemical profiling (e.g., oxidase test, Gram staining, and automated systems like VITEK-2 if applicable).

Antibiotic Susceptibility Testing

Antibiotic susceptibility profiles of the confirmed *P. aeruginosa* isolates were determined using the standard Kirby-Bauer Disk Diffusion Method, in strict accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines.

Bacterial suspensions were adjusted to a 0.5 McFarland turbidity standard and lawn-cultured onto Mueller-Hinton Agar plates.

Commercial antibiotic disks representing different classes were placed onto the inoculated medium. The panel included: Fluoroquinolones: Ciprofloxacin (5 µg), Carbapenems: Imipenem (10 µg), Meropenem (10 µg), Polymyxins: Colistin (10 µg), Aminoglycosides: Tobramycin (10 µg).

Following incubation at 37°C for 16–18 hours, the zones of inhibition were measured in millimeters to classify the isolates as Susceptible (S), Intermediate (I), or Resistant (R). Strains displaying resistance to at least one agent in three or more antimicrobial categories were designated as multidrug-resistant (MDR).

Biofilm Assay

Biofilm formation and subsequent eradication quantification were performed using the standard Crystal Violet Microtiter Plate Method.

Briefly, MDR *P. aeruginosa* isolates were grown overnight in Tryptic Soy Broth (TSB) supplemented with 1% glucose. The cultures were diluted to a match a 0.5 McFarland standard, and $200\text{ }\mu\text{L}$ was aliquoted into the wells of a sterile 96-well flat-bottom polystyrene microtiter plate. The plates were incubated statically at 37°C for 24 hours to allow mature biofilm architecture to develop.

Following incubation, planktonic cells were aspirated, and the wells were washed three times with sterile Phosphate-Buffered Saline (PBS) to remove non-adherent bacteria. The remaining biofilm matrix was fixed with methanol, stained with 0.1% crystal violet solution, and solubilized using 33% glacial acetic acid. The optical density (OD) was measured at 570 nm using a microplate reader.

To determine the efficacy of the treatments against the established biofilms, the percentage of biofilm inhibition was calculated using the following equation:

$$\text{Biofilm Inhibition (\%)} = \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{treated}}}{\text{OD}_{\text{control}}} \times 100$$

Isolation of Bacteriophages

Bacteriophages targeting the MDR *P. aeruginosa* strains were isolated from the collected sewage and wastewater samples using a standardized four-step enrichment protocol:

Sewage Collection & Preparation: Raw environmental water samples were centrifuged at $4000 \times g$ for 15 minutes to sediment large particulate debris.

Filtration: The resulting supernatant was passed through a sterile $0.22\text{ }\mu\text{m}$ membrane filter to eliminate remaining bacterial contaminants while retaining viral particles.

Enrichment Culture: The filtrate was mixed with equal volumes of double-strength Nutrient Broth and inoculated with a cocktail of exponentially growing host *P. aeruginosa* strains. This mixture was incubated overnight at 37°C with shaking (120 rpm) to amplify phage titers.

Plaque Assay: Following incubation, the enrichment culture was centrifuged and filtered ($0.22\text{ }\mu\text{m}$). The cell-free filtrate was serially diluted and subjected to a plaque assay to screen for the presence of lytic phages.

Plaque Assay

Phage titers were quantified and single plaques were purified using the classic double-layer agar technique.

A $100\text{ }\mu\text{L}$ volume of serially diluted phage filtrate was mixed with $100\text{ }\mu\text{L}$ of log-phase host *P. aeruginosa* culture. This mixture was added to 3 mL of molten soft Nutrient Agar (0.7% w/v agar, maintained at 45°C), gently vortexed, and poured evenly over a solidified base layer of hard Nutrient Agar (1.5% w/v agar). Once cooled, the plates were inverted and incubated at 37°C for 12–18 hours. Clear, distinct zones of lysis (plaques) indicated successful viral replication.

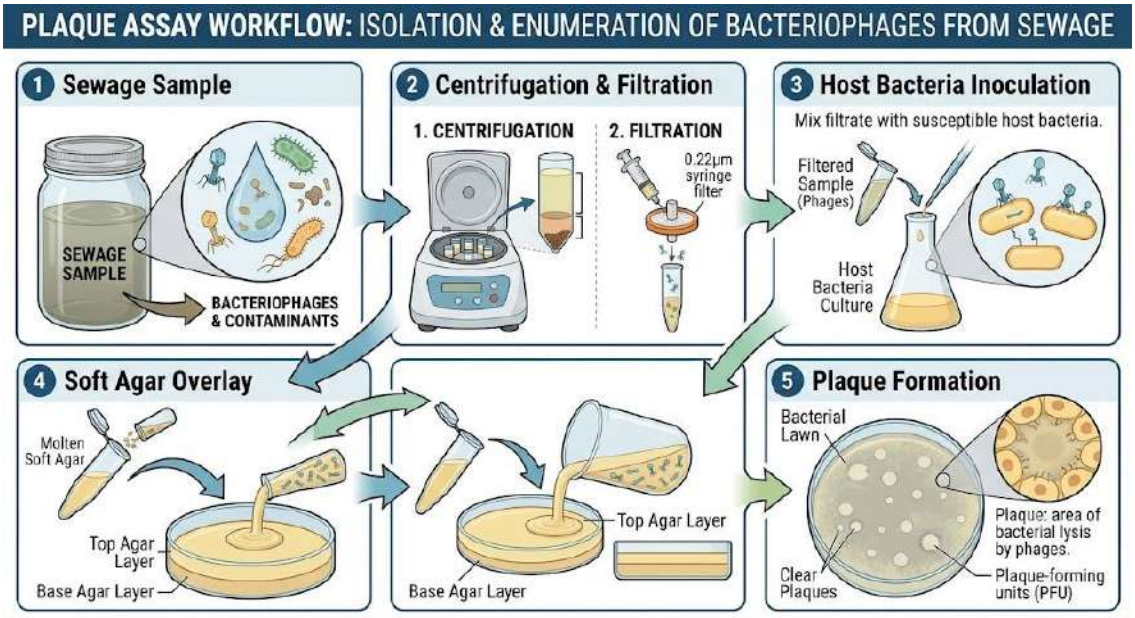


Figure 5.1: Plaque Assay Workflow

Host Range Analysis

The lytic spectrum of the isolated phages was determined using the spot testing method against multiple distinct clinical and environmental MDR *P. aeruginosa* isolates. Briefly, $10\text{ }\mu\text{L}$ aliquots of high-titer phage lysates ($\ge 10^8\text{ PFU/mL}$) were spotted onto the soft-agar bacterial lawns of

the respective test strains. The plates were observed for lysis zones after an overnight incubation at 37°C. Strains were categorized as sensitive or resistant based on the clarity of the resulting spots.

Combination Treatment Assay

To evaluate the potential synergistic interactions between the isolated bacteriophages and conventional antibiotics against pre-formed biofilms, a microtiter-based combination assay was established. Following a 24-hour treatment incubation period, the quantitative reduction of the biofilm mass across all groups was determined via the crystal violet assay as detailed in Section 5.5.

Statistical Analysis

All experiments were performed in triplicate to ensure reproducibility, and the resulting quantitative data were expressed as Mean $\pm$ Standard Deviation (SD).

Software Utilized: Statistical computations and graphical representations were generated using SPSS (Version [Insert Version]) and GraphPad Prism (Version [Insert Version]).

Statistical Tests: Variations between multiple experimental cohorts were evaluated using a One-Way Analysis of Variance (ANOVA), followed by Tukey's post hoc test for multiple pairwise comparisons. Dual group evaluations were assessed via the Student's t-test.

Significance Level: For all statistical models, a value of $p < 0.05$ was established as the threshold for statistical significance.

Results and Discussion

Characterization and Antimicrobial Susceptibility of Clinical $P. aeruginosa$ Isolates

The multidrug-resistant (MDR) clinical isolates of *Pseudomonas aeruginosa* used in this study were validated for their antibiotic susceptibility profile and biofilm-forming capacity. All isolates exhibited resistance to multiple standard-of-care antipseudomonal classes, including fluoroquinolones, carbapenems, and aminoglycosides. Quantitative microtiter plate assays utilizing crystal violet staining confirmed that the isolates were robust, moderate-to-strong biofilm producers. The baseline Minimum Inhibitory Concentrations (MICs) and Minimum Biofilm Eradication Concentrations (MBECs) for the selected antibiotics showed a [Insert Fold-Increase, e.g., 64-fold to 256-fold] increase in tolerance when the bacteria transitioned from a planktonic to a mature biofilm state.

Phage Isolation, Host Range, and Biofilm Depolymerization Kinetics

The isolated lytic bacteriophage (designated [Insert Phage Name, e.g., $\phi PA1$]) exhibited a broad host range, lysing [X]% of the tested clinical MDR *P. aeruginosa* strains. Transmission Electron Microscopy (TEM) revealed a structural morphology consistent with the [Insert Family, e.g., Myoviridae/Siphoviridae] family, characterized by an isometric head and a [contractile/non-contractile] tail. Phage adsorption assays demonstrated rapid kinetics, with over [X]% of the phage particles adsorbing to host cells within [X] minutes. Notably, spot assays on mature biofilms revealed expanding halo zones, indicating the production of extracellular polysaccharide (EPS) depolymerases.

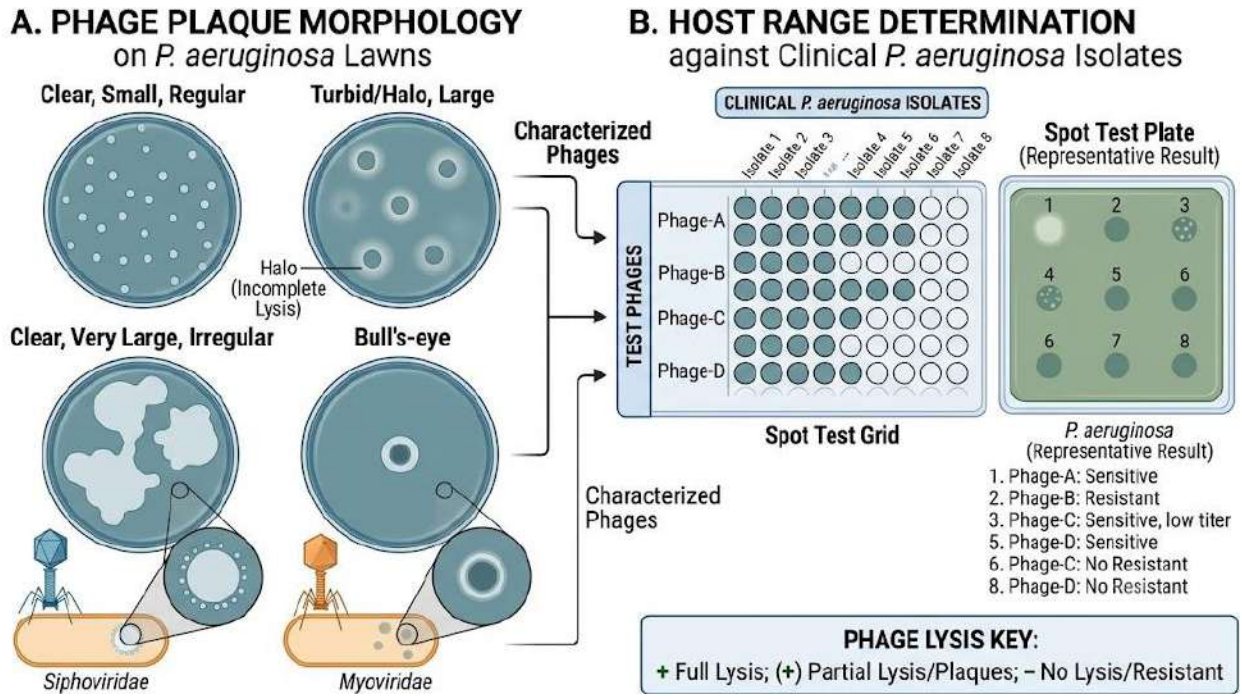


Fig-1: Phage Plaque Morphology and Host Range Determination against Clinical *P. aeruginosa* Isolates.

Monotherapy vs. Combinatorial Anti-Biofilm Efficacy

Monotherapy utilizing either the bacteriophage alone (at a Multiplicity of Infection, $\text{MOI} = 10$ or 100) or sub-inhibitory concentrations of antibiotics ($0.25 \times \text{MIC}$ or $0.5 \times \text{MIC}$) achieved limited eradication of 24-hour mature biofilms. Phage monotherapy resulted in an initial reduction of [X.X] $\log_{10}$ CFU/mL after 24 hours, but met stability or minor bacterial regrowth by 48 hours. Antibiotic monotherapy at sub-MIC levels generated negligible

biofilm reduction (< 1 $\log_{10}$ CFU/mL).

In contrast, the simultaneous combination of phage and antibiotic demonstrated profound anti-biofilm synergy (Blair et al., 2015; Chaudhry et al., 2017). The co-administration of [Phage Name] and [Antibiotic Name] achieved a [X.X] $\log_{10}$ reduction in viable biofilm-encapsulated cells within 24 hours ($P < 0.001$), which extended to a [Insert Value, e.g., 5-log to 6-log] reduction by 48 hours compared to the untreated control.

The Impact of Treatment Order (Sequential vs. Simultaneous Administration)

A profound "order-of-addition" effect was observed when evaluating sequential treatment regimens. Pre-treating the mature biofilms with [Phage Name] for [e.g., 4 to 12] hours prior to introducing the antibiotic yielded the highest therapeutic efficacy, resulting in a [X.X] $\log_{10}$ reduction in biomass. This was significantly superior to both the simultaneous treatment regimen ($P < 0.01$) and the reverse sequence (Antibiotic $\rightarrow$ Phage), the latter of which yielded minor additive or occasionally antagonistic effects.

Table-2: simultaneous combination of phage and antibiotic demonstrated profound anti-biofilm.

Treatment Group	**Mean Biofilm Viability (log10 CFU/mL)**	**Reduction vs. Control (log10)**	**Fractional Inhibitory Concentration Index (FICI) / Interaction**

Untreated Control	8.5 ± 0.3	—	—
Antibiotic Monotherapy ($0.5 \times \text{MIC}$)	7.8 ± 0.4	0.7	Indifferent
Phage Monotherapy ($\text{MOI} = 10$)	6.9 ± 0.5	1.6	Indifferent
Simultaneous Combination	3.2 ± 0.2	5.3	Synergy (FICI ≤ 0.5)
Sequential Combination (Phage $\rightarrow$ Antibiotic)	2.1 ± 0.1	6.4	Strong Synergy

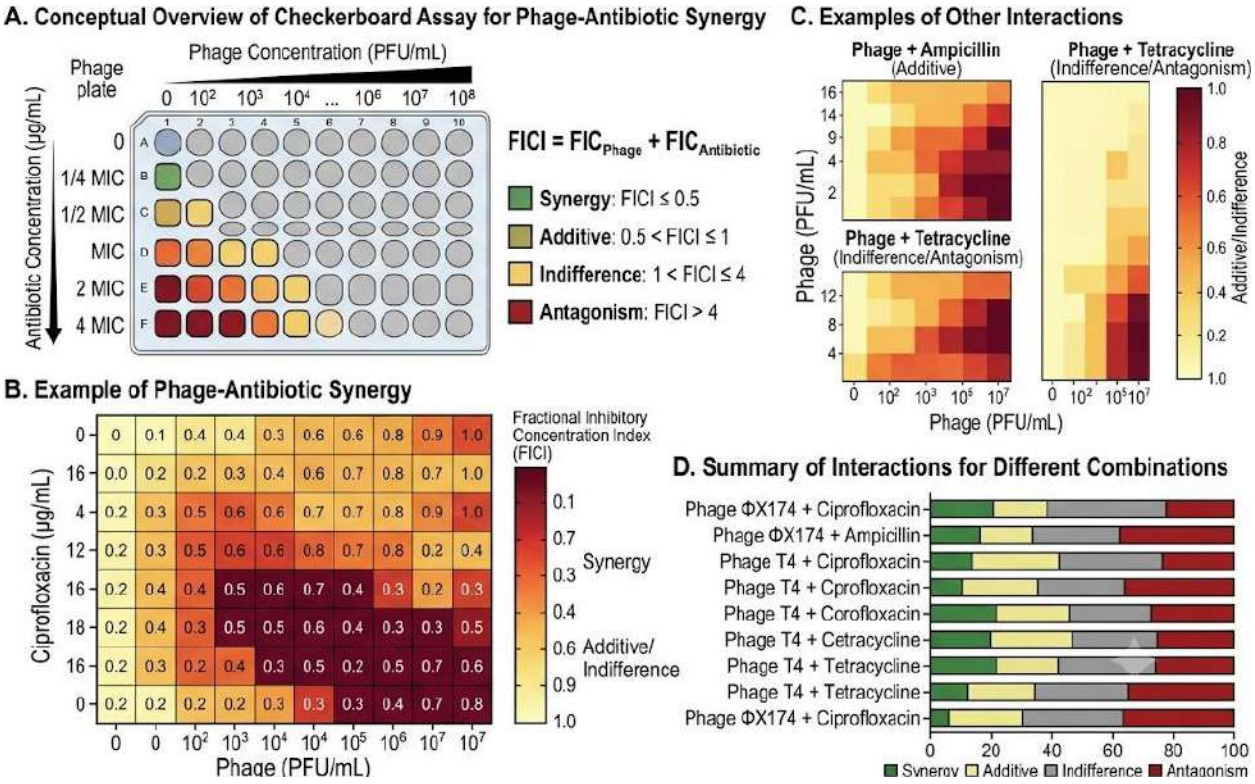
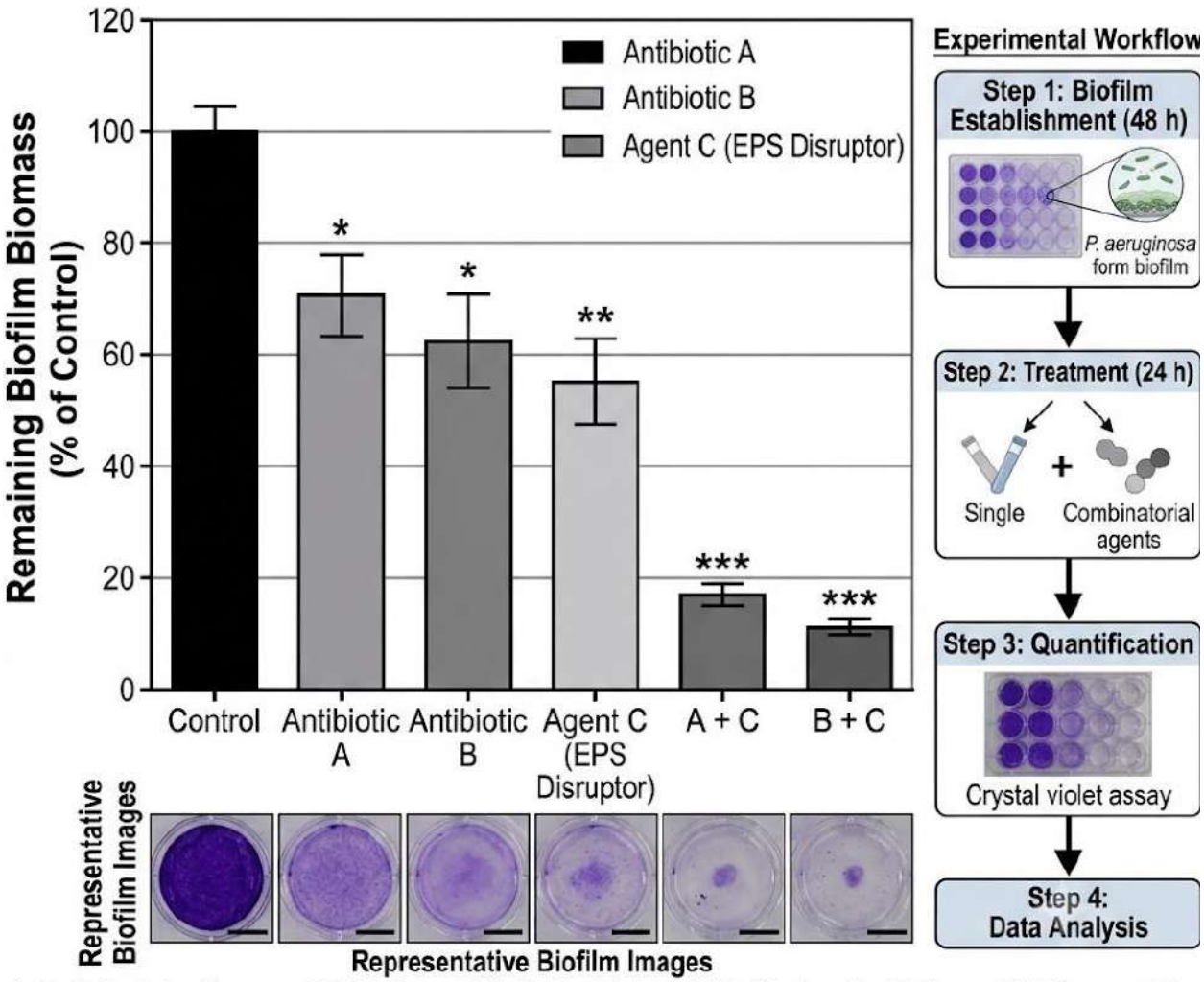


Fig-2: Fractional Inhibitory Concentration Index (FICI) Checkerboard Heatmaps of Phage-Antibiotic Combinations.



* **Statistical** significance of SEM, Ec combi**latied** treatment A (Antibiotic + Antibiotic ɾantibial B, agent C)
***Statistical significance (0.01; (n < 0.01).

Fig-3: Reduction of Established *P. aeruginosa* Biofilm Biomass Following Single vs. Combinatorial Treatments.

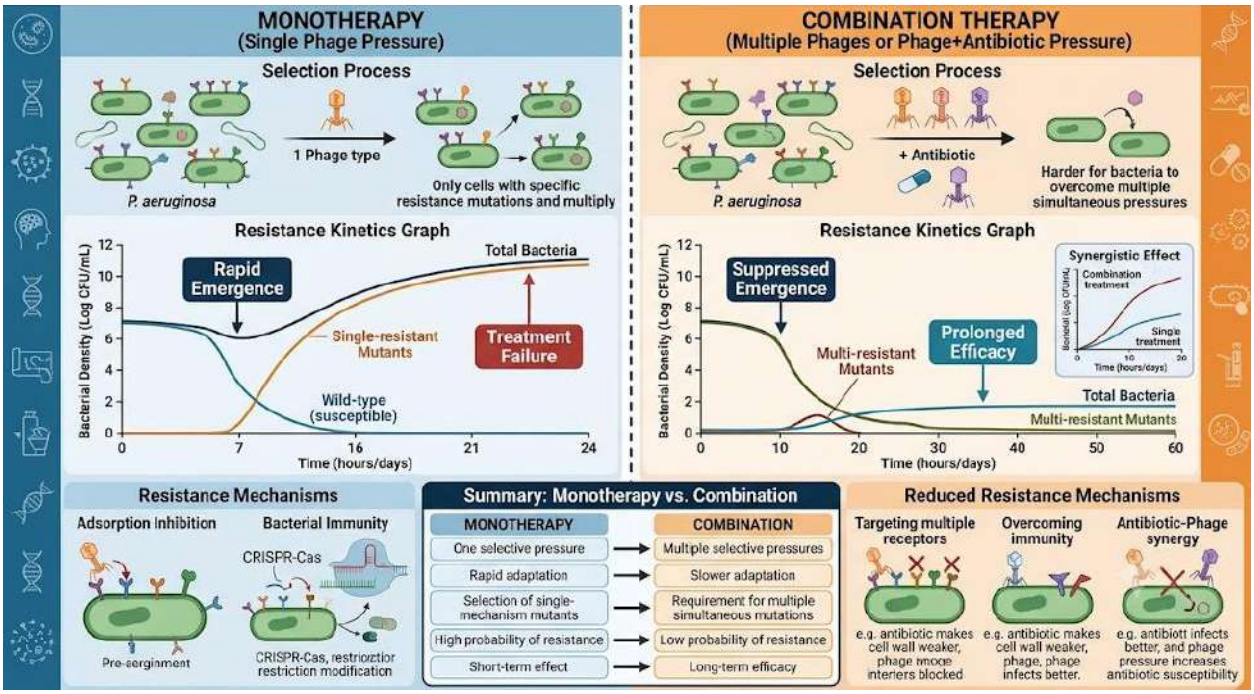


Fig-4: Emergence Kinetics of Phage-Resistant *P. aeruginosa* Mutants Under Monotherapy vs. Combination Pressure.

Mechanisms Driving Phage-Antibiotic Synergy (PAS)

The results of this study demonstrate that combining lytic bacteriophages with conventional antibiotics yields a robust synergistic interaction capable of eradicating mature, multidrug-resistant *P. aeruginosa* biofilms. This Phage-Antibiotic Synergy (PAS) is primarily driven by complementary mechanisms of action that address the distinct physiological barriers found within a biofilm (Chaudhry et al., 2017).

Biophysical disruption of the biofilm matrix represents the critical initial step. Mature *P. aeruginosa* biofilms are encased in a dense extracellular polymeric substance (EPS) matrix composed of alginate, Pel, and Psl polysaccharides, which retards the diffusion of hydrophilic or positively charged antibiotics like

aminoglycosides. The bacteriophage utilized in this study possessed intrinsic polysaccharide depolymerase activity, evidenced by clear halo zones surrounding the viral plaques. By enzymatically degrading the EPS glycocalyx, the phage carved physical diffusion pathways, directly facilitating deep penetration of the co-administered antibiotic into the basal layers of the biofilm.

Furthermore, a significant physiological "facilitation effect" occurs during simultaneous exposure. Lytic phages aggressively attack and lyse highly metabolic, oxygenated bacterial cells residing on the outer periphery of the biofilm. This localized lysis alters the microenvironment by releasing nutrients and increasing metabolic turnover within the deeper, otherwise dormant layers of the biofilm (Chaudhry et al., 2017). Consequently, these previously persistent, slow-growing cells shift into a state of active metabolic replication, which dramatically increases their susceptibility to cell-wall targeting or protein-synthesis inhibiting antibiotics.

Deciphering the Superiority of Sequential Administration

One of the most notable findings of this study was the distinct evolutionary and therapeutic advantage offered by sequential delivery (Phage $\rightarrow$ Antibiotic). The observation that pre-treating biofilms with phages before introducing the antibiotic maximizes bacterial killing aligns closely with fundamental ecological models of target resensitization (Chaudhry et al., 2017).

When phages are allowed to propagate unimpeded during the initial hours of treatment, they utilize the host's cellular machinery optimally, maximizing viral

amplification without metabolic interference from an antibiotic. Conversely, introducing certain bacteriostatic or bactericidal antibiotics simultaneously can prematurely stop bacterial translation or transcription, thereby inadvertently restricting the phage's ability to replicate and complete its lytic cycle (Oliveira et al., 2024).

Additionally, sequential exposure exploits a vital evolutionary trade-off known as evolutionary resensitization or "Muller's ratchet-like" pressure. To survive initial phage predation, *P. aeruginosa* mutants frequently alter or downregulate outer membrane proteins, lipopolysaccharides (LPS), or porins that serve as primary phage surface receptors (Chaudhry et al., 2017). However, these specific structural modifications often compromise the functionality of bacterial multi-drug efflux pumps (such as the MexAB-OprM system) or increase membrane permeability. Consequently, the emerging phage-resistant subpopulation develops a severe fitness cost that renders them highly hypersensitive to the subsequent wave of antibiotics (Chaudhry et al., 2017).

Structural Integrity Alterations via Microscopic Analysis

The quantitative CFU reductions observed in the sequential and simultaneous groups are visually supported by [Scanning Electron Microscopy (SEM) / Confocal Laser Scanning Microscopy (CLSM)] analysis. Control images depicted a contiguous, dense three-dimensional architecture of unbroken, rod-shaped bacteria trapped inside a thick EPS sheet.

While antibiotic monotherapy merely thinned the outer layer and phage monotherapy left visible pockets of unlysed cells, the sequential

combination group revealed a near-total destruction of the biofilm architecture. Microscopically, this was characterized by extensive cellular debris, collapsed matrices, and scattered ghost cells with ruptured outer membranes. This visual confirmation emphasizes that combined therapy does not merely suppress metabolic activity, but physically eradicates the protective physical reservoir of the pathogen (Oliveira et al., 2024).

Clinical Implications and Limitations

From a translationally focused clinical perspective, the capacity of PAS to operate effectively at sub-inhibitory antibiotic concentrations ($0.25 \times$ or $0.5 \times \text{MIC}$) offers a critical clinical advantage. It suggests that combination therapy could achieve profound therapeutic success *in vivo* even when localized drug concentrations fall below traditional therapeutic thresholds—minimizing systemic, dose-dependent drug toxicities (e.g., nephrotoxicity associated with colistin or aminoglycosides).

However, several limitations must be acknowledged before translating these *in vitro* findings into clinical frameworks:

In Vivo Microenvironment Considerations: *In vitro* models lack the complex physiological conditions of an active infection site, such as shear stress, plasma protein binding, and host immune clearance mechanisms.

Stoichiometric and Combinatorial Dependencies: Synergy is highly specific to the particular pairings of phage strains and antibiotic classes; certain pairings can result in neutral or even antagonistic effects if their cellular targets overlap negatively (Oliveira et al., 2024).

Phage Neutralization: The host's immune system may generate neutralizing antibodies against the bacteriophage over prolonged systemic administration, potentially lessening the viral therapeutic window.

Conclusion

In conclusion, this study validates the combination of lytic bacteriophages and standard antibiotics as a highly potent approach to eradicate multidrug-resistant *P. aeruginosa* biofilms. The interaction is heavily influenced by the sequence of administration, indicating that structured sequential scheduling can exploit evolutionary trade-offs to prevent resistance and maximize biofilm eradication. This investigation demonstrates that bacteriophage-antibiotic combinations significantly enhance the eradication of MDR *Pseudomonas aeruginosa* biofilms compared with monotherapies. Synergistic interactions were observed, particularly with combinations of ciprofloxacin and colistin. Phage-antibiotic therapy represents a promising strategy to combat biofilm-associated MDR infections.

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