To evaluate the susceptibility of multidrug-resistant (MDR) Gram-negative bacteria to lytic bacteriophages isolated from environmental sources.

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Abstract-

The rapid rise of multidrug-resistant (MDR) Gram-negative bacteria has turned into a significant worldwide health issue, diminishing the efficacy of traditional antibiotics and leading to more treatment failures. Bacteriophage therapy has resurfaced as a potential alternative method because of its targeted nature and capacity to destroy antibiotic-resistant bacteria. The current research sought to assess the vulnerability of multidrug-resistant Gram-negative bacteria to lytic bacteriophages obtained from environmental sources. MDR Gram-negative bacterial strains, such as Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Acinetobacter baumannii, were collected from clinical specimens and analyzed using standard microbiological techniques. The Kirby–Bauer disk diffusion method was utilized for antibiotic susceptibility testing according to CLSI guidelines to verify multidrug resistance. Lytic bacteriophages were obtained from environmental sources like sewage and hospital effluent through enrichment methods. Detection and purification of phages were performed using the double agar overlay plaque method. The vulnerability of MDR bacterial isolates to the obtained bacteriophages was evaluated through spot tests and plating efficiency assays. The findings showed diverse phage susceptibility across various MDR isolates, with certain bacteriophages displaying a wide host range and robust lytic efficacy. These results emphasize the promise of environmental bacteriophages as powerful defenders against MDR Gram-negative pathogens. This research demonstrates the viability of using bacteriophage-based methods as a substitute or complement to antibiotic treatment and offers essential foundational data for advancing phage therapy techniques for multidrug-resistant infections

Keywords-
Multidrug-resistant bacteria; Gram-negative pathogens; Bacteriophages; Phage therapy; Lytic phage's; Environmental isolates; Antibiotic resistance; Host range, soil sample

Introduction –

The growing occurrence of multidrug-resistant (MDR) Gram-negative bacteria has surfaced as a significant global health issue, greatly restricting the efficacy of current antibiotic treatments. Pathogens like Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Acinetobacter baumannii cause numerous infections in hospitals and communities and have shown resistance to various antibiotic classes, such as β-lactams, fluoroquinolones, and aminoglycosides (World Health Organization, 2023; Tacconelli et al., 2022). The swift spread of MDR strains has led to higher morbidity, mortality, and healthcare expenses globally (Murray et al., 2022).

Traditional antibiotic development has not matched the increasing challenge of antimicrobial resistance, leading to revived interest in alternative treatment approaches. Among these, bacteriophage therapy has attracted significant interest as a potential strategy to manage MDR bacterial infections. Bacteriophages are viruses that uniquely target and rupture bacterial cells, distinguished by their strong host specificity, the ability to replicate autonomously at the infection site, and limited effects on the usual microbiota (Kortright et al., 2021; Pirnay et al., 2023). Recent clinical and experimental research has shown the efficacy of lytic bacteriophages against MDR Gram-negative pathogens, emphasizing their promise as possible antimicrobial agents (Schooley et al., 2022).

Environmental sources like sewage, hospital wastewater, and surface water are identified as abundant sources of various bacteriophages that can specifically attack clinically significant bacteria. The extraction of bacteriophages from these settings presents an economical and efficient method for discovering phages that could have therapeutic applications against MDR Gram-negative bacteria (Abedon et al., 2022; Jurczak-Kurek et al., 2024). Assessing the resistance patterns of MDR bacteria to lytic bacteriophages is crucial for comprehending phage-host dynamics and choosing suitable phages for treatment purposes.

Although phage therapy shows great promise, issues like limited host range, development of phage resistance, and absence of standardized assessment protocols persist. Hence, a systematic evaluation of bacteriophage susceptibility in MDR Gram-negative bacteria is essential to create baseline information and aid in the formulation of phage-oriented treatment approaches (Lin et al., 2023). This study seeks to assess the vulnerability of MDR Gram-negative bacterial isolates to lytic bacteriophages obtained from environmental sources, adding to the increasing evidence that supports phage therapy as a substitute or complement to antibiotics.

Antimicrobial resistance (AMR) has surfaced as a significant threat to global public health, with multidrug-resistant (MDR) Gram-negative bacteria being especially worrisome due to their swift dissemination and restricted treatment alternatives. Gram-negative pathogens like Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Acinetobacter baumannii are significant contributors to hospital-acquired and community-acquired infections, including urinary tract infections, bloodstream infections, pneumonia, and wound infections. These organisms have both inherent and developed resistance strategies, such as decreased membrane permeability, active efflux pumps, enzymatic breakdown of antibiotics, and horizontal gene transfer, which together enhance their capacity to withstand various types of antibiotics (Tacconelli et al., 2022; Murray et al., 2022).

The extensive misuse and excessive use of antibiotics in healthcare, farming, and livestock management have sped up the emergence and spread of MDR Gram-negative bacteria globally. Infections from these pathogens lead to extended hospital admissions, elevated healthcare expenses, and increased rates of morbidity and mortality (World Health Organization, 2023). Worryingly, the creation of new antibiotics has significantly declined in recent decades, resulting in a crucial disparity between the increasing threat of resistance and the accessibility of effective antimicrobial treatments. The increasing crisis has required the investigation of alternative or supplementary therapeutic methods to address MDR bacterial infections (Ventola et al., 2021).

Bacteriophages, commonly referred to as phages, are viruses that target bacteria and offer a promising substitute for traditional antibiotics. Phages occur naturally, exhibit high specificity for their bacterial hosts, and can replicate themselves at the infection site. Of the various forms of bacteriophages, lytic phages are especially well-suited for therapeutic use as they infect and quickly kill bacterial cells without incorporating into the host genome (Kortright et al., 2021; Abedon et al., 2022). In contrast to broad-spectrum antibiotics, phage therapy lessens the disturbance of the normal microbiota and diminishes selective pressure on non-target bacteria, presenting it as an appealing approach for managing MDR infections.

A resurgence of interest in phage therapy has been fueled by recent experimental and clinical research showcasing its efficacy against MDR Gram-negative infections. Effective cases of phage therapy have been documented when traditional antibiotic treatments were unsuccessful, especially for infections due to carbapenem-resistant and extensively drug-resistant bacteria (Schooley et al., 2022; Pirnay et al., 2023). Moreover, phages have demonstrated possible synergistic benefits when combined with antibiotics, improving bacterial elimination and minimizing the development of resistance (Siopi et al., 2024).

Environmental sources like sewage, hospital effluent, river water, and soil act as abundant reservoirs for bacteriophages because of the significant bacterial diversity found in these environments. The extraction of phages from environmental sources is viewed as a practical and economical method for discovering lytic phages that are effective against clinically significant MDR Gram-negative bacteria (Jurczak-Kurek et al., 2024). Due to the natural co-evolution of phages and bacteria in these settings, they frequently demonstrate significant lytic activity towards resistant strains. Nonetheless, phage susceptibility can differ greatly between bacterial species and even within strains of the same species, underscoring the necessity for a systematic assessment of phage–host interactions.

Although phage therapy shows great promise, various challenges impede its broad clinical use, such as limited host range, emergence of phage resistance, absence of standardized susceptibility testing methods, and regulatory hurdles. Thus, assessing the susceptibility patterns of MDR Gram-negative bacteria to lytic bacteriophages is crucial for choosing effective phages, formulating phage cocktails, and creating baseline data for future therapeutic uses (Lin et al., 2023). The current investigation seeks to assess the vulnerability of multidrug-resistant Gram-negative bacteria to lytic bacteriophages obtained from environmental sources, thus adding to the expanding evidence that supports bacteriophage therapy as a feasible alternative or complement to antibiotic treatment.

Material and method

1. Collection of Bacterial Isolates

The MDR Gram-negative bacterial isolates were obtained from clinical samples such as urine, pus, wound swab, and sputum collected from a tertiary care hospital. The samples were processed using standard laboratory technique. The isolates were identified based on Gram staining, colony morphology, and biochemical tests. Isolates showing resistance to three or more classes of antibiotics were considered MDR.

2. Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was performed using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar plates. Results were interpreted according to CLSI guidelines. Isolates resistant to multiple antibiotic classes were selected for phage susceptibility testing.

3. Soil Sample Collection

Soil samples were collected from areas with high microbial activity such as hospital surroundings, sewage disposal sites, and agricultural land. Using sterile spatulas, 10–20 g of soil was collected from the top 5–10 cm layer and transferred into sterile containers. Samples were transported to the laboratory and processed within 24 hours.

4. Processing of Soil Samples

Soil samples were mixed with sterile phosphate-buffered saline (PBS) in a 1:5 ratio and vortexed thoroughly to release bacteriophages into the liquid phase. The mixture was allowed to settle and then centrifuged to remove soil particles. The supernatant was filtered through a 0.22 µm membrane filter to remove bacterial cells.

5. Isolation and Enrichment of Bacteriophages

The filtered soil extract was mixed with exponentially growing MDR bacterial cultures in Luria–Bertani (LB) broth and incubated at 37 °C for 18–24 hours with shaking. After incubation, the mixture was centrifuged and filtered to obtain bacteriophage-containing lysates.

6. Detection of Bacteriophages

Bacteriophages were detected using the double agar overlay plaque assay. Soft agar containing the host bacterial culture was overlaid on agar plates, and phage lysates were added. Plates were incubated at 37 °C for 18–24 hours and examined for clear zones (plaques), indicating lytic activity.

7. Purification of Bacteriophages

Individual plaques were picked using sterile tips and suspended in SM buffer. The purification process was repeated three times to obtain pure lytic bacteriophage preparations.

8. Phage Susceptibility Testing

Phage susceptibility of MDR Gram-negative isolates was evaluated using the spot test method. A lawn culture of bacteria was prepared on agar plates, and 10 µL of purified phage suspension was spotted onto the surface. Plates were incubated at 37 °C for 18–24 hours and observed for zones of lysis.

9. Host Range Determination

The host range of isolated bacteriophages was determined by testing their lytic activity against different MDR Gram-negative bacterial isolates. The results were recorded based on the presence or absence of lysis.

Result and discussion –

1. Collection of Bacterial Isolates

MDR Gram-negative bacterial isolates was obtained from sun diagnostic lab Kolhapur. Those clinical samples such as urine, blood, pus collected from a tertiary care hospital. The samples were processed using standard microbiological techniques. Bacterial isolates were identified based on Gram staining, colony morphology, and routine biochemical tests. Isolates showing resistance to three or more classes of antibiotics were considered multidrug-resistant

2. Antibiotic Susceptibility Testing

Antibiotic susceptibility testing of the Gram-negative bacterial isolates was performed using the Kirby–Bauer disk diffusion method following CLSI guidelines.

3. Soil Sample Collection

Soil samples were collected from Siddhivinayak Hospital, Kolhapur. hospital areas with high microbial activity such as hospital surroundings, sewage disposal sites, and agricultural land. Using sterile spatulas, 20-30 g of soil was collected from and transferred into sterile containers. Samples were testing for to the laboratory work.

4. Processing of Soil Samples

Soil samples was mixed with sterile phosphate-buffered saline (PBS) in a 1:5 ratio and vortexed thoroughly to release bacteriophages into the liquid phase. The mixture was allowed to settle and then centrifuged to remove soil particles. The supernatant was filtered through a 0.22 µm membrane filter to remove bacterial cells.

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Image 1. MDR bacterial cultures

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Image 2 The appearance of a clear zone on the agar plate indicated lytic activity of bacteriophages against the MDR bacterial isolate

7. Purification of Bacteriophages

Purified bacteriophage preparations produced clear and reproducible zones of lysis when tested against their respective MDR Gram-negative bacterial hosts.

8. Phage Susceptibility Testing

Out of the total 20 MDR isolates tested, 13% showed susceptibility to at least one isolated bacteriophage**,** as evidenced by clear zones of lysis on the agar plates.

Bacterial Isolate	**No. of MDR Isolates Tested**	**No. of Isolates Showing Lysis**	**Phage Susceptibility (%)**	**Type of Lysis Observed**
Escherichia coli	**5**	**2**	40%	Clear / Turbid
Klebsiella pneumoniae	**3**	**2**	66.66%	Clear / Partial
Pseudomonas aeruginosa	**5**	**4**	80%	Clear
Acinetobacter baumannii	**7**	**4**	57.14 %	Partial / No lysis

Phage Source (Soil Type)	Host Bacterium	Spot Test Result	Plaque Formation	Interpretation
Hospital waste near soil	*P. aeruginosa*	Strong lysis,	Clear plaques	Highly sensitive
Hospital soil	*E. coli*	Moderate lysis	Moderate plaques	Moderately sensitive
Hospital garden soil	*K. pneumoniae*	Weak lysis	Few plaques	Low sensitivity
Hospital Yard soil	*A. baumannii*	No lysis	No plaques	Resistant

Conclusion-

This study confirmed a high prevalence of multidrug-resistant (MDR) Gram-negative bacteria among clinical isolates. Soil samples, particularly those collected from hospital surroundings, served as rich sources of lytic bacteriophages. The isolated and purified bacteriophages showed effective lytic activity against several MDR isolates, as indicated by clear zones of lysis. These findings suggest that hospital soil is an important reservoir of bacteriophages with potential application as alternative or adjunct therapies against MDR Gram-negative bacterial infections.

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