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Brief Report

Eighteen-month Stability of *Myoviridae* Bacteriophages under Refrigeration Temperatures for Therapeutic Applications

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Abstract

Background: Rising antimicrobial resistance, especially in *Pseudomonas aeruginosa*, has renewed interest in Myoviridae phages as therapeutic agents. Their long-term stability under refrigeration remains a key challenge. This study was conducted to examine the stability and therapeutic potential of three *Myoviridae* bacteriophages (PA45, PA32, and PA6) stored at refrigeration temperatures (approximately 4°C with minor variations) over 18 months.

Methods: The refrigerator temperature was checked weekly using a manual thermometer. Regular enrichment was performed on a monthly basis to maintain phage infectivity. Phage titers were measured using the double-layer agar method.

Results: Results indicated that jumbo phage PA32 retained over 90% of its infectivity, PA45 retained about 85%, and PA6 retained approximately 70% of its initial level.

Conclusion: These findings highlight refrigeration as a viable and practical approach for long-term phage storage, particularly in resource-limited settings.

Keywords: Refrigeration, Therapeutic, *Myoviridae*, Bacteriophages

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Introduction

Antimicrobial resistance (AMR) is a growing global health crisis, contributing to an estimated 1.27 million deaths worldwide in 2019 (1). Among resistant pathogens, *Pseudomonas aeruginosa* presents particular challenges due to multidrug resistance and prevalence in healthcare settings. Conventional antibiotics are increasingly ineffective against these strains, necessitating alternative treatment strategies (2). Phage therapy has emerged as a promising antibacterial strategy, using bacteriophages that specifically target pathogenic bacteria while sparing the normal human microbiota. Phages in the *Myoviridae* family have shown effectiveness against *P. aeruginosa*, but the long-term preservation of these phages is essential to maintain therapeutic efficacy (3).

Preservation strategies can significantly influence phage viability. Factors such as temperature, pH, and storage buffer all play roles in stability. Although previous studies have reported the storage of phages at various temperatures (including deep freezing and lyophilization), limited information exists on the prolonged stability of *Myoviridae* phages under standard refrigeration temperatures encountered in routine laboratory practice (4,5). This study investigated the stability of three *Myoviridae* bacteriophages stored under refrigeration temperatures (4 °C with minor fluctuations) for an extended period of 18 months, to inform practical preservation methods for phage therapy.

Materials and Methods

Phage Stocks

Three *Myoviridae* bacteriophages, PA6 (vB_PaeM_GUMS6), PA45 (vB_PaeM_GUMS45), and PA32 (vB_PaeM_GUMS32) (Table 1), were isolated from environmental samples, including soil and sewage, based on their lytic activity against *P. aeruginosa*. (6,7).

Phage Preparation and Enrichment

Phages were amplified using *P. aeruginosa* cultures in the logarithmic growth phase. Cultures were incubated in LB

broth, and lysates were obtained by centrifugation at 4000 rpm for 20 minutes, followed by filtration through a 0.45 µm membrane filter. Stocks were enriched monthly by infecting fresh bacterial cultures to maintain high titers.

Purification and Storage

After enumeration, phage suspensions were further purified and stored at 4 °C. This process was repeated over an 18-month study period.

Storage Conditions

Phage suspensions were stored in a standard laboratory refrigerator at typical refrigeration temperatures (nominally 4 °C, ranging approximately from 2 to 8 °C with minor fluctuations). The refrigerator temperature was manually checked weekly using a calibrated thermometer to ensure that the temperature remained within this range.

Phage Enumeration

Phages were amplified using logarithmic-phase *P. aeruginosa* cultures in LB broth, followed by centrifugation at 4000 rpm for 20 minutes and filtration through a 0.45 µm membrane filter. Stocks were enriched monthly by re-infecting fresh bacterial cultures to maintain high titers. After each enrichment cycle, phage suspensions were purified and stored under refrigeration conditions.

Results

Throughout the 18-month storage period, the phages showed variable but generally high stability: PA32 retained over 90% of its initial titer, PA45 retained about 85%, and PA6 retained approximately 70% of its initial level (Table 2 and Figure 1). No significant contamination or loss of viability was observed due to the monthly enrichment strategy.

Discussion

The growing interest in phage therapy to combat AMR underscores the importance of reliable preservation strategies. Our results confirm that refrigeration temperatures around 4°C with weekly manual checks can preserve *Myoviridae* phages for 18 months, comparable to earlier reports by Sarker et al (8), Tremblay et al (9) and Alvi et al (10), which showed stable phage titers over shorter durations. Unlike some studies suggesting that freezing at -20 °C or -80 °C is superior (10-12), our findings support that refrigeration, ombined with monthly enrichment, maintains infectivity without specialized equipment. Clark and Geary (13) found high titer losses at freezing, reinforcing the practicality of our approach. Furthermore, the remarkable stability of PA32 suggests capsid robustness, consistent with observations by Tremblay et al (9) about jumbo phages. However, PA6 showed a moderate decline, highlighting variability across phages, as described by Zhai et al (5). This debate suggests that while refrigeration works well for some phages, strain-specific preservation protocols remain necessary. Future research should identify molecular factors linked to long-term viability and test modern stabilizers for broader applications.

Conclusion

Refrigeration with monthly enrichment and weekly temperature checks effectively preserves the infectivity of *Myoviridae* phages PA45, PA32, and PA6 over at least 18 months. This approach is practical and cost-effective for maintaining phage banks intended for therapeutic use against antibiotic-resistant pathogens.

Authors' Contribution

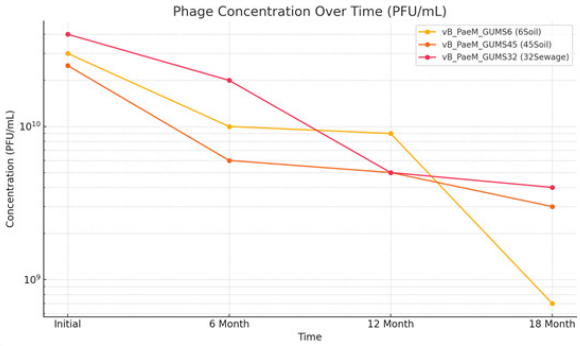

Figure 1. Long-term Concentration Profiles (PFU/mL) of PA6, PA45, and PA32 Over an 18-Month Refrigeration Storage Period

Table 1. Characteristics of the *Myoviridae* Bacteriophages Used in this Study, Including Plaque Morphology and Isolation Source

Phage Number	Source of Isolation	Plaque Type	Halo Formation Around the Plaque	Plaque Size (mm)
vB_PaeM_GUMS6 (6Soil)	Soil	Tiny clear	No	1
vB_PaeM_GUMS45 (45Soil)	Soil	Tint clear	No	1.5
vB_PaeM_GUMS32 (32Sewage)	Sewage	Clear	No	2

Table 2. Phage Titer (PFU/mL) Measured at Initial, 6-Month, 12-Month, and 18-Month Time Points during Refrigeration Storage

Phage Name	Stock duration			
	Initial Concentration (Pfu/mL)	6 Months (Pfu/mL)	12 Months (Pfu/mL)	18 Months (Pfu/mL)
vB_PaeM_GUMS6 (6Soil)	$3*10^{10}$	$1*10^{10}$	$9*10^{9}$	$7*10^{8}$
vB_PaeM_GUMS45 (45Soil)	$25*10^{9}$	$6*10^{9}$	$5*10^{9}$	$3*10^{9}$
vB_PaeM_GUMS32 (32Sewage)	$4*10^{10}$	$2*10^{10}$	$5*10^{9}$	$4*10^{9}$

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Competing Interests

The authors declare no conflict of interests.

Ethical Approval

This study was approved by Research Ethics Committees of Ramsar Campus- Mazandaran University of medical sciences(Ethic no: IR.MAZUMS.RIB.REC.1403.038).

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