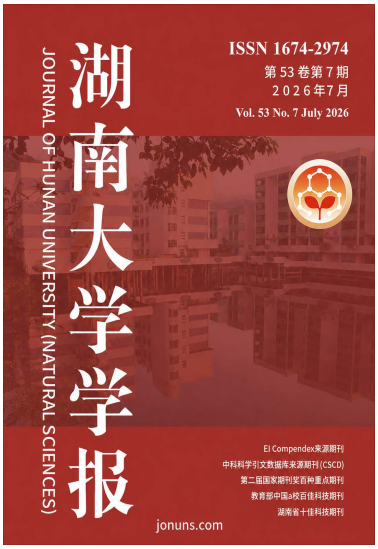


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Characterization of Bacteriophages from Sewage Water and Their Lytic Activity Against *Mycobacterium smegmatis* ATCC 14468

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Abstract: Bacteriophage-based inhibition of Mycobacterium tuberculosis offers a potential alternative for managing tuberculosis. In vitro testing generally utilizes Mycobacterium smegmatis as a surrogate model. This study aimed to isolate and characterize bacteriophages from wastewater capable of lysing M. smegmatis ATCC 14468, and to determine the optimal lytic activity from the combination of bacteriophage type and Multiplicity of Infection (MOI) doses. This research employed a 3x3 factorial randomized block design incorporating bacteriophage type and the MOI levels (0.01, 0.1, and 1) as independent variations. The dependent variable, lytic activity (PhageScore), was analyzed using a two-way ANOVA with 95% significance and Duncan's Multiple Range Test (DMRT) for post-hoc analysis. Three types of bacteriophages, namely Kc, Cm, and Mt, were successfully isolated from wastewater. These three bacteriophages shared similar characteristics: they were specific to M. smegmatis ATCC 14468, unable to infect host cells during the non-logarithmic (lag) phase and were only stable at pH 7. The stability of bacteriophage Mt could reach a temperature of 45°C, while bacteriophages Kc and Cm were stable at 37°C. The highest lytic activity was exhibited by bacteriophage Cm at a Multiplicity of Infection (MOI) of 1, yielding a PhageScore of 0.6920.

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Keywords: Lytic Activity; Mycobacteriophage; Multiplicity of Infection; PhageScore; Phage Therapy; Tuberculosis; Wastewater.

污水中噬菌体的特性及其对耻垢分枝杆菌 ATCC 14468 的裂解活性

摘要：噬菌体抑制结核分枝杆菌为结核病的治疗提供了一种潜在的替代方案。体外试验通常以耻垢分枝杆菌作为替代模型。本研究旨在从废水中分离和鉴定能够裂解耻垢分枝杆菌 ATCC 14468 的噬菌体，并确定噬菌体类型和感染复数（MOI）组合的最佳裂解活性。本研究采用 3×3 析因随机区组设计，以噬菌体类型和 MOI 水平（0.01、0.1 和 1）作为独立变量。因变量裂解活性（噬菌体评分）采用双因素方差分析（ANOVA）进行分析，显著性水平为 95%，并采用 Duncan 多重比较检验（DMRT）进行事后分析。从废水中成功分离出三种噬菌体，分别为 Kc、Cm 和 Mt。这三种噬菌体具有相似的特征：它们均特异性针对耻垢分枝杆菌 ATCC 14468，在非对数期（滞后期）无法感染宿主细胞，且仅在 pH 7 时稳定。噬菌体 Mt 的稳定性可达 45° C，而噬菌体 Kc 和 Cm 在 37° C 时稳定。噬菌体 Cm 在感染复数（MOI）为 1 时表现出最高的裂解活性，其噬菌体评分（PhageScore）为 0.6920。

关键词：溶菌活性；分枝杆菌噬菌体；感染复数；噬菌体评分；噬菌体疗法；结核病；废水。

1. Introduction

Tuberculosis (TB) remains a critical bacterial infectious disease caused by *Mycobacterium tuberculosis*, with an annual mortality rate of approximately 1.25 million people. Since 2021, more than 10 million new TB cases have occurred each year, with an increase of 4.6% between 2022 and 2023 [1]. Challenges in treating TB are primarily attributed to the high prevalence of antibiotic resistance. The estimated number of individuals developing drug-resistant tuberculosis remained consistent between 2022 and 2023, at around four hundred thousand individuals. The proportion of people with multidrug-resistant tuberculosis (MDR-TB) is 3.2% among new cases and 16% among those previously treated. This makes TB a serious public health threat. According to the Centers for Disease Control and Prevention (CDC) and WHO, MDR infections are projected to become the leading cause of human death worldwide by 2050 [1]. Declining antibiotic efficacy has underscored the urgent need for new approaches to combating bacterial infections.

Beyond resistance issues, challenges in developing TB therapies also stem from the prolonged testing period, as *M. tuberculosis* grows slowly, requiring 3 to 4 weeks. Moreover, direct studies involving *M. tuberculosis* carry significant pathogenic risks. [2]. Therefore, surrogate bacteria are commonly employed to study the biological activity of TB-causing bacteria, such as *Mycobacterium smegmatis*. Several reasons support the use of *M. smegmatis* as a model organism in TB research, including its rapid growth rate of about 3 to 4 days, its non-pathogenic nature to humans and animals, and the presence of 9 out of 13 genes homologous to *M. tuberculosis* [3].

Bacteriophage therapy represents a promising alternative to address bacterial infections, having been widely explored since its first application by d'Herelle in 1917 to treat dysentery and gaining renewed attention in the post-antibiotic era. The use of bacteriophages as therapeutic agents offers several advantages over antibiotics, most notably their ability to co-evolve with host cells, which enables them to adapt to emerging bacterial resistance [4]. A study by Little et al. [5] demonstrated that bacteriophage therapy successfully improved the condition of a patient suffering from a skin infection caused by *M. chelonae* within the first two weeks of treatment following the failure of ten different antibiotics, thereby underscoring the potential of bacteriophages as effective agents against *Mycobacterium*.

To facilitate the application of phage therapy, bacteriophages are continuously isolated from diverse environments owing to their ubiquitous nature. These viruses can be found wherever their bacterial hosts inhabit, including freshwater, seawater, soil, air, and even the human body [6]. However, for therapeutic purposes targeting human pathogens, bacteriophages

are typically isolated from aqueous samples, particularly wastewater from hospitals and residential areas, which serve as major reservoirs rich in bacterial hosts [7]. According to Ballesté et al. [8] viral particles (mostly bacteriophages) in wastewater generally range in concentration between 10^8 and 10^{10} particles·mL^{-1}. For example, bacteriophage vB_KpnP_IME337, capable of lysing carbapenem-resistant *Klebsiella pneumoniae*, was previously isolated from hospital wastewater in China [9]. Similarly, Pereira et al. [10] isolated bacteriophages from domestic wastewater samples in Educandos, Amazonas, demonstrating their ability to infect *M. smegmatis*.

Characterization is a crucial initial step following isolation to evaluate potential applications, particularly in a therapeutic context. Phage therapy involves the direct administration of virulent bacteriophages to patients to lyse pathogenic bacteria causing infections. Consequently, evaluating the lytic activity of bacteriophages is paramount. Lytic activity represents the effectiveness of bacteriophages in infecting and subsequently lysing their host cells. Several factors influence lytic activity, including the expression and diffusion of holin proteins, multiplicity of infection (MOI), and the compatibility of bacteriophage DNA with the molecular machinery of the bacterial cell [11, 12]. Therefore, the exploration of bacteriophages through isolation and characterization is a fundamental step required for the development of phage therapy to address tuberculosis caused by *Mycobacterium tuberculosis*.

While numerous mycobacteriophages have been isolated from soil environments worldwide, research targeting diverse wastewater reservoirs in high-tuberculosis (TB) burden regions, such as Jakarta, Indonesia, remains sparse. The present study addresses this gap by isolating novel mycobacteriophages from clinical and urban wastewater and providing a rigorous comparative evaluation of their lytic efficiency through the application of the PhageScore model. This approach offers a distinct methodological advantage by providing precise quantitative measurements of lytic performance, transcending traditional qualitative spot tests and facilitating a more data-driven selection of potential therapeutic candidates.

Therefore, the objective of this study was to isolate and characterize novel bacteriophages from hospital and urban sewage in Jakarta, Indonesia, using *Mycobacterium smegmatis* ATCC 14468 as a surrogate model. Furthermore, the research evaluated optimal lytic activity by analyzing the interaction between phage isolates and various Multiplicity of Infection (MOI) levels. Ultimately, this study provides critical insights into the potential development of environmental bacteriophages as alternative control agents against mycobacterial infections by establishing a robust mathematical framework for lytic assessment.

2. Methods

2.1 Bacterial Strain and Growth Conditions

Mycobacterium smegmatis ATCC 14468 (obtained from the National Research and Innovation Agency - BRIN collection, Cibinong, Indonesia), along with its rifampicin-resistant (ATCC 14468^{RIF}), and isoniazid-resistant (ATCC 14468^{INH}) mutants, were employed in this study. Cultures were cultivated at 37°C in Middlebrook 7H9 broth supplemented with glycerol and Tween 80.

2.2 Wastewater Sampling and Enrichment

Wastewater samples (25 mL each) were collected in the morning (between 06:00 and 09:00) from ten distinct locations in Jakarta, Indonesia, representing both hospitals and urban areas. The hospital sources included Pasar Minggu Regional General Hospital (Pm), Harapan Bunda Hospital (Hb), Fatmawati General Hospital (Rf), Jakarta Islamic Hospital (Ij), and Cipto Mangunkusumo National General Hospital (Cm). Urban wastewater sources, representing domestic waste confluence, were collected from Matraman (Mt), Rawamangun (Rw), Kalimalang (Km), Kali Cakung (Kc), and Kali Ciliwung (Cl). A single collection event was conducted at each location, where samples were pooled from three distinct points to ensure representative screening. Each of these primary samples was processed in triplicate, resulting in a robust environmental assessment of bacteriophage presence. The samples were enriched with an early log phase *M. smegmatis* culture (1:1 v/v) and 2.5 mL $CaCl_2$ (1 mM) [13]. Following a 7-day incubation at room temperature, the mixtures were centrifuged (10,000×g, 4°C, 10 min) and filtered through a 0.22 μm membrane. Filtrates were stored at 4°C.

2.3 Phage Isolation and Purification

Bacteriophages were isolated employing the double-layer agar method. Briefly, 4.5 mL of filtrate was mixed with 0.5 mL of early-log-phase host culture and 0.5 mL of SM buffer, followed by incubation at 37°C for 18 h. The suspension was then plated with 7H9 soft agar (0.65% w/v). The plates were incubated 37°C for 72h, and individual plaques were purified through three sequential rounds of isolation [14]. Final lysates were stored at 4°C.

2.4 Small-Scale Phage Amplification

Amplification was conducted by incubating 4.5 mL lysate with 0.5 mL host culture and 0.5 mL SM buffer at 37°C for 18h, followed by filtration (0.22 μm) [2].

2.5 Phage Titer and Plaque Size Determination

Bacteriophage titers were expressed as plaque-forming units per milliliter (PFU·mL^{-1}) [2]. The titers following amplification were calculated using the following formula:

$$PFU/mL = \frac{(\text{number of plaques} \times \text{dilution factor})}{\text{volume of inoculum (mL)}}$$

Additionally, plaque diameters were quantified using digital image analysis software from high-resolution photographs. For each isolate, the diameters of at least 10 representative plaques were measured across triplicate plates to determine the mean diameter.

2.6 Host Range Assay

The host range was evaluated against *M. smegmatis* ATCC 14468, as well as the rifampicin-resistant (ATCC 14468RIF) and isoniazid-resistant (ATCC 14468INH) strains using the double-layer agar assay. The presence or absence of plaque formation was recorded after 72 h of incubation at 37°C.

2.7 Lysis of Non-Log-Phase Hosts

The lytic activity against lag-phase hosts was tested using the double-layer method, with log-phase hosts serving as controls. The lag phase was experimentally confirmed through periodic OD_{600} monitoring, which showed that host cells remained in a stationary lag phase for up to 15 hours post-inoculation. Plaque formation was compared after a 72-h incubation period.

2.8 pH Stability Assay

Phage suspensions were exposed to SM buffer adjusted to pH 1, 3, 5, 7, and 9 for 60 min at 37°C [15]. Treated suspensions were tested by plaque assay.

2.9 Thermal Stability Assay

Phages were incubated at 37, 45, 50, 55, and 60°C for 60 min [15]. The residual infectivity was assessed via plaque assay. Stability at 37°C was defined as the maintenance of the original bacteriophage titer without significant loss of plaque-forming ability.

2.10 Lytic Activity at Different MOIs

Early-log-phase cultures were inoculated into 7H9 broth and incubated to reach the required density. Lysates were then added at multiplicities of infection (MOI) of 0.01, 0.1, and 1 [11]. Lytic activity was monitored by measuring the optical density (OD_{600}) hourly for 32 h.

2.11 Data Collection and Statistical Analysis

The lytic activity of wastewater-derived bacteriophages at different multiplicities of infection (MOIs) was evaluated by monitoring the turbidity of *M. smegmatis* cultures. Optical density at 600nm (OD_{600}) was measured every 60 min for 32 h. Lytic activity was quantified using the PhageScore (Ps) model as described by Konopacki et al. [11], with parameters (y_0, x_0, a, d, L, b, c, k, h) obtained through non-linear estimation curve fitting of time–OD data using Statistica software. The curve fitting procedure employed the Levenberg-Marquardt algorithm to achieve the best fit for the time–OD data. The PhageScore (P_S) is a quantitative metric that integrates the area under the lytic curve compared to the growth curve of uninfected host cells. The calculation effectively measures the gap between the bacterial growth in the presence and absence of phages. P_S values are normalized to a range of 0 to 1, where a value of 0 indicates no lytic activity (growth identical to control), and a value of 1 represents maximum lytic efficiency (complete inhibition of host growth). The resulting PhageScore values were analyzed using two-way ANOVA at a 95% confidence level in SPSS, where the experimental replicates were designated as the blocking factor to account for inter-assay variability. Prior to analysis, data were tested for normality and homogeneity of variance. When significant differences were detected, mean separation was performed with Duncan's Multiple Range Test (DMRT). To ensure reliability and reproducibility, all experimental procedures were performed with three biological and technical replicates.

3. Results and Discussion

3.1 *Mycobacterium smegmatis* Growth

As a surrogate for *Mycobacterium tuberculosis*, *Mycobacterium smegmatis* is widely utilized in research due to its safety and significant genomic homology. Various studies have demonstrated that many mycobacteriophages exhibit a broad host range, with the ability to infect multiple species within the genus. The use of *M. smegmatis* as a model bacterium is highly suitable, even when the research aims to identify bacteriophages targeting *M. tuberculosis*. For instance, Kalapala et al. [16] reported that bacteriophages D29 and TM4, which use *M. smegmatis* as a host, can effectively infect *M. tuberculosis*. Additionally, Broxmeyer et al. [17] reported that bacteriophage TM4 was able to reduce the intracellular population of *M. tuberculosis* inside macrophages. Similarly, bacteriophage Celfi, which was identified using *M. smegmatis* as a host, is also capable of infecting and lysing *M. tuberculosis* [18].

The M. smegmatis strains employed in this study were obtained from the collection of the National Research and Innovation Agency (BRIN) Cibinong. These included the antibiotic-susceptible ATCC (American Type Culture Collection) 14468 parental strain, along with its rifampicin-resistant (ATCC 14468RIF) and isoniazid-resistant (ATCC 14468INH) derivatives. The resistant phenotypes were established by BRIN researchers through sequential drug pressure cultivation.

To determine the optimal timing for phage inoculation, the growth dynamics of M. smegmatis ATCC 14468 were first monitored. Bacterial cultures

grown in Middlebrook 7H9 broth supplemented with glycerol and Tween 80 showed typical growth dynamics consisting of lag, exponential, and stationary phases. The experimental results confirmed that the host cells remained in the lag phase for approximately 15 hours post-inoculation before transitioning into the exponential growth phase. These observations are consistent with the established growth kinetics reported by Tran and Cook [19]. Under the experimental conditions (28°C with agitation at 120 rpm), uniform and predictable growth was prioritized to ensure reproducibility in subsequent infection assays.

Bacteria within the genus Mycobacterium spp., including M. smegmatis, typically exhibit an optimal growth temperature of 37°C, though some studies report growth at temperatures up to 42°C. However, in this study, agitation was prioritized over maintaining the optimal growth temperature; consequently, incubation was carried out at room temperature (28°C). Achieving uniform growth was essential for the experimental design, even at the expense of a faster growth rate. As a result, the logarithmic phase of M. smegmatis extended beyond 20 hours, whereas it typically lasts only 10 hours under optimal temperature conditions.

The application of Tween 80 presents a distinct challenge in bacteriophage research. Tween 80 is instrumental in achieving uniform and unclumped host growth in Middlebrook 7H9 broth, providing the homogenous cultures required for accurate experiments involving Mycobacterium species, such as optical density measurements and total plate count (TPC) analysis Non-uniform M. smegmatis cultures can introduce significant experimental inaccuracies; for example, clumping can cause cells to settle at the bottom of the medium, leading to underestimation during optical density quantification. A similar problem arises in TPC analysis, as cellular aggregation may lead to multiple cells forming a single colony-forming unit, thereby artificially reducing the calculated TPC. Although advantageous for host cultivation, the use of Tween 80 can inhibit bacteriophage infection. Acting as a surface tension agent, it interferes with the attachment of bacteriophage tails to bacterial cell surface molecules, thereby impeding infection and subsequent host lysis. This was confirmed in this study (data not shown), as no bacteriophage activity was detected when Tween 80 was incorporated into the test medium. Tween 80 was exclusively used during the initial cultivation of M. smegmatis to achieve clump-free logarithmic phase cultures. Subsequently, Tween 80 was strictly excluded from all media used for lytic activity assays, including broth, solid agar, and semi-solid agar. This omission was critical to prevent any surface-tension interference with bacteriophage adsorption and infection processes in both liquid and solid environments.

3.2 Wastewater Source Characteristics

Hospital and urban wastewater samples were utilized to obtain virulent bacteriophages against *M. smegmatis*. Systematic wastewater sampling was conducted by collecting 10 samples from hospitals treating drug-resistant tuberculosis (DR-TB) patients and from densely populated urban open water systems. The selection of hospitals was based on the hypothesis that healthcare facilities environments are more likely to contain bacterial species from the genus *Mycobacterium*, thereby increasing the likelihood of finding adapted bacteriophages. A similar rationale was applied to the selection of urban water bodies, which represent convergence points of domestic wastewater and are therefore likely to contain *Mycobacterium* spp. from either environmental or human sources. According to Thakali et al. [20], wastewater, particularly that from densely populated urban areas and healthcare facilities, serves as a reservoir with high microbial diversity, including pathogenic bacteria and their associated bacteriophages. The concentration of virus-like particles (VLPs) in wastewater can reach up to 10^9 particles per milliliter [13], significantly higher than other aquatic environments, making wastewater an ideal source for bacteriophage exploration.

The characteristics of the wastewater samples utilized in this study are summarized in Table 1. Samples collected from hospital sources including Pasar Minggu Regional General Hospital (Pm), Harapan Bunda Hospital (Hb), Fatmawati General Hospital (Rf), Jakarta Islamic Hospital (Ij), and Cipto Mangunkusumo National General Hospital (Cm) exhibited similar characteristics: a clear appearance, little to no debris, and no noticeable odor. In contrast, wastewater from densely populated urban water bodies comprising Matraman (Mt), Rawamangun (Rw), Kalimalang (Km), Cakung River (Kc), and Ciliwung River (Cl) was characterized by a turbid appearance and an unpleasant odor. Additionally, while urban wastewater sources were characterized by active flow, hospital wastewater was stagnant at the time of collection.

Table 1. Characteristics of the wastewater used

Wastewater source	Turbidity	Odor	Flow type
Pm	Clear	Odorless	Stagnant
Hb	Clear	Odorless	Stagnant
Rf	Clear	Odorless	Stagnant
Ij	Clear	Odorless	Stagnant
Cm	Clear	Odorless	Stagnant
Mt	Highly turbid	Foul odor	Slow
Rw	Turbid	Foul odor	Slow
Km	Turbid	Foul odor	Fast
Kc	Turbid	Foul odor	Slow
Cl	Highly turbid	Foul odor	Fast

Note: Pm: Pasar Minggu Regional General Hospital; Hb:

Harapan Bunda Hospital; Rf: Fatmawati General Hospital; Ij: Jakarta Islamic Hospital; Cm: Cipto Mangunkusumo National General Hospital; Mt: Matraman; Rw: Rawamangun; Km: Kalimalang; Kc: Kali Cakung; Cl: Kali Ciliwung.

samples, (b) enrichment of wastewater samples, (c) general isolation of viral particles, (d) specific isolation of virulent (lytic cycle) bacteriophages against *M. smegmatis*, and (e) purification of bacteriophages.

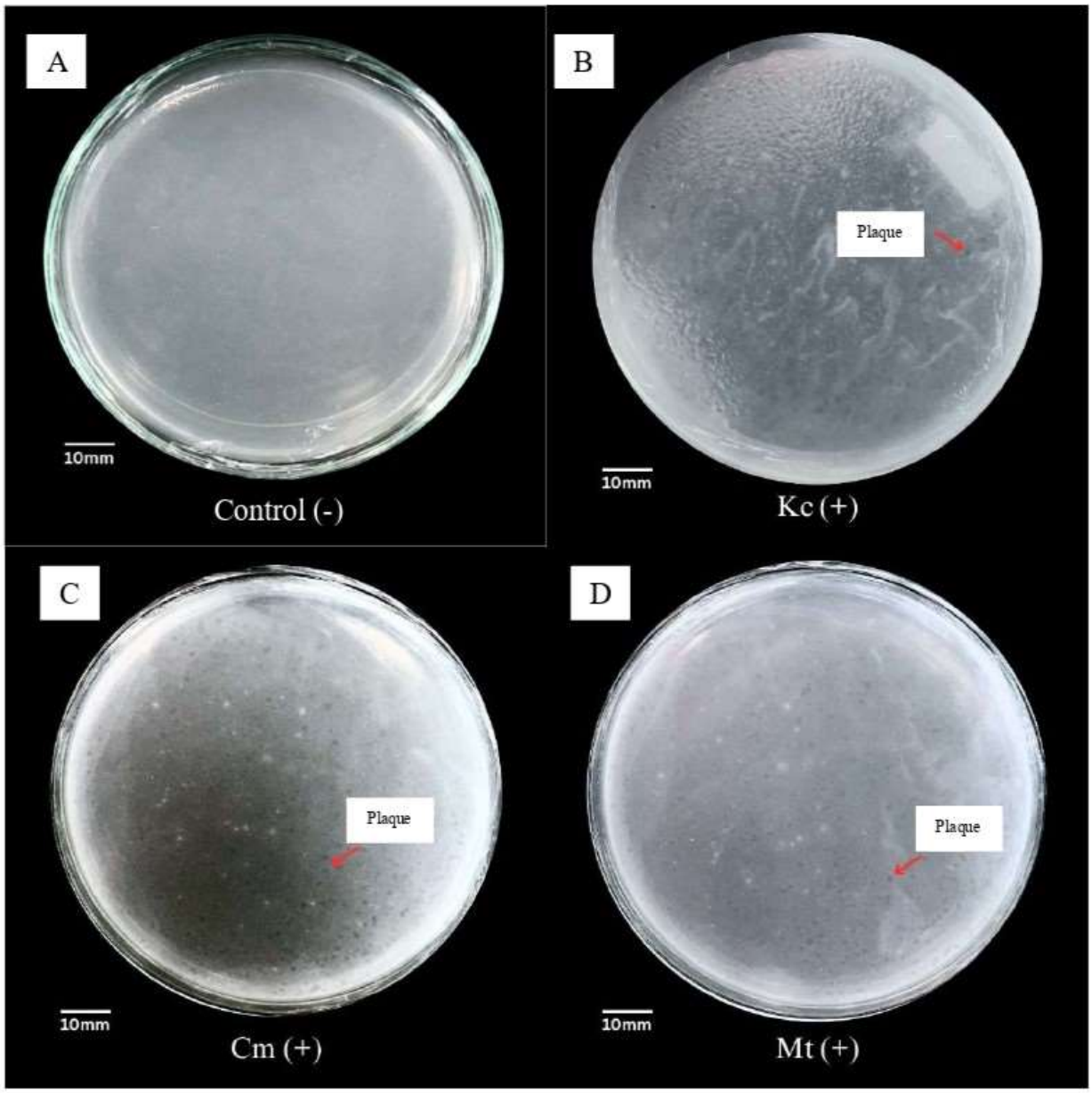


Figure 1. Clear zones (indicated by arrows) on *M. smegmatis* lawns formed due to infection by bacteriophages (B) Kc: Kali Cakung, (C) Mt: Matraman, and (D) Cm: Cipto Mangunkusumo National General Hospital.

3.3 Phage Isolation and Purification

Wastewater samples were screened for the presence of bacteriophages capable of infecting *M. smegmatis*. Plaque assays revealed the presence of three distinct bacteriophage isolates, which were designated as Kc, Cm, and Mt. Two phages originated from urban open-water wastewater, Kc from the Cakung River and Mt from Matraman, while one isolate was recovered from hospital wastewater, namely phage Cm from Cipto Mangunkusumo National General Hospital. The isolation process involved five main stages: (a) collection of wastewater

The enrichment process was performed on wastewater samples prior to isolation by inoculating the host M. smegmatis ATCC 14468 cells. Enrichment serves not only to increase the overall viral titer but also acts as a selective amplification process for the desired viral particles, in this case, M. smegmatis bacteriophages. A 1mM $CaCl_2$ solution was used in the enrichment process and in each assay aimed at detecting lytic activity of bacteriophages against the host cells. Calcium ions play a critical role in the bacteriophage infection process. Numerous studies

have demonstrated the importance of calcium ions in phage-host interactions; these functions include acting as essential cofactors in infection, stabilizing the infectious conformation, attaching the central tail needle to the cell membrane, and neutralizing the negative charge on bacterial cell surfaces. This neutralization facilitates phage genome entry by reducing electrostatic repulsion between the negatively charged cell membrane and nucleic acids, while also stabilizing capsid structure by mediating interactions between protein subunits [21].

Viral particle isolation from wastewater samples was carried out using a membrane filtration method, employing syringe filters with a pore size of 0.22 μm. This size-exclusion filtration effectively separates viral particles from bacterial contaminants. After filtration, the resulting solution, referred to as the lysate, is assumed to contain only viral particles and be free from other microorganisms such as bacteria or fungi.

The isolation of M. smegmatis-specific virulent bacteriophages was essentially a screening process aimed at identifying the desired phages within the wastewater samples. Enriched and filtered wastewater samples may contain a diverse range of viruses, not limited to bacteriophages. In the double-layer assay, positive results were indicated by the formation of clear zones (plaques) within the *M. smegmatis* bacterial lawn as shown by the red arrows in Figure 1. These clear zones provide visual evidence of the successful bacteriophage lytic cycle.

Based on the isolation results, only 3 out of 10 wastewater sources tested positive for virulent *M. smegmatis* bacteriophages (Table 2). This 30% success rate aligns with the reported range typically observed in phage isolation studies, suggesting that the methodology employed was effective and valid. Negative results from the remaining seven samples do not necessarily imply the complete absence of bacteriophages, but rather the absence of virulent phages specific to the *M. smegmatis* ATCC 14468 strain under the experimental conditions. These samples may still contain phages targeting other bacterial species or those with a temperate (lysogenic) lifestyle. Temperate phages remain undetectable by the double-layer assay, which relies exclusively on plaque formation resulting from lytic cycles. Hatfull [22] previously noted that the majority of bacteriophages infecting *M. smegmatis* are temperate. Therefore, the 30% success rate reflects the isolation of highly specific virulent bacteriophages against *M. smegmatis*, rather than serving as an absolute measure of the total phage population in the sampled wastewater. These findings are comparable to those of Abusalah et al. [23], who reported a 31.7% success rate in isolating *Klebsiella pneumoniae*-specific bacteriophages from hospital wastewater, a figure comparable to this study.

Table 2. Detection of *M. smegmatis* bacteriophages as indicated by plaque formation

Wastewater Sources	Plaque in Double Layer Assay
Pm	-
Hb	-
Rf	-
Ij	-
Cm	+
Mt	+
Rw	-
Km	-
Kc	+
Cl	-

Notes. Symbol (–) indicates a negative result, while symbol (+) indicates a positive result for the presence of virulent *M. smegmatis* bacteriophages in wastewater samples. Pm: Pasar Minggu Regional General Hospital; Hb: Harapan Bunda Hospital; Rf: Fatmawati General Hospital; Ij: Jakarta Islamic Hospital; Cm: Cipto Mangunkusumo National General Hospital; Mt: Matraman; Rw: Rawamangun; Km: Kalimalang; Kc: Kali Cakung; Cl: Kali Ciliwung.

The three wastewater sources found to harbor virulent *M. smegmatis* bacteriophages were Kali Cakung (Kc), Cipto Mangunkusumo National General Hospital (Cm), and Matraman (Mt). No definitive common characteristics were identified across these three sites that could be established as the primary factor for the presence of these phages. While the water flow at these locations was consistently slow-moving or stagnant, other sampled sources shared these physical traits without yielding any detectable mycobacteriophages. None of the other environmental parameters were consistently shared among the three positive samples. Consequently, these findings suggest that visible wastewater characteristics alone are not reliable determinants for predicting bacteriophage presence.

3.4 Phage Morphology and Titer

The characteristics of each bacteriophage are detailed in Table 3. All three phages produced clear plaques, confirming their classification as virulent phages rather than temperate (lysogenic) phages. The main difference was observed in the plaque edges: Kc displayed smooth edges, whereas both Cm and Mt exhibited irregular edges. Bacteriophage Kc formed plaques with a diameter of 1.3 mm, which was larger than the 1.0 mm plaques produced by Cm and Mt. Although plaque size and edge morphology provide strong phenotypic characteristics to distinguish Kc from Cm and Mt, all isolates fall into the same size category of small plaques (< 2 mm), according to Shende et al. [24]. Further differentiation among these phages is provided by their bacteriophage titers, which reflect the initial concentration of phage particles immediately after enrichment but prior to large-scale

amplification. Among the three, bacteriophage Cm showed the highest titer at 1.6×10^3 PFU·mL^{-1}, surpassing Kc (5.5×10^2 PFU·mL^{-1}) and Mt (4.5×10^2 PFU·mL^{-1}). The higher titer observed for Cm may indicate greater replication efficiency, as all wastewater samples were subjected to enrichment under the same conditions and duration. This elevated titer is indicative of its superior efficiency in lysing host *M. smegmatis* cells.

Table 3. Characteristics of *M. smegmatis* bacteriophages isolated from wastewater

Bacteriophage	Plaque Morphology	Plaque Diameter (mm)	Bacteriophage Titer (PFU·mL^{-1})
Kc	Clear plaque, smooth edges	1.3	5.5×10^2
Cm	Clear plaque, irregular edges	1.0	1.6×10^3
Mt	Clear plaque, irregular edges	1.0	4.5×10^2

Notes. Bacteriophage titer refers to the concentration of phages in the lysate at the initial stage of isolation and before subsequent amplification stages.

The titers of the isolated bacteriophages presented in Table 3 reflect the initial concentrations recovered immediately following isolation and purification, before any large-scale amplification. These relatively low initial titers, ranging from 4.5×10^2 to 1.6×10^3 PFU·mL^{-1}, are typical for environmental isolates prior to laboratory propagation. To meet the required concentrations for lytic activity assays, these primary lysates underwent subsequent amplification as described in the Methods section. Following this, the amplified lysates were titrated using the double-layer assay to verify the increase in phage concentration. This amplification process was conducted iteratively until the bacteriophage titers reached a target level of 10^5 PFU·mL^{-1}, ensuring a consistent and sufficient viral density for all subsequent lytic assessments.

3.5 Host Specificity of Isolated Bacteriophages

The host range of the isolated bacteriophages was evaluated using three bacterial strains with varying antibiotic resistance: *M. smegmatis* ATCC 14468 (non-resistant), *M. smegmatis* ATCC 14468^{RIF} resistant to rifampicin (RIF), and M. smegmatis ATCC 14468INH resistant to isoniazid (INH). As summarized in Table 4, plaque formation was exclusively observed on the wild-type strain, while no lytic activity was detected on the resistant mutants. These results indicate a high degree of host specificity, suggesting that the lytic spectrum of these isolates is restricted to the parental strain under the conditions tested. Such specificity is typically governed by precise receptor recognition on the bacterial cell surface.

Table 4. Ability of bacteriophages to lyse other *M. smegmatis* strains

Bacteriophage	*Strain Mycobacterium smegmatis*		
	ATCC 14468	ATCC 14468^{RIF}	ATCC 14468^{INH}
Kc	+	-	-
Cm	+	-	-
Mt	+	-	-

Notes. Symbol "–" indicates negative result, while "+" indicates positive bacteriophage lysis of *M. smegmatis*.

The inability of the phages to infect antibiotic-resistant strains may be linked to host-mediated modifications in core metabolic or structural pathways. Resistance to rifampicin, for instance, involves mutations in the β-subunit of RNA polymerase [25], which could potentially interfere with the phage's dependence on host transcriptional machinery for protein synthesis. Similarly, resistance to isoniazid often involves mutations in the *ndh* gene, leading to an altered NADH/NAD$^+$ ratio [26]. Since many bacteriophages, such as the well-studied phage T4, require host NAD$^+$ to modify cellular enzymes during the molecular hijacking process [27], the metabolic shifts associated with drug resistance might serve as an inadvertent defense mechanism against viral replication. However, as molecular evidence was not collected in this study, these biochemical interpretations remain suggestive and require further validation through genomic and proteomic analysis.

The host specificity analysis in this study was intentionally focused on a select panel of *M. smegmatis* strains to provide a preliminary assessment of phage specificity. However, it is important to emphasize that these results are limited to the three strains tested. While the phages demonstrated clear specificity for the wild-type strain, their broader host range potential remains to be determined. Future studies employing a more extensive and diverse library of mycobacterial hosts, including various clinical and environmental isolates, are necessary to fully map the lytic breadth of these phages.

3.6 Lysis of Non-Log-Phase Hosts

The purpose of testing the ability of bacteriophages to infect host cells at different growth phases was to determine whether the three bacteriophages (Kc, Cm, and Mt) were still capable of infecting and lysing host cells under suboptimal growth conditions. The results showed that all three bacteriophages successfully lysed bacteria in the logarithmic phase (active growth phase) but were unable to lyse bacteria in the lag phase (Table 5). The host's lag phase was experimentally confirmed through periodic OD_{600} monitoring, which showed that the host cells remained in a lag phase for up to 15 hours post-inoculation. These experimental data points were found to be consistent with the growth kinetics reported by

Tran and Cook [19] for this strain under similar environmental conditions. Consequently, host cells harvested within this 15-hour window were used to represent the lag-phase population in the lytic assays.

Table 5. Ability of bacteriophages to lyse host cells at different growth phases

Bacteriophage	Host Cell Growth Phase	
	Log	Lag
Kc	+	-
Cm	+	-
Mt	+	-

Notes. Symbol "–" indicates a negative result, while "+" indicates a positive result for bacteriophage lysis of *M. smegmatis*.

The inability of bacteriophages to lyse lag phase cells indicates an obligate dependence on the physiological state and metabolic activity of the host. In the logarithmic phase, host cells facilitate maximal rates of transcription, translation, and cellular component synthesis, providing abundant resources that bacteriophages can exploit to replicate their genomes and synthesize viral proteins rapidly. As explained by Kasman and Porter [28], bacteriophages lack their own biosynthetic machinery, such as ribosomes and metabolic pathways for energy production, and therefore must fully hijack the metabolic apparatus of the host cells to support their replication cycle.

Most bacteriophages, such as the widely studied mycobacteriophage D29, exhibit limited infectivity toward host cells at suboptimal growth stages. The three isolates in the present study appear to share similar characteristics with D29 regarding their inability to lyse M. smegmatis during the lag phase, a failure that likely occurs during the replication stage rather than during initial adsorption. In contrast, certain bacteriophages like TM4 are capable of infecting hosts in suboptimal phases because they possess tail-associated peptidoglycan hydrolases that function similarly to resuscitation-promoting factors (Rpf), which can activate host metabolism to support viral replication [29].

Dependence on metabolically active host cells presents a limitation for the therapeutic application of these phages in tuberculosis treatment. A hallmark of M. tuberculosis pathogenesis is its ability to enter a dormant or latent state within the host [30]. Based on the results of this study, it can be concluded that bacteriophages Kc, Cm, and Mt are likely ineffective against dormant *M. tuberculosis*. Nevertheless, these bacteriophages could still be developed for therapeutic strategies targeting active tuberculosis cases, where the majority of the bacterial population is undergoing active replication.

3.7 pH Stability of Bacteriophages

The stability of bacteriophages across different temperature and pH ranges is a key characteristic analyzed after the isolation process to evaluate their potential application in therapy. Bacteriophages are particles composed of proteins and nucleic acids, with the capsid structural integrity formed by proteins that are highly vulnerable to denaturation under extreme pH (Table 6) and temperature conditions.

Table 6. pH stability of *M. smegmatis* bacteriophages

Bacteriophage	pH				
	1	3	5	7	9
Kc	-	-	-	+	-
Cm	-	-	-	+	-
Mt	-	-	-	+	-

Notes. Symbol "–" indicates negative result, while "+" indicates positive bacteriophage lysis of *M. smegmatis*.

The stability of bacteriophages under different pH conditions was evaluated. The results indicated that the lytic activity of all three bacteriophages was highest under neutral pH conditions (pH 7) and showed a complete loss of infectivity under acidic (pH 1, 3, 5) and alkaline (pH 9) conditions. This indicates that the protein capsid structures of these bacteriophages are highly sensitive to pH variations. According to Jończyk et al. [31], conformational changes induced by protonation or deprotonation of amino acid functional groups at non-neutral pH can render bacteriophages incapable of infecting host cells.

This extreme sensitivity to pH presents a limitation for therapeutic applications. The narrow pH stability of these bacteriophages restricts potential delivery routes. Phages that are only stable at neutral pH (pH 7) eliminate certain therapeutic options. For instance, oral administration becomes impossible due to the highly acidic gastric environment (pH 1.5–3.5). Therefore, advanced formulation strategies, such as encapsulation or pH-buffered delivery systems, would be essential to protect the phage particles and maintain their lytic activity in clinical settings.

Based on the pH stability tests, these bacteriophages are relatively fragile compared to many others reported in previous studies. For instance, bacteriophages infecting Gram-positive bacteria; for instance, phages targeting S. aureus in the study by Kwiatek et al. [32] remained stable across pH 4 to 10.

3.8 Thermal Stability of Bacteriophages

The thermal stability of the isolated bacteriophages was evaluated by incubating phage suspensions at various temperatures, followed by measuring their lytic activity. This assay aimed to determine whether the bacteriophages retained their ability to infect and lyse host cells after exposure to specific temperatures for 60 minutes. All

bacteriophages were subsequently tested on their host M. smegmatis at 37°C, the optimal growth temperature for the host. Therefore, testing was conducted at temperatures at and above 37°C to assess their potential application as therapeutic agents at human body temperature (~37°C).

The results (Table 7) indicated that the three bacteriophages exhibited different levels of thermal tolerance. Bacteriophage Mt maintained detectable lytic activity at temperatures up to 45°C, whereas bacteriophages Kc and Cm showed optimal stability only at 37°C. Notably, stability at 37°C corresponds to human physiological temperature, which is a favorable characteristic for therapeutic applications. Stability was defined as the maintenance of the original bacteriophage titer without significant loss of plaque-forming ability after 60 minutes of incubation. At higher temperatures, a reduction in lytic activity was observed, suggesting partial inactivation of phage particles.

Table 7. Temperature stability of *M. smegmatis* bacteriophages

Bacteriophage	Temperature (°C)				
	37	45	50	55	60
Kc	+	-	-	-	-
Cm	+	-	-	-	-
Mt	+	+	-	-	-

Notes. Symbol "–" indicates negative result, while "+" indicates positive bacteriophage lysis of *M. smegmatis*.

Although unstable at higher temperatures, these bacteriophages likely remain stable and active at lower temperatures. Some studies have reported bacteriophages capable of retaining activity even at elevated temperatures, such as the phages infecting *Mycobacterium* spp. described by Lopez-Davis et al. [33], which remained active up to 60°C. However, many bacteriophages are strictly mesophilic. For example, Komijani et al. [34] reported that a *K. pneumoniae* phage was stable only up to 40°C.

3.9 Effect of MOI Doses on Lytic Activity

The lytic activity of the three bacteriophages in this study (Kc, Cm, and Mt) was quantitatively evaluated using the PhageScore (Ps) at three Multiplicity of Infection (MOI) doses (0.01, 0.1, and 1). To ensure accurate dosing, host M. smegmatis density was standardized to 10^5 CFU·mL^{-1}, with bacteriophage titers adjusted to 103, 104, and 10^5 PFU·mL^{-1} for each respective MOI.

PhageScore is a quantitative method developed by Konopacki et al. [11] to analyze the capacity of bacteriophages to lyse their host cells This metric provides superior sensitivity in discriminating differences in lytic ability among bacteriophages more sensitively compared to other methods such as the Lysis Score, Virulence Index, or the Streak-based Method. In addition, the application of PhageScore facilitates the analysis of lytic activity at specific MOI doses, allowing for accurate comparative analysis of bacteriophage activity and enabling the selection of appropriate bacteriophages for potential therapeutic applications. PhageScore values range from 0 to 1, where values approaching 1 indicate greater lytic capability of the bacteriophage against its host cells.

The lytic activity of the three bacteriophages at three different MOI doses (0.01, 0.1, and 1) is summarized in Table 8. Statistical analysis using two-way ANOVA confirmed that both bacteriophage type ($F_{(2,18)} = 1664.227, p < 0.001$) and MOI dose ($F_{(2,18)} = 522.530, p < 0.00$) significantly influenced the lytic activity. Furthermore, a significant interaction between phage type and MOI was observed ($F_{(4,18)} = 54.722, p < 0.001$), indicating that the lytic performance of each isolate was dependent on the initial MOI. The treatment with the highest lytic activity was bacteriophage Cm at MOI 1, with a PhageScore value of 0.6920, which was significantly different from all other treatments (as denoted by different superscript letters). Specifically, the treatments of Cm at MOI 0.01 and 0.1 yielding the second-best PhageScore values and were not significantly different from each other, with both remaining above 0.6.

Table 8. Lytic activity (PhageScore) of *M. smegmatis* bacteriophages from wastewater at different multiplicity of infection (MOI) doses

Bacteriophage	Multiplicity of Infection		
	0.01	0.1	1
Kc	0.3328 ± 0.0079^{a}	0.4599 ± 0.0089^{d}	0.5362 ± 0.0033^{f}
Cm	0.6146 ± 0.0014^{g}	0.6282 ± 0.0028^{g}	0.6920 ± 0.0012^{h}
Mt	0.3970 ± 0.0068^{b}	0.4340 ± 0.0017^{c}	0.5054 ± 0.0025^{e}

Notes. Different superscript letters indicate significant differences according to DMRT post-hoc test at 95% significance.

Bacteriophage Cm consistently exhibited the highest lytic activity across all tested MOI doses, indicating that this bacteriophage had stronger lytic ability compared to the other types, Kc and Mt. The PhageScore values for bacteriophage Cm were comparable to those of lytic bacteriophages reported in previous studies, suggesting that Cm has a lytic capacity equivalent to other promising bacteriophage candidates. For example, bacteriophage Pf-10 in the study by Kazantseva et al. [35] demonstrated a PhageScore of approximately 0.7 at MOI 1.

The lowest PhageScore was observed for bacteriophage Kc at MOI 0.01 (0.3328), followed by bacteriophage Mt at the same MOI (0.3970). These results suggest that the specific bacteriophage type has a more dominant effect than the MOI dose on lytic activity. A decrease in MOI from 1 to 0.01 generally resulted in a reduction of PhageScore across all bacteriophages tested. At a low MOI (0.01), only a small proportion of the bacterial population is initially infected, necessitating multiple replication cycles before substantial host lysis occurs. Conversely, at a high MOI (1), the probability of each bacterium being infected by at least one phage particle increases significantly, leading to faster bacterial lysis, as reflected by higher PhageScore values. Kazantseva et al. [35] reported a similar trend, where bacteriophage Pf-10 exhibited decreasing PhageScore values correlated with decreasing MOI.

The total lytic activity of the three bacteriophages is presented in Table 9. Results from the DMRT post-hoc test indicated that isolate Cm had the highest Total PhageScore (0.6449), which was significantly different from isolates Kc (0.4430) and Mt (0.4455). The total lytic activity between Kc and Mt did not differ significantly. These results suggest that bacteriophage Cm possesses more potent biological characteristics, being more efficient in infecting and lysing host cells.

Table 9. Total lytic activity (Total PhageScore) of *M. smegmatis*

Bacteriophage	Total PhageScore
Kc	0.4430 ± 0.0064^{a}
Cm	0.6449 ± 0.0011^{b}
Mt	0.4455 ± 0.0022^{a}

Notes. Different superscript letters indicate significant differences according to DMRT post-hoc test at 95% significance. Values after the ± symbol indicate standard error.

This finding is further supported by the fact that bacteriophage Cm exhibited higher titers than Kc and Mt when propagated with its host cell, M. smegmatis. The enhanced lytic activity of bacteriophages can be attributed to a combination of factors, such as a faster adsorption rate, shorter latent period, larger burst size, or higher efficiency within the holin-endolysin lysis system in degrading bacterial cell walls. The superior performance of bacteriophage Cm suggests that this isolate may possess higher adsorption efficiency, faster replication dynamics, or more robust lytic capability compared with the other isolates. However, while these results are encouraging, it is premature to label isolate Cm as therapeutically promising. Comprehensive genomic sequencing is required to confirm the absence of lysogeny-related genes, toxins, or antimicrobial resistance genes. Furthermore, since this study exclusively used M. smegmatis as a non-pathogenic surrogate, direct lytic assays against pathogenic M. tuberculosis strains are essential to validate its true clinical potential.

3.10 Therapeutic Potential, Limitations, and Future Perspective

The isolation of bacteriophages capable of infecting *M.* smegmatis from wastewater highlights the potential of environmental samples as sources of phages for mycobacterial research. However, it is imperative to interpret the therapeutic implications of these findings with caution, as this study utilized M. smegmatis ATCC 14468 as a non-pathogenic surrogate model. While M. smegmatis is a valuable tool for preliminary screening due to its rapid growth and genomic similarities to the pathogen, the lytic efficacy of isolates Kc, Cm, and Mt against Mycobacterium tuberculosis was not directly evaluated. Despite the metabolic similarities, differences in cell wall composition and the absence of specific virulence factors in M. smegmatis may influence phage-host interactions. Consequently, lytic activity observed against the model organism does not always guarantee identical performance against M. tuberculosis. Furthermore, M. smegmatis does not fully replicate the complex dormancy and intracellular survival mechanisms of the pathogen. Therefore, while these results provide a critical preliminary screening, direct validation against pathogenic clinical isolates remains a necessary step for future therapeutic development.

The present study provides a valuable preliminary screening of wastewater-derived mycobacteriophages. However, some limitations must be acknowledged. The lack of Transmission Electron Microscopy (TEM) prevented the morphological classification of the isolates into specific viral families. Additionally, the absence of whole-genome sequencing limits our understanding of their genetic safety and lytic mechanisms. Future research should prioritize molecular characterizations and expand the host range testing to include a broader spectrum of clinical *Mycobacterium* isolates to fully assess their viability for phage-based interventions.

4. Conclusion

The study successfully isolated three bacteriophages from wastewater, designated as Kc, Cm, and Mt, that are capable of infecting and lysing Mycobacterium smegmatis ATCC 14468. The novelty of this research is established through the isolation of these novel bacteriophages combined with a rigorous quantitative assessment of their MOI-dependent lytic activity using the PhageScore model. However, the study concludes that these findings represent only a preliminary stage of discovery.

The three isolates exhibited similar host specificity toward M. smegmatis and showed differences in

environmental stability. Bacteriophages Kc and Cm remained stable at temperatures up to 37°C, whereas bacteriophage Mt retained detectable lytic activity at temperatures up to 45°C. All three bacteriophages demonstrated the highest activity under neutral pH conditions.

Among the tested isolates, bacteriophage Cm exhibited the highest lytic activity against *M. smegmatis* ATCC 14468 at a multiplicity of infection (MOI) of 1, achieving a PhageScore of 0.6920. These findings indicate that wastewater environments can serve as potential sources of bacteriophages capable of infecting mycobacterial hosts and may provide useful candidates for further investigation in phage-based antimicrobial strategies.

However, several limitations should be considered when interpreting the results of this study. First, the experiments were conducted using *M. smegmatis* as a model organism rather than pathogenic *Mycobacterium tuberculosis*, therefore the results cannot directly represent phage activity against the tuberculosis pathogen. Second, the experiments were conducted under controlled in vitro laboratory conditions, which may not fully reflect environmental or clinical conditions where additional biological factors could influence phage activity. In addition, the study did not include Transmission Electron Microscopy (TEM) for morphological classification or whole-genome sequencing to ensure the absence of lysogeny-related and toxin genes. While these findings suggest a potential for future therapeutic research, its application remains subject to comprehensive genomic validation and safety testing against pathogenic mycobacteria.

Declarations

Author Contributions

Conceptualization, R.H.P. and A.L.Y.; methodology, R.H.P. and A.L.Y.; software, A.L.Y.; validation, R.H.P., T.H.K., and A.L.Y.; formal analysis, R.H.P., T.H.K., and A.L.Y.; investigation, R.H.P. and A.L.Y.; resources, R.H.P. and A.L.Y; data curation, R.H.P., T.H.K., and A.L.Y.; writing-original draft preparation, A.L.Y. and R.H.P.; writing—review and editing, R.H.P. and A.L.Y.; visualization, A.L.Y.; supervision, R.H.P., T.H.K., and A.L.Y.; project administration, R.H.P.; funding acquisition, R.H.P. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

All data generated or analyzed in this study are fully available within the article.

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Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

References

[1] WORLD HEALTH ORGANIZATION [WHO]. Global tuberculosis report 2024. World Health Organization; 2025.

[2] SPARKS I.L., DERBYSHIRE K.M., JACOBS JR W.R., et al. *Mycobacterium smegmatis*: the vanguard of mycobacterial research. J Bacteriol. 2023;205(1):e00337-22. https://doi.org/10.1128/jb.00337-22

[3] LELOVIC N., MITACHI K., YANG J., et al. Application of *Mycobacterium smegmatis* as a surrogate to evaluate drug leads against *Mycobacterium tuberculosis*. J Antibiot. 2020;73(11):780-789. https://doi.org/10.1038/s41429-020-0320-7

[4] KOSKELLA B., BROCKHURST M.A. Bacteria–phage coevolution as a driver of ecological and evolutionary processes in microbial communities. FEMS Microbiol Rev. 2014;38(5):916–31. https://doi.org/10.1111/1574-6976.12072

[5] LITTLE J.S., DEDRICK R.M., FREEMAN K.G., et al. Bacteriophage treatment of disseminated cutaneous *Mycobacterium chelonae* infection. Nat Commun. 2022;13(1). https://doi.org/10.1038/s41467-022-29689-4

[6] ALHARBI N.M., ZIADI M.M. Wastewater as a fertility source for novel bacteriophages against multi-drug resistant bacteria. Saudi J Biol Sci. 2021;28(8):4358–64. https://doi.org/10.1016/j.sjbs.2021.04.025

[7] NAIR A., GHUGARE G.S., KHAIRNAR K. An appraisal of bacteriophage isolation techniques from environment. Microb Ecol. 2022;83(3):519–35. https://doi.org/10.1007/s00248-021-01782-z

[8] BALLESTÉ E., BLANCH A.R., MUNIESA M., et al. Bacteriophages in sewage: Abundance, roles, and applications. FEMS Microbes. 2022;3:xtac009. https://doi.org/10.1093/femsmc/xtac009
[9] GAO M., WANG C., QIANG X., et al. Isolation and characterization of a novel bacteriophage infecting carbapenem-resistant *Klebsiella pneumoniae.* Curr Microbiol. 2020;77(5):722–9. https://doi.org/10.1007/s00284-019-01849-8
[10] PEREIRA R.M.M., OLIVEIRA H.V.C., ANDRADE S.L., et al. Isolation of a mycobacteriophage against *Mycobacterium smegmatis.* European Journal of Biology and Biotechnology. 2021;2(1):34–7. https://doi.org/10.24018/ejbio.2021.2.1.147
[11] KONOPACKI M., GRYGORCEWICZ B., DOŁĘGOWSKA B., et al. PhageScore: A simple method for comparative evaluation of bacteriophages lytic activity. Biochem Eng J. 2020;161:107652. https://doi.org/10.1016/j.bej.2020.107652
[12] WONG C.L., SIEO C.C., TAN W.S., et al. Evaluation of a lytic bacteriophage, Φ st1, for biocontrol of *Salmonella enterica* serovar Typhimurium in chickens. Int J Food Microbiol. 2014;172:92–101. https://doi.org/10.1016/j.ijfoodmicro.2013.11.034
[13] VIAZIS S., AKHTAR M., FEIRTAG J., et al. Isolation and characterization of lytic bacteriophages against enterohaemorrhagic *Escherichia coli*. J Appl Microbiol. 2011;110(5):1323–31. https://doi.org/10.1111/j.1365-2672.2011.04989.x
[14] SHAHIN K., BOUZARI M., WANG R. Isolation, characterization and genomic analysis of a novel lytic bacteriophage vB_SsoS-ISF002 infecting *Shigella sonnei* and *Shigella flexneri*. J Med Microbiol. 2018;67(3):376–86. https://doi.org/10.1099/jmm.0.000683
[15] TOAQUIZA-VILCA B., QUITO-AVILA D., MALDONADO-ALVARADO P., et al. Physicochemical, genomic, and phenotypic characterization of *Escherichia* phage BME3. Microbiol Spectr. 2025;13(7): e01301-24. https://doi.org/10.1128/spectrum.01301-24
[16] KALAPALA Y.C., SHARMA P.R., AGARWAL R. Antimycobacterial potential of mycobacteriophage under disease-mimicking conditions. Front Microbiol. 2020;11: 583661. https://doi.org/10.3389/fmicb.2020.583661
[17] BROXMEYER L., SOSNOWSKA D., MILTNER E., et al. Killing of *Mycobacterium avium* and *Mycobacterium tuberculosis* by a mycobacteriophage delivered by a nonvirulent *Mycobacterium*: a model for phage therapy of intracellular bacterial pathogens. J Infect Dis. 2002;186(8):1155–1160. Available from: https://doi.org/10.1086/343812
[18] PAYASLIAN F., GRADASCHI V., RONDÓN SALAZAR L., et al. Isolation and characterization of vB_MsmS_Celfi: a new *Mycobacterium tuberculosis* bacteriophage. PHAGE. 2021;2(1):43–49. https://doi.org/10.1089/phage.2020.0030
[19] TRAN S.L., COOK G.M. The F1Fo-ATP synthase of *Mycobacterium smegmatis* is essential for growth. J Bacteriol. 2005;187(14):5023–5028. https://doi.org/10.1128/jb.187.14.5023-5028.2005
[20] THAKALI O., WAN S., KABIR M.D.P., et al. A call to wastewater researchers to support phage therapy in the global fight against antibiotic resistance. ACS ES&T Water. 2024;4(4):1177–9. https://doi.org/10.1021/acsestwater.3c00844
[21] FERNANDES S., LABARDE A., BAPTISTA C., et al. A non-invasive method for studying viral DNA delivery to bacteria reveals key requirements for phage SPP1 DNA entry in *Bacillus subtilis* cells. Virology. 2016;495:79–91. https://doi.org/10.1016/j.virol.2016.05.004
[22] HATFULL G.F. Mycobacteriophages: From Petri dish to patient. PLoS Pathog. 2022;18(7):e1010602. https://doi.org/10.1371/journal.ppat.1010602
[23] ABUSALAH M.A.H.A., ABUSALAH M.A.H.A., YEAN YEAN C., et al. Isolation and characterization of bacteriophage against clinical isolates of AmpC beta lactamase–producing *Klebsiella pneumoniae* from hospital wastewater. PLoS One. 2025;20(2):e0315079. https://doi.org/10.1371/journal.pone.0315079
[24] SHENDE R.K., HIRPURKAR S.D., SANNAT C., et al. Isolation and characterization of bacteriophages with lytic activity against common bacterial pathogens. Vet World. 2017;10(8):973–978. https://doi.org/10.14202/vetworld.2017.973-978
[25] TUPIN A., GUALTIERI M., ROQUET-BANÈRES F., et al. Resistance to rifampicin: At the crossroads between ecological, genomic and medical concerns. Int J Antimicrob Agents. 2010;35(6):519–23. https://doi.org/10.1016/j.ijantimicag.2009.12.017
[26] KOCH A., MIZRAHI V., WARNER D.F. The impact of drug resistance on *Mycobacterium tuberculosis* physiology: what can we learn from rifampicin?. Emerg Microb Infect 2014;3(1):1-11. https://doi.org/10.1038/emi.2014.17
[27] WANG I.N. Lysis timing and bacteriophage fitness. Genetics. 2006;172(1):17-26. https://doi.org/10.1534/genetics.105.045922
[28] KASMAN L.M., PORTER L.D. Bacteriophages. In: StatPearls. StatPearls Publishing, 2022.
[29] SWIFT B.M.C., GERRARD Z.E., HUXLEY J.N., et al. Factors affecting phage D29 infection: A tool to investigate different growth states of mycobacteria. PLoS One. 2014;9(9):e106690. https://doi.org/10.1371/journal.pone.0106690
[30] ALLUÉ-GUARDIA A., SARANATHAN R.,

CHAN J., et al. Mycobacteriophages as potential therapeutic agents against drug-resistant tuberculosis. Int J Mol Sci. 2021;22(2):735. https://doi.org/10.3390/ijms22020735
[31] JOŃCZYK E., KŁAK M., MIĘDZYBRODZKI R., et al. The influence of external factors on bacteriophages—review. Folia Microbiol (Praha). 2011;56(3):191–200. https://doi.org/10.1007/s12223-011-0039-8
[32] KWIATEK M., PARASION S., MIZAK L., et al. Characterization of a bacteriophage, isolated from a cow with mastitis, that is lytic against *Staphylococcus aureus* strains. Arch Virol. 2012;157(2):225–34. https://doi.org/10.1007/s00705-011-1160-3
[33] LOPEZ-DAVIS C., KEDY C., ALI A.K., et al. Characterization of mycobacteriophage Fulbright isolated from Oklahoma soil. Proc Okla Acad Sci. 2022;102:132–41.
[34] KOMIJANI M., BOUZARI M., RAHIMI F. Detection and characterization of a novel lytic bacteriophage (vB-KpneM-Isf48) against *Klebsiella pneumoniae* isolates from infected wounds carrying antibiotic-resistance genes (TEM, SHV, and CTX-M). Iran Red Crescent Med J. 2017;19(2): e34475. https://doi.org/10.5812/ircmj.34475
[35] KAZANTSEVA O.A., BUZIKOV R.M., PILIPCHUK T.A., et al. The bacteriophage Pf-10—A component of the biopesticide "Multiphage" used to control agricultural crop diseases caused by *Pseudomonas syringae.* Viruses. 2021;14(1):42. https://doi.org/10.3390/v14010042

参考文:

[1] WORLD HEALTH ORGANIZATION [WHO]. 全球结核病报告. 2024. World Health Organization; 2025.
[2] SPARKS I.L., DERBYSHIRE K.M., JACOBS JR W.R., et al. 耻垢分枝杆菌：分枝杆菌研究的先锋. J Bacteriol. 2023;205(1):e00337-22. https://doi.org/10.1128/jb.00337-22
[3] LELOVIC N., MITACHI K., YANG J., et al. 利用耻垢分枝杆菌作为替代菌株评价抗结核分枝杆菌的先导药物. J Antibiot. 2020;73(11):780-789. https://doi.org/10.1038/s41429-020-0320-7
[4] KOSKELLA B., BROCKHURST M.A. 细菌-噬菌体协同进化是微生物群落生态和进化过程的驱动力. FEMS Microbiol Rev. 2014;38(5):916–31. https://doi.org/10.1111/1574-6976.12072
[5] LITTLE J.S., DEDRICK R.M., FREEMAN K.G., et al. 噬菌体治疗播散性皮肤龟分枝杆菌感染. Nat Commun. 2022;13(1). https://doi.org/10.1038/s41467-022-29689-4
[6] ALHARBI N.M., ZIADI M.M. 废水作为新型噬菌体对抗多重耐药菌的营养来源. Saudi J Biol Sci. 2021;28(8):4358–64. https://doi.org/10.1016/j.sjbs.2021.04.025
[7] NAIR A., GHUGARE G.S., KHAIRNAR K. 对从环境中分离噬菌体的技术进行评价. Microb Ecol. 2022;83(3):519–35. https://doi.org/10.1007/s00248-021-01782-z
[8] BALLESTÉ E., BLANCH A.R., MUNIESA M., et al. 污水中的噬菌体：丰度、作用和应用. FEMS Microbes. 2022;3:xtac009. https://doi.org/10.1093/femsmc/xtac009
[9] GAO M., WANG C., QIANG X., et al. 分离和鉴定一种感染耐碳青霉烯类肺炎克雷伯菌的新型噬菌体. Curr Microbiol. 2020;77(5):722–9. https://doi.org/10.1007/s00284-019-01849-8
[10] PEREIRA R.M.M., OLIVEIRA H.V.C., ANDRADE S.L., et al. 分离抗耻垢分枝杆菌的噬菌体. European Journal of Biology and Biotechnology. 2021;2(1):34–7. https://doi.org/10.24018/ejbio.2021.2.1.147
[11] KONOPACKI M., GRYGORCEWICZ B., DOŁĘGOWSKA B., et al. PhageScore：一种用于比较评价噬菌体裂解活性的简便方法. Biochem Eng J. 2020;161:107652. https://doi.org/10.1016/j.bej.2020.107652
[12] WONG C.L., SIEO C.C., TAN W.S., et al. 评价溶菌噬菌体 Φst1 对鸡体内鼠伤寒沙门氏菌的生物防治效果. Int J Food Microbiol. 2014;172:92–101. https://doi.org/10.1016/j.ijfoodmicro.2013.11.034
[13] VIAZIS S., AKHTAR M., FEIRTAG J., et al. 分离和鉴定针对肠出血性大肠杆菌的裂解性噬菌体. J Appl Microbiol. 2011;110(5):1323–31. https://doi.org/10.1111/j.1365-2672.2011.04989.x
[14] SHAHIN K., BOUZARI M., WANG R. 分离、鉴定和基因组分析一种感染宋内氏志贺氏菌和福氏志贺氏菌的新型裂解性噬菌体 vB_SsoS-ISF002. J Med Microbiol. 2018;67(3):376–86. https://doi.org/10.1099/jmm.0.000683
[15] TOAQUIZA-VILCA B., QUITO-AVILA D., MALDONADO-ALVARADO P., et al. 大肠杆菌噬菌体 BME3 的理化、基因组和表型特征. Microbiol Spectr. 2025;13(7): e01301-24. https://doi.org/10.1128/spectrum.01301-24
[16] KALAPALA Y.C., SHARMA P.R., AGARWAL R. 在模拟疾病条件下，分枝杆菌噬菌体具有抗分枝杆菌的潜力. Front Microbiol. 2020;11:

583661. https://doi.org/10.3389/fmicb.2020.583661
[17] BROXMEYER L., SOSNOWSKA D., MILTNER E., et al. 利用无毒分枝杆菌递送的分枝杆菌噬菌体杀灭鸟分枝杆菌和结核分枝杆菌：胞内细菌病原体噬菌体疗法的模型. J Infect Dis. 2002;186(8):1155–1160. Available from: https://doi.org/10.1086/343812
[18] PAYASLIAN F., GRADASCHI V., RONDÓN SALAZAR L., et al. vB_MsmS_Celfi 的分离和鉴定：一种新的结核分枝杆菌噬菌体. PHAGE. 2021;2(1):43–49. https://doi.org/10.1089/phage.2020.0030
[19] TRAN S.L., COOK G.M. 耻垢分枝杆菌的 F1Fo-ATP 合酶对生长至关重要. J Bacteriol. 2005;187(14):5023–5028. https://doi.org/10.1128/jb.187.14.5023-5028.2005
[20] THAKALI O., WAN S., KABIR M.D.P., et al. 呼吁废水研究人员支持噬菌体疗法，以对抗全球抗生素耐药性。. ACS ES&T Water. 2024;4(4):1177–9. https://doi.org/10.1021/acsestwater.3c00844
[21] FERNANDES S., LABARDE A., BAPTISTA C., et al. 一种用于研究病毒 DNA 向细菌递送的非侵入性方法揭示了噬菌体 SPP1 DNA 进入枯草芽孢杆菌细胞的关键要求. Virology. 2016;495:79–91. https://doi.org/10.1016/j.virol.2016.05.004
[22] HATFULL G.F. 分枝杆菌噬菌体：从培养皿到患者. PLoS Pathog. 2022;18(7):e1010602. https://doi.org/10.1371/journal.ppat.1010602
[23] ABUSALAH M.A.H.A., ABUSALAH M.A.H.A., YEAN YEAN C., et al. 从医院废水中分离并鉴定针对产 AmpC β-内酰胺酶肺炎克雷伯菌临床分离株的噬菌体. PLoS One. 2025;20(2):e0315079. https://doi.org/10.1371/journal.pone.0315079
[24] SHENDE R.K., HIRPURKAR S.D., SANNAT C., et al. 分离和鉴定对常见致病菌具有裂解活性的噬菌体. Vet World. 2017;10(8):973–978. https://doi.org/10.14202/vetworld.2017.973-978
[25] TUPIN A., GUALTIERI M., ROQUET-BANÈRES F., et al. 利福平耐药性：生态、基因组和医学问题的交汇点. Int J Antimicrob Agents. 2010;35(6):519–23. https://doi.org/10.1016/j.ijantimicag.2009.12.017
[26] KOCH A., MIZRAHI V., WARNER D.F. 耐药性对结核分枝杆菌生理的影响：我们可以从利福平中学到什么？. Emerg Microb Infect 2014;3(1):1-11. https://doi.org/10.1038/emi.2014.17
[27] WANG I.N. 裂解时间和噬菌体适应性. Genetics. 2006;172(1):17-26. https://doi.org/10.1534/genetics.105.045922
[28] KASMAN L.M., PORTER L.D. 噬菌体. In: StatPearls. StatPearls Publishing, 2022.
[29] SWIFT B.M.C., GERRARD Z.E., HUXLEY J.N., et al. 影响噬菌体 D29 感染的因素：一种研究分枝杆菌不同生长状态的工具. PLoS One. 2014;9(9):e106690. https://doi.org/10.1371/journal.pone.0106690
[30] ALLUÉ-GUARDIA A., SARANATHAN R., CHAN J., et al. 分枝杆菌噬菌体作为抗耐药结核病的潜在治疗剂. Int J Mol Sci. 2021;22(2):735. https://doi.org/10.3390/ijms22020735
[31] JOŃCZYK E., KŁAK M., MIĘDZYBRODZKI R., et al. 外部因素对噬菌体的影响——综述. Folia Microbiol (Praha). 2011;56(3):191–200. https://doi.org/10.1007/s12223-011-0039-8
[32] KWIATEK M., PARASION S., MIZAK L., et al. 从患有乳腺炎的奶牛中分离出一种噬菌体，该噬菌体对金黄色葡萄球菌菌株具有裂解作用，并对其进行表征。. Arch Virol. 2012;157(2):225–34. https://doi.org/10.1007/s00705-011-1160-3
[33] LOPEZ-DAVIS C., KEDY C., ALI A.K., et al. 对从俄克拉荷马州土壤中分离出的分枝杆菌噬菌体 Fulbright 进行表征. Proc Okla Acad Sci. 2022;102:132–41.
[34] KOMIJANI M., BOUZARI M., RAHIMI F. 检测和鉴定一种新型裂解性噬菌体（vB-KpneM-Isf48），该噬菌体可对抗携带抗生素耐药基因（TEM、SHV 和 CTX-M）的感染伤口中的肺炎克雷伯菌分离株。. Iran Red Crescent Med J. 2017;19(2): e34475. https://doi.org/10.5812/ircmj.34475
[35] KAZANTSEVA O.A., BUZIKOV R.M., PILIPCHUK T.A., et al. 噬菌体 Pf-10 是生物农药“Multiphage”的组成部分，该农药用于控制由丁香假单胞菌引起的农作物病害。. Viruses. 2021;14(1):42. https://doi.org/10.3390/v14010042

Appendix A

Statistical Analysis Results of Bacteriophage Lytic Activity

Tabel 11. PhageScore Values Based on Konopacki et al.

		Group 1			Group 2			Group 3		
		Kc	Cm	Mt	Kc	Cm	Mt	Kc	Cm	Mt
MOI	1	0.5345	0.6940	0.5092	0.5425	0.6898	0.5008	0.5316	0.6921	0.5062
	0.1	0.4450	0.6225	0.4312	0.4757	0.6307	0.4371	0.4590	0.6313	0.4335
	0.01	0.3224	0.6126	0.3855	0.3482	0.6139	0.3967	0.3278	0.6174	0.4090

A. Standardized Residual Normality Test

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistics	df	Sig.	Statistics	df	Sig.
Standardized Residual	0.146	27	0.146	0.948	27	0.192

[a]Lilliefors Significance Correlation

Interpretation:

If the significance value is > 0.05, then the standardized residual values are normally distributed.

If the significance value is < 0.05, then the standardized residual values are not normally distributed.

Result: The treatments are normally distributed and can proceed to the homogeneity test.

B. Homogeneity Test

		Levene Statistic	df1	df2	Sig.
Lytic Activity	Based on Mean	2.240	8	18	0.074
	Based on Median	1.057	8	18	0.433
	Based on Median and with adjusted df	1.057	8	7.725	0.472
	Based on trimmed Mean	2.155	8	18	0.084

Test the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Dependent variable: Lytic Activity
b. Design : Intercept + Lysate + MOI + Lysate*MOI

Interpretation:

If the significance value is > 0.05, then the variable variance is homogeneous.

If the significance value is < 0.05, then the variable variance is not homogeneous.

Result: The treatments have homogeneous variable variances; therefore, the analysis can proceed to the Analysis of Variance (ANOVA).

C. Two-way Analysis of Variance (ANOVA) Test

Source	Type III Sum of Square	df	Mean Square	F	Sig.
Corrected Model	0.334[a]	8	0.042	574.050	0.000
Intercept	7.053	1	7.053	97106.211	0.000
Lysate	0.242	2	0.121	1664.227	0.000
MOI	0.076	2	0.038	522.530	0.000
Lysate*MOI	0.016	4	0.004	54.722	0.000
Error	0.001	18	7.263E-5		
Total	7.388	27			
Corrected Total	0.335	26			

[a] R Squared = 0.996 (Adjusted R Squared = 0.994)

If the significance value is < 0.05, there is a significant difference in the variable results.

If the significance value is > 0.05, there is no significant difference in the variable results.

Result: The lytic activity results are influenced by the type of bacteriophage and the Multiplicity of Infection (MOI) dose, and there is a significant interaction between these variables.

D. Post Hoc Duncan's Multiple Range Test (DMRT)

DMRT Total PhageScore

Duncan[a,b]

		Subset for alpha = 0,05	
Lysate	N	1	2
Kc	9	0.442945	
Mt	9	0.445460	
Cm	9		0.644923
Sig.		0.593	1.000

Means for groups in homogenous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 7.263E-5
a. Uses Harmonic Mean Sample Size = 9.000
b. Alpha = 0.05

DMRT Interaction between Bacteriophage and MOI Dose

Duncan[a]

		Subset for alpha = 0.05							
Treatment	N	1	2	3	4	5	6	7	8
Kc_MOI 0.01	3	0.332780							
Mt_MOI 0.01	3		0.397024						
Mt_MOI 0.1	3			0.433949					
Kc_MOI 0.1	3				0.459866				
Mt_MOI 1	3					0.505406			
Kc_MOI 1	3						0.536189		
Cm_MOI 0.01	3							0.614634	
Cm_MOI 0.1	3							0.628155	
Cm_MOI 1	3								0.691981
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	0.068	1.000

Means for groups in homogenous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 9.000