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Preliminary Exploration of Bacteriophages Targeting *Mycobacterium smegmatis* from Hospital Wastewater in Bekasi, Indonesia

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Tuberculosis (TB) remains a major global health problem, exacerbated by the emergence of multidrug-resistant strains. Bacteriophages, particularly lytic phages, are gaining increasing attention as alternative antimicrobial agents due to their specificity and low resistance potential. This is a pilot study aimed at isolating lytic bacteriophages from hospital and domestic wastewater that exhibit activity against *Mycobacterium smegmatis* (*M.sm*) as a model organism for *Mycobacterium tuberculosis* (*M.tb*). Wastewater samples were collected from five locations and tested for lytic activity using a spot test on *M.sm*. The results confirmed the presence of phages in four of the five samples, as evidenced by plaque formation with varying morphology. Plaques from samples coded ABR and ABM were mostly clear and large, indicating strong lytic activity, while samples coded IS and VB showed more diverse plaque shapes, including turbid and small plaques. This difference reflects the influence of environmental origin on phage diversity. The highest plaque diversity was observed in samples with high organic content and microbial richness, supporting the hypothesis that phage-host interactions are shaped by environmental factors.

Keywords: Bacteriophage; Lytic; *Mycobacterium*; Tuberculosis; Waste

INTRODUCTION

Tuberculosis (TB) remains one of the leading infectious diseases worldwide, with increasing concern regarding the emergence of multidrug-resistant tuberculosis (MDR-TB). Although conventional anti-tuberculosis drugs remain the primary treatment strategy, prolonged antibiotic exposure has contributed to the development of resistant bacterial populations and environmental dissemination of antimicrobial residues (WHO, 2024). Consequently, alternative biological control strategies such as bacteriophage therapy have attracted renewed scientific attention due to their host specificity, self-replicating nature, and ability to lyse antibiotic-resistant bacteria (Zeynali kelishomi et al., 2022; Prasad et al., 2018).

Bacteriophages are the most abundant biological entities in aquatic ecosystems and play major roles in regulating microbial population dynamics, horizontal gene transfer, and nutrient cycling (Wen et al., 2020). In wastewater environments, phage–host interactions are strongly shaped by ecological and physicochemical factors including bacterial density, organic matter availability, antimicrobial residues, and environmental stressors (Mtetwa et al., 2022). These conditions create selective pressures that influence phage diversity, infectivity, and evolutionary adaptation.

Hospital wastewater is increasingly recognized as a critical ecological hotspot for bacteriophage diversity because it contains high concentrations of pathogenic and non-pathogenic bacteria, residual antibiotics, disinfectants, and organic waste materials. Continuous exposure to antimicrobial compounds can enrich bacterial communities with resistant phenotypes, thereby promoting co-evolutionary dynamics between bacteria and bacteriophages (Ahmed et al., 2024). In addition, high organic loads and suspended particles support dense microbial populations that facilitate phage replication and persistence in aquatic environments (Aghaee et al., 2021). Previous environmental microbiology studies have demonstrated that sewage and hospital effluents represent productive reservoirs for isolating lytic phages with potential biotechnological and therapeutic applications (Mtetwa et al., 2022; Aghaee et al., 2021).

Among mycobacterial models, *Mycobacterium smegmatis* is widely used as a surrogate host for *Mycobacterium tuberculosis* because of its non-pathogenic characteristics, rapid growth, and genetic similarity to pathogenic mycobacteria (Xie et al., 2023). The use of *M. smegmatis* enables safer preliminary screening and ecological exploration of mycobacteriophages without the biosafety limitations associated with *M. tuberculosis*. Several studies have successfully isolated mycobacteriophages from wastewater and sewage environments, demonstrating considerable diversity in plaque morphology, host specificity, and lytic activity (Guerrero-Bustamante et al., 2021; Gudlavalleti et al., 2020).

However, despite the growing number of studies on phage isolation from wastewater, important gaps remain unresolved. Most previous studies have primarily focused on therapeutic potential or genomic characterization, while relatively few have examined how environmental characteristics of hospital wastewater influence plaque diversity and lytic phenotypes of mycobacteriophages. In addition, data from tropical urban wastewater ecosystems, particularly in Indonesia, remain limited. Comparative observations linking wastewater physicochemical appearance, organic load indications, and phage plaque characteristics are still rarely discussed in the context of environmental selection pressure and phage ecology.

Therefore, this is a pilot study aimed at isolating lytic bacteriophages from hospital and domestic wastewater that exhibit activity against *Mycobacterium smegmatis* (*M.sm*) as a model organism for *Mycobacterium tuberculosis* (*M.tb*). The specific contribution of this study lies in its ecological approach, emphasizing the relationship between environmental wastewater characteristics and variations in phage lytic morphology. This work provides preliminary evidence supporting hospital wastewater as a selective ecological niche for diverse mycobacteriophages and contributes baseline data for future exploration of environmentally adapted phages for therapeutic and biotechnological applications.

METHODS

Sampling Design and Wastewater Collection

Wastewater samples were collected from five different environmental sources in Bekasi, Indonesia, consisting of hospital drainage systems and domestic wastewater channels, namely ABR, ABM, VB, IS, and MM. Sampling was conducted during the dry season in July 2025 to minimize fluctuations caused by rainfall dilution. Each sampling point was sampled independently in triplicate on three different collection occasions at one-week intervals to improve environmental representation and reduce temporal bias, following recommendations for environmental phage surveillance studies (Mtetwa et al., 2022).

At each location, approximately 500 mL of wastewater was collected aseptically using sterile polypropylene bottles, resulting in a total collected volume of 1.5 L per sampling site. Samples were transported to the laboratory in insulated containers at 4°C and processed within 6 hours after collection to preserve phage viability (Aghaee et al., 2021). Visual environmental characteristics including turbidity, odor intensity, and suspended particles were recorded as preliminary indicators of microbial and organic load conditions.

The selected sampling sites represented environments with different ecological pressures, including hospital-associated antibiotic exposure, domestic organic waste accumulation, and mixed anthropogenic contamination. Such heterogeneous wastewater conditions are recognized as important ecological reservoirs for bacteriophage diversity and phage–host co-evolution (Ahmed et al., 2024; Wen et al., 2020).

Phage Isolation and Spot Test Assay

The host bacterium used in this study was *Mycobacterium smegmatis* mc²155 (ATCC 14468) obtained from the Indonesian Culture Collection (InaCC), BRIN Cibinong. Bacterial cultures were grown in 7H9 broth supplemented with Tween 80 and incubated at 37°C for 48 h until reaching the logarithmic growth phase. For phage enrichment, 6 mL of wastewater sample was mixed with 6 mL of *M. smegmatis* culture and incubated at 37°C for 24 h. The enrichment mixture was centrifuged at 4,500 rpm for 40 min, and the supernatant was filtered through a 0.22 µm syringe filter to remove bacterial cells and obtain crude phage filtrates.

Prior to spot testing, all filtrates were adjusted to the same initial enrichment condition without serial dilution to ensure consistency among samples. The spot assay was performed by mixing 100 µL of enriched host culture with 3 mL of molten top agar (0.65% agar) supplemented with 100 µL of 0.1 mM $CaCl_2$, then overlaying onto solidified base agar plates. Subsequently, 10 µL aliquots of each filtrate were spotted onto the bacterial lawn surface. Each wastewater sample was tested in triplicate technical replicates using independent agar plates under identical incubation conditions. Plates were incubated at 37°C for 48 h and examined for plaque formation. Plaque observations included presence or absence of lysis zones, plaque diameter, clarity, edge morphology, and approximate plaque abundance.

Although the spot assay is primarily qualitative, semi-quantitative plaque observations were recorded to compare relative lytic activity among environmental samples, consistent with previous exploratory bacteriophage screening studies (Guerrero-Bustamante et al., 2021; Gudlavalleti et al., 2020). Plaque counts reported in this study therefore represent approximate average numbers observed across technical replicates rather than absolute phage titers. Quantitative assays such as double-layer plaque assays and PFU enumeration are planned for subsequent investigations.

RESULT AND DISCUSSION

Wastewater Sample Characteristics

The visual characteristics of wastewater collected from the five sampling locations (ABR, ABM, VB, IS, and MM) demonstrated considerable environmental variation (Table 1, Figure 1). Samples from ABR and ABM showed high turbidity, darker coloration, pungent odors, and abundant suspended particles, indicating elevated organic contamination and microbial activity. In contrast, IS and MM appeared relatively clearer with lower visible particulate content and milder odor intensity. The VB sample exhibited intermediate characteristics with grayish coloration and visible suspended organic matter.

Table 1.
Visual Characteristics of Wastewater Samples Based

NO	CODE	LOCATION	COLOR	ODOR	SUSPENDED PARTICLES
1.	ABR	Drainage near hospital and swamp area	Green	Strong pungent odor	Visible particles, food waste, and swamp water
2.	ABM	Drainage near hospital and food stalls	Grayish turbid	Strong pungent odor	Particles, food waste, and live bacteria
3.	IS	Household drainage with domestic waste	Clear yellowish	Pungent odor	Organic matter and live bacteria
4.	VB	Hospital drainage near food stalls	Grayish turbid	Pungent odor	Organic matter, small particles, and live bacteria
5.	MM	Hospital drainage channe	Relatively clear	Mild odor	Fine sand

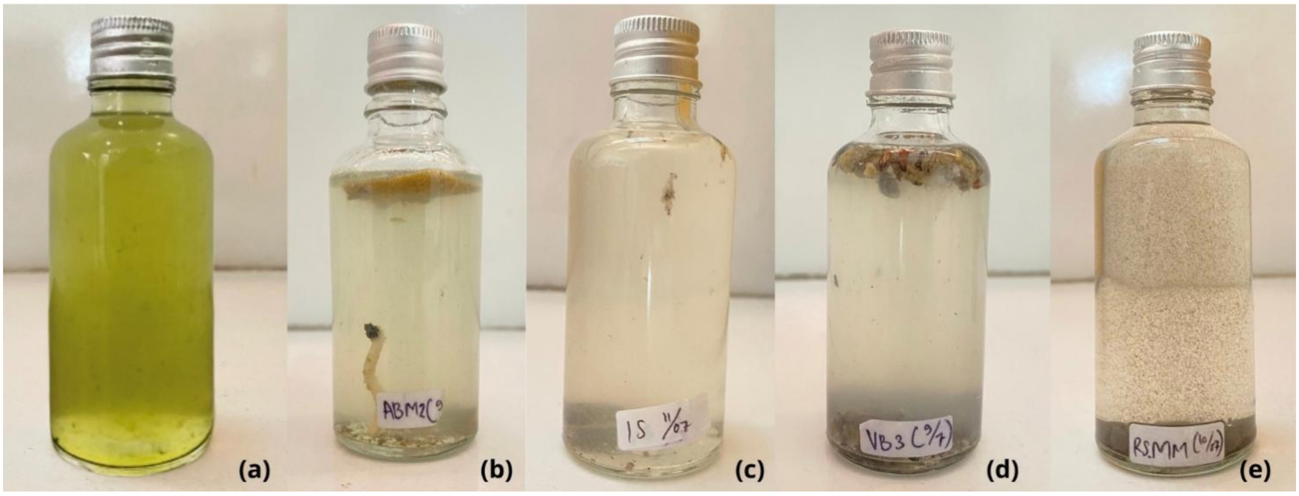

Figure 1.
Visual Appearance of Wastewater Samples

Note: [a]The ABR point is located near AB Hospital, close to a swamp area.
[b]The ABM point is located near AB Hospital, close to a food waste disposal site.
[c]The VB point is located near VM Hospital, Bekasi City.
[d]The IS point is located in a household domestic waste area in Bekasi Regency.
[e]The MM point is located near M Hospital, Bekasi City.

Although these observations were based primarily on visual assessment and did not include physicochemical measurements such as pH, chemical oxygen demand (COD), total suspended solids (TSS), or dissolved oxygen, the recorded environmental features still provide important preliminary ecological context regarding wastewater heterogeneity. Previous studies have shown that wastewater turbidity, organic load, and suspended particles are strongly associated with microbial abundance and bacteriophage persistence in aquatic systems (Ahmed et al., 2024; Wen et al., 2020). Environments rich in organic matter generally support denser bacterial populations, thereby increasing opportunities for phage replication and phage–host interactions (Aghaee et al., 2021).

The absence of physicochemical parameter measurements represents a limitation of the present study. Quantitative environmental analyses such as COD, TSS, nutrient concentration, and pH profiling would provide a more comprehensive understanding of ecological selection pressures influencing phage diversity and lytic activity. Future studies should therefore integrate microbiological and physicochemical analyses to better characterize environmental determinants of mycobacteriophage distribution in wastewater ecosystems.

Spot assay observations demonstrated detectable lytic activity in wastewater samples ABR, ABM, IS, and VB, as indicated by the appearance of plaque-like lysis zones on *Mycobacterium smegmatis* lawns (Table 2, Figure 2). No visible plaques were observed in sample MM or the negative control. The plaques varied in diameter, clarity, edge morphology, and abundance, suggesting differences in phage–host interactions among wastewater environments.

Spot Test Results and Plaque Morphology

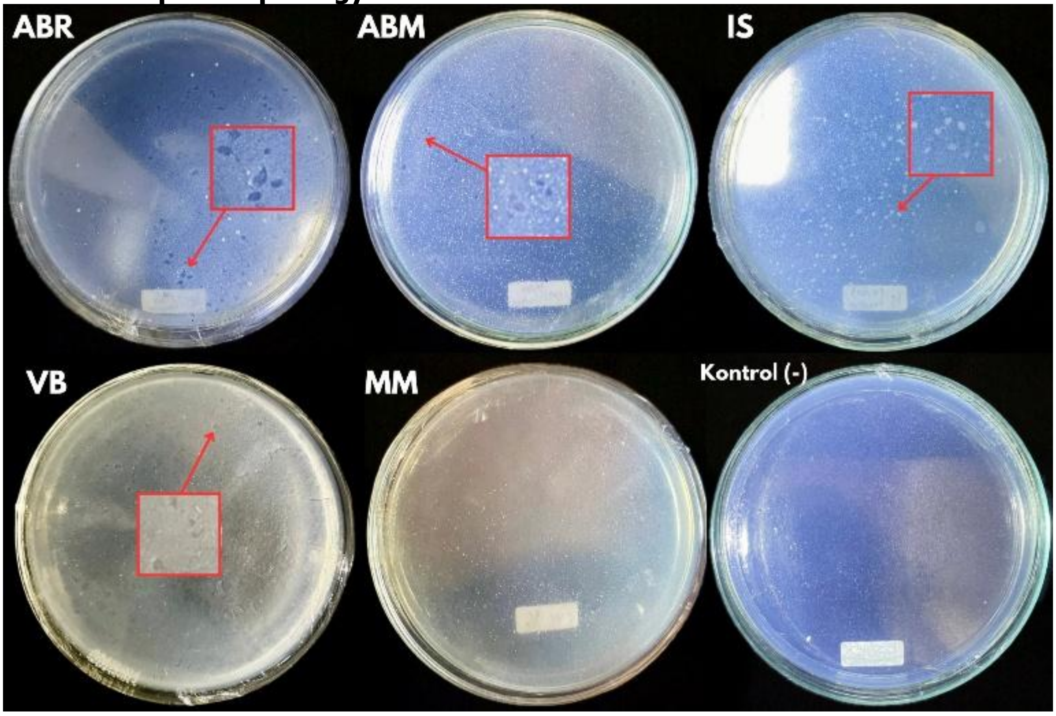

Figure 2.
Spot Test Results Showing Plaques (arrows indicate lysis zones)

Table 2.
Visual Characteristics of Observed Plaques

No.	Code	Clear Zone	Zone Size	Clarity	Zone Shape	Number of Plaques
1.	ABR	Present	3 ± 0.2 mm	Clear	Irregular	78 plaques
2.	ABM	Present	2 ± 0.2 mm	Semi-clear	Irregular	39 plaques
3.	VB	Present	1.5 ± 0.2 mm	Semi-clear	Irregular	18 plaques
4.	IS	Present	2 ± 0.2 mm	Turbid	Round, Regular	134 plaques
5.	MM	Not Present	-	-	-	-
6.	Control (-)	Not Present	-	-	-	-

The ABR sample produced relatively large and clear plaques with irregular edges, whereas IS generated a greater number of smaller and turbid plaques with smoother margins. Samples ABM and VB showed intermediate plaque characteristics, including semi-clear lysis zones and moderate plaque abundance. Variations in plaque morphology are commonly associated with differences in phage adsorption efficiency, diffusion rate, latent period, burst size, and host susceptibility (Gudlavalleti et al., 2020; Valdez et al., 2025).

Clear plaques are generally associated with virulent lytic phages capable of complete bacterial lysis, whereas turbid plaques may indicate incomplete lysis, reduced infection efficiency, or possible lysogenic tendencies (Gudlavalleti et al., 2020). However, in the present study, the interpretation of "strong lytic activity" was limited to phenotypic plaque appearance and should therefore be considered preliminary. Plaque clarity and size alone are insufficient to conclusively determine phage virulence because additional parameters such as burst size, adsorption rate, latent period, and efficiency of plating were not evaluated. Consequently, the present findings should be interpreted as indicative of relative lytic potential rather than definitive evidence of superior phage infectivity.

Similarly, differences in plaque abundance among sampling locations should be interpreted cautiously. Although all spot assays were performed under standardized laboratory conditions using the same host strain and enrichment protocol, plaque number comparisons may still be influenced by variations in initial phage concentration, enrichment efficiency, and localized host density within the agar overlay. Spot assays are primarily qualitative screening methods and do not provide absolute quantification of phage particles (Guerrero-Bustamante et al., 2021). Therefore, the reported plaque counts represent approximate relative observations rather than direct comparisons of environmental phage concentration.

Despite these limitations, the observed plaque diversity supports the hypothesis that wastewater environmental conditions influence bacteriophage ecology and phage–host dynamics. Wastewater environments with high organic content and complex microbial communities may facilitate greater phage diversity by increasing the availability of susceptible bacterial hosts and promoting selective adaptation under environmental stress conditions (Mtetwa et al., 2022). These findings are consistent with previous environmental microbiology studies reporting that hospital and sewage wastewater serve as important reservoirs for diverse lytic bacteriophages with potential therapeutic and biotechnological applications (Aghaee et al., 2021; Ahmed et al., 2024).

The present study provides preliminary ecological evidence linking wastewater environmental characteristics with differences in mycobacteriophage plaque morphology. Nevertheless, further investigations involving plaque purification, host range analysis, quantitative plaque-forming unit (PFU) assays, transmission electron microscopy, and genomic characterization are necessary to validate phage identity and better understand the biological significance of the observed phenotypic diversity.

This study represents a preliminary ecological exploration of bacteriophages infecting *Mycobacterium smegmatis* isolated from hospital and domestic wastewater environments. Several limitations should therefore be acknowledged when interpreting the findings. First, the present study relied primarily on spot test assays as an initial screening method for detecting lytic activity. Although spot assays are widely used for rapid phage detection, they are inherently qualitative and cannot provide accurate quantification of phage concentration or infectivity (Guerrero-Bustamante et al., 2021). Second, host range testing was not performed in this study. The isolated phages were evaluated only against *M. smegmatis* mc²155 as a surrogate host for *M. tuberculosis*. As a result, the spectrum of bacterial susceptibility and the potential cross-infectivity of the isolated phages remain unknown. Third, genomic confirmation and molecular characterization of the isolated bacteriophages were not conducted. Future investigations should therefore incorporate whole-genome sequencing, transmission electron microscopy, and comparative genomic analysis to validate phage identity and biological properties.

In addition, environmental characterization in this study was limited to visual observations such as turbidity, odor, and suspended particles. Quantitative physicochemical analyses including pH, total suspended solids (TSS), biological oxygen demand (BOD), chemical oxygen demand (COD), and antibiotic residue measurements were not performed. These parameters could provide stronger ecological evidence regarding environmental selection pressures affecting phage diversity and phage–host dynamics in wastewater systems (Mtetwa et al., 2022; Ahmed et al., 2024).

Despite these limitations, this study provides important preliminary evidence that hospital and domestic wastewater environments in Bekasi, Indonesia, harbor diverse bacteriophages capable of infecting *M. smegmatis*. This preliminary plaque characterization provides a foundation for selecting candidate phages for further testing such as host range assays and genomic analysis. Considering the correlation between wastewater conditions and phage diversity, strategic sampling from extreme and varied environments may enhance the discovery of novel or highly active phages for biocontrol or phage therapy applications.

CONCLUSIONS

This study confirmed the presence of bacteriophages capable of infecting *Mycobacterium smegmatis* in wastewater samples collected from hospital and domestic environments in Bekasi, Indonesia. The observed variations in plaque morphology, including differences in size, clarity, edge structure, and abundance, indicate the presence of diverse phage–host interactions influenced by environmental conditions. Wastewater sources with higher organic content and visible microbial richness tended to exhibit greater plaque diversity, supporting the ecological role of wastewater as a reservoir for bacteriophage diversity.

The findings provide preliminary ecological evidence that hospital and domestic wastewater environments may serve as selective niches for mycobacteriophages with different lytic characteristics. However, the present study was limited to initial phenotypic screening using spot assays and did not include host range analysis, quantitative infectivity assays, or genomic characterization. Therefore, interpretations regarding phage virulence and biological activity should remain cautious.

Although the isolated phages may have prospective relevance for future biotechnological or therapeutic exploration, claims regarding therapeutic applicability remain preliminary at this stage. Further investigations involving plaque purification, quantitative plaque-forming unit (PFU) analysis, host specificity testing, whole-genome sequencing, and safety evaluation are required before considering potential therapeutic applications. This study nevertheless provides important baseline data for future environmental and mycobacteriophage research in tropical wastewater ecosystems.

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