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RESEARCH PAPER

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Isolation, Quantification, and Plaque Morphology Analysis of Lytic Bacteriophages from River Water Targeting Clinical MDR *Klebsiella pneumoniae* Using the Double-Layer Agar Method

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ABSTRACT

Antimicrobial resistance is a growing global health threat and is projected to cause up to 10 million deaths per year by 2050. Klebsiella pneumoniae is a priority pathogen due to its multidrug resistance (MDR) mechanisms, such as extended-spectrum β-lactamases and carbapenemases, which significantly limit therapeutic options and increase the need for antimicrobial alternatives. This study aimed to isolate and quantify active lytic bacteriophages capable of infecting clinical MDR K. pneumoniae from river water samples. Water samples were processed by centrifugation and membrane filtration to remove debris and bacterial cells, then incubated with MDR K. pneumoniae in Luria broth at 37°C to enhance phage adsorption and amplification. Phage detection and enumeration were performed using the double-layer agar method. Plaque morphology was observed to confirm lytic activity, while serial dilutions were used to determine phage titer. Several lytic bacteriophages were successfully isolated from river water samples. The plaques formed were clear, spherical, and well-defined, with some exhibiting halos indicative of possible depolymerase activity. Phage titers ranged from 1.28 × 10^3 to 2.00 × 10^6 PFU/mL, indicating efficient replication against MDR K. pneumoniae without repeated enrichment processes. River water is a potential source of lytic bacteriophages capable of infecting MDR K. pneumoniae. These findings emphasize the role of aquatic environments as natural reservoirs of phages with potential use in the development of future antimicrobial or biocontrol strategies and support the need for further studies on the host range, stability, and therapeutic applications of the isolated phages.

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I. INTRODUCTION

iThe WHO Global Antimicrobial Resistance Surveillance System (GLASS) has been monitoring priority pathogens, including *Klebsiella pneumoniae,* since 2015. Implementation of GLASS recommendations in Africa shows variable progress, with *K. pneumoniae* recovered from 68.3% of blood samples and 68.1% of urine samples in surveillance studies. However, no studies tested all recommended pathogen-antibiotic combinations [1]. Antimicrobial resistance (AMR) represents a critical global health crisis, projected to cause 10 million deaths annually by 2050 without effective interventions[2],[3]. Currently, AMR contributes to approximately 4.95 million deaths each year, with potential economic losses up to US$100 trillion [4]. More recent estimates indicate annual economic burdens of up to US$730 billion for managing AMR-related diseases [5]. These figures emphasize that AMR is not only a medical challenge but also a socio-economic threat requiring immediate solutions. One pathogen prioritized by the World Health Organization is *Klebsiella pneumoniae*, due to its rising resistance and its role as a major cause of healthcare-associated infections [6]. This pathogen possesses multiple resistance mechanisms, including extended-spectrum β-lactamase (ESBL) production, efflux pumps, and carbapenemases, all of which significantly limit therapeutic options [7],[8]. The situation is further aggravated by the emergence of hypervirulent strains that increase morbidity and mortality [6]. According to WHO GLASS (2018), ESBL-producing *E. coli* and *K. pneumoniae* are leading causes of

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bacteremia and nosocomial infections in Southeast Asia, including Indonesia [9]. MDR *K. pneumoniae* prevalence in the region reaches 55%, with 27% being ESBL producers [10]. Outbreaks in India involving carbapenem-resistant *K. pneumoniae* (CRKP) harboring blaOXA-48 and blaNDM genes demonstrated high mortality rates and rapid inter-hospital spread [11]. These findings highlight the urgent need for novel strategies to combat antibiotic-resistant *K. pneumoniae*.

Phage therapy has emerged as a promising alternative other than herbal medicines in fighting drug-resistant pathogens[12],[13],[14]. Lytic bacteriophages specifically target and lyse bacteria without disturbing the host microbiota [15]. Bacteriophages show significant promise in combating *Klebsiella pneumoniae* biofilms, offering alternatives to conventional antibiotic treatments. Characterized phage PG14, which demonstrated effective biofilm inhibition and disruption against carbapenem-resistant *K.pneumoniae*, with rapid adsorption (>90% within 12 minutes) and no antimicrobial resistance genes [16]. Similarly, phages vB_KpnA_GBH014 and vB_KpnM_GBH019 significantly prevented and disrupted biofilms from clinical *K. pneumoniae* isolates. These phages were successfully incorporated into alginate hydrogels, creating antibiofilm surfaces with dramatically reduced bacterial counts (from 10^8 to 10^4–10^2 cfu/ml) compared to controls[17]. Both in vitro and in vivo studies have shown phage effectiveness against MDR *K. pneumoniae*, with advantages including self-replication, co-evolution with bacterial hosts to prevent resistance, and minimal disruption of commensal flora [18],[19]. Recent research has focused on isolating and characterizing bacteriophages targeting *K. pneumoniae* from various water sources. Fang *et al.* isolated two lytic phages, P24 and P39, which demonstrated strong activity against carbapenem-resistant *K. pneumoniae*, exhibiting large burst sizes and potential for intestinal decolonization in mice [20]. Combining phages with antibiotics has further enhanced efficacy and, in some cases, reversed resistance [21]. Recent reviews reinforce the therapeutic promise of phages, whether as phage cocktails, phage-derived enzymes, or synergistic antibiotic combinations [22],[23].

In Indonesia, research on therapeutic phage applications remains limited. A review of available literature revealed no published studies describing the isolation of *Klebsiella pneumoniae* phages from river or wastewater samples[24],[25],[26],[27],[28]. Existing investigations have primarily focused on environmental phage isolation targeting other clinical pathogens like *Escherichia coli* [28] and *Staphylococcus aureus* [25] or in vitro characterization without therapeutic validation. This highlights a significant research gap in the discovery and application of indigenous bacteriophages as potential therapeutic agents against *K. pneumoniae* infections in Indonesia. An essential first step in phage therapy development is the isolation and characterization of bacteriophages from the environment. Several studies have successfully isolated highly lytic phages against MDR *K. pneumoniae* [23],[29],[30],[31]. Environmental sources such as rivers, hospital wastewater, and contaminated water systems are known to harbor diverse phages with significant potential against clinical MDR isolates [32],[33],[34],[35]. Likewise, the widespread presence of MDR *K. pneumoniae* in surface waters, river estuaries, and wastewater strengthens the urgency of isolating phages from these environments [29],[36],[37]. Thus, exploring natural phage reservoirs is not only scientifically relevant but also essential for developing alternative therapeutic approaches against MDR *K. pneumoniae*.

Despite these advances, many countries have reported environmental phages active against MDR K. pneumoniae, Indonesia lacks baseline data on natural lytic phages against clinical MDR strains, particularly from natural water sources in Banten Province, Indonesia, from the Cisadane River, which is highly exposed to hospital and community waste. This indicates a clear research gap in identifying naturally occurring bacteriophages in this region, especially those capable of lysing multidrug-resistant (*MDR*) *K. pneumoniae*. Previous studies conducted in other areas have demonstrated that river and freshwater environments serve as rich reservoirs of bacteriophages active against *MDR K. pneumoniae* when isolated using the double-layer agar (*DLA*) method. For instance, Balcão *et al.* successfully isolated a novel lytic bacteriophage from river water employing the DLA technique, which exhibited strong lytic activity and stability under varying environmental conditions [38]. Similarly, Peng *et al.* reported the isolation of bacteriophage vB_Kp_XP4 from freshwater sources using the same approach, showing potent infectivity against hypervirulent and *MDR K. pneumoniae* strains [19]. Nepal *et al.* also utilized the DLA assay to isolate and quantify bacteriophages infecting *MDR Klebsiella* and other Enterobacteriaceae from environmental sources, confirming the robustness of this method for phage detection and enumeration [33]. Therefore, this study aims to isolate, quantify, and characterize lytic bacteriophages from Cisadane River water as a potential reservoir for phage therapy development in Indonesia. This research directly addresses a critical knowledge gap by employing the DLA method to detect and quantify lytic bacteriophages from river water using clinical isolates of *K. pneumoniae* as hosts, without prior enrichment. Establishing baseline data from this location is essential not only for expanding the understanding of natural phage reservoirs in Indonesia but also for providing a foundation for future applications in phage therapy. The contributions of this study are 1) isolation of lytic bacteriophages from

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river water contaminated with hospital effluents in Tangerang, Banten, 2) determination of bacteriophage titers and plaque morphology against clinical *K. pneumoniae* isolates, 3) identification of potential depolymerase activity indicated by halo zones surrounding plaques, and 4) providing baseline data for bacteriophage therapy and environmental surveillance in Indonesia. This study is structured as follows: Section II discusses the theoretical background, data acquisition, data processing, and data analysis. Section III displays the results of bacteriophage isolation and titration. Section IV discusses the interpretation and comparison of the results with other studies, as well as the limitations. Section V displays conclusions which rewrite the objectives, main findings, and future works.

II. MATERIALS AND METHOD

A. Theoretical Background

Bacteriophages are viruses that specifically infect bacteria, acting as natural bacterial predators and potential alternatives to antibiotics for multidrug-resistant infections. The double-layer agar technique remains the gold standard for phage isolation and enumeration, as it enables visualization of phage-induced bacterial lysis as plaques. This method provides both qualitative and quantitative data regarding phage activity and concentration, serving as a reference approach for phage screening, propagation, and titration [39],[40].

B. Data Acquisition

Clinical isolates of *Klebsiella pneumoniae* were obtained from human clinical specimens provided by a collaborating hospital laboratory. Environmental water samples were collected from the Sukamandi segment of the Cisadane River, located in Neglasari, Tangerang, Indonesia. This site receives mixed discharges from hospital and community wastewater sources, providing a potential reservoir of bacteriophages targeting multidrug-resistant bacteria.

Each sample (500 mL) was collected aseptically in sterile plastic bottles, stored at 4 °C, and processed within 24 h to minimize phage degradation and bacterial overgrowth. River water samples were centrifuged at 10,000 × g for 10 min to sediment large particles and cells [41]. The resulting supernatant was filtered through regenerated cellulose (RC) membranes with a 0.22 µm pore size to remove residual bacterial cells and debris. The clarified filtrate was then mixed with exponential-phase *K. pneumoniae* host cultures adjusted to a turbidity equivalent to 0.5 McFarland standard in nutrient broth supplemented with 10 mM $CaCl_2$ and 10 mM $MgSO_4$ to facilitate phage adsorption. The mixture was combined with molten soft agar (0.7% w/v, ~45 °C) and poured onto Luria–Bertani (LB) agar plates. Plates were incubated at 37 °C for 18–24 h. The formation of clear plaques on the bacterial lawn indicated the presence of lytic bacteriophages.

C. Data Processing and Data Analysis

Lytic activity was confirmed by observing plaque formation on bacterial lawns. Quantitative titration was conducted through serial dilution using the DLA method as follows: phage lysates were diluted from 10^{-1} to 10^{-5}, and 100 µL of each dilution was mixed with 200 µL of host culture (log phase) and 3 mL of molten soft agar. The mixture was overlaid onto LB agar and incubated at 37 °C for 18–24 h. Plates containing 30–300 plaques were selected for counting. Phage titer (PFU/mL) was calculated using the standard formula:

$$\text{Titer (PFU/mL)} = \frac{N \times F}{V}$$

Description:
N = number of plaques,
F = dilution factor (e.g., 10^5 for 10^{-5}),
V = plated phage volume (mL).

For this study, dilutions were prepared up to 10^{-5} to determine final titers. Phage titers (PFU/mL) were determined from plaque counts obtained through standard double-layer agar assays across serial dilutions. Because only single primary isolates were tested without biological or technical replicates, no inferential statistical comparisons were performed. However, to express the stochastic uncertainty associated with individual plaque counts, 95% confidence intervals were calculated under a Poisson model, assuming plaque formation events occur independently [33]. The lower and upper bounds were derived using the exact Poisson method based on chi-square quantiles and then converted into PFU/mL using the corresponding dilution factor and plated volume according to:

$$\text{PFU/mL lower} = \frac{L \times F}{V},\ \text{PFU/mL upper} = \frac{U \times F}{V}$$

Where *L* and *U* are the lower and upper Poisson confidence limits, respectively.

III. RESULTS

A. Confirmation of Bacteriophage Presence (Qualitative Detection)

The presence of bacteriophages in one out of five river water samples was confirmed by the appearance of plaques on the bacterial lawn of *K. pneumoniae* after 18–24 h of incubation at 37 °C, as shown in Fig. 1. In addition to plaques on overlay agar, quadrantal streak assays revealed lytic zones forming streak patterns, further confirming phage activity. Plaques were typically round with distinct edges, and their clarity indicated complete bacterial lysis. Plaque morphology varied slightly in diameter (0.5–2 mm), suggesting possible heterogeneity in phage particle size or adsorption kinetics, as shown in **Error! Reference source not found.**. The results indicate that *K. pneumoniae*-specific lytic phages were

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successfully isolated from environmental samples collected from the Cisadane River, Neglasari, Tangerang.

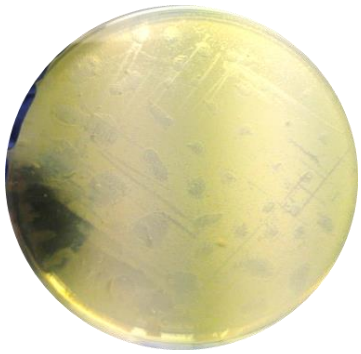

Fig. 1. Representative clear plaques formed on K.pneumoniae lawns by the DLA method, and lytic zones observed in the quadrant-streak assay, confirming phage activity.

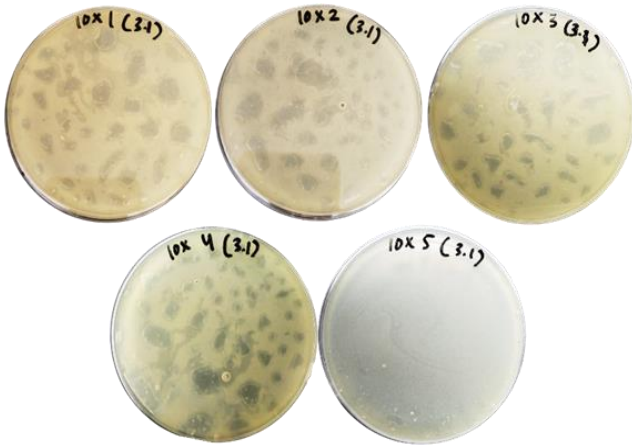

Fig. 2. Variation in plaque size and density across dilution series (10^{-1}–10^{-5}).

B. Phage Titration (Quantitative Assessment by DLA)

Phage titration yielded plaque counts across serial dilutions (10^{-1}–10^{-5}), with results summarized in Table 1. Quantification was performed using the double-layer agar (DLA) method, and plaque counts were converted to phage titers expressed as PFU/mL.

Table 1. Plaque counts and calculated phage titers against *K. pneumoniae* (dilution series 10^{-1}–10^{-5}).

Plate Code	Plaque Count (N)	Dilution Factor (F)	Phage Titer (PFU/mL)
10^{-1}	32	10	1.28×10^3
10^{-2}	28	10^2	1.12×10^4
10^{-3}	35	10^3	1.40×10^5
10^{-4}	40	10^4	1.60×10^6
10^{-5}	5	10^5	2.00×10^6

As shown in Table 1, plaque counts ranged from 5 to 40, yielding phage titers between 1.28×10^3 and 2.00×10^5 PFU/mL. The maximum titer was obtained at the 10^{-5} dilution, while the lowest titer appeared at the 10^{-1} dilution. These findings suggest that the isolated bacteriophage exhibited moderate infectivity toward *K. pneumoniae*.

C. Statistical Evaluation Using Poisson Distribution

To evaluate the consistency and reliability of the plaque count data, Poisson distribution analysis was applied, as this model assumes random, independent events of phage infection on the bacterial lawn. The standard deviation (SD) for each dilution was estimated using the square root of the observed plaque number ($SD = \sqrt{N}$), and 95% confidence intervals (CI) were calculated using $N \pm 1.96 \times \sqrt{N}$. The results are summarized in Table 2, showing that the coefficient of variation (CV = SD / N × 100%) ranged from 7.8% to 44.0%, indicating good reproducibility for plaque counts within the range of 20–40 plaques. This finding supports the reliability of the titration results, particularly for dilutions 10^{-3} and 10^{-4}, which fall within the ideal countable range (20–200 plaques).

Table 2. Statistical analysis of plaque counts using Poisson distribution (95% CI).

Plate Code	Plaque Count (N)	√N	95% CI (N ± 1.96 × √N)	CV (%)
10^{-1}	32	5.66	1.28×10^3	17.7
10^{-2}	28	5.29	1.12×10^4	18.9
10^{-3}	35	5.92	1.40×10^5	16.9
10^{-4}	40	6.32	1.60×10^6	15,8
10^{-5}	5	2.24	2.00×10^6	44.8

These statistical results demonstrate that plaque counts at moderate dilutions (10^{-2} to 10^{-4}) showed low variability and acceptable statistical precision (CV < 20%). The high CV at 10^{-5} is attributed to the low plaque number, which increases counting uncertainty. Overall, the calculated titers and Poisson analysis confirm that the bacteriophage isolated from the sample produced consistent and countable plaques suitable for further characterization.

IV. DISCUSSION

A. Isolation and Detection of Bacteriophages

The successful isolation of lytic bacteriophages from Cisadane River water demonstrates that naturally

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occurring phages persist in aquatic environments exposed to both hospital and community wastewater. The presence of clear plaques on *K. pneumoniae* lawns (Fig. 1) confirmed active lytic infection, aligning with previous studies that environmental waters serve as rich reservoirs of bacteriophages against multidrug-resistant (MDR) pathogens [1],[42],[19], [38],[43]. Quantitatively, phage titers obtained via the double-layer agar (DLA) method ranged from 1.28 × 10^3 to 2.00 × 10^5 PFU/mL (Table 1). Statistical evaluation using the Poisson distribution indicated moderate variation at higher dilutions (Table 2), reflecting the stochastic distribution of phage particles within environmental samples rather than technical error. These results underscore the natural heterogeneity of phage populations in river waters, consistent with previous observations of variable environmental phage loads [3],[37],[44]. Moreover, differences in plaque size and density suggest the presence of multiple phage morphotypes, possibly reflecting distinct adsorption kinetics, latent periods, or burst sizes. Such heterogeneity may influence downstream applications, including phage therapy formulation and environmental biocontrol [29],[30].

B. Environmental and Clinical Relevance

The isolation of phages capable of lysing MDR *K.* pneumoniae from the Cisadane River highlights the potential of local aquatic environments as reservoirs of therapeutically relevant bacteriophages. Rivers receiving hospital effluents provide ecological niches for the co-evolution of phages and antibiotic-resistant bacteria, enhancing phage diversity and potentially selecting for broad-spectrum lytic activity [4],[5]. Plaque morphology observed in the double-layer agar (DLA) assays provided insight into the phenotypic diversity and functional potential of the isolated phages. Most plaques displayed clear lysis zones surrounded by peripheral halos, a pattern frequently associated with phage-encoded depolymerases. These specialized enzymes, typically located on phage tail fibers, degrade bacterial capsular polysaccharides, thereby generating the characteristic halo zones around plaques [45]. Functional domain analyses in previous studies have identified motifs within these depolymerases, such as pectin lyase-like and glycosidase domains, that facilitate capsule degradation and determine host specificity[45]. The observed halo structures in this study, although not biochemically confirmed, are consistent with such depolymerase activity and suggest the potential presence of enzymes capable of degrading the capsular or exopolysaccharide layers of Klebsiella pneumoniae. This activity is particularly important, as capsule degradation enhances phage access to bacterial cell surfaces, disrupts biofilms, and improves antibacterial efficacy [7],[46],[47]. Differences in plaque size, edge clarity, and halo intensity may further reflect variations in adsorption rate, burst size, or enzymatic efficiency among individual phages [48],[49]. Although biochemical confirmation was not performed in this study, the halo structures observed are consistent with reports where phage depolymerases enhanced biofilm clearance, an important consideration for therapeutic applications against biofilm-associated *K. pneumoniae* infections [16],[17],[50].

C. Variability Across Samples and Statistical Considerations

The observed variability in phage titers across serial dilutions underscores the critical importance of incorporating statistical considerations when interpreting data derived from environmental phage isolations. Such variability likely reflects both intrinsic factors, such as the heterogeneity of initial phage densities in environmental matrices [51], and extrinsic technical factors introduced during the double-layer agar procedure. Variations in bacterial lawn uniformity, mixing efficiency, and pipetting accuracy may further influence plaque formation and enumeration. According to established plaque assay standards, only plates yielding 30–300 plaques are recommended for robust titer estimation, as they provide a balance between statistical precision and countability [52]. In this study, lower dilutions (10^{-1}) often produced confluent lysis and uncountable plaques due to crowding effects, whereas intermediate dilutions (10^{-3}–10^{-4}) yielded discrete, well-defined plaques with coefficients of variation below 20%. These findings indicate that moderate dilutions offer the most reliable representation of phage abundance and activity [53], reinforcing the need for methodological standardization when assessing environmental phage populations.

Beyond methodological optimization, the observed variability has broader implications for the reproducibility and scalability of phage research. Accurate and consistent quantification is fundamental to ensuring the reliability of downstream applications, including phage biobanking, comparative host range studies, and therapeutic screening [53],[54]. When phage titers are measured within the statistically optimal range, inter-sample comparisons become more meaningful and biologically interpretable. Furthermore, extending future studies to include replicate sampling across multiple environmental sites and time points would enable a more comprehensive understanding of temporal and spatial fluctuations in phage **abundance**. Such systematic approaches would enhance the representativeness of environmental phage libraries and facilitate the identification of robust, high-potency candidates for therapeutic development, particularly those active against clinically significant pathogens such as *Klebsiella pneumoniae.*

D. Implications for Phage Therapy

The isolated lytic bacteriophages represent promising candidates for phage therapy, particularly against MDR *K. pneumoniae*, a WHO-priority pathogen [6]. Environmental phages may offer advantages such as adaptation to local bacterial strains, potentially increasing efficacy in region-specific applications [9],[10]. The presence of halo-forming phages further suggests potential anti-biofilm activity, critical for combating persistent infections associated with indwelling medical devices or chronic

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wounds[7],[16],[17]. One notable limitation of lytic phages is their inherently narrow host range, which confines their activity to specific bacterial targets, such as Klebsiella spp., and may limit broader therapeutic applicability. However, in precision medicine, this high specificity is beneficial [55]. When used in vivo, such targeted activity minimizes collateral effects on the normal microbiota, thereby reducing the risk of dysbiosis and preserving microbial homeostasis [55],[56]. Comprehensive host range testing across diverse *K. pneumoniae* isolates and other clinically relevant Enterobacteriaceae is necessary to assess the spectrum of activity and ensure specificity [1],[57]. The phage also shows potential for application as a biocontrol agent against multidrug-resistant *Klebsiella pneumoniae* in wastewater treatment facilities prior to discharge into natural water bodies [58]. Because the phage was isolated from the environment, it is likely to exhibit enhanced stability and persistence under environmental conditions, supporting its efficacy as a biocontrol agent [59]. However, its high host specificity presents a limitation, as it would selectively target *K. pneumoniae* while leaving other clinically relevant Enterobacteriaceae that may also require control unaffected. This limitation can be addressed by employing a phage cocktail composed of multiple lytic phages with complementary host ranges, thereby broadening the overall spectrum of activity [60].

E. Study Limitations and Future Directions

While this study demonstrates the feasibility of isolating lytic phages from river water, several limitations must be addressed in future research:

1. Single-point sampling: Only one location and sampling time were assessed, limiting ecological inference. Multi-site and temporal studies are recommended to evaluate phage abundance dynamics.
2. Absence of molecular confirmation: Phage identity, genome content, and potential lysogeny were not verified. Whole-genome sequencing and bioinformatics analyses are essential in ensuring phage safety for therapeutic use [61]. This process helps to identify and exclude any phages carrying undesirable genes, particularly those associated with lysogeny (such as integrases, repressors, or excisionases) that enable the phage to integrate into the bacterial chromosome and potentially transfer harmful genetic material [62],[63]. It also screens for virulence or toxin-related genes that could enhance bacterial pathogenicity or pose risks to patients[64].
3. Host range and functional activity: The current study focused on a single *K. pneumoniae* host. Expanded host range testing, alongside depolymerase and antibiofilm assays, would strengthen translational relevance. Functional assays for depolymerase activity would provide further evidence for clinical or biocontrol applications.
4. Environmental stability: Factors such as temperature, pH, and river flow may affect phage survival and efficacy. Controlled studies to evaluate environmental stability will inform practical applications.

Addressing these aspects will enhance the translational value of environmental phage isolates and support the development of phage-based therapeutics in Indonesia. Establishing a local phage library could facilitate personalized therapy and contribute to antimicrobial stewardship programs, particularly in regions facing high MDR *K. pneumoniae* prevalence [23].

V. CONCLUSION

This study aimed to isolate and characterize lytic bacteriophages from the Cisadane River in Tangerang, targeting multidrug-resistant *Klebsiella pneumoniae* clinical isolates. Lytic phages were successfully isolated by the double-layer agar (DLA) method, producing clear plaques with halo zones, and titers ranging from 1.28×10^3 to 2.00×10^5 PFU/mL. The Poisson distribution analysis showed moderate variability in plaque counts, indicating natural distribution rather than technical errors. The presence of halos implies potential depolymerase activity, which could enhance phage therapeutic efficacy. This work represents the first isolation of *K. pneumoniae* phages from an environmental source in Banten Province. Future research should involve multi-site sampling, host-range testing, genome sequencing to confirm the absence of virulence genes, and functional enzyme assays to confirm depolymerase activity for therapeutic and biocontrol applications.

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