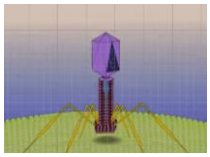

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Efficient Reduction of *Pseudomonas aeruginosa* Biofilms Using the Myoviridae Lytic Bacteriophage vBPaeM *MLG*

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ABSTRACT: *Pseudomonas aeruginosa biofilm infections pose significant challenges in clinical settings due to their increased resistance to conventional antibiotics. Bacteriophages, viruses that infect and kill bacteria, have emerged as promising agents for combating biofilm-related infections. This study aimed to isolate and characterize a potent bacteriophage with antibiofilm activity against P. aeruginosa. Hospital sewage was utilized to isolate a bacteriophage targeting P. aeruginosa. Quantification of phages was conducted through spot tests and double-layer agar methods. The stability of the isolated phage was assessed under varying pH and temperature conditions. Furthermore, the bacteriophage's ability to reduce bacterial growth and exhibit antibiofilm activity was evaluated at different Multiplicity of Infection (MOI) levels. The isolated bacteriophage, named MLG, was identified as a member of the Myoviridae family within the Caudovirales order. MLG effectively reduced bacterial growth over a 14-hour period. It displayed tolerance to a pH range of 5 to 9 and temperatures spanning 25 to 60°C. Moreover, MLG demonstrated efficient inhibition of biofilm formation across various MOI levels. Given its demonstrated in vitro capacity for bacterial growth reduction and antibiofilm activity, MLG holds potential for combatting P. aeruginosa biofilm infections. This study suggests a promising avenue for the development of alternative antibiofilm strategies using bacteriophages.*

Keywords: Antibio film activity, bacteriophage, biofilm, phage therapy, Myoviridae

INTRODUCTION

Pseudomonas aeruginosa, a known opportunistic pathogen, plays a prominent role in approximately 11% of all nosocomial infections (Aloush et al., 2006). This bacterium frequently gives rise to life-threatening illnesses in individuals with weakened health conditions. Notably, it stands as the predominant cause of burn-related infections, contributing to roughly 50% of fatalities in burn patients. The World Health Organization (WHO) highlights the high prevalence of multidrug-resistant bacteria in intensive care units and surgical wards, intensifying the urgency for physicians and clinicians to address this clinical challenge posed by superbugs(Khawaja et al., 2016). These resistance capabilities stem from various sources, including resistant genes encoded on chromosomes, multidrug efflux pumps, and the protective shield of exopoly saccharide-based biofilm formation (Shafique et al., 2017). Additionally, instances of resistance arising from genetic mutations or horizontal gene transfer have been reported (Shafique et al., 2017). In the face of these complex resistance mechanisms, effectively countering *P. aeruginosa* infections has become a critical concern in the medical field.

Various alternative strategies are currently under development for treating infections caused by extremely drug-resistant pathogens, and among these strategies, phage therapy emerges as one of the most potent options against drug-resistant pathogenic bacteria (Brüssow 2005; Khawaja et al., 2016). The application of phages as a therapeutic solution for humans originated in 1919, marking their first use in this capacity (Cisek et al., 2017). Bacteriophages, which are viruses specifically targeting bacteria, have gained attention for their ability to effectively combat bacterial infections. This is achieved through lytic phages and their associated gene products (Khawaja et al., 2016; Shafique et al., 2017) including phage enzymes and lytic proteins such as endolysins, holins, and spanins, which serve as bio-agents in the fight against bacterial infections (Drulis-Kawa et al., 2015).

Bacteriophages present an alternative therapeutic approach to antibiotics due to their unique advantages. These advantages include a narrow spectrum of action, ensuring the preservation of normal flora, as well as the ability to replicate at the site of infection (Pirisi 2000; Khawaja et al., 2016; Shafique et al., 2017; Tian et al., 2021). Additionally, bacteriophages exhibit

qualities such as non-toxicity and cost-effective production. Extensive research confirms their effectiveness against multidrug-resistant pathogenic bacteria in human subjects (Sulakvelidze et al., 2001; Yin et al., 2017). Notably, phages possess a high degree of specificity, targeting only specific bacterial strains (Alvi et al., 2020), a trait that is largely species-specific in reported cases (Yoong et al., 2004). The formation of biofilms stands as a significant contributor to bacterial resistance against antibiotics (Chang, 2018). Numerous investigations have demonstrated that phages can effectively dismantle *P. aeruginosa* biofilms by penetrating the protective exopolysaccharide layer (Sutherland, et al., 2004; Donlan, 2009; Shafique et al., 2017). This study aims to assess the antibiofilm potential of an isolated bacteriophage against *P. aeruginosa*, focusing on its ability to counter biofilm formation.

MATERIAL AND METHODS

Host bacterial strain

A total of 28 strains of multi-drug resistant *P. aeruginosa* (N=28) were acquired from the Institute of Microbiology and Molecular Genetics at the University of the Punjab in Lahore, Pakistan. These strains were originally isolated from clinical specimens (Alvi et al., 2021). Among these strains, the PA-1 strain (with the NCBI Accession number MG763232) was selected as the host for the isolation of the bacteriophage.

Isolation and purification of bacteriophage

The bacteriophage vB-PaeM-*MLG* was isolated from the sewage of the District Head Quarter (DHQ) hospital in Haripur, using a previously described method (Bibi et al., 2016; Alvi et al., 2020). In brief, a sewage sample (40 mL) was combined with 5X LB broth (10 mL), followed by the addition of 500 µL of log-phase PA-1 culture. The mixture was then incubated with continuous shaking at 37°C overnight. Subsequently, the mixture underwent centrifugation at 4000 × g for 10 minutes, and the resulting supernatant was filtered through 0.22 µm syringe filters (Merck, Millipore). Filtrate was applied to LB plates with a PA-1 lawn in order to detect the formation of plaques.

For quantification of the bacteriophage titer, a double-layer agar assay was employed (Asif et al. 2020).The filtrate was subjected to serial dilution in LB broth, followed by the addition of log-phase PA-1 culture. After incubating the mixture at 37°C for 5-10 minutes and

combining it with soft agar medium (4 mL), the resulting mixture was poured over a solid agar layer in plates. Plaques that formed were selected and subjected to successive re-propagation, up to 10 times. The bacteriophage titer within the lysate was calculated as plaque forming units per milliliter (pfu/mL).

Morphological studies of bacteriophages

To conduct morphological investigations of the bacteriophage MLG, transmission electron microscopy (TEM) was employedusing an FEI T20 transmission electron microscope (FEI, Hillsboro, OR). The procedure involved applying the concentrated phage lysate onto carbon-coated copper grids along with phosphotungstic acid. Subsequently, micrographs were obtained at an accelerating voltage of 200V.

Bacterial growth reduction assay

Two conical flasks containing a log-phase bacterial culture, comprising 1.5×10^8 CFU (50 mL) of the host bacterial strain PA-1, were prepared. In one flask, 1.5×10^7 pfu of the phage lysate was introduced as an inoculum, while the other flask was designated as a negative control and remained phage-free. Both flasks were then incubated at 37°C for a duration of 24 hours within a shaking incubator. During this period, the optical density ($O.D_{600}$) of both cultures was measured at regular intervals of 2 hours over the course of 24 hours (Tahir et al., 2017). This entire process was conducted in triplicate, and the resultant readings were averaged to generate a graphical representation of the mean values.

Determination of bacteriophage host range

The host range of the *MLG* phage was assessed through a spot assay, involving a range of multi-drug resistant *P. aeruginosa* isolates (N=28), along with *Escherichia coli* (N=2), *Klebsiella pneumoniae* (N=3), and *Staphylococcus aureus* (N=3).

For the assay, a log-phase bacterial culture (100 µL) was pouredonto LB agar medium, which was mixed with semisolid LB agar (3-5 mL) and allowed to solidify at room temperature. Subsequently, 5 µL of the phage lysate containing 1.5×10^5 pfu of the *MLG* bacteriophage was applied. The appearance of a lytic zone on the bacterial lawn was observed after an overnight incubation at 37°C (Obeso et al., 2010; Alvi et al., 2020). A positive result was indicated by the appearance of a cleared zone on the bacterial lawn, signifying a successful spot test. Conversely, the absence of clearance

denoted a negative spot test. A positive spot test result indicates that the phage *MLG* is capable of infecting the strain on which it produced the positive spot.

Determination of Bacteriophage Thermal and pH stability

The stability of the bacteriophage under varying temperatures and pH conditions was assessed using a previously established methodology (Asif et al. 2020). To assess the impact of temperature on phage activity, a known titer of the bacteriophage (3×10^9 pfu/mL) was subjected to incubation separately at 25°C, 37°C, 40°C, and 60°C for a duration of 1 hour, all under a pH of 7.0 in LB broth. Similarly, the phage titer (3×10^9 pfu/mL) was subjected to different pH conditions (5, 6,7, 8, and 9) at a constant temperature of 37°C for 1 hour. Afterward, the pH was restored to 7.0, and the phage titer was determined using the double-layer agar method (Bibi et al., 2016; Tabassum et al., 2018). The pH adjustments were made using 1N HCl for lowering the pH and 1N NaOH for raising it. These experiments were conducted in triplicate, and the mean values of the obtained readings were graphed to illustrate the results.

Genome Extraction and Characterization of Phage Genome

The extraction of the bacteriophage genome was carried out using the Phenol Chloroform Isoamyl Alcohol (PCI) method, as previously documented (Sambrook and Russell 2006; Tabassum et al., 2018). To outline the procedure briefly, a filtered bacteriophage lysate (1 mL) underwent incubation with DNase I (1 U) and RNase A (100 µg) at 37°C for 4 hours. Subsequently, a 75°C water bath was employed to denature DNase I and RNase A. Proteinase K (20 mg/mL, 2.5 µL), SDS (10%, 50 µL), and EDTA (0.5 M, 40 µL) were added to the lysate, and the mixture was incubated at 55°C for 1 hour, with intermittent gentle mixing every 20 minutes.

The lysate was then divided into two microcentrifuge tubes, with equal amounts of PCI (Thermo Fisher Scientific Cat #15593049) added to each. Centrifugation at 12000× g for 10 minutes followed, and the upper layer was cautiously transferred to new microcentrifuge tubes. Chilled sodium acetate (3M, 50 µL) and ice-chilled 95% ethanol (1 mL) were introduced into the tubes. After a 5-minute period on ice, centrifugation at 12000 × g for 10 minutes ensued. The supernatant was gently decanted, and the resulting pellet

was washed with 500 µL of 70% ethanol, followed by centrifugation at 12000 × g for 5 minutes. The supernatant was once again decanted, and the tubes were inverted on blotting paper to facilitate drying. Subsequently, the DNA pellet was reconstituted in DNase/RNase-free water (50 µL). The extracted genome was visualized, in 1 % agarose gel with 1% TAE buffer, by UV trans-illuminator (Khawaja et al., 2016).

Determination of Antibiofilm Activity of Bacteriophage

The experiment involved the following steps: Bacterial cultures were prepared in LB broth (200 µL in each well) and dispensed into a 96-well microtiter plate. Bacteriophages were added to columns 3, 4, 5, 6, and 7 at different multiplicities of infection (MOI) values: 0.01, 0.1, 1, 10, and 100, respectively. Column 1 served as a blank control with no additions), while column 2 was the control with only bacteria. In rows A, B, C, D, E, and F, bacteriophages were added after 0 hour, 1 hour, 12 hours, 24 hours, 48 hours, and 72 hours, respectively, along with the bacteria.

After a total of 96 hours, the extent of biofilm formation was quantified using crystal violet (CV) staining, following an established protocol (Shafique et al., 2017). Briefly, the planktonic cells were removed, and the wells were washed three times with 0.85% saline. Subsequently, 0.1% CV solution (200 µL) was added to each well and incubated for 10 minutes. The CV solution was then removed, and the wells were washed again to eliminate any unbound stain. Following drying, 33% glacial acetic acid (200 µL) was added to each well. The absorbance of CV was measured at 580 nm using a microplate reader.

The biomass of the treated samples and controls was determined, and the mean values along with their standard deviations were used to generate a graph. Statistical analysis was performed using the multiple t-test feature already integrated within Graph Pad Prism 6.

RESULTS

MLG produced Circular Transparent Plaques

The bacteriophage *MLG* demonstrated lytic activity against the *Pseudomonas aeruginosa* strain PA-1. It generated distinct, transparent plaques with a diameter ranging from 2 to 3mm, as depicted in Fig. 1A. The titer of the *MLG* bacteriophage was determined to be 1.5×10^9 pfu/mL (Fig. 1B).

Isolated Phage showed Bacterial Growth Reduction upto 14 Hours

In terms of their efficacy, bacterial growth reduction is an important criterion for lytic bacteriophages. Bacteriophage *MLG* displayed a notable reduction in *P. aeruginosa* growth within the initial 14-hour period, followed by a subsequent gradual increase in bacterial growth. The slight uptick in growth observed in the bacteriophage-treated bacterial culture might be due to the emergence of *MLG* resistant bacterial mutants. At the 24-hour mark, the MLG-treated group exhibited diminished growth (OD_{600}=0.48) in comparison to the control group (OD600=1.8) (Fig. 1C).

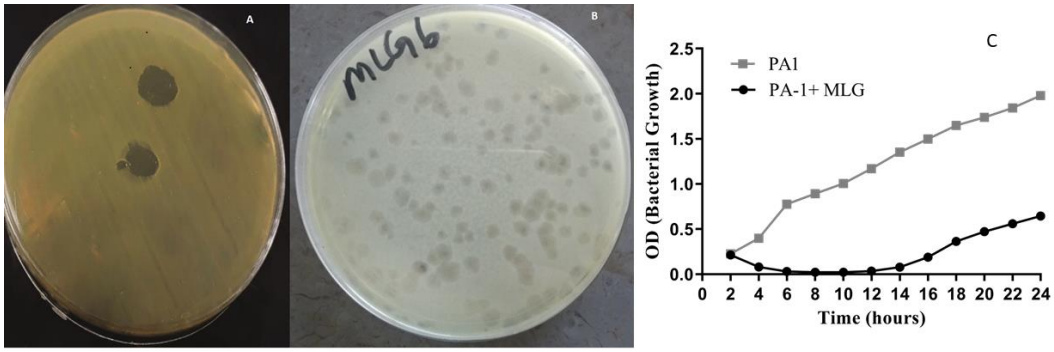

Fig. 1: Plaque of *MLG* on PA-1 lawn and Bacterial Reduction Assay. (A) Positive spot test (B) Plaques formed by *MLG* by double layer agar overlay method on PA-1 lawn (C) The bacterial growth in the absence of bacteriophage (control), and bacterial growth in the presence of bacteriophage *MLG* for 24 hours

Phage *MLG* Showed a host range of 71%

The *MLG* phage successfully infected and lysed 20 out of the 28 strains of *P. aeruginosa* tested. However, it did not exhibit lytic activity against any of the other bacteria tested from different genera, including *E. coli*, *K. pneumoniae*, and *S. aureus* (Table 1). These findings suggest that the phage *MLG* has a narrow spectrum of antibacterial activity and can only infect selective *P. aeruginosa* strains.

Table 1: Host Range of the bacteriophage *MLG* against different bacterial strains. The host range was determined by spot assay using 1.5×10^5 *pfu* and confirmed by double layer agar overlay method

Strain #	Lytic Activity by*MLG*	Strain #	Lytic Activity by*MLG*	Strain #	Lytic Activity by*MLG*
PA-1	+	*PA*-13	+	*PA*-25	+
PA-2	+	*PA*-14	+	*PA*-26	-
PA-3	-	*PA*-15	+	*PA*-27	-
PA-4	+	*PA*-16	+	*PA*-28	+
PA-5	+	*PA*-17	-	*EC-1*	-
PA-6	-	*PA*-18	+	*EC-2*	-
PA-7	+	*PA*-19	+	*KP-1*	-
PA-8	+	*PA*-20	-	*KP-2*	-
PA-9	-	*PA*-21	+	*KP-3*	-
PA-10	+	*PA*-22	-	*SA-1*	-
PA-11	+	*PA*-23	+	*SA-2*	-
PA-12	+	*PA*-24	+	*SA-3*	-

PA = Pseudomonas aeruginosa, EC = Escherichia coli, KP = Klebsiella pneumoniae, SA = Staphylococcus aureus

MLG Depicted *Myoviridae* Morphology

The electron micrograph of *MLG* displayed an icosahedral head measuring 83 nm in diameter, with an elongated tail spanning a length of 279 nm. Based on its morphology, *MLG* is indicative of belonging to the *Myoviridae* family within the *Caudovirales* order (Fig. 2A).

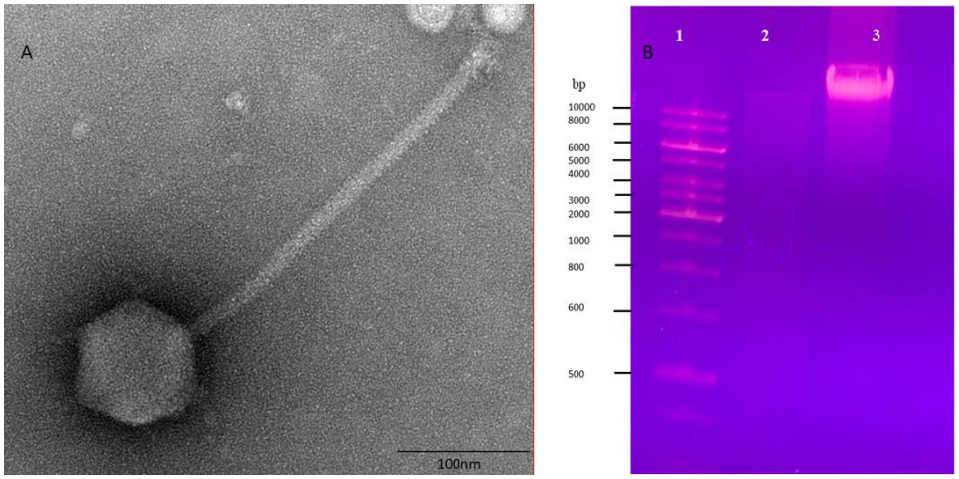

Fig. 2: (A)The electron micrograph of *MLG* revealed an icosahedral head having a diameter of 83 nm and a long tail of 279 nm in length. The morphology of *MLG* suggested it as a member of the family *Myoviridae* of the order *Caudovirales.* (B)The genome of *MLG* on agarose gel (lane 3) in the presence of a DNA ladder (lane 1). The size of *MLG* genome was above 10 kb. The lane 2 was loaded with a DNase treated *MLG* genome and indicated that the genome is DNA.

MLG was Found Stable at variable pH and Temperature

The *MLG* phage exhibited stability across a broad pH range (from 5 to 9), with the optimal pH being 7, during which no decline in *MLG* titer was noted. At pH 6 and 8, a marginal reduction in log titer was observed, while at pH 9 and 5, reductions of 1 log and 2 logs, respectively, were observed (Figure 3A). *MLG* Similarly, the *MLG* phage maintained stability at temperatures of 25°C, 37°C, and 40°C, displaying no decrease in titer. However, following exposure to a thermal stress of 60°C, a twofold decrease in titer was recorded (Fig. 3B).

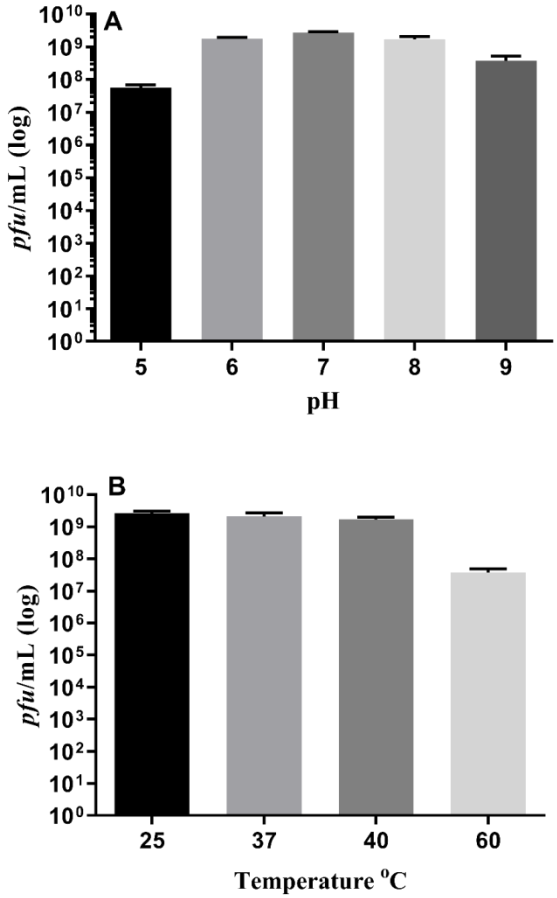

Fig. 3: A pH and thermal stability of bacteriophage *MLG* at.(A)*MLG* was kept at 5, 6, 7, 8 and 9 pH for 1 hour duration and the phage titer was determined using double layer agar overlay method. (B) *MLG* was kept at 25, 37, 40 and 60°C for 1 hour duration and the phage titer was determined using double layer agar overlay method.

Genome of *MLG* was found to be DNA

The genetic material of phage *MLG* was identified as DNA, substantiated by its complete degradation when exposed to DNases, resulting in the absence of any bands on the agarose gel (Fig. 2B, Lane 2). Conversely, treatment with RNases produced a distinct band on gel electrophoresis. Furthermore, the DNA gel analysis indicated that the size of the phage genome exceeded 10 kb (Fig. 2B).

***MLG* inhibited *P. aeruginosa* Biofilm Formation**

Phages must possess the capability to infiltrate biofilms for effective

integration into bacteriophage therapy. The microbial community's microenvironment undergoes shifts during biofilm development. When exposed to simultaneous challenges from*MLG* phages across various MOIs (100, 10, 1, 0.1, 0.01), no biofilm formation occurred (Fig. 4). MOI 100 exhibited the highest efficacy, preventing biofilm formation even when phages were introduced 24 hours post incubation and assessed after 96 hours. However, minimal biofilm was evident upon phage introduction after 72 hours of bacterial incubation (Fig. 4). Among the MOIs, 0.01 proved the least effective in inhibiting biofilm formation. Nonetheless, a notable disparity was observed between the control and phage-treated groups at MOI 0.01, particularly when bacteriophages were introduced after 6 hours ($P<0.0001$), 12 hours ($P<0.0001$), and 24 hours ($P=0.0001$) of bacterial incubation. No noteworthy decrease in bacterial growth was noted at MOI 0.01 when *MLG* was introduced at 0 hours, 1 hour, or 72 hours. While MOI 100 resulted in significant bacterial reduction across all scenarios, the most substantial biofilm reduction occurred when phages were introduced after 24 hours of bacterial incubation. These findings indicate that the optimal timeframe for biofilm reduction falls within 24 to 72 hours following incubation at MOI 0.1, 1, 10, and 100. However, growth reduction at MOI 1 and 10 yielded comparable impacts. Overall, *MLG* infection emerged as an inhibitor or reducer of biofilm formation and bacterial growth.

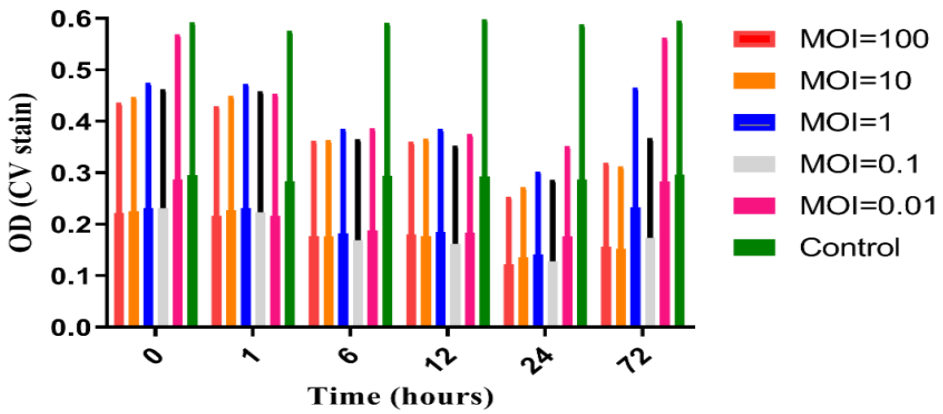

Fig. 4: Antibiofilm activity of *MLG* on PA-1 biofilm at different MOI. Phage was added at 0, 1, 6, 12, 24 and 72 hours post inoculation. The bars showed the mean value along with the standard deviation

DISCUSSION

The commencement of the post-antibiotic era has undermined the urgency of developing effective alternative therapies to combat infections caused by antimicrobial resistance (AMR) in pathogens (Alanis, 2005). Numerous research groups are currently exploring various alternative treatment strategies. These strategies encompass the search for novel antimicrobial compounds derived from plant extracts nano particles, the utilization of green nano particles and short antimicrobial peptides, as well as molecular techniques like the CRISPR-Cas system to target resistance genes. Another avenue being explored is the use of natural bacterial predators known as bacteriophages (Alvi et al., 2020). Bacteriophages are increasingly gaining prominence among these alternatives due to their distinct advantages over traditional antibiotics. These advantages include lower production costs, inherent presence, in-situ replication at the infection site, and specificity against the target pathogen. The bacteriophage *MLG* against *P. aeruginosa* was isolated from hospital sewage located in Haripur, Pakistan. Hospital sewage serves as a reservoir for pathogenic bacteria, and *P. aeruginosa*, being ubiquitous, is commonly found in such environments. Notably, bacteriophages targeting *P. aeruginosa* are naturally present in hospital sewage. Previous instances have shown the successful isolation of various bacteriophages active against *P. aeruginosa* and other pathogenic bacteria from hospital sewage sources (Rigvava et al., 2013; Khawaja et al., 2016; Shafique et al.. 2017).

The electron micrograph of bacteriophage *MLG* unveiled its distinct morphology—an elongated tail with an icosahedral head (Fig. 2A). This *MLG* structural configuration firmly classifies *MLG* as a member of the *Myoviridae* family. Notably, the *Myoviridae* family has previously yielded phages targeted against *P. aeruginosa* in multiple investigations (Heo et al., 2009; Pires et al., 2015). For instance, the phages MPK1 and MPK6, both *Myoviridae* family members, exhibit head diameters of approximately 70 nm and contractile tails measuring around 110 nm(Heo, Lee et al. 2009). However, studies have also revealed the isolation and characterization of bacteriophages from the *Podoviridae* (Alvi et al., 2020, 2021) and *Siphoviridae* families (Knezevic et al., 2009; Khawaja et al., 2016) targeting *P. aeruginosa*.

Throughout the course of its interaction, *MLG* effectively curbed bacterial proliferation until the 14-hour mark,

after which bacterial growth exhibited a resurgence (Fig. 1C). This resurgence could be ascribed to the emergence of phage-resistant mutants within the bacterial population. Similar instances of phage-resistant mutants developing after 10-12 hours of incubation have been documented in previous studies (Haq et al., 2012; Kwiatek et al., 2017). Furthermore some phages restrict bacterial growth for many hours and then bacteria develop resistance to phages after a few hours (Chaudhry et al., 2014; Bibi et al., 2016; Pallavali et al., 2017). The ability of the phage *MLG* to reduce bacterial growth for the initial 14hours can be beneficial and holds promising implications for its therapeutic application. During this timeframe, the immune system could potentially eliminate the slow-growing pathogens. Additionally, employing phage preparations in cocktails might aid in mitigating the emergence of phage-resistant mutants.

The bacteriophage *MLG* exhibited a notably broad host range of 71% against the tested strains of *P. aeruginosa* (Table 1), while its efficacy spanned a narrower spectrum when it came to other genera. Various bacteriophages targeting *P. aeruginosa* have been documented to exhibit host specificity, solely infecting their intended host without affecting other genera (Knezevic et al., 2009; Alvi et al., 2021). Nevertheless, some bacteriophages have showcased the ability to infect other genera as well (Khawaja et al., 2016). Knezevic, et al. (2009) studied many bacteriophages having high host range against *P. aeruginosa* only but no lytic activity against other genera. On the other hand Khawaja et al. (2016), reported a bacteriophage JHP isolated against *P. aeruginosa* having lytic activity against other genera, too (Khawaja et al., 2016). The specificity of bacteriophages towards particular bacterial genera or species holds promising implications for treating pathogenic bacteria without perturbing the natural microflora. In some scenarios, treating several bacterial pathogens (super infections) necessitates a strategy involving bacteriophage cocktails (Chaudhry et al., 2014). To ascertain the host range, a titer of 10^5 was chosen for the spot assay, specifically to avert the phenomenon termed "Lysis from without"(Abedon, 2011). Prior research has highlighted that using a high bacteriophage titer for the spot assay can trigger non-specific lysis or inhibition of bacterial cells due to the overcrowding of bacteriophages at a single site (Kutter 2009).

Evaluating phage infectivity across a spectrum of pH levels holds promise for their potential application in phage therapy. Bacteriophage *MLG* demonstrated considerable stability within the pH range of 5 to 9, showcasing its optimal activity at a neutral pH of 7. Across pH 6 and 8, the phage titer (log) remained consistently unaltered. However, a reduction of 1 log in titer occurred at pH 9, and a more significant decrease of 2 logs was observed at pH 5 (Fig. 3A). Comparable findings regarding the pH stability of bacteriophages have been reported in numerous other studies (Fan et al., 2017; Smolarska et al., 2018), underscoring that the majority of phages remain stable within the pH range of 5 to 9. This wide pH stability bears significance for the preservation and clinical utilization of phages as therapeutic agents (Capra et al., 2006; Alvi et al., 2020). In the context of certain bacterial infections, alterations in the environment of human organs, including shifts in pH, are commonplace. Phages capable of enduring variable pH stresses thus hold potential as therapeutic candidates. This adaptability could prove crucial for effectively targeting infections within the dynamic pH environments of human organs.

The stability assessment of phage *MLG* encompassed a range of tested temperatures (25, 37, 40, and 60°C), revealing robustness across all conditions. Specifically, the titer of *MLG* remained steady at 25, 37, and 40°C. However, a modest 2-fold reduction in titer was noted at 60°C (Fig. 3B). The resilience of phage *MLG* to varying temperatures carries significant implications for their storage and transportation. Enhancing the practicality of these aspects could consequently contribute to a reduction in the overall cost associated with phage therapy (Bibi et al., 2016; Alvi et al., 2021). Numerous previous investigations have underscored the stability of bacteriophages within the temperature range of 37°C to 60°C (Yang et al., 2010; Haq et al., 2012; Alvi et al., 2020, 2021). In a notable study, the stability of bacteriophage *PaeP-SaPL*across a temperature span of 25-60°C was reported (Alvi et al., 2021).

Biofilm formation stands as a significant virulence factor for bacteria, as it provides a shield through exopolysaccharides, allowing bacteria to evade immune responses and rendering them impervious to antibiotics (Liu et al., 2022). Notably, bacteriophages present a promising avenue for

combatting biofilm-forming bacteria, exhibiting an advantage over conventional antibiotics. Remarkably, treatment with bacteriophage *MLG* yielded a notable reduction in the biofilm-forming capacity of the bacterial strain PA-1 across all tested multiplicities of infection (MOI) levels (100, 10, 1, 0.1, and 0.01 (Fig. 4). Particularly encouraging outcomes were observed at MOI levels of 1 and 10. Previously, a study on bacteriophage PB-1 infecting *P. aeruginosa* revealed that when PB-1 was used at MOI 0.01, 69% reduction in biofilm biomass was observed after 24 hours of infection (Coulter et al., 2014). This underscores the potential of bacteriophages in efficiently curtailing biofilm formation, particularly when administered at optimal MOI levels.

The findings of this study emphasize the impact of bacteriophage exposure duration on biofilm formation. Notably, compelling outcomes emerged when bacterial cultures were subjected to bacteriophages after 24 and 72 hours post-incubation. According toShafique, Alvi et al. (2017) the bacteriophage JHP has the least reduction of biofilm after 24 hours of exposure, which contradicts our findings.. At both 6- and 12-hourspost exposure, the biofilm reduction remained consistent for MOI 100 and 10, respectively. However, a notable distinction emerged at the 72-hour post-exposure, where the efficacy of MOI 10 surpassed that of MOI 100. This discrepancy might be attributed to the aggregation of phages, potentially competing for bacterial receptors. This phenomenon could contribute to the observed variations in efficacy between the two MOI levels.

CONCLUSION

The application of bacteriophages for treating biofilms proves to be an effective strategy against biofilm-producing bacteria. Infections caused by bacterial pathogens that form biofilms pose significant treatment challenges, as conventional antibiotics often struggle to permeate these biofilms. As a result, bacterial cells within biofilms remain relatively unaffected or only marginally impacted by antibiotics. The utilization of bacteriophages, however, presents a viable solution for biofilm elimination. In this study, the isolated phage *MLG* exhibited robust lytic activity against *P. aeruginosa*, targeting both planktonic cells and biofilm structures. The in vitro efficacy of *MLG* to reduce bacterial growth, thermal and pH stability, specificity and antibiofilm potential make this phage a candidate for phage therapy against *P. aeruginosa* infections, after performing necessary animal model studies.

Conflict of interest

The authors declare there is no conflict of interest.

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