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ORIGINAL ARTICLE

Evaluation of the Efficacy of a Recombinant Phage-Lysin Hydrogel on Multi-Drug Resistant *Pseudomonas aeruginosa*

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ABSTRACT

Background: *Multidrug-resistant (MDR) Pseudomonas aeruginosa infections represent a grave public health concern. Bacteriophages (phages) or their lysins can be used as alternatives or complements to antibiotics.* ***Objective:*** *This study was designed to isolate and characterize specific lytic bacteriophages for P. aeruginosa and development of lysine-thermosensitive hydrogel be used as a biomedicine application.* ***Methodology:*** *VITEK-2 Compact System was used for identification of isolated organisms while Kirby-Bauer disc diffusion method was used for antibiotics sensitivity testing. Phages were isolated and their biological kinetic and phenotypic properties were discovered by spotting test and transmission electron microscopy. Gene cloning and lysin effects were tested. A lysin hydrogel formulation was developed.* ***Results:*** *Two specific P. aeruginosa bacteriophages were isolated (BP-PS1) and (BP-PS2). They belong to the Myoviridae family, order Caudovirales. The infectivity rates of BP-PS1 and BP-PS2 were 83% and 91%, respectively. While, a phage cocktail has achieved full infectivity at 100% on the tested MDR isolates. The expressed and purified lytic lysins have been derived from these bacteriophages. They exhibited potent bactericidal activity against P. aeruginosa. The developed lysin hydrogel demonstrated exceptional stability and uniform drug content. The hydrogel exhibited favorable ex vivo skin diffusion characteristics with 84.87% permeation rate, indicating a favorable sustained and controlled drug release. The gel optimal pH level of 6.16 ± 0.1 displayed a pseudo-plastic flow behavior and proved to be easy to apply.* ***Conclusion:*** *This study highlights the immense potential that projects bacteriophage lysin hydrogels as an enzybiotic therapy wherewith to effectively address MDR P. aeruginosa infections. Given the urgency of the antibiotics resistance crisis, the development of innovative treatments like lysin hydrogels offers hope in mitigating the impact of drug-resistant bacterial infections.*

INTRODUCTION

According to Church *et al.* [1], burn wound infections have become increasingly common among third-degree burn patients, impacting their chances of survival. Prolonged hospital stays render these patients susceptible to post-burn infections caused by multidrug-resistant (MDR) microorganisms. Immuno-compromised individuals are at greater risk, as burn wounds destroy the protective stratum corneum layer, leading to immunosuppression and vulnerability to microbial infections. Moreover, burn wounds provide a highly conducive environment for bacterial growth, since they contain necrotic tissues and protein wound exudate[2].

Among the opportunistic pathogens, *Pseudomonas aeruginosa* is of significant concern, causing acute or chronic infections in immune-compromised individuals, patients suffering from cystic fibrosis, the elderly and hospitalized patients. The bacterium manifests resistance to various antibiotic families, including beta-lactams, aminoglycosides, and quinolones, owing to acquired, adaptive, and intrinsic resistance mechanisms. The escalating global issue of wide spread resistance to antibiotics poses a major threat to human health (and animal health) due to the rising prevalence of infections caused by antibiotic-resistant bacteria[3].

In the battle against infectious diseases, bacteriophages, also known as ‘phages’ and their lytic enzymes (lysins or endolysins) offer an alternative antibacterial approach. Lysins, produced at the end of the phage cycle, form part of the lysis cassette and exhibit high specificity and activity. The multitude of phages in the biosphere makes phage enzymes particularly promising for an enzybiotic application. Enzybiotic is a promising replacement to the

conventional antimicrobial approach or antibiotics involves the use of bacteriophage-derived protein(s) or endolysins [4].

Wound healing is a complex process comprising four stages: hemostasis, inflammation, repair and remodeling. However, infections and external factors can disrupt the ordered plasticity of wound healing especially if the infection is with multi-drug resistant bacteria. Topical application of lytic phage-containing ointments, creams, and lotions can accelerate wound healing. These solutions (topical applications) offer higher stability and do not require frequent treatments, thereby making them simple to use[5].

Thus, the enzybiotic approach to treat infected burned wounds with multi-drug resistant bacteria was the aim of the current study. In order to isolate specific *P. aeruginosa* phages, a multi-drug resistant *P. aeruginosa* was isolated from infected burned wounds of hospitalized patients. Consequently, the antibacterial activity of the specific *P. aeruginosa* phages and their lytic lysins were investigated. To facilitate topical delivery of the lysins, a novel lysin hydrogel formulation was developed.

METHODOLOGY

Bacterial Isolation and Identification

A total of 173 clinical isolates of *P. aeruginosa* were collected from hospitalized patients with burn wound infections at Al-Imamein Al-Kadhimein, and Medical City Hospitals in Baghdad, Iraq. The isolates were collected in the period from September 2021 to May 2022. To determine the exact genus and species of the bacteria that were isolated, the VITEK-2 Compact System (bioMerieux, Marcy l'Etoile, France) was utilized.

Antibiotic Sensitivity Test

The antibiotic susceptibility of *P. aeruginosa* isolates was determined using the Kirby-Bauer disc diffusion method on Muller Hinton agar plates. *P. aeruginosa* isolates were cultured on Tryptic Soy Broth (TSB) medium and incubated at 37°C overnight. After preparing a 0.5 McFarland standard dilution, the cultures were spread on Muller Hinton agar plates. Twelve antibiotic discs from Hi Media were placed on the plates and incubated at 37°C for 18–24 hours. The antibiotic discs were as follows: Ceftazidime (30µg), Cefepime (30µg), Piperacillin-tazobactam (100/10µg), Aztreonam (30µg), Gentamicin (10µg), Amikacin (30µg), Tobramycin (10µg), Ciprofloxacin (5µg), Levofloxacin (5µg), Imipenem (10µg), Meropenem (10µg), and Colistin. The results were interpreted in accordance with the recommendations of the Clinical and Laboratory Standards Institute[6].

Bacteriophage Isolation and Purification

Waste water samples from different locations in Baghdad city were collected for the isolation of *P. aeruginosa* specific bacteriophages using modified methods[7]. Primary bacteriophages were then isolated by mixing environmental specimens with target bacteria. Later, the lytic bacteriophages were identified through a bacteriophage-spotting test on nutrient agar plates. The plaque assay was performed by combining diluted phage suspension and *P. aeruginosa* culture on LB agar plates, which were then incubated overnight to count and differentiate among the bacteriophages.

Determination of the Bio-kinetics of Isolated Bacteriophages

The burst size, burst time, and infection percentage of isolated bacteriophages were calculated using a specific method[8]. Phage-bacteria mixtures were incubated to allow phage entry into bacterial hosts, and then centrifuged to remove extracellular phages. Serial dilutions were spotted on target bacterial lawns at various time intervals, enabling counting of plaques in order to determine the infection percentage, burst time, and burst size.

One-Step Growth

Pseudomonas aeruginosa cultures were then centrifuged to remove bacterial cells, and the pellet was combined with phage suspension. After phage adsorption, the mixture was again centrifuged to eliminate remaining phages. The pellet was then re-suspended with temperature maintained at 37°C. Samples were taken at intervals to calculate phage titers[9].

Host Range

Bacterial strains were sub-cultured in Brain Heart Infusion (BHI) broth and transferred to BHI agar plates. Phage suspensions were spotted on host strains, and lysis zones were observed after incubation. Thermal stability was determined by incubating phage suspensions at designated temperatures, and the half-life was calculated[10].

Transmission Electron Microscopy of Phage Morphology

Lysate samples were placed on copper grids, centrifuged, and negatively stained with phosphor-tungstic acid. The samples were examined using a TEM 900 to visualize phage morphology[11].

Lysin Genes Cloning

The cloning procedure for lysin genes was performed in accordance with a method that has been previously reported[7].

Protein Expression and Purification

Putative endolysins were expressed in *Escherichia coli* and provoked with Isopropyl β-d-1-thiogalactopyranoside (IPTG). Bacterial cultures were centrifuged, and the pellets were frozen for processing. Different IPTG concentrations and lower temperatures

were examined for induction, and protein purification and determination of concentration were conducted[7].

Antimicrobial Activity of Endolysins and MIC/MBC Determination

Bactericidal activity of recombinant lysins against *P. aeruginosa* was investigated using dose-dependent assays. Serial dilutions were used to evaluate minimum inhibitory concentration (MIC) and minimum bactericidal activity (MBC) of the purified lysins[12].

Preparation of Lysin Hydrogel

A topical protein hydrogel was prepared by incorporating lysine (LYS)-prepared lyophilized protein into a 2% W/V carbopol 934 gel base. The pH level was adjusted using triethanolamine[13].

Physical Appearance

The prepared lysin hydrogel was visually assessed for phase separation, color, homogeneity, grittiness, consistency, and pH[14].

Viscosity Determination

The viscosity of lysin hydrogel was measured using a Brookfield Digital viscometer at different rpm values[15].

Determination of the spread ability

The spread-ability of lysin hydrogel was evaluated by measuring the diameter of a circle formed under certain specific conditions[16].

Lysin Content Determination

Lysin hydrogel samples were analyzed by High performance liquid chromatography (HPLC) to quantify the percentage of drug content[17].

***Ex Vivo* Skin Diffusion**

Lysin hydrogel diffusion was assessed using a Franz cell with rat skin as a barrier between donor and receptor compartments. Samples were taken at regular intervals to evaluate drug concentration[18].

***In Vitro* Antibacterial Activity of the Tested Hydrogels**

The antibacterial activity of tested hydrogels was evaluated using the cup-plate method on Mueller-Hinton agar plates. The presence of a zone of inhibition indicated antibacterial activity[18].

Statistical Analysis

Statistical analysis was performed by using the SPSS Statistical package (Version 26; SPSS, IBM) and Microsoft Office Excel (2010) for drawing the figures. The statistical significance threshold (P – value) was accepted at $P < 0.01$ and Data were expressed as mean ± SD.

RESULTS

Antibiotic Susceptibility Test (AST)

The study aimed to assess the prevalence of antibiotics resistance profiles of *P. aeruginosa* isolated from infected wounds. The estimation of MDR, extensively drug-resistant (XDR), and Pan Drug-Resistant (PDR), among these isolates was determined. The results revealed that out of 173 samples, 21 isolates (12.1%) exhibited MDR, while 3 isolates (1.7%) displayed XDR, and 1 isolate (0.6%) was classified as PDR. Furthermore, 152 isolates (87.9%) were found to be sensitive to the tested antibiotics, the analysis revealed varying levels of antibiotics resistance in *P. aeruginosa*. as presented in table 1.

Table 1: The percentages of resistance of *P. aeruginosa* isolates to different antibiotics.

No.	Name of the antibiotics	Percentage of resistance (%)
1	Ciprofloxacin	77.5%
2	Levofloxacin	72.3%
3	Cefepime	70.5%
4	Imipenem	69.4%
5	Tobramycin	68.2%
6	Piperacillin-tazobactam	68.2%
7	Gentamicin	67.1%
8	Ceftazidime	65.3%
9	Meropenem	64.7%
10	Amikacin	61.3%
11	Aztreonam	27.2%
12	Colistin	7.5%

Bacteriophage Isolation

Bacteriophage isolation involved spot lysis and plaque assays to confirm infection with 12 MDR *P. aeruginosa*. **Figure 1** shows the results of two lytic putative bacteriophages.

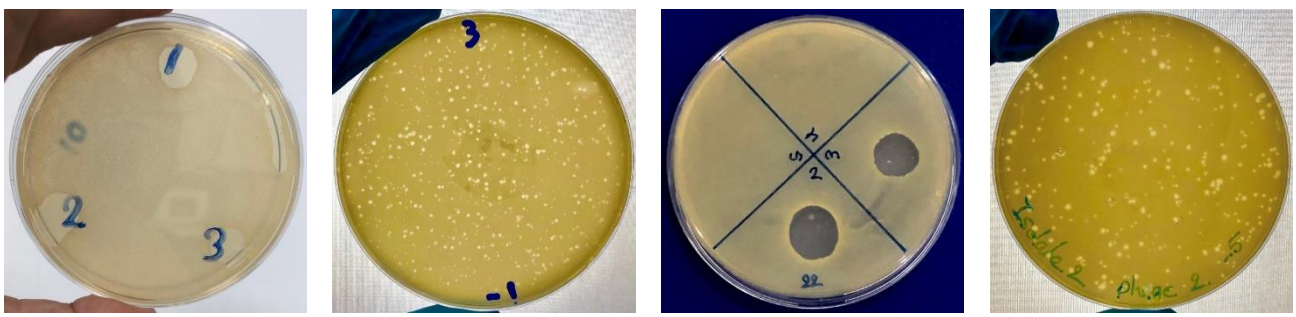

Fig. 1: Lytic putative bacteriophages infecting targeted bacteria.

Bacteriophage Infectivity Rate

The isolated bacteriophages exhibited high lytic activity, forming distinct clear zones on 12 targeted MDR *P. aeruginosa*. Bacteriophage- *P. aeruginosa* (BP-PS1) displayed an infectivity rate of 83% (10/12), while BP-PS2 showed an infectivity rate of 91% (11/12). When both bacteriophages were combined (phage cocktail), their infectivity rates overlapped, resulting in a 100% (12/12) infectivity rate.

Transmission Electron Microscope (TEM) Analysis

TEM analysis was employed to classify the isolated bacteriophages. This was done based on their morphological characteristics which enabled them to be assigned to specific viral families and orders. Negative staining revealed that the phages belonged to *Myoviridae* family within the order of *Caudovirales* (**Figure 2)**.

BP-PS1 and BP-PS2 are *Myoviridae* viruses with an average head diameter of 72 and 78 nm, respectively, and a long tail with length of 85 and 94 nm, respectively. The width size of their tails is 10.2 and 11.5 nm, respectively. These bacteriophages possess contractile tails that are relatively long, thick, and rigid, consisting of a central core composed of stacked rings of six subunits, surrounded by a helical contractile sheath, which is separated from the head by a neck.

Fig. 2: Transmission Electron Micrograph confirming that the isolated bacteriophages belong to Myoviridae family, order Caudovirales.

One-Step Growth Experiment

The latent period and burst size of BP-PS bacteriophages were measured in a one-step growth experiment. This exercise resulted in a curve that displayed three phases, namely: latent, log, and stationary phases. The burst size was found to be 360phages/cell and the latent duration was 20 minutes **(Figure 3)**. The burst size was determined by calculating the ratio of the mean yield of the phages used to infect the bacteria to the mean of the phage particles released after infection.

The lytic activity of the recombinant *Pseudomonas* lysine (PS-Lys) against bacterial cells was examined, and the OD_{600} values of the bacterial cells treated with PS-Lys significantly decreased from 0.98 to 0.33, while in the control group it showed no change. Furthermore, treatment with PS-Lys resulted in a considerable reduction in bacterial counts of *P. aeruginosa* at 2 and 4 hours, compared with the control group. These findings highlight the potent bactericidal effect of PS-Lys on *P. aeruginosa* **(Figure 4)**.

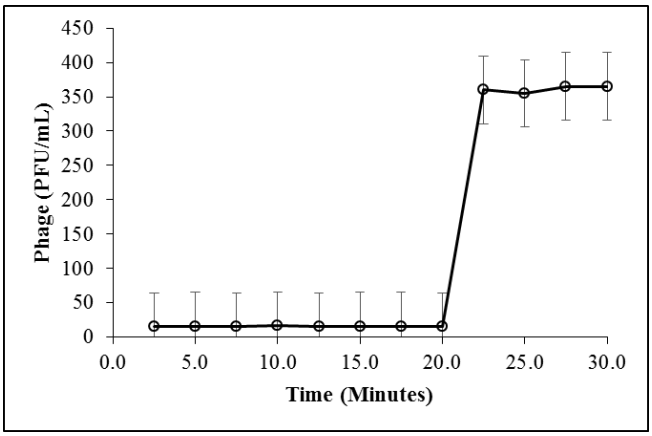

Fig. 3: Lytic activity of the purified Pseudomonas lysine (PS-Lys) against P. aeruginosa.

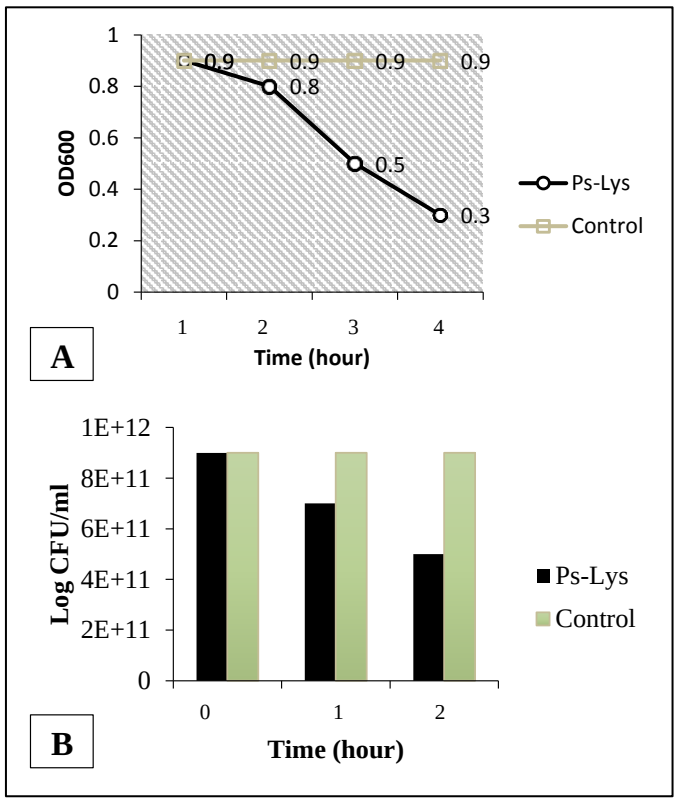

Fig. 4: The Lytic activity of purified PS-Lys against P. aeruginosa. (A) The OD_{600} values of bacterial culture following PS-Lys treatment significantly decreased from 0.98 to 0.33, while the control group showed no change. (B) PS-Lys treatment led to a considerable reduction in bacterial counts of P. aeruginosa at different time points compared with the control group.

PS-Lys MIC and MBC against *Pseudomonas aeruginosa*

In order to investigate PS-Lys as a potential molecule to combat *P. aeruginosa*, the MIC was initially determined by subjecting the bacterium to various enzyme concentrations and observing the decline in OD600. The results indicated an MIC of 100 µg/mL for PS-Lys. The bactericidal activity of PS-Lys led to a remarkable reduction in bacterial count by over 3 log10, demonstrating its potential bactericidal properties and suggesting its potential as an antibacterial agent.

Physical Characteristics of PS-Lysin hydrogel

The formulated hydrogel containing PS-Lys exhibited a clear, homogenous, and white appearance, possessing an acceptable consistency without precipitation. A pH value of 6.16 ± 0.1 was determined, indicating the hydrogel's skin-friendly nature, making it suitable for topical delivery.

Viscosity Analysis of Lysin Hydrogel Formula

The viscosity of the lysin hydrogel formula was analyzed at various shear stress levels, the results of which are presented in (**Table 2**). The study revealed that the lysin hydrogel exhibited a pseudo-plastic flow behavior. With an increase in shear rate, the apparent viscosity decreased promptly due to the alignment of normally disorganized gelling agent molecules in the flow direction, reducing internal resistance and resulting in a lower viscosity[19]. The results indicate that the viscosity of the lysin hydrogel decreases as the shear stress increases. At the lowest shear stress of 1 rpm, the viscosity is highest at 79207±7.5 centipoise, and as the shear stress increases, the viscosity progressively decreases, reaching 3842±6.9 centipoise at 100 rpm. This pseudo-plastic flow behavior of the hydrogel is advantageous for topical delivery due to its ease of application and spreading on the skin[20].

Table 2: Shows the viscosity (in centipoise) of the lysin hydrogel measured at room temperature under different shear stress levels.

Speed (rpm)	Viscosity ± SD
1	79207 ± 7.5
5	37780 ± 1.5
20	15756 ± 8.7
60	5450 ± 9.1
100	3842 ± 6.9

Spread ability determination

The spread ability of the prepared lysin hydrogel was measured by assessing the difference in the diameter of the circle formed before and after the application of 500 g, resulting in 4.5 cm ± 0.08. This indicates that the lysin hydrogel spreads easily over the affected area of the skin when applied topically, as its bioavailability is highly dependent on the spreading value[21].

Drug content determination

The drug (protein) content of the prepared lysin hydrogel formula was found to be 98.1 ± 1.3, which falls within the acceptable range of U.S. Pharmacopeia (USP) Reference Standards (85–115%). This demonstrates that the preparation has a high capability and uniformity in content[22].

***Ex Vivo* skin diffusion**

Utilizing the Franz diffusion cell, the percentage of lysin hydrogel permeation through abdominal rat skin was determined to be 84.87% ± 0.19 **(Figure 5)**. The incorporation of lyophilized lysin powder in the gelling agent (carpool 934) resulted in sustained protein release for 24 hours, facilitated by the torus cross-linked structure of the gelling agent that promotes gradual protein release. This allows for easy and efficient application of the prepared protein solution on the skin[23].

***In Vitro* Antibacterial Activity of the Tested Hydrogels**

Inhibition zones were observed surrounding the cups containing the tested hydrogel, phage BP-PS1, BP-PS2, or lysate (positive control), but not the control hydrogel (negative control), which lacked any inhibition zones.

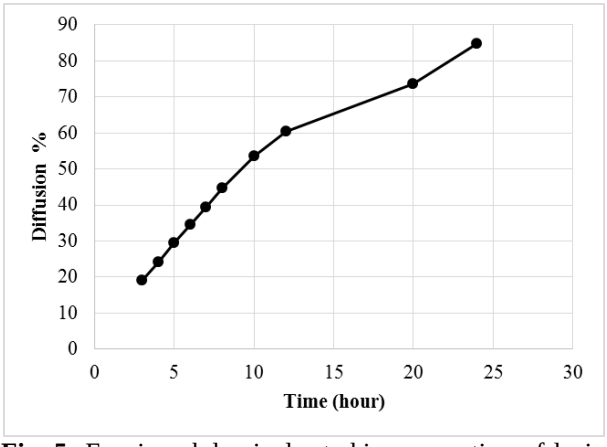

Fig. 5: Ex vivo abdominal rat skin permeation of lysin hydrogel in 50 mL PBS 7.4 diffusion cell. The graph shows the percentage of lysin hydrogel abdominal rat skin permeation over time, demonstrating sustained protein release for 24 hours.

DISCUSSION

Pseudomonas aeruginosa, a notoriously opportunistic pathogen, exhibits a high capacity to rapidly develop resistance against various antibiotics, posing substantial challenges in clinical management[24]. The global surge in skin and soft tissue infections caused by MDR pathogens raises serious concerns, exacerbated by limited treatment protocols and guidelines[25]. The findings of the current study underscore the alarming prevalence of resistance to antibiotics among *P. aeruginosa*, with MDR isolates (21 out of 173 isolates) exhibiting resistance to multiple classes of antibiotics, thereby compromising therapeutic efficacy. The presence of XDR (3 out of 173 isolates) and PDR (only one isolate) further heightens the urgency, as these strains defy all tested antibiotics, leaving available very limited therapeutic options. These findings highlight the concerning levels of drug resistance among *P. aeruginosa* isolates. The high prevalence of MDR indicates that these bacteria have developed resistance to multiple classes of antibiotics, limiting the effectiveness of commonly used treatment options. Furthermore, the presence of PDR isolates raises additional concerns, as these bacteria are resistant to all antibiotics for which tests were conducted. Although the count of PDR isolates is relatively low in comparison with the total isolates, it remains an alarming issue that requires close monitoring. The emergence of even a single PDR isolate is of significant concern, as it poses a serious challenge in clinical management and infection control.

Notably, Ciprofloxacin showed the highest level of resistance (77.5%), indicating the need for cautious use of this fluoroquinolone in *P. aeruginosa* infections. The resistance observed against Cephalosporins, such as Ceftazidime (65.3%) and Cefepime (70.5%), highlights the challenges in treating *P. aeruginosa* using these commonly prescribed antibiotics.It is concerning to observe significant resistance to Carbapenems, including Imipenem (69.4%) and Meropenem (64.7%), as these antibiotics are often reserved for severe cases. Additionally, resistance to aminoglycosides, such as Gentamicin (67.1%) and Amikacin (61.3%), further emphasizes the need for finding alternative options of treatment. By contrast, the Lipopeptide antibiotic Colistin demonstrated relatively lower resistance (7.5%). However, it is crucial to carefully monitor and preserve the effectiveness of Colistin, considering its role as a last-resort treatment for MDR *P. aeruginosa* infections. The usage of bacteriophage in treating MDR bacteria is gaining more interest in the scientific field. The treatment with bacteriophage poses the advantage of fewer side effects with efficiency to illuminate the resistant bacteria without disturbance to the normal microbiota of the treated area[26].

The current investigation explored bacteriophage-based therapies as a potential solution. Phages have demonstrated potent bactericidal activity against MDR *P. aeruginosa* (BP-PS1 showed 83% infectivity rate, while BP-PS2 showed 91% infectivity rate). The source of the isolated phages was wastewater from Baghdad city the infectivity rate of the combined two phages revealed 100% efficiency to illuminate the target MDR *P. aeruginosa*. The phenotypic characteristic of the isolated two phages demonstrated that the phages belong to *Caudovirales* order under *Myoviridae* family with high lytic activity and large burst size.

Additionally, the development of thermo sensitive lysin hydrogel facilitates facile topical application. The hydrogel exhibits fluidity at lower temperatures, enabling precise and quantitative use, while swiftly transitioning into a solid state at the wound site under elevated temperatures, creating a durable antibacterial environment. The results showed that the incorporation of phage-lysin into the hydrogel not only had a considerable influence on the bacterial killing efficiency of phage-lysin, but also act as a depot to maintain higher titer at the infectious site for a prolong period for more effective treatment. The efficiency of using thermosensitive hydrogels in disease treatment was investigated by many studies as the most promising and practical drug delivery systems one of them is in wound healing[27] [28]. The present results came in harmony with other study which demonstrated the potency of phage-loaded thermosensitive hydrogel to treat wounds infected with MDR *Acinetobacter baumannii* and consider it as a simple and promising phage formulation for controlling wound infections[25].

Optimal candidates for phage therapy necessitate specific attributes, including natural stability, obligate lytic activity, and a wide range of hosts[29]. The restricted host range of BP-PS1 and BP-PS2 lysins, as evidenced by their activity against MDR *P. aeruginosa* isolates, indicates their specificity for *P. aeruginosa*, suggesting their potential as therapeutic agents with minimal impact on beneficial microbiota[30]. Furthermore, the two lysin hydrogels displayed remarkable efficacy against MDR bacteria, accentuating their potential as phage lysin hydrogels for therapeutic applications.

The effective delivery of bacteriophage lysins is pivotal in achieving successful treatment of infection[31]. Previous studies have investigated various formulations, including buffers, hydrogels, or creams, for local skin delivery of bacteriophages, yielding valuable insights into their therapeutic prospects[32]. Besides taking into consideration the promising drug release properties of the present developed lysine hydrogel, these favorable physical attributes of the drug make the hydrogel an attractive candidate for the treatment of topical wounds. Furthermore, extensive antibacterial testing against MDR *P. aeruginosa* isolates showcased the lysin hydrogel's remarkable efficacy. Thus, it provided a potentially significant alternative therapeutic approach for combatting drug-resistant infections effectively.

The preserving of the recombinant lysine for a longer time is considered as a potential limitation of this study. Hence, using of this recombinant lysine as enzybiotic could be as the only way or life-saving approach for many patients suffering from infections with MDR *P. aeruginosa*. However, further investigations are warranted to explore and optimize the clinical efficacy and safety of this promising enzybiotic therapy for eventual and speedy translation into clinical practice.

CONCLUSION

This study emphasizes the urgent need to address and quell the prevalence of drug-resistant *P. aeruginosa* infections. The lytic bacteriophages, BP-PS1 and BP-PS2, and their potential bactericidal activity offers hope in combating MDR infections. The lysin hydrogel formulation shows promising efficacy against MDR *P. aeruginosa*, making it a potential, effective, therapeutic option for topical wound treatment. Bacteriophage lysin hydrogels present an enzybiotic approach to tackle drug-resistant infections. Further research is needed to optimize their clinical use, but their development offers promise in mitigating the impact of antibiotic resistance. Comprehensive strategies and surveillance programs are crucial in combating drug resistance effectively and in ensuring effective treatment outcomes.

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