

Topical Polyethylene Glycol-Phage Ointment as a Therapy to Treat Burn-Wound Infection Using Mice Model

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ABSTRACT: Background: One of the most significant problems facing public health today is antimicrobial resistance. *Pseudomonas aeruginosa* is considered one of the most prevalent causes of healthcare-associated illnesses. **Objective:** The use of phages as a potential alternative therapeutic method for treating bacterial infections has been the subject of significant research to solve this predicament. **Methods:** The *P. aeruginosa* isolates that caused infections of burn wounds were collected from three hospitals in Baghdad. The VITEK system diagnosed and examined the isolates for their antibiotic sensitivity. As well as the phages were isolated and purified from sewage water samples from different sewer stations. After these steps, phages were examined by transmission electron microscopy to ensure the order and the families of these phages, and the tests of lytic activities of phages on the *P. aeruginosa* isolates were done to determine the best lytic ability of them to use for treatment wounds infections. **Results:** Burn-wound infections swabs culture showed a positive culture for 109 isolates as the following *P. aeruginosa* in 76(69.72%), *Staphylococcus aureus* 16(14.67%), *Acinetobacter baumannii* 9(8.25%), *Klebsiella pneumoniae* 5(4.58%), *Escherichia coli* 3(2.75%) obtained from burn wound infections, had a significant level of resistance to several antibiotics. Four bacteriophages were isolated: KM1, KM2, KM3, KM4 and KM5. KM5 is a mixture of phages referred to as a cocktail. The four phages and their cocktail exhibited significant lytic activity against *P. aeruginosa*. A total of four monophages and a cocktail were utilized in the preparation of the PEG-phage ointment. **Conclusions:** All monophages and their cocktail could completely clear bacterial infection in 17 days. KM3 and KM4 PEG-phage ointments demonstrated more potent healing activity than standard, which required 12 days for full recovery, while the cocktail took 15 days (KM5). However, KM1 and KM2 took the same time as the standard, which is 17 days for burn wound infection treated with PEG-phage ointments, while the control, which did not receive any treatment, took 23 days to recover. KM1 and KM2 show less activity for healing burn wound infections from all five phage ointments.

KEYWORDS: Phage; Phage therapy; *P. aeruginosa*; burn wound infection; PEG-phage ointment

INTRODUCTION

An injury to the flesh or epidermis that can be caused by radiation, heat, electricity, or chemicals is referred to as a burn. Around 11 million people need medical attention every year, and around 300,000 people lose their lives as a result of burns [1]. Despite major breakthroughs in burn wound care and infection management practices, infection continues to be the leading cause of mortality [1]. In burn survivors, burns can be classed based on the severity of the burn, the method of injury that caused the burn, the extent of the burn, and any related injuries [2]. *P. aeruginosa* is an opportunistic Gram-negative bacteria that has the ability to cause both acute and chronic infections in patients [3].

Studies have shown that it is responsible for up to 77% of burn wound fatalities [4]. As a result, it is acknowledged as a significant contributor to both mortality and morbidity in burn patients [4]. *P. aeruginosa* can encourage cross-transmission and outbreaks within hospitals, which can result in

localized epidemics in burn treatment facilities if it circulates through those locations and subsequently spreads through contaminated areas [5]. When treating microorganisms that are multi-drug resistant, phage therapy provides a significant substitute for antibiotics. It has been proven by numerous researchers that bacterial infections can be prevented or treated by the injection of bacteriophage before the widespread use of antibiotics [6], [7]. Studies from Poland and the former Soviet Union have so far demonstrated substantial success in the application of phage technologies in the fields of treatment, diagnosis, and prophylaxis of different bacterial illnesses [8]. Using *in vitro* and/or *in vivo* settings, researchers have shown that phages are significantly effective against *Acinetobacter* spp., *Pseudomonas* spp., *Escherichia coli*, and *Staphylococcus aureus* [2], [9]. While antibiotic therapy exhibited more resistance and side effects, bacteriophage therapy showed higher specificity, a narrower spectrum of activity, higher safety, better tolerability, and an affordable process [10]. The majority of the bacteriophages that are known to infect *P. aeruginosa* belong to the order Caudovirales, which is characterized by their head-and-tail form and possession of double-stranded DNA (dsDNA). The phages under discussion can be classified into three distinct categories: *Siphoviridae*, characterized by a lengthy and flexible tail; *Myoviridae*, distinguished by a tail that contracts; and *Podoviridae*, recognized by a short and stubby tail [11]. Ointments, creams, and lotions that contain lytic phages can be applied topically to speed up the healing of wounds. These treatments offer more stability and don't require repeated applications, and they are simple to use and take off [12]. Phage treatment for bacterial infections like *P. aeruginosa* has typically been validated by animal research for its usefulness and safety [13], [14]. Other research on *P. aeruginosa* involved therapies for patients and animals, both of which showed encouraging outcomes [15], [16]. It was discovered that treatment with phages significantly reduced mortality in mice infected with inoculum of 10^6 CFU of highly virulent *P. aeruginosa*. This was demonstrated in a study using burn-wounded laboratory mice that underwent phage therapy. The recent findings proved that phage therapy is effective *in vitro* and provided evidence for the viability of *in vivo* phage therapy. The study's selection of the bacterial isolate P46 revealed a highly resistant profile to the tested antibiotics [17]. The effectiveness of topical bacteriophages administration in burns and infected wounds has been evaluated in a few studies [18], [19]. In this research, a polyethylene glycol (PEG) ointment base containing phages was prepared, and its therapeutic potential was evaluated to treat burn infected wounds caused by *P. aeruginosa* in mice [20]. There are scientific papers used phage therapy as an alternative treatment for burn wound infections [15], [21]–[24].

MATERIALS AND METHODS

Bacterial Isolates

A total of 118 cotton swab specimens from infected burn wound patients were collected from October 2021 to March 2022 from AL-Imam Ali Hospital, Al-Karama Hospital, and Burn Specialist Hospital in the Medical City directory in Baghdad. 76 *P. aeruginosa* (64.4%) isolates were isolated from burn wound infections. The isolates were purified and identified using standard biochemical assays, slide morphology, colonial appearance of selective and differential media, and the VITEK 2 automated system (bioMérieux, France).

Diagnosis of the Bacterial Isolates by using VITEK 2 Compact System

The study focused on the identification and susceptibility testing of prominent bacteria in clinical settings. Specifically, various isolates of *P. aeruginosa* were identified at the species level using the VITEK-2 compact system. This was achieved by employing identification Gram Negative Bacteria (ID-GNB) cards in accordance with the manufacturer's instructions. The system comprises of the VITEK-2 small instrument, a computer, and a printer. The VITEK-2 Compact system is equipped with software that encompasses analytical and data-management modules. The outcomes were presented in terms of several categories provided by the manufacturer, ranging from excellent identification (96% to 100%), very good identification (93% to 95%), good identification (89% to 92%), and acceptable identification (85% to 88%) [25].

Sewage Water Collection

During the time period of January 2022 to April 2022, several crude samples for phage isolation were taken from various places of sewer station in Baghdad. Ten water samples were filtered by a 0.22 μ m Millipore filter and each sample incubated with one isolate of *P. aeruginosa* in a shaker incubator

at 37°C and 200 rpm [26]. After 24-48 hrs. of incubation, the culture was centrifuged at 7000 rpm for 10 min. and assayed for the presence of lytic phages by spot lysis method [27].

Phage Titration

Until the colony's optical density (O.D.) reached (0.5), a single *P. aeruginosa* isolate was grown in Brain Heart Infusion Broth (Himedia, India). The Phage Titration was measured using the Top Agarose Method. To avoid solidification, three milliliters of top agarose were placed in flat tubes and given time to equilibrate at 45 °C. Tubes were filled with 10 ml of mid-log phase *P. aeruginosa* culture and 1 ml of phage preparation. Each was serially diluted using the same top agarose tubes that were equilibrated. Once the tubes were cooled, 1ml of the mixture from each tube was poured onto the top of the brain heart agar. After being refrigerated, the plates were incubated for 18 hours at 37 °C. Phage titration was measured using plaque forming units (PFU) per milliliter of phage preparation [28].

Analysis of Transmission Electron Microscope: For each phage, 120 µl of each lysate was placed in an airfuge (Beckman) tube, which was then centrifuged at 20 Pounds per inch square (psi) for 15 minutes while the carbon stabilizer formal was applied to a 200-mesh copper grid. After centrifugation, the resulting pellet was then negatively stained with 2 phosphotungstic acid. Following that, each sample was examined with a TEM EM 900 T (Zeiss) at magnifications ranging from 12000x to 80000x [29].

PEG-Phages Ointment Preparation Method

Mixing the phage solution in the amount of 10 ml with 1 ml of propylene glycol, with stirring on a hot plate magnetic stirrer dissolve vaseline with white paraffin in a water bath and mixing the phages solution with vaseline, while continuing to mix with the magnetic stirrer, at a temperature of less than 40 °C Fill the ointment in plastic containers and leave in the refrigerator to cool. An agar lawn plate of *P. aeruginosa* was prepared, and five serial dilutions of PEG ointment were syringed onto the surface of the lawn to test the stability of lytic phages in PEG ointments. At 37 °C, plates were incubated for 24 hours.

In vivo Phage Therapy of Mice Infected with *Pseudomonas aeruginosa*

In these experiments used albino *Mus musculus* males of mean body weight 23 ± 1.8 g per mouse, and the age of these mice was 1.5 months. In mice, a second-degree burn wound infection model was created using *P. aeruginosa*, employing Dale *et al.*'s method [30]. In a nutshell, mice under anesthesia had their back hair trimmed and their dorsal area's skin shaved. Mice were given ether anesthesia before a burn was inflicted on them for 45 seconds (s) using a hot brass bar (10 mm by 10 mm by 100 mm) arranged in an annular shape. All the mice received an intraperitoneal injection of 0.5 ml of sterile physiological saline to restore lost fluids and prevent overt shock. Acetaminophen (0.25 mg/ml) was also administered as a post-burn painkiller in drinking water. *P. aeruginosa* was inoculated in nutrient broth and incubated at 37 °C overnight to create the bacterial inoculum. The *P. aeruginosa* was wiped on the back area and left the area until the second day, then the mice were treated with the PEG-phages ointment topically. The experiment was carried out in triplicate [31]. These group which burned and treated topically with phage ointment for (KM1, KM2, KM3, KM4, KM5) KM5 as cocktail of phages in five mice, the sixth one was control which still without any treatment and the last one considered standard which treated with Hamazine cream which considered as a choice for treatment burn wounds for human in the hospitals In Iraq. The ointment was given daily in one time every day for 17 days and monitor the health statement of mice.

Statistical Analysis

The repeated measure between the tested concentration and the control was examined using the ANOVA test. The letters (A, B, C, and D) LSD for rows and (a, b, c, and d) for columns signified the degrees of significance, with the last one being the least significant. The LSD test was used to compute the significant differences between the tested means. Similar letters indicate that the tested means are not significantly different from one another. Values of $p > 0.05$ were deemed statistically non-significant, while $p < 0.05$ and 0.01, 0.001 were deemed significantly different, respectively. Using SPSS (v 20), the statistical analysis was performed.

RESULTS AND DISCUSSION

Isolation of Bacteria

From A total of 118 swabs, 109 isolates gave a positive growth. Gram negatives were in majority 85.32% and Gram positives were 14.6%. The most common pathogen of burn-wound infection was *Pseudomonas aeruginosa* (69.72%), whereas the less common was *Escherichia coli* (2.75%), as shown Table 1.

Table 1. Percentage of single clinical isolates which have a positive growth from burn wound infection samples

n= 109	
Isolates	n (%)
<i>P. aeruginosa</i>	76(69.72%)
<i>Staphylococcus aureus</i>	16(14.67%)
<i>Acinetobacter baumannii</i>	9 (8.25%)
<i>Klebsiella pneumonia</i>	5 (4.58%)
<i>Escherichia coli</i>	3 (2.75%)

The antibiogram of *P. aeruginosa*

The antimicrobial susceptibility pattern of the *P. aeruginosa* isolates revealed that most of the isolates were resistant to different groups of antibiotics, the Table 2 shows the results of antibiotics sensitivity by vitek device.

Table 2. Percentage of antibiotic resistance of *P. aeruginosa* isolated from burn-wound infections

Antibiotic	R %	S %	I %
CTX	100	0	0
IPM	21	79	0
AK	43.4	47.3	9.3
CRO	100	0	0
CAZ	86.8	0	0
ATM	47.3	0	0
MEM	14.4	0	0
AMC	100	0	0
AZM	43.5	36.8	19.7
TZP	61.8	23.6	14.6
LEV	26.3	73.7	0
CIP	85.5	14.5	0
TE	100	0	0
E	86.8	13.2	0
CN	89.4	10.6	0

Amoxicillin/Clavulanic acid =AMC, Ceftriaxone= CRO, Ceftazidime=CAZ, Cefotaxime=CTX, Tetracycline= TET, Aztreonam =ATM,Erythromycin=E, Azithromycin = AZM, Piperacillin/ Tazobactam= TZP, Gentamicin= CN, Ciprofloxacin= CIP, Levofloxacin= LVX, Amikacin =AMK , Imipenem =IPM, meropenem= MEM.

Phage isolation

Four phages active against four isolates of *P. aeruginosa* were isolated and purified. All the isolated phages formed clear plaques on lawn of *P. aeruginosa*, as shown in Figure 1.

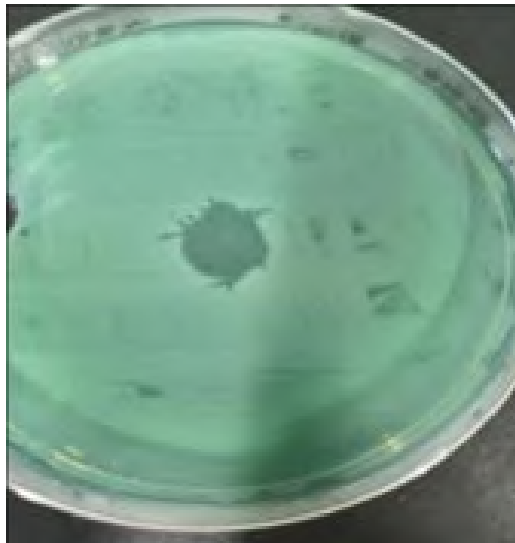


Figure 1. The plaque of KM1 phage on bacterial lawn of *P. aeruginosa* in spot lysis method

Phage Plaque Morphology

The four isolated phages showed varying plaque properties Figure 2 and Table 3.



Figure 2. plaques morphology of KM3 phage on *P. aeruginosa* lawn by top agarose method

Table 3. plaques morphology of phages

Plaques shape	Plaques clarity	Plaques size (mm)	Margin cut	Phage	Bacterial isolate
Oval	Semi-clear	0.8	Irregular	KM1	P 72
Oval	Cloudy	1.5	Irregular	KM2	P 62
Circular	Clear	0.3	Irregular	KM3	P 74
Circular	Clear	3.0	Regular	KM4	P 76

Phage Morphology

The investigation for the ultrastructure of the four bacteriophages revealed that KM-1 phage had an icosahedral-isometric head with a diameter of roughly 80 nm. Tail with a length of around 170 nm that is long, thin, non-contractile, and flexible. The phage is 200 nm in length overall. Phage KM-1 was identified by examination of this data as belonging to *Siphoviridae* family [29], Group B, Morphotype B1 [32], and Caudovirales order. It is a virus with double -stranded DNA (Figure 4).



Figure 3. The phage KM1's ultrastructure consists of an 80 nm-diameter, icosahedral, isometric head. Tail that is long, thin, flexible, non-contractile, and measures about 170 nm in length. (Transmission electron microscope, 105.47k ×)

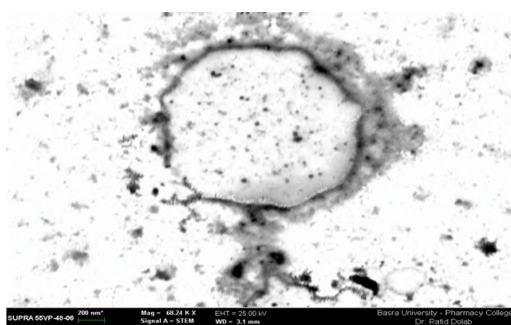


Figure 4. The icosahedral-isometric head of the phage KM2 has an approximate diameter of 60 nm. Tail is long, thin, non-contractile, flexible, and measures about 120 nm. transmission electron microscope, 68.247k ×

The KM2 phage has icosahedral-isometric head with a diameter of around 60 nm. About 120 nm long, flexible, and non-contractile tail. The phage is roughly 200 nm long overall. This bacteriophage is also a double stranded DNA virus since it belongs to the Caudovirales order, *Siphoviridae* family [32], Group B, and Morphotype B1 [33], [34], as depicted in Figure 4. The KM3 phage has icosahedral-isometric head with a diameter of roughly 80 nm. A lengthy, stiff, and contractile tail of about 90 nm in length is attached to a neck measuring about 20 nm in length. A clearly discernible exterior contractile sheath and an interior tube make up the tail. About 190 nm is the length of the whole phage. Clearly visible were the base-plate and the tail-tube that protruded from the constricted tail. This phage belongs to the *Myoviridae* family of the Order Caudovirales [32], [33]. Additionally, this virus has double-stranded DNA illustrated in Figure 5. The icosahedral-isometric head of the KM-4 phage measures about 60 nm in diameter, approximately 20 nm long neck. About 80 nm is the whole length of the phage. Clearly visible were the base-plate and the tail-tube that protruded from the contracted tail. Because of its unique tail pattern, this phage was identified as belonging to the *Myoviridae* family of the Order Caudovirales [32], Group A, and Morphotype A1 [35]. Furthermore, the DNA of this virus is double-stranded, as depicted in Figure 6.

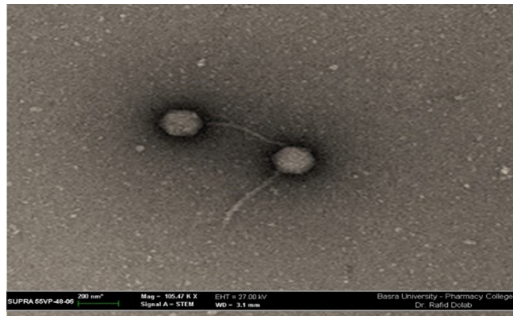


Figure 5. The icosahedral-isometric head of the phage KM3, which has a diameter of about 80 nm. Tail is long, thin, contractile, flexible, and measures about 90 nm. (Transmission electron microscope, 232.13k ×)



Figure 6. The icosahedral-isometric head of the phage KM4 has an approximate diameter of 60 nm. possess a base plate, a tail tube, and a neck with a length of around 20 nm that protrude from the contracted tail. (Transmission Electron Microscope, 615.73k ×)

Phage Therapy of Mouse Model of Burn-Wound Infection

When treating mice that were burnt and infected with *P. aeruginosa*, PEG-phage ointment was made for all monophage and cocktail and administered once daily. The results showed that all monophage and their cocktail were able to completely eradicate bacterial infection in 17 days while the control took 23 days for recovery (Figure 7). Clear zones on the agar plate's surface demonstrated the lytic potential of phages. Additionally, the thermo-stability and pH variations of ointment bases were examined. The creams were kept at 4°C and 20–25°C [21]. From Figure 8, KM3 and KM4 PEG-ointments demonstrated more potent healing activity than standard, which required 12 days for full recovery, while the cocktail needed 15 days for full recovery (KM5). However KM1 and KM2 took the same amount of time as usual 17 days for burn wounds infection treated with PEG-ointments of that were thought to be less effective for healing burn wounds from the all five phages ointments.

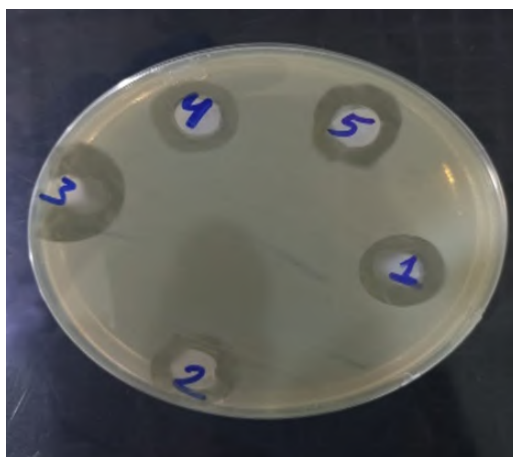


Figure 7. PEG-phages ointment activities on bacterial lawn *P. aeruginosa*

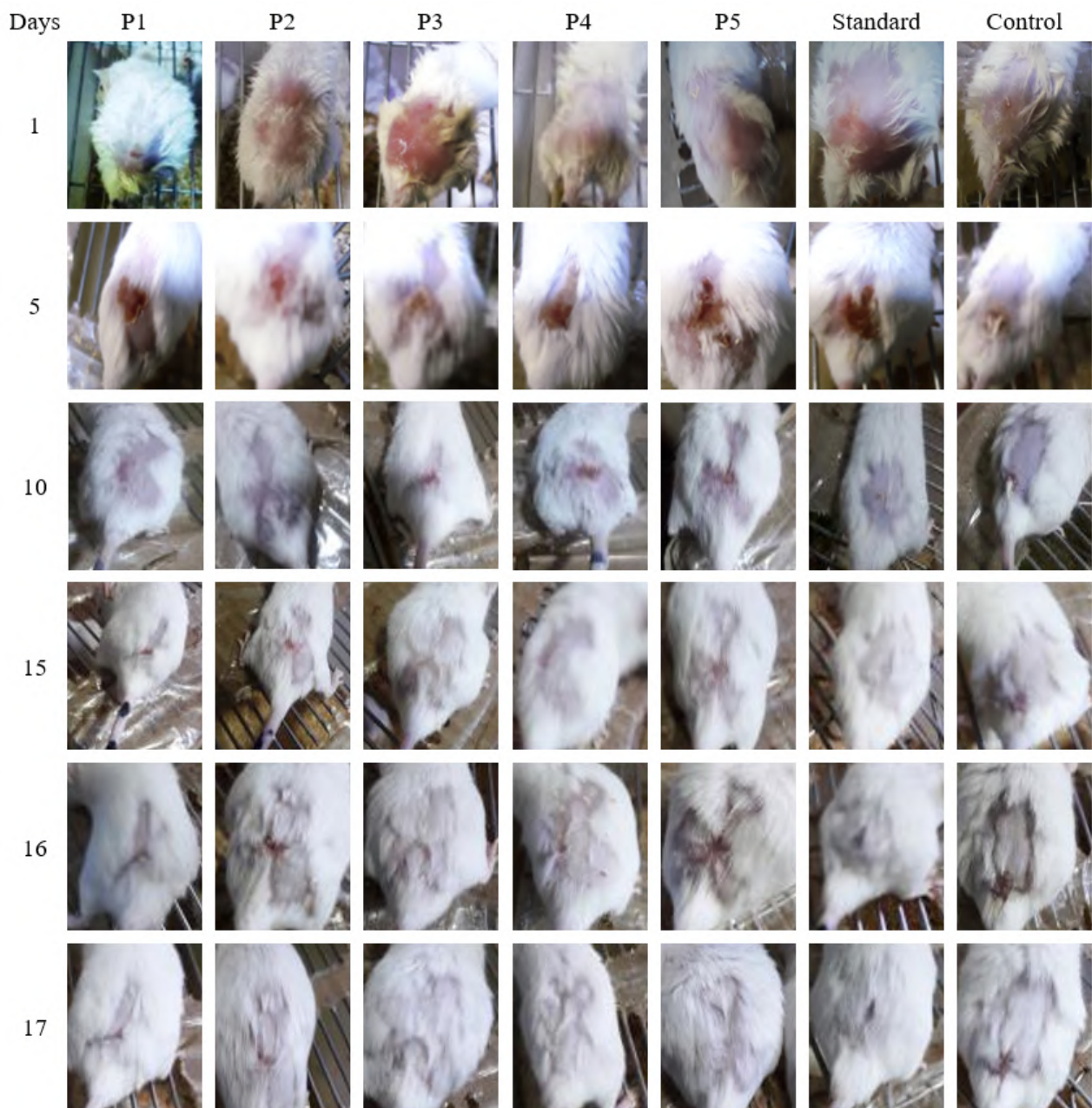


Figure 8. PEG-phage ointment activity on burned mice; the experiment took 17 days for five PEG-phage ointments (KM1, KM2, KM3, KM4, and KM5) as a topical treatment for five mice with the standard treated with hamazin and the control which have no treatment the experiment has done duplicate

Phage-host-range

The four phages cocktail showed lytic activity against 76 of *P. aeruginosa* isolates. The range of %inhibition of the four phages (KM1, KM2, KM3, KM4) for *P. aeruginosa* isolates as showed in Table 4.

Table 4. The statistical analysis of the lytic activity of the four phages and their cocktail against the 76 *P. aeruginosa* isolates by microtiter plate (SPSS. v 20)

Pseudo/76	KM1	KM2	KM3	KM4	KM5	p value
Mean	D 27.58 A	C 29.32 A	B 34.01 a	D 29.81 a	A 39.32 a	
SE of Mean	0.9	0.81	1.15	0.9	1.2	
Median	25.8	28.42	32.22	28.18	37.6	0.001
SD	7.87	7.14	10.03	7.84	10.6	
Min	18.56	19.38	17.09	20.76	21.9	
Max	67.66	65.67	78.52	62.13	78.4	

The letters (A, B, C, and D) LSD for rows and (a, b, c, and d) for columns signified the degrees of significance, with the final one being the least significant. The LSD test was used to compute the significant differences between the tested means. Similar letters indicate that the tested means are not significantly different from one another.

Transmission electron microscopy (TEM) is one of the most versatile and potent experimental techniques for imaging and diffraction of structures on the micrometer scale, with sub-angstrom resolution now routinely achievable with modern aberration-corrected instruments. In transmission electron microscopy, sample structure information is encoded in the scattering of electron waves by the specimen's full electromagnetic potential, which is dominated by the atomic electrostatic potentials [36]. These potentials include both the contribution of the screened nuclear nuclei and the valence electron density of the sample. Since valence electrons are responsible for holding the material together, their study is of great scientific interest [36].

According to the results as shown in Figure 7, KM3 and KM4 were more potent and successful in treating *P. aeruginosa* infections than other treatments, and they also provided faster healing times than the standard. These findings imply that (KM3, KM4) phages, which are members of the *Myoviridae* family and are well recognized for their powerful capacity to infect and lyse bacterial cells, have higher healing activity. They have certain genetic traits and morphologies that contribute to this capacity. The unique shape of *Myoviridae* phages enables them to effectively infect and lyse bacterial cells [37]. They may adhere to certain receptors on the surface of bacterial cells and inject their genetic material into the cell. The genome is introduced into the cytoplasm through the cell envelope when the tail sheath contracts. The phage's potency may be increased due to the ability to package a lot of genetic material due to the size of its head and tail [38]. Overall, *Myoviridae* phages are potential candidates for use in phage therapy to treat bacterial infections due to their powerful capacity to infect and lyse bacterial cells and their complex genetic makeup. Because of being a member of the *Siphoviridae* family, the KM1 and KM2 phages, however, demonstrated less strong healing action against *P. aeruginosa* infection. Bacteriophages of the *Siphoviridae* family, which belong to the Caudovirales order, are distinguished by their protracted and non-contractile tails. *Siphoviridae* phages that belong to the subgroup Morphotype B1 have a similar shape and genetic make-up [39]. These phages feature a linear double-stranded DNA genome, a long, flexible tail, and a short, isometric head. Because *Siphoviridae* phages have a lengthy, non-contractile tail that is utilized to connect to the surface of the bacterial cell and start infection, this family was shown in the current investigation to have less effective topical healing activity than the *Myoviridae* family. The phage's tail is made up of a flexible, helical shape that may help it bend and adapt to various cell surface configurations. Although *Myoviridae* and *Siphoviridae* phages have both been extensively studied for their potential use in phage therapy, both families have been shown to be effective against a wide range of bacterial strains and both of them given a good activity for eliminating bacterial infection as compared with control and Hamazine treatment. The differences in tail structure between the two families suggest that the cause of different potency for these single phage [40]. The activity of the phages cocktail for treating wounds was intermediate between the two families, with the cocktail being more potent from the *Siphoviridae* phages family and less potent from the *Myoviridae* phages family, as can be seen in Figure 8. These findings suggested that: Firstly, using a single phage strain ensures that the phage can target the particular bacterial strain causing the infection more effectively and making the chances of a successful treatment are increased. Second, using a single phage strain prevents possible phage cocktail interference. The various phage strains in a cocktail of phages may interact with one another in unforeseen ways, decreasing the treatment's efficiency [41]. This possibility of interference is removed by employing a single phage strain. The use of numerous phages in a cocktail may not

always result in synergistic results; in certain cases, it may even result in antagonistic consequences. The efficiency of utilizing a single phage and phage cocktail to kill bacteria in mice was investigated, as well as the possibility of using phage therapy in addition to antibiotics to heal burn wounds [42]. The findings demonstrated that the mono phage was highly effective in eliminating bacteria, and within two days of phage delivery, bacterium levels began to gradually fall. However, both the single phage and phage cocktail treatment groups showed full recovery of the mice. Between single phage and phage cocktail treatments, there were no discernible changes in the *in vivo* results of the response to phage therapy. A phage cocktail can improve treatment effectiveness and delay the emergence of phage-resistant genotypes, according to earlier research. This is so that bacteria resistant to one phage are still vulnerable to other phages in the phage cocktail, which comprises phages that employ several receptors [41], [43]. Overall, the study demonstrates that mono- or multi-phage treatment effectively eliminates bacterial infections when used at deficient and safe concentrations. A special receptor-specific phage cocktail may also partially overcome other potential challenges to the use of phage therapy, such as the limited host range of phage, host bacterial resistance to phage, the potential for immune system inactivation of the patient, and safety of phage preparations in humans [44]. The study also discovered that when monitored for 24 hours before the experiment and for 20 days, the mice in all treatment groups exhibited no signs of discomfort. This implies that phages have the potential to be a topical treatment with no side effects. Phage therapy has the potential to be used in addition to antibiotics rather than as a replacement, although this is less likely. Anticipating the assessment of potential applications of a semisolid phage formulation in combination with antibiotics for the management of burn injuries in forthcoming research endeavors.

CONCLUSION

The study demonstrated that mono-phage and phage cocktail treatments were highly efficient at eliminating bacterial illnesses when utilized at deficient and secure concentrations. The *in vivo* outcomes of the single phage and phage cocktail treatments on the response to phage therapy showed no appreciable differences. According to the results of this study, PEG-phage ointment can be used to treat burn wound infection in humans.

SUPPLEMENTARY MATERIAL

None.

AUTHOR CONTRIBUTIONS

Methodology, writing—review, and editing, Sadeq Abdulridha Gatea Kaabi; Software, validation, and formal analysis, Karrar Jabbar Mansoor; Validation review and editing of the manuscript were carried out jointly by Karrar Jabbar Mansoor and Sadeq Abdulridha Gatea Kaabi.

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DATA AVAILABILITY STATEMENT

None.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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