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Therapeutic potential of selected sewage bacteriophage against multidrug-resistant *Acinetobacter baumannii*: an overwhelming health issue

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Abstract
Acinetobacter baumannii being a multidrug-resistant (MDR) pathogen poses great threats to health with mortality ranging from 8% to 35%. As it is resistant to the broad spectrum of antimicrobial drugs, there is a need for alternative therapeutic methods to combat its propagation. Bacteriophages being the natural killer of bacteria are an effective therapeutic agent against *A. baumannii*. In this research work, a sewage bacteriophage was tested for its therapeutic potential against MDR *A. baumannii*. To achieve this, albino mice were infected with the bacterium (*A. baumannii*) and bacteriophage with the appropriate dose, and the mice were observed for 12 days and their hematological and histopathological studies were performed. Hematological studies show that erythrocyte, leukocyte, and platelet count remain comparable with healthy individuals. While histopathological analysis of mice lung tissues also supports these results, bacteriophage is involved in clearing out the infection. So, from these results, it is confirmed that the isolated bacteriophage could act as an effective therapeutic agent against multidrug-resistant *A. baumannii*. In the future, further research will be designed on using this phage in combination with other *A. baumannii*-specific bacteriophages and with other antimicrobial drugs for a more potent therapeutic agent against all the variants of *A. baumannii*.

Key Words: MDR, Hematological, Histopathological, bacteriophage

Introduction

Acinetobacter baumannii, an opportunistic microbe is a non-fermentative, gram negative, aerobic, pleomorphic catalase positive, oxidase negative, non-motile, ubiquitous, and non-fastidious pathogenic bacteria. Multidrug-resistant bacteria are isolated from healthcare departments. The Infectious Disease Society of America declared six significant pathogens named as "ESKAPE group" as a global problematic nosocomial threat, and *Acinetobacter baumannii is* one of them(1, 2). A present, for the healthcare department *A. baumannii* is a nightmare due to its multidrug resistance ability, mortality range from 8% to 35%, increased hospitalization, and treatment expenses(3).

Several infections are associated with *A. baumannii* such as urinary tract infections, skin and wound infections, meningitis, respiratory tract infections, bacteremia, BSI (Bloodstream infection), and pneumonia more specifically VAP (ventilator-associated pneumonia) (4, 5). It is particularly common in immunocompromised and ICU patients. The incidence of infection is associated with premature birth, burns, ventilation, long-term antimicrobial therapy or antibiotics, and indwelling foreign devices. *A baumannii* acquires resistance against broad-spectrum antibiotics leading to its outbreak in healthcare units, mostly it happened in ICUs (6, 7). During the COVID-19 outbreak, a significant number of people were admitted to the ICU and given antimicrobial treatment, which led to the *A. baumannii* and other multidrug-resistant pathogens outbreak. The targeted drugs for cytokines also increased the risk of infection by such pathogens(8, 9).

Acinetobacter baumannii associated with the production of oxa-48β-lactamase was first identified in New York's hospitals, and then its transmission and outbreak due to the usage of contaminated surgical equipment, and from

person-to-person contact. The prevalence of *A. baumannii* from the Middle East, Asia, Africa, Latin America, Europe, and the US, among all other infections linked to hospitals is 4.6%, 3.6%, 2.5%, 1.9%, 1.6%, and 0.7%, respectively. The multidrug resistant infections are more common in ICU as compared to general wards(10).

Virulence of *A. baumannii* is multifactorial leading to its pathogenesis in different ways like biofilm formation, adherence, killing the host by apoptosis, invasion serum-resistance, and in-vivo survival strategies (11). For biofilm formation, it needs BAP (biofilm-associated protein) leads to biofilm formation and it also increases its adherence to the host cell so, by controlling BAP expression, the infection can be inhibited. For its colonization, it needs metallic homeostasis from which it acquires iron and zinc enhancing its virulence. K1 capsular polysaccharide protects the bacteria from phagocytosis by macrophages, this bacterial factor promotes its persistence and indirectly enhances its virulence. Different bacterial proteins like RecA, phospholipase C, and D, and Omp38 are considered virulence factors as they are engaged in the apoptosis of host cells(12, 13).

Among its various drug-resistance variants, Carbapenem drug-resistant *Acinetobacter baumannii* needs special attention are announced by the WHO (World Health Organization)(5, 14). This bacillus is resistant to a wide variety of antibiotics including quinolones, tetracyclines, β-lactams, and aminoglycosides. It has multiple defense mechanisms to resist various drugs such as by the alteration of membrane proteins, efflux pumps, β-lactamase (hydrolytic enzyme), carbapenemases, metallo- β-lactamase, serine oxacillinase production, changes in the expression and binding affinity of proteins involved in drug binding, as happened in case of penicillin, changes in binding site and efflux pumps are involved in case of quinolones resistance, 16S rRNA and different modifying enzymes take part in resistance against aminoglycosides(15, 16). The propagation of *A. baumannii* increases to a critical level when metallo-β-lactamases are located on mobile genetic elements (17, 18).

The high prevalence rate of multidrug-resistant *A. baumannii* poses serious threats to the health and socio-economic state of a developing as well as a developed country. As the patient needs more healthcare and admittance in hospital for a very long time that requires more cost. This long-term hospitalization leads to more investigation and treatment with expensive antibiotics and bacteria develop resistance against these treatments due to the overuse of antibiotics(19). Although, multidrug resistant bacteria have a serious impact on developing and developed countries middle-class, low-income countries, where there are already a limited number of facilities are devastated socially and economically by such outbreaks(1, 20).

As the multidrug-resistant bacteria emerged, it posed serious issues for the development of novel medicine/antibiotics and effective treatment methods. In this research work, the use of bacteriophages for the neutralization of *A. baumannii* effect is our intriguing approach. The therapeutic use of bacteriophages was common in the 1930 to 1940 era but after the discovery of antibiotics, this therapy was considered outdated(21). So we are going to study the therapeutic effect of *A. baumannii*-specific bacteriophage on infected mice.

Methods

Bacterial strain

The bacterium *A. baumannii* was acquired from Intensive Care Unit (ICU) of a tertiary care hospital and well maintained on blood agar and MacConkey agar under optimized conditions in our laboratory. From MacConkey agar, the bacterial colony was grown in nutrient broth overnight at 37 °C. The bacteria were harvested by centrifugation, washed, and diluted with 0.2 M PBS to the desired concentration.

Isolation and purification of phage

The bacteriophage specific for *A. baumannii* was isolated from sewage samples, and the phage titer was estimated on agar. For the sake of phage plaque assay, bacterial culture was mixed with phage in a 2:1 ratio and incubated for 10 minutes at 37 °C. The mixture was then mixed with agar and poured into petri plates. The plates were incubated overnight at 37 °C, and plaques were obtained. Then, from the agar plate, phage plaques were harvested and purified by recommended procedures. The specificity of the phage for the bacteria was estimated by the spot test method against 21 bacterial isolates. For this purpose, the bacteria were cultured on MacConkey agar and blood agar plates, and 15 µl of isolated phage was added; the plates were then incubated at 37 °C. A clear zone appeared on the bacterial plate, indicating phage activity.

Selection of Experimental Organism and In vivo studies

Pathogen-free albino mice, six weeks old and weighing 25g, were selected for this study. All experiments on animal models were performed after approval from the Animal Ethical and Experimental Committee. Phage toxicity in mice was also tested using Soothill's method (1992). The albino mice were divided into five groups: G1, G2, G3, G4, and G5, with six mice in each group. The lethal bacterial dose was estimated before proceeding with the experiment. The phage dose was varied to determine the most effective dose against multidrug-resistant *A. baumannii*. Before the experiment, the mice's temperature, weight, and other parameters were carefully recorded. For experimental purposes, the mice were divided into five groups and given different treatments.

i.Mice Infected with *A. baumannii*

The mice in Group 1 (G1) were given a dose of *A. baumannii*. The initial dose was 10^2 CFU/ml, which was then increased until a dose that induced 100% infection was reached. The optimum dose (10^8) was selected, and the animals were observed for the next 12 days. On post-infection days 4, 8, and 12, cardiac blood was collected in EDTA vials for analysis, and two mice were sacrificed on each selected post-infection day (4th, 8th, and 12th). The lungs of the sacrificed mice were removed aseptically, and their weight was recorded. The lungs were then homogenized in PBS buffer for further tissue-level studies.

ii.Mice without any Treatment

The mice in Group 2 (G2) were not given any bacterial or phage dose and were kept as a control group.

iii.Mice Treated with *A. baumannii* and phage (PFU: 10^8)

The experimental group 3 (G3) was selected for the evaluation of bacteriophage potential for resolving the effect of *A. baumannii*. For this purpose, mice were infected with 100 µl of *A. baumannii* at 10^8 CFU, and after 2 hours, the mice were treated with 200 µl of phage at 10^8 PFU. Then, the mice were observed for the next 12 days, and on selected days (4th, 8th, and 12th), two mice were sacrificed, and their blood samples and lungs were collected.

iv.Mice Treated with *A. baumannii* and phage (PFU: 10^7)

Group 4 (G4) was also infected with 100 µl of *A. baumannii* at 10^8 CFU and 2 hours post-infection treated with 200 µl of phage at 10^7 PFU, a different dose of lysed phage to find out the optimum dose of bacteriophage. After treatment, the mice were observed for the next 12 days and on selected days (4th, 8th, and 12th), they were sacrificed, and the required samples were collected for further analysis.

v.Mice Treated with *A. baumannii* and phage (PFU: 10^6)

Group 5 (G5) also consisted of six mice, which were infected with 100 µl of *A. baumannii* at 10^8 CFU and 2 hours post-infection treated with 200 µl of phage at 10^6 PFU. The mice were observed, and sample specimens were collected similarly to the other groups.

Mice Hematology Studies and Histopathological Studies

The blood samples of mice, which were kept in EDTA vials, were used to estimate various hematological parameters such as erythrocyte count, hemoglobin concentration, leukocyte count, and platelets count using a Medonic hematology analyzer. The histopathological analysis of homogenized lung tissues and renal tissues of mice was also carried out and micrographed.

Statistical Analysis

The results of various hematological parameters were critically analyzed using Two-way ANOVA and Tukey's HSD test, implemented in RStudio (version 2024.04.0+735). A significance level of $p < 0.05$ was considered statistically significant across all categories.

Results

As the experiment was based on five groups, including G1 (Positive Control: infection with *A. baumannii* at 10^8 CFU), G2 (Negative Control), G3 (infected with 100 µl of *A. baumannii* at 10^8 CFU and 2 hours post-treated with 200 µl of phage at 10^8 PFU), G4 (infected with 100 µl of *A. baumannii* at 10^8 CFU and 2 hours post-treated with 200 µl of phage at 10^7 PFU), and G5 (infected with 100 µl of *A. baumannii* at 10^8 CFU and 2 hours post-treated with 200 µl of phage at 10^6 PFU). Then, after 4 days, different parameters were calculated, such as anorexia, fever, and changes in body weight due to infection, as shown in Table 1. As we can observe, mice in G1 developed all the disease symptoms, as they were only infected with *A. baumannii*. G2 organisms did not develop any symptoms (negative control). G3 and G4 mice exhibited anorexia and fever on the 4th and 8th days, respectively, but recovered by the 12th day. In G5, mice developed disease symptoms, indicating that the phage dose of 10^6 PFU was not effective against the bacterium.

Table 1: Disease parameters on Day Post-Infection

Groups		**G 1**			**G 2**			**G 3**			**G 4**			**G 5**		
Days Post Infection		**4th**	**8th**	**12th**	**4th**	**8th**	**12th**	**4th**	**8th**	**12th**	**4th**	**8th**	**12th**	**4th**	**8th**	**12th**
Parameters	**Anorexia**	-	++	++	-	-	-	+	-	-	+	+	-	+	+	+
	Fever	+	+	+	-	-	-	+	-	-	+	+	-	+	+	+
	Loss in body weight	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+

On Day Post-Infection (4th, 8th, and 12th), blood samples were collected, and various hematological parameters were calculated using a hematology analyzer. Table 2 shows the mean values of the Red Blood Cell (RBC) count and standard deviation. Different alphabets in superscripts of the mean are assigned, indicating that the particular results in a row are statistically different at $p < 0.05$. The RBCs in the negative control group and experimental group 3 (G3) are almost comparable, indicating that the effective dose of phage is 10^8 PFU. Figure 1 shows the graphical representation of the mean values of Erythrocyte count and Day Post-Infection, which also indicates that the RBC count of G2 and G3 is almost the same.

Table 2: Erythrocyte Count (M/ µl) of Experimental Mice

Days	Experimental Groups (Mean range of RBCs)				
	G1	**G 2**	**G 3**	**G 4**	**G 5**
4th	6.316 ± 0.1^{f}	8.310 ± 0.04^{a}	8.219 ± 0.1^{a}	7.022 ± 0.02^{e}	6.429 ± 0.06^{f}
8th	7.312 ± 0.03^{c}	8.300 ± 0.01^{a}	8.305 ± 0.05^{a}	7.031 ± 0.01^{e}	7.071 ± 0.05^{de}
12th	7.541 ± 0.11^{b}	8.199 ± 0.01^{a}	8.265 ± 0.03^{a}	7.237 ± 0.01^{cd}	6.317 ± 0.1^{f}

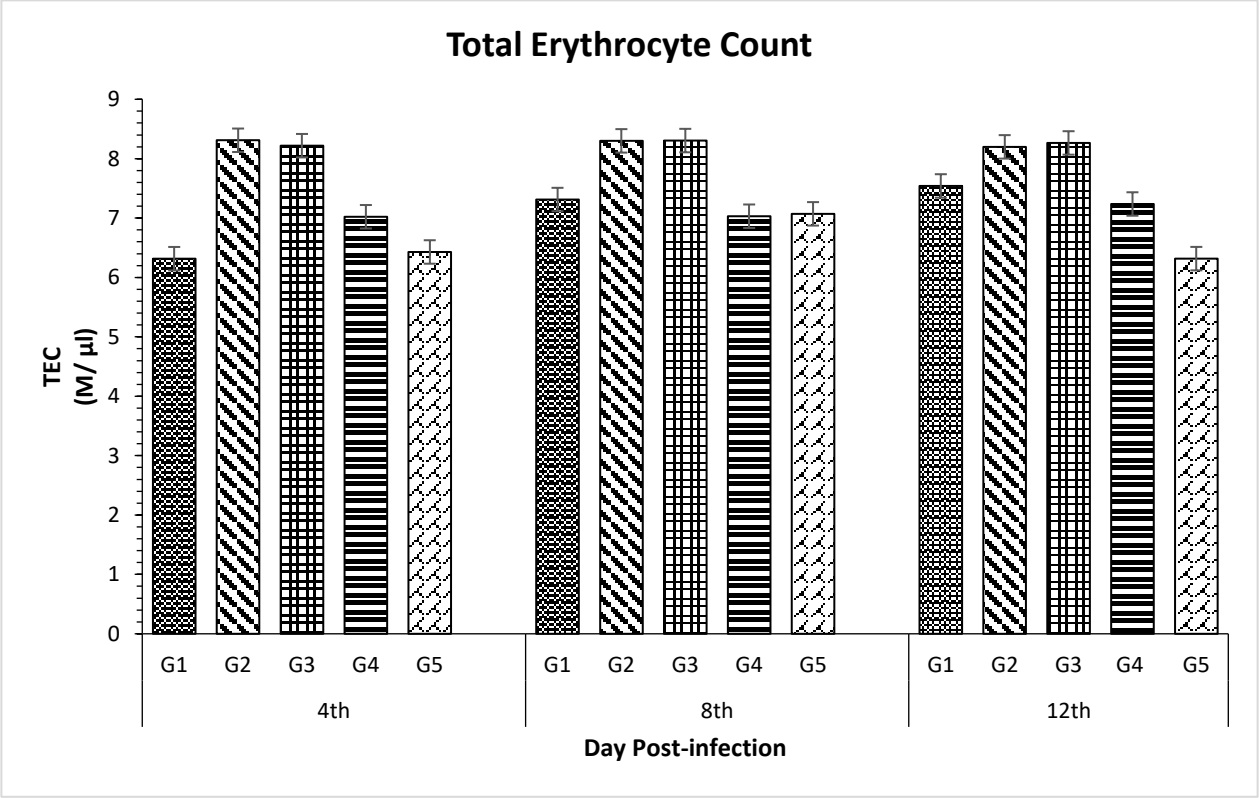


Figure 1: Graphical representation of Total Erythrocyte count after infection with *A. baumannii*.

The hemoglobin concentration of experimental mice was also estimated. Table 3 shows the mean values of hemoglobin concentration for the five groups with respect to post-infection days. Different alphabets in the rows highlight that the results are statistically different at $p < 0.05$. The graphical visualization of hemoglobin concentration and day post-infection is represented in Figure 2.

Table 3: Hemoglobin Concentration (g/dl) of Experimental Mice

Days	Experimental Groups				
	G1	**G 2**	**G 3**	**G 4**	**G 5**
4th	13.03 ± 0.07^{cde}	13.63 ± 0.4^{a}	12.65 ± 0.06^{f}	12.59 ± 0.10^{f}	13.15 ± 0.02^{bcd}
8th	12.87 ± 0.2^{def}	13.52 ± 0.12^{ab}	13.16 ± 0.10^{def}	12.86 ± 0.03^{def}	13.02 ± 0.08^{cde}
12th	12.64 ± 0.2^{ef}	13.32 ± 0.12^{abc}	13.44 ± 0.06^{cdef}	13.00 ± 0.05^{cdef}	12.98 ± 0.04^{cdef}

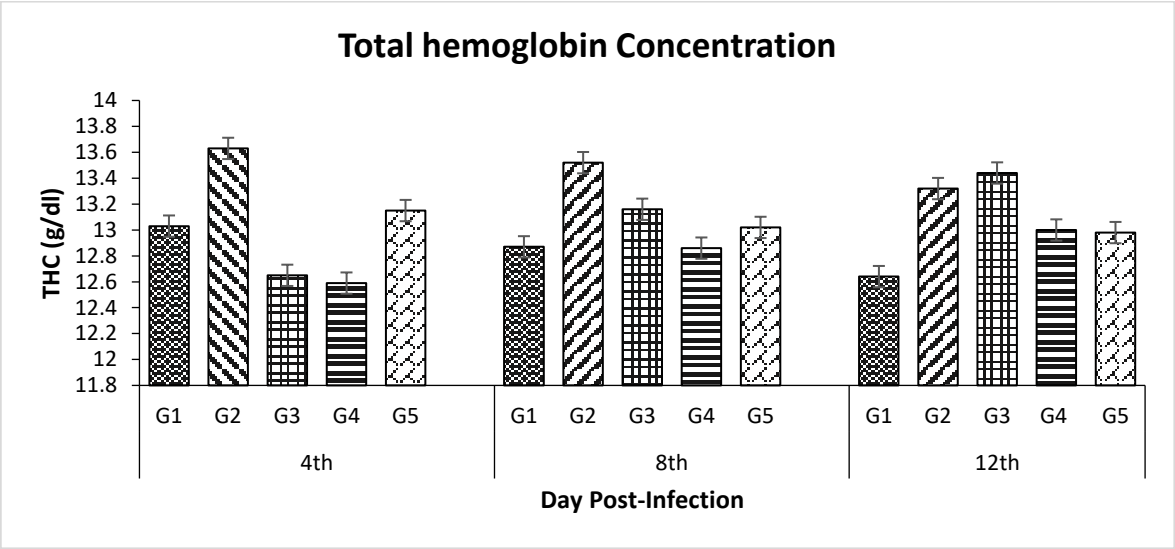


Figure 2: Graphical representation of Total Hemoglobin concentration after infection with *A. baumannii*.

The White Blood Cell (WBC) count was also carried out, and the results are illustrated in Table 4. Different alphabets indicate statistical differences among the mean values in a row at $p < 0.05$. As we can see from the results, the White Blood Cell (leukocyte) count increases in Group 1, which shows the activation of the immune system against *A. baumannii*. In G3 and G4, after 4 days, leukocytes increased slightly as an immune response against the bacteria and phage, but after 8 and 12 days, the WBC count of both groups was comparable to G2 (the control group). In the case of G5, the increase in leukocytes shows the infection, but the leukocyte count is still lower compared to G1. Figure 3 shows the graphical representation of the Total Leukocyte count with reference to post-infection days.

Table 4: Leukocyte Count (10^3/µl) of Experimental Mice

Days	Experimental Groups				
	G1	**G 2**	**G 3**	**G 4**	**G 5**
4th	13.14 ± 0.06^{c}	7.52 ± 0.06^{k}	9.92 ± 0.07^{f}	8.76 ± 0.02^{g}	10.84 ± 0.05^{e}
8th	14.97 ± 0.02^{b}	7.69 ± 0.04^{ij}	7.35 ± 0.06^{l}	8.05 ± 0.05^{h}	11.64 ± 0.03^{d}
12th	19.62 ± 0.10^{a}	7.56 ± 0.02^{jk}	7.56 ± 0.04^{jk}	7.75 ± 0.03^{i}	11.62 ± 0.01^{d}

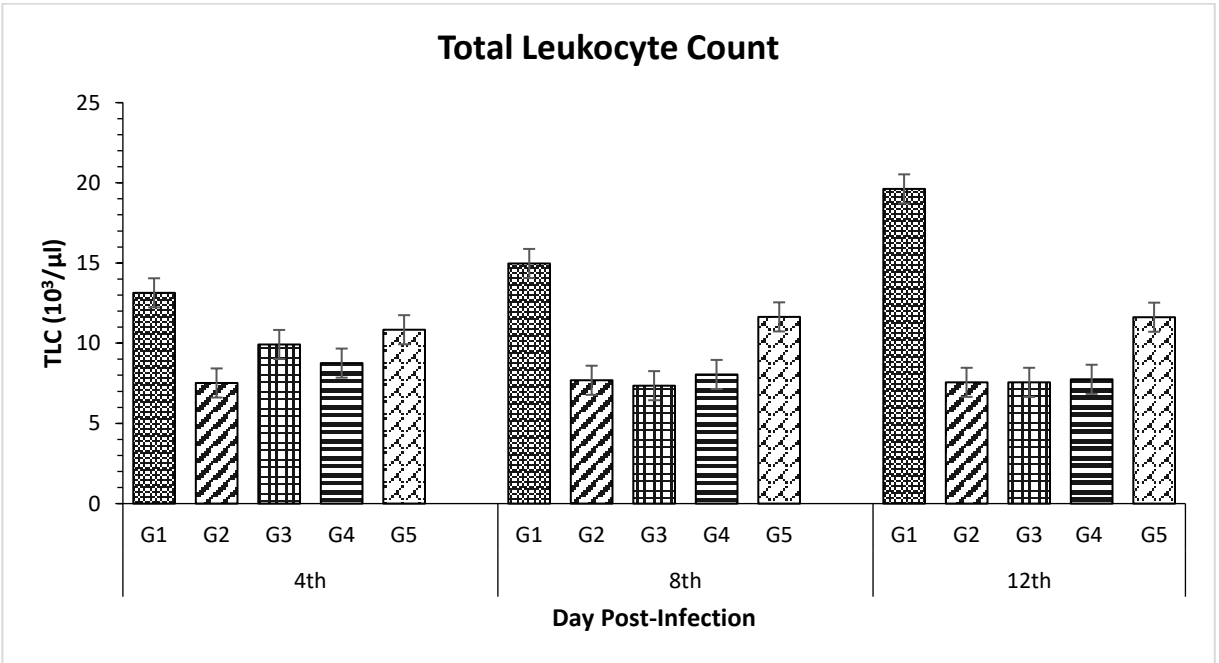
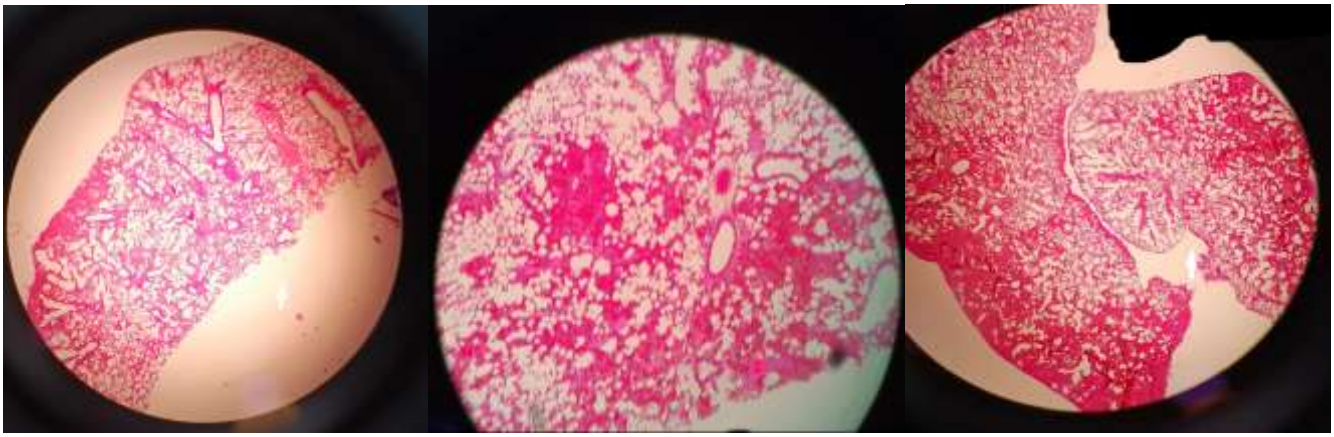


Figure 3: Graphical representation of Total Leukocyte Count after infection with *A. baumannii*.

The platelet count of the experimental groups was also performed. Table 5 shows the mean values of the platelet count of all five experimental groups with respect to post-infection days. Compared to the negative control group G2, the platelet count of all other groups G1, G3, G4, and G5 is slightly less but still comparable. The graphical representation of the Total platelet count versus Day post-infection is demonstrated in Figure 4.

Table 5: Platelet Count (10^3/µl) of Experimental Mice

Days	Experimental Groups				
	G1	**G 2**	**G 3**	**G 4**	**G 5**
4th	565.16 ± 0.04[j]	675.08 ± 0.03[a]	595.83 ± 0.02[g]	542.03 ±0.04[i]	549.66 ± 0.06[k]
8th	587.12 ± 0.5[h]	618.42 ± 0.03[e]	587.12 ±0.06[h]	587.12 ±0.03[h]	579.07 ±0.08[i]
12th	632.86 ± 0.01[c]	654.86 ± 0.09[b]	627.54 ±0.04[d]	627.54 ± 0.02[d]	598.62 ±0.04[f]



Photomicrographs of healthy lung of Mice

Photomicrographs of lungs of Mice infected with *A. baumannii*

Photomicrographs of lungs of Mice infected with *A. baumannii* and treated with bacteriophage

Figure 4: Photomicrographs of Lungs of experimental mice.

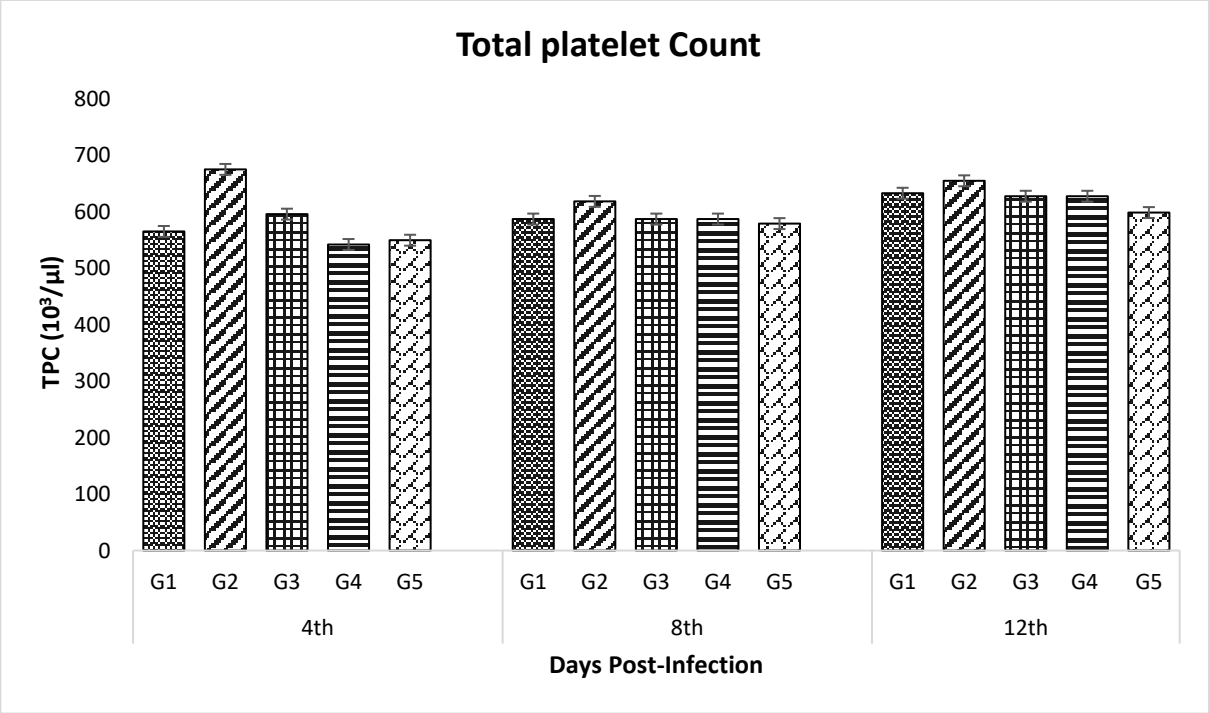


Figure 5: Graphical representation of Total Platelet Count after infection with *A. baumannii*.

Histopathological studies were also performed on the lungs isolated after sacrificing the mice. The micrograph of healthy mice lungs shows normal lung tissue, with no significant pathological changes. In contrast, the lungs of mice infected with *A. baumannii* appeared inflamed and congested. Infiltration of neutrophils and macrophages were observed in the alveoli of the mice lungs. However, in the case of mice infected with *A. baumannii* and treated with phage, the micrograph of the lung shows only mild inflammation, with bronchial walls that are not much congested. Figure 5 shows the micrographs of the lungs of healthy, infected, and recovered mice.

Discussion

Acinetobacter baumannii, a multidrug-resistant nosocomial pathogen was initially treated with antibiotics but the continued overuse of antibiotics against it and other pathogens leads to resistance against many of commonly used antibiotics. It is resilient to traditional sterilization processes so easily invade the hospital equipments and surfaces to play its role in hospital acquired infections. It is also found to be resistant against the last resort drugs like meropenem and colistin(22). As multidrug resistant organisms are evolving and continually linked with the increased expenditure of its treatment in healthcare units as well as socio-economic impacts there is a need to revamp the therapeutic strategies against such pathogens including *A. baumannii*. At present time, bacteriophages are considered promising candidates for the treatment against multidrug-resistant bacteria as these are abundant in natural niches(23). Bacteriophage therapy seems to be highly specific, cost effective, and easily available as compared to modern antibiotics(24).

The prevalence of *A. baumannii* induced infections is 47% to 93% due to its smart acquisition of resistance pattern (25). So our study is based on the use of bacteriophage as a therapeutic agent against *A. baumannii*. For this purpose, *A. baumannii*-specific bacteriophage was isolated from sewage water, and plaque assay and single spot method were used to find out the phage activity. Our in-vivo experiment was based on five groups of mice, three experimental groups and two control groups (positive and negative control). The mice in three experimental groups were given the similar dosage of bacteria (CFU) for infection but different bacteriophage dosage (PFU) to find out the effective dose for the neutralization of infection caused by *A. baumannii*. G5 was given very low dose of bacteriophage, while G3 and G4 had an effective dose against 10^8 CFU units of bacteria as the erythrocyte, leukocyte, and platelet counts support our findings. The same conditions on Positive control (G1, mice only infected with *A. baumanni*) and negative control (G2, mice without any infection) were given and it also supports our results. Apart from hematological studies, histopathological studies of the lungs of mice have also given satisfactory results. The mice infected with *A. baumannii* and treated with bacteriophage show the recovery stages. So, our results predict that bacteriophage therapy could be an effective treatment against multidrug resistant *A. baumannii*.

A study was performed on the therapeutic effects of bacteriophages against *Staphylococcus aureus* for mastitis disease. This antibiotic resistant bacterium was treated with phage therapy and they tried ten different strains of bacteriophages for confirmation, each of the lytic phages showed a dominant eliminating effect against bacteria which supports our hypothesis of treating a multidrug resistant bacterium with phage therapy(26, 27).

A similar study was performed by scientists in Iraq, in which they treated *A. baumannii* infected mice with individual lytic phages as well as with cocktail phages. In the case of individual lytic phage treatment, some *A. baumannii* infected mice were recovered but when this phage activity was compared with cocktail phage activity it was more successful in the elimination of all types of *A. baumannii* infection. Although Cocktail phage activity is more effective than single phage activity still it supports our phage activity for the treatment of *A. baumannii* infection(28).

Zhang et al., 2024 performed a study on a novel lytic bacteriophage as a therapeutic agent against ten different isolates of multidrug resistance *A. baumannii*. This phage was found to form partially transparent haloes around the core of *A. baumannii*. It is involved in the inhibition of the biofilm form of the bacterium as well as it has depolymerase that is involved in the degradation of polysaccharides around the bacterium, hence it is involved in treating *A. baumannii* infection(29). Similarly in our study, phage therapy is involved in the treatment of *A. baumannii* infection in mice by lysis of bacterial walls and the inhibition of biofilm formation(30).

However, the treatment with bacteriophages is challenging due to the biofilms, and new phage resistant bacteria may emerge but this situation can be overcome by using enzymes of bacteriophages, a cocktail of different phages, and using phage in combination with other antimicrobial drugs(31). The phage used in this research work could be used in combination with other phages to form a cocktail to treat in a better way as the therapeutic activity of this phage is promising.

Conclusion

From this research work, it is concluded that this bacteriophage is a promising therapeutic candidate against *Acinetobacter baumannii* infection. Hematological and histopathological studies of experimental mice's blood and lung tissues strongly suggest that the phage treatment leads to a recovery process. However, further research will

be more focused on the use of this phage in combination with other promising phages for a more effective treatment against all other variants of *A. baumannii*.

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