

Characteristic and Potential Therapeutic Effect of Isolated MDR-Acinetobacter Baumannii Lytic Phage

Behnam Sisakhtpour

Isfahan University of Medical Sciences

Arezoo Mirzaei

Isfahan University of Medical Sciences

Vajihe Karbasizadeh

Isfahan University of Medical Sciences

Nafise Sadat Hosseini

Isfahan University of Medical Sciences

Mehdi Shabani

Isfahan University of Medical Sciences

Sharareh Moghim (✉ moghim@med.mui.ac.ir)

Isfahan University of Medical Sciences

Research

Keywords: Acinetobacter baumannii, Bacteriophage, Phage Therapy, Antibiotic Resistance, Endolysin activity

Posted Date: September 8th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-73134/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: *Acinetobacter baumannii* is a major pathogen in the hospital, especially in Intensive Care Units (ICU) and the resistance to multiple drugs as a major contributor to hospital infection. Bacteriophages are viruses that attack bacteria and kill them that could be used for clinical treatment. The aim of the study is in evaluating the function of bacteriophage specificity of multi-drug resistant *Acinetobacter baumannii*, to be used as a useful method for treating of Acinetobacter Infections.

Methods: Cross-sectional study during the year 2017, from patients admitted to the ICU, First, 48 isolates of *Acinetobacter baumannii* were identified by phenotypic method and amplified with blaOXA-51 gene. Then, the sensitivity of phages to pathogens namely ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp) evaluated. DNA of the phage was extracted using the Viral Nucleic Extraction Kit II (Geneaid, Taipei, Taiwan) according to the manufacturer's instructions. Then for protein analysis, PEG-precipitated purified phages were subjected directly to SDS-PAGE, and protein bands were visualized by coomassie Blue G-250 staining method. Finally for cell survival assay we investigated the toxicity of the isolated phage to Hela cells.

Results: In the bacterial resistance pattern, the highest resistance belongs to ciprofloxacin. In optimal phage test, at dilution of 1 (MOI 1) it produced the best effect on bacteria in 30 minutes. Phage sensitivity to different hosts performed by double layer agar method, the phage was treated with ESKAPE bacteria and after 24 hours' incubation at 37°C, only for *Acinetobacter baumannii* Plaque created. The genome analysis indicated that phage plsf-AB2 has a double-stranded DNA genome. In bacterial control, all cells were killed by *A. baumannii*, and no live-cell was seen. The cells remained in control of the phage, and the phage did not affect the cells.

Conclusion: Our findings support the potential application of the phage with potent endolysin activity against MDR *A. baumannii* and give useful information for its further study and use.

Background

Acinetobacter baumannii is responsible for many health care infections, especially burn and wound infection [1]. This non-fermentative, non-motile and aerobic gram-negative bacterium is listed as one of the most six dangerous pathogens namely ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp). [2]. ESKAPE pathogens are resistant to antibiotics and are responsible for the majority of nosocomial infections [2, 3]. Recently, some strains of *A. baumannii* were found to be resistant to nearly all known antibiotics [4, 5]. Therefore, alternative treatments for these infections are urgently needed. In recent years phage therapy is of particular importance since antimicrobial resistance is rising [6]. In addition to using active phages in bacterial infections [7], new phage-derived antimicrobial substances, such as endolysin, are being identified to be a potent antimicrobial agent in vitro and animal models can be used as a

successful treatment for bacterial infections. Thus isolation and characterization of lytic phages is a potential strategy to fight MDR *A. baumannii* [8, 9].

Recently we have isolated two lytic phages from hospital wastewater [10]. In this study, we characterized newly isolated bacteriophage and evaluated the endolysin activity of the phage against isolated MDR *A. baumannii*. We also assess the efficacy of phage on MDR *A. baumannii* clinical isolates. Our findings support the potential application of the phage with potent endolysin activity against MDR *A. baumannii* and give useful information for its further study and use.

Materials And Methods

Bacterial isolation

All *A. baumannii* clinical isolates, comprising 48 strains, were collected from ICUs of Isfahan Medical University hospitals during 2016–2018. All 48 specimens were inoculated initially on blood and MacConkey agar (Merck) and incubated for 24 h at 37 °C. All isolates were identified by conventional biochemical methods [11] and confirmed by PCR. DNA of the strains was extracted by the boiling process. The Primers were used to amplify the *blaOXA-51* gene OXA-F 5'-TAATGCTTTGATCGGCCTTG – 3', and OXA-R5'-TGGATTGCACTTCATCTTGG-3'. The PCR was performed, as described previously [12].

Antibiotic susceptibility testing

Agar disk diffusion method was used to determine the susceptibility of the isolates to various antibiotics (amikacin, cefepime, ceftazidime, ciprofloxacin, and rifampin). The inhibition of bacterial growth was measured as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2017).

Isolation, purification, and titration of lytic phages

For isolating phages, sewage samples were collected from various water sources in Alzahra General Hospital (Isfahan, Iran). The phages were isolated by enrichment method [13]. Briefly, 50 ml of centrifuged sewage supernatant was filtered through a 0.45 µm pore size membrane and mixed with an equal volume of 2x nutrient broth containing 1 ml exponential phase MDR *A. baumannii*. After incubation at 35 ° C for 24 hours with shaking at 160 rpm, the mixture was centrifuged for 10 min at 13000 rpm, and the supernatant was filtered through 0.45 µm membrane size filter. Subsequently, 200 µl of the filtrate was mixed with 2.5 ml of 0.7% soft nutrient agar in the presence of 100 µl of the log-phase bacterial indicator. Then, the mixture was poured onto a solid medium of 1.5% nutrient agar and incubated for 24 hours at 37 ° C. Double-layer agar method was used to detect plaque formation by phage. Single plaques were picked. A double-layer agar method was performed To obtain purified phage strains, several times and plaque picking was repeated until single-plaque morphology was observed [14]. The titer of phage was determined by standard plaque assay [15].

Phage concentration and storage

Each single purified plaque was added into 5 ml of nutrient broth containing the MDR-AB indicator (OD₆₀₀ = 0.6) and cultured at 37 °C for 24 h. Then, the suspension was transferred into 500 ml of nutrient broth and shaken overnight at 35 °C. Chloroform was then added to the to a final concentration 0.1% mixed gently and allowed to stand at room temperature for 15 min to kill the bacteria. Solid NaCl was added to the culture to a final concentration of 1 M, mixed and dissolved, and the culture was incubated in an ice bath for one hour. After centrifugation at 10,000 × g for 10 min, and solid PEG6000 was added to the supernatant to a final concentration of 10% (w/v) mixed and dissolved at room temperature. The solution was incubated overnight on ice to precipitate the phage particles. The pellet was obtained by centrifugation (10,000 × g) for 10 min at 4 °C, and suspended in 5 ml of SM buffer (50 mM Tris-Cl, 100 mM NaCl, 8 mM MgSO₄, pH 7.5) [45]. An equal volume of chloroform was then added to separate the phage particles from PEG6000. Following centrifugation at 3,000 × g for 10 min, the aqueous phase was collected and filtered through a 0.22 µm pore-size membrane filter and stored at 4 °C [16].

Examination phage morphology by transmission electron microscopy

Negative staining and TEM electron microscopy were used to observe bacteriophages. A drop of concentrated phage was placed onto a copper mesh grid surface and negatively stained with 2% phosphotungstic acid (PTA). The grid was examined by transmission electron microscopy (Zeiss-EM10c, Germany) at an operating voltage of 100 kV.

pH, thermal and chloroform stability

For pH stability test, 10¹⁰ PFU/ml of the phage was incubated at different pH buffers (3, 5, 7.9, and 11) at 37 °C in SM buffer for 1 hour. The phage titer was determined by the double-layer agar method, as described above. For thermal stability, the phage aliquots were collected after 5 min, 15 min, 45 min, 1-hour intervals at different temperatures (37, 50, and 70 °). To determine chloroform stability, 1 ml (1 × 10¹⁰ PFU) of the phage was mixed with 0.4 ml chloroform, and phage was collected and titered after 1-hour incubation at room temperature [17].

Determination of MOI

MDR *A. baumannii* cultures in OD₆₀₀ = 0.1 (10⁸ CFU/ml) were divided into identical aliquots and infected with a serial dilution of bacteriophage stocks and let to adsorb for 15 min. then, free phages were removed by centrifugation at 5000 g for 10 min. Precipitants were resuspended in NB medium, and bacteriophages were tittered after 4 hours of incubation at 30 °C by double-layer method[18].

One-step growth curve

For the one-step growth curve experiment, one milliliter of the MDR *A. baumannii* (OD₆₀₀ = 0.1) in the exponential phase was mixed with the phage at an MOI 0.01 and let to adsorb for 10 min. The unadsorbed phages were removed by brief centrifugation (6000 g, 10 min) and 50 µl of the pellet was transferred to 50 ml of NB medium and placed at 37 ° C on a shaker (160 rpm). Samples were collected

every 10 min over a time period of 120 min, and the number of phages was immediately assessed by the double-layer agar method [18]. This experiment was done in triplicate.

Phage genome isolation and analysis by restriction enzymes

DNA of the phage was extracted using the Viral Nucleic Extraction Kit II (Geneaid, Taipei, Taiwan) according to the manufacturer's instructions. DNA of the phage was digested with the HindIII restriction enzyme (sigma aldrich) according to the manufacturer's protocol. The digested DNA was analyzed by 0.8% agarose gel electrophoresis with 0.5% TBE (Tris-Borate EDTA) running buffer [18].

Phage protein analysis

For protein analysis, PEG-precipitated purified phages were subjected directly to SDS-PAGE, and protein bands were visualized by coomassie Blue G-250 staining method [19]. In order to determine the protein of the phage with a lytic activity, we used SDS-PAGE under non-denaturing conditions [20]. Briefly, phage lysates were centrifuged at $14000 \times g$ at 4°C for 30 minutes. Then, the supernatant was filtered through a $0.22 \mu\text{m}$ filter and concentrated through centrifugation in $10000 g$. The specimen was mixed with protein loading buffer without β -mercaptoethanol. The samples were then loaded on an SDS-PAGE without boiling. After electrophoresis (80 V), the resolved gel was placed onto an agar coated plate, in which soft agar mixed with the MDR AB was previously poured onto the gel [21].

Bacteriophage host range

For determining the phage host range, 3 ml of 0.6% melted agar (50°C) was mixed with $200 \mu\text{l}$ of 10^8 CFU/ml of the phage and poured onto a plate which was previously coated with 1.5% solid agar. After agar was solidified, $10 \mu\text{l}$ of the filtered phage was spotted on each plate with *A.baumannii* clinical isolates, *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), *K. pneumonia* (ATCC 10031). The appearance of lysis plaques was investigated after 12 h [22].

Bacterial reduction assay

For this assay, we used a previously described method [23]. One milliliter $\text{OD}_{600} = 0.1$ culture of the bacterial indicator was inoculated to two separated flasks containing 100 mL nutrient agar. One vial was inoculated the isolated phage, and the other one was taken without phage as a negative control. The cultures were incubated at 35°C at 160 rpm. The optical density (OD_{600}) of samples was measured at 20 min intervals for 4 hours [24].

Determination of Phage Titer

Bacteriophage dilutions of 10 serial dilutions were prepared, and then each dilution was equally mixed with bacteria and placed in an incubator at 35°C and 160 rpm for absorbance. Then centrifugation was performed for 10 minutes, and the precipitate was remixed in the Nutrient Broth medium. After 8 hours of incubation at 37°C , the bacteriophage titer was determined [15].

Cells survival assay

We investigated the toxicity of the isolated phage to HeLa cells. HeLa cell line (ATCC CCL-2) was obtained from the National Cell Bank of Iran, Pasteur Institute of Iran (Tehran, I.R. Iran). The cells were plated (1.5×10^4 well/plate) in 96-well plate in the presence of 100 μ l Dulbecco's modified eagle's medium (DMEM, Gibco, USA) supplemented with 5% fetal calf serum (FCS; Gibco, USA), at 37 °C in 5% CO₂ (15). When cells reached 70–80% confluent, 10^6 CFU of *A. baumannii* (AB-2) was added to each well, followed by the addition of the phage at different MOI (0, 0.01, 0.1, 1, 10). As a control, HeLa cells were treated with 10^8 PFU of phage without the addition of *A. baumannii*. In a separate experiment, the cells were first infected with 10^6 CFU of AB-2. After 2 hours, the phage was added to the infected wells. The cells were incubated for an additional 24 hours, and the number of living cells was counted using trypan blue [18].

Results

Bacterial identification and antibiotic susceptibility pattern

All 48 isolates, which were phenotypically identified as *A. baumannii*, harbored the *bla*_{OXA-51} gene (Fig. 1).

Based on the pattern of *A. baumannii* (Fig. 2), 82% showed resistance to amikacin, 97% to cefepime, 96% to ceftazidime, 99% to ciprofloxacin, and 82% to rifampin. Few samples had the intermediate resistance pattern, while susceptibility was not found among them.

Isolation of lytic phages

MDR *A. baumannii* strain, MDR-AB2, isolated from catheter of a patient with pneumonia at Alzahra hospital, was resistant to several antibiotics (table 1). The bacteria were used as an indicator to screen bacteriophages in sewage samples of the same hospital. The isolated phage named plsf-AB2 forms clear, round, 2–3 mm plaques in the double-layer agar (Fig. 3). The morphology of plsf-AB2 was examined by negative staining of the phage and observation by electron microscopy. The phage had an icosahedral head of 70 ± 10 nm and a tail of about 60 nm (Fig. 4). The phage belongs to the order Caudovirales and family Myoviridae following the current guidelines of the ICTV (International Committee on Taxonomy of Viruses, <http://ictv.global/taxonomyRelease.s.asp>).

Phage Growth Curve

One-step growth experiment showed that the latent period of plsf-AB2 was about 30 min and was followed by the lysis phase, which lasted for 70 min. The burst size was 120 PFU per infected cell (Fig. 5).

The optimal phage titer

For determining the optimal MOI of plsf-AB2, different MOIs of the phage were inoculated into AB-2 (10^8 CFU/ml). As shown in the Fig. 6 The plsf-AB2 with MOI of 1 reduces MDR AB-2 from 10^8 CFU/ml to 10^3 CFU/ml in 30 min. Lower MOIs (0.1 and 0.01) decreased the virus titer to the same point in 1.5-2 h. The results indicate that although higher MOI reduced *A. baumannii* more quickly, but is not necessary for lysis.

Reduction bacterial assay

Infections of *A. baumannii* with high titer of lysate was monitored for 7.5 h. Phage infection significantly decreased the *A. baumannii* culture turbidity in comparison to control. However, an increase of the turbidity (OD600) was observed after about 4 hour of culture incubation. This increase of turbidity was most probably due to the growth of phage-resistant bacteria (Fig. 7).

Phage stability

The stability of the plsf-AB2 to different pH, chloroform, and the temperature was tested. The phage plsf-AB2 lost its infectivity at pH 3 and 11, while pH 7 is the most suitable condition to maintain the phage. The phage was stable at different pH ranging from - 20 to 25 °C. However, the phage titer was slightly dropped at 50 °C and reduced dramatically at 70 °C. The activity of the virus was not affected by chloroform treatment.

Bacteriophage host range

Host range spectrum surveyed on forty eight *A. baumannii* strains and showed that the plsf-AB2 phage was able to infect 56.3% of the *A. baumannii* strains. The results demonstrated that phage was specific for the *A. baumannii* and didn't have any effect on *Klebsiella*, *Pseudomonas*, *E. coli*.

Genome size and protein profiles

The genome analysis indicated that phage plsf-AB2 has a double-stranded DNA genome (approximately 12.6 kb). The genome of plsf-AB2 could be digested by *HindIII* endonuclease (fig.8). It was found that *HindIII* has three cutting sites. Although, endonucleases, *HincII* have no cutting site. The purified phages were denatured in loading buffer (containing SDS and 1% β -Mercaptoethanol) and heated in boiling water for 5 min. The phage proteins were separated in SDS-PAGE. The results plsf-AB2 has nine structural protein bands in 10% SDS-PAGE, with a molecular weight ranging from 14.5 to 150 kDa. The most abundant protein band in the gel above 35 kDa, which was assumed to be the phage putative capsid protein (fig.9).

Phage endolysin activity

The proteins of plsf-AB2 were loaded on SDS-PAGE without boiling as described in the materials and methods. The AB-2 overlay on SDS-PAGE showed a clear band at 15 kDa (Fig. 10).

Cell survival assay under bacterial infection

The safety of phage was examined in the Vero cells line in the presence of MDR-AB-2. In 12 primary wells, we added phage in different dilutions MOI = 0, 100, 1, 0.1, 0.01, separately to each well, and the bacterium was combined with a concentration of half Mac Farland. A well was considered as a cell control, a phage control well, and a well was considered as control of bacteria.

The plate was incubated for 24 hours, then counted by neobar cell count; in all dilutions, the living cells were observed, and by decreasing the dilution of the phage, the number of live cells decreased, so the lowest cell viability was found at dilution of 0.01. In bacterial control, all cells were killed by *A. baumannii*, and no live-cell was seen. The cells remained in control of the phage, and the phage did not affect the cells (diagrams 1 and 2).

Discussion

The aim of our study was to demonstrate a new approach for the usage of the bacteriophage for treating a life-threatening MDR *A. baumannii* infection. Growth under challenging conditions and prolonged shelf-life in dry conditions, as well as resistance to most commonly used antibiotics, *A. baumannii* has become an essential and effective pathogen in the hospital [25]. Similarly, this bacterium is found as the cause of the spread of several diseases worldwide [26]. The importance of this bacterium and the increasing resistance to antimicrobial agents necessitates more verifications. In this study, 48 isolates were tested with an antibiogram and tested for resistance patterns using the CLSI guidelines. They had multidrug resistance (MDR), with the highest resistance to 100% ampicillin, 99% ciprofloxacin, 97% cefepime, and Ceftazidime being 96%; resistance to amikacin was 82%, and rifampin was 86%. According to a study by Sadiqifard et al. in 2006 in Tehran, resistance to *A. baumannii* has been 100% to ceftazidime and 52% to amikacin [27]. Another study by Henwood et al. in the United States, of 649 isolates from different hospitals; 595 isolates of *A. baumannii* were diagnosed and ciprofloxacin and ceftazidime resistance, was 30% and 70% respectively [28]. In our study, the resistance to these antibiotics was 99% and 96%, respectively. The results of the antibiotic resistance pattern obtained in this study are in contrast to other studies, which can be due to the diverse clinical samples, therapeutic methods in different regions, and the time of the survey.

In similar studies in different locations, the MDR rate has increased in all countries, and resistance of *A. baumannii* to antibiotics has been rising, exhibiting that resistance levels vary across different geographic areas. In the study of Simhon et al., *A. baumannii* resistance to ciprofloxacin from 49% in 1990 increased to 87% in 2000, which shows elevating the antibiotic resistance [29].

Bacteriophage, commonly known as phage, is defined as viruses that infect bacteria. Phages are present everywhere and require a bacterial host for this presence [30]. Phage therapies were used for human beings for the first time in 1915 by d'Herelle, which received significant results from early phlebotomy experiments [31]. The use of bacteriophages for phage therapies is the use of lytic phages that infect bacterial cells and eliminate them by lysis [32]. Unlike antibiotics that may affect body flora, phages target the host bacteria and do not affect other bacterial species, which present the specificity of this [33].

Most categorized Acinetobacter phages are tailed viruses with double-stranded DNA genomes. They are arranged into three families, including Myoviridae, Podoviridae, and Siphoviridae that are in the order of Caudovirales [18, 23].

Tailed bacteriophages make their progeny to protrude the cells and infect other cells with endolysins which cause to breaking down the bacterial cell wall. In a study by Regeimbal JM et al. in the United States, they were able to separate the *A. baumannii* particular phage from the sewage and, by examining the electron microscope, stated that the phage had a shaft-shaped head and a contractile tail, and was in Myoviridae group [34]. In the study we performed, we were able to isolate the phage from sewage by hosting *A. baumannii*. Placing a phage in a plate on an *A. baumannii*, a zone of the plaque was formed and examined by morphology with an electron microscope, a 6-capsid, fractured, and constricted phage of the myoviridae family in which morphological results overlapped with Regeimbal JM studies. Another study by Merabishvili M in Belgium in 2014, the phage of *A. baumannii* was isolated from the river. By microscopic examination, a 6-capsid head and taut phage were introduced from the myoviridae family [10].

A study by Kusradze in 2016, the bacteriophage separated from *A. baumannii* from sewage, was introduced by microscopic examination as the myoviridae family. They also investigated the phage secretion stability at different temperatures, pH, and chloroform, which stated that after 24 hours, the phage incubation was maintained at 37 ° C, 100% of the phages. At 50, 90% of the phage was retained, and at 70 ° C, the phage was not observed. Also, it was stable in exposure to chloroform and had no phages at pH 5 and 11 (19).

Broadly speaking, high pH stability and high thermal resistance made phage remarkably pledged for practical usage in deracination of *A. baumannii* contaminations and or treatment of *A.baumannii* infections. In our study, we investigated phage at different temperatures, pH and chloroform stability, and after 24 hours of inoculation, the phage exhibited a steady-state for chloroform; and at 37 ° C temperature, the best effect was on bacteria which the phage titer was not diminished; in different pH analyzes, phages at pH 5 and 11 disappeared. These results are consistent with the results of the Kusradze study. Thermal resistant phages were usually isolated from extreme thermal habits [31, 32], but they could also be found in other environments. Recently, thermal resistant phages have been isolated and characterized from various dairy products [33, 34]. There are several possible explanations for thermal and pH resistance of phages that one of the main reason is lysin (endolysin). Most double-stranded DNA phages accomplish host cell lysis through the holin-endolysin system. The similarity of bacteriophage endolysin genes is essential for structural analysis, which contributes to the potential of using endolysin as an antimicrobial agent.

The endolysins antibacterial activity is generally attribute to their enzymatic function, which make, rupturing of the covalent bonds in peptidoglycan. However, some endolysins, especially those from phages of Gram-negative bacteria, are capable of affecting bacterial cells by means of a mechanism completely independent of their enzymatic activity (During et al. 1999; Orito et al. 2004). in our study the

protein that caused lysis estimated with SDS PAGE about 15 kDa that corroborate with previous results. The lysin is utterly stable and constant over a wide range of pHs. A number of concerns have been raised about the potential toxicities and the practicality of bacteriophage therapy for MDR bacterial infections. However, in this particular case, we overcame these hurdles and did not observe any discernible adverse clinical events.

Conclusion

The results of this study highlighted that *A. baumannii* resistance has been increased, and treatment of infections caused by it was painful and should be considered as an appropriate alternative to antibiotics. Considering the results of the effect of the phage on *A. baumannii*, which caused the bacterial lysis in the plate as well as the results of cell culture, the phage treated the bacteria infected with the cells, as well as other experiments including optimal phage titers for reducing the concentration of the bacteria, during 30 minutes, significantly reduced the level of bacteria. According to these results, bacteriophages can be used as a useful therapeutic alternative to antibiotics to combat MDR bacterial infections.

Abbreviations

MDR: multi drug resistance, ICU: Intensive Care Units, ESKAPE:(*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp), MOI: multiplicity of infection, OD: optimal density, PFU: plaque forming unit, CFU: colony forming unit, pH: potential of hydrogen, TEM: transmission electron microscopy, NB: nutrient broth, TBE: tris-boric acid- EDTA, ATCC: American type culture collection,.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

Funding

This study was financially supported by Isfahan University of Medical Sciences, Isfahan, I.R. Iran through the Grant No 396188.

Authors' contributions

BS was a significant contributor to doing and writing the manuscript. AM , MS , NH and VK collaborated in doing the thesis that results in the paper. SM Designed and supervised the manuscript. All authors read and approved the final manuscript.

Acknowledgments

This research benefited from a grant from Isfahan University of medical sciences.

References

1. Bassetti M, Righi E, Esposito S, Petrosillo N, Nicolini L: **Drug treatment for multidrug-resistant *Acinetobacter baumannii* infections.** 2008.
2. Rice LB: **Progress and challenges in implementing the research on ESKAPE pathogens.** *Infection Control & Hospital Epidemiology* 2010, **31**(S1):S7-S10.
3. Navidinia M: **The clinical importance of emerging ESKAPE pathogens in nosocomial infections.** 2016.
4. Gong Y, Shen X, Huang G, Zhang C, Luo X, Yin S, Wang J, Hu F, Peng Y, Li M: **Epidemiology and resistance features of *Acinetobacter baumannii* isolates from the ward environment and patients in the burn ICU of a Chinese hospital.** *Journal of Microbiology* 2016, **54**(8):551-558.
5. Huang G, Yin S, Gong Y, Zhao X, Zou L, Jiang B, Dong Z, Chen Y, Chen J, Jin S: **Multilocus sequence typing analysis of carbapenem-resistant *Acinetobacter baumannii* in a Chinese burns institute.** *Frontiers in microbiology* 2016, **7**:1717.
6. Adlhart C, Verran J, Azevedo NF, Olmez H, Keinänen-Toivola MM, Gouveia I, Melo LF, Crijns F: **Surface modifications for antimicrobial effects in the healthcare setting: A critical overview.** *Journal of Hospital Infection* 2018, **99**(3):239-249.
7. Schooley RT, Biswas B, Gill JJ, Hernandez-Morales A, Lancaster J, Lessor L, Barr JJ, Reed SL, Rohwer F, Benler S: **Development and use of personalized bacteriophage-based therapeutic cocktails to treat a patient with a disseminated resistant *Acinetobacter baumannii* infection.** *Antimicrobial agents and chemotherapy* 2017, **61**(10):e00954-00917.
8. Fan J, Zeng Z, Mai K, Yang Y, Feng J, Bai Y, Sun B, Xie Q, Tong Y, Ma J: **Preliminary treatment of bovine mastitis caused by *Staphylococcus aureus*, with trx-SA1, recombinant endolysin of *S. aureus* bacteriophage IME-SA1.** *Veterinary microbiology* 2016, **191**:65-71.

9. Parisien A, Allain B, Zhang J, Mandeville R, Lan C: **Novel alternatives to antibiotics: bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides.** *Journal of applied microbiology* 2008, **104**(1):1-13.
10. Merabishvili M, Vandenheuvel D, Kropinski AM, Mast J, De Vos D, Verbeken G, Noben J-P, Lavigne R, Vaneechoutte M, Pirnay J-P: **Characterization of newly isolated lytic bacteriophages active against *Acinetobacter baumannii*.** *PloS one* 2014, **9**(8).
11. Adams-Haduch JM, Paterson DL, Sidjabat HE, Pasculle AW, Potoski BA, Muto CA, Harrison LH, Doi Y: **Genetic basis of multidrug resistance in *Acinetobacter baumannii* clinical isolates at a tertiary medical center in Pennsylvania.** *Antimicrobial agents and chemotherapy* 2008, **52**(11):3837-3843.
12. Safari M, Saidijam M, BAHADOR A, Jafari R, Alikhani MY: **High prevalence of multidrug resistance and metallo-beta-lactamase (MbetaL) producing *Acinetobacter baumannii* isolated from patients in ICU wards, Hamadan, Iran.** 2013.
13. Standardization IOF: **Water Quality: Detection and Enumeration of Bacteriophages:** ISO; 2000.
14. Kropinski AM, Mazzocco A, Waddell TE, Lingohr E, Johnson RP: **Enumeration of bacteriophages by double agar overlay plaque assay.** In *Bacteriophages*. Springer; 2009: 69-76.
15. Kusradze I, Karumidze N, Rigvava S, Dvalidze T, Katsitadze M, Amiranashvili I, Goderdzishvili M: **Characterization and testing the efficiency of *Acinetobacter baumannii* phage vB-GEC_Ab-M-G7 as an antibacterial agent.** *Frontiers in microbiology* 2016, **7**:1590.
16. Lockington RA, Attwood GT, Brooker JD: **Isolation and characterization of a temperate bacteriophage from the ruminal anaerobe *Selenomonas ruminantium*.** *Appl Environ Microbiol* 1988, **54**(6):1575-1580.
17. Capra M, Quiberoni A, Reinheimer J: **Phages of *Lactobacillus casei/paracasei*: response to environmental factors and interaction with collection and commercial strains.** *Journal of applied microbiology* 2006, **100**(2):334-342.
18. Shen G-H, Wang J-L, Wen F-S, Chang K-M, Kuo C-F, Lin C-H, Luo H-R, Hung C-H: **Isolation and characterization of ϕ km18p, a novel lytic phage with therapeutic potential against extensively drug resistant *Acinetobacter baumannii*.** *PloS one* 2012, **7**(10).
19. Laemmli UK, Favre M: **Maturation of the head of bacteriophage T4: I. DNA packaging events.** *Journal of molecular biology* 1973, **80**(4):575-599.
20. Jyothisri K, Deepak V, Rajeswari MR: **Purification and characterization of a major 40 kDa outer membrane protein of *Acinetobacter baumannii*.** *FEBS letters* 1999, **443**(1):57-60.
21. Cha K, Oh HK, Jang JY, Jo Y, Kim WK, Ha GU, Ko KS, Myung H: **Characterization of two novel bacteriophages infecting multidrug-resistant (MDR) *Acinetobacter baumannii* and evaluation of their therapeutic efficacy in vivo.** *Frontiers in Microbiology* 2018, **9**:696.
22. Lee C-N, Tseng T-T, Lin J-W, Fu Y-C, Weng S-F, Tseng Y-H: **Lytic myophage Abp53 encodes several proteins similar to those encoded by host *Acinetobacter baumannii* and phage phiK02.** *Appl Environ Microbiol* 2011, **77**(19):6755-6762.

23. Ghajavand H, Esfahani BN, Havaei A, Fazeli H, Jafari R, Moghim S: **Isolation of bacteriophages against multidrug resistant *Acinetobacter baumannii***. *Research in pharmaceutical sciences* 2017, **12**(5):373.
24. Haq IU, Chaudhry WN, Andleeb S, Qadri I: **Isolation and partial characterization of a virulent bacteriophage IHQ1 specific for *Aeromonas punctata* from stream water**. *Microbial ecology* 2012, **63**(4):954-963.
25. Nordmann P: ***Acinetobacter baumannii*, le pathogène nosocomial par excellence**. *Pathologie biologique* 2004, **6**(52):301-303.
26. Villegas MV, Hartstein AI: ***Acinetobacter* Outbreaks, 1977–2000**. *Infection Control & Hospital Epidemiology* 2003, **24**(4):284-295.
27. SADEGHIFARD NK, RANJBAR R, GHASEMI A, Pakzad I, ZAEIMI YJ, Zaheri A, HEMATIAN A, Ghafourian S: **A study of antimicrobial resistance of *acinetobacter baumannii* and non-*acinetobacter baumannii* strains isolated from three hospitals in Tehran**. 2006.
28. Henwood CJ, Gatward T, Warner M, James D, Stockdale MW, Spence RP, Towner KJ, Livermore DM, Woodford N: **Antibiotic resistance among clinical isolates of *Acinetobacter* in the UK, and in vitro evaluation of tigecycline (GAR-936)**. *Journal of Antimicrobial Chemotherapy* 2002, **49**(3):479-487.
29. Simhon A, Rahav G, Shazberg G, Block C, Bercovier H, Shapiro M: ***Acinetobacter baumannii* at a tertiary-care teaching hospital in Jerusalem, Israel**. *Journal of clinical microbiology* 2001, **39**(1):389-391.
30. Doss J, Culbertson K, Hahn D, Camacho J, Barezki N: **A review of phage therapy against bacterial pathogens of aquatic and terrestrial organisms**. *Viruses* 2017, **9**(3):50.
31. Haq IU, Chaudhry WN, Akhtar MN, Andleeb S, Qadri I: **Bacteriophages and their implications on future biotechnology: a review**. *Virology journal* 2012, **9**(1):9.
32. Nakai T, Park SC: **Bacteriophage therapy of infectious diseases in aquaculture**. *Research in microbiology* 2002, **153**(1):13-18.
33. Laanto E, Sundberg L-R, Bamford JK: **Phage specificity of the freshwater fish pathogen *Flavobacterium columnare***. *Appl Environ Microbiol* 2011, **77**(21):7868-7872.
34. Regeimbal JM, Jacobs AC, Corey BW, Henry MS, Thompson MG, Pavlicek RL, Quinones J, Hannah RM, Ghebremedhin M, Crane NJ: **Personalized therapeutic cocktail of wild environmental phages rescues mice from *Acinetobacter baumannii* wound infections**. *Antimicrobial agents and chemotherapy* 2016, **60**(10):5806-5816.

Figures

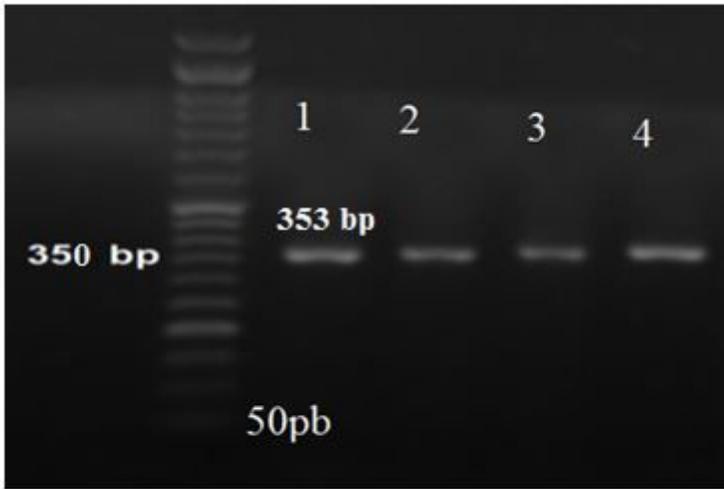


Figure 1

PCR of blaOXA-51 gene

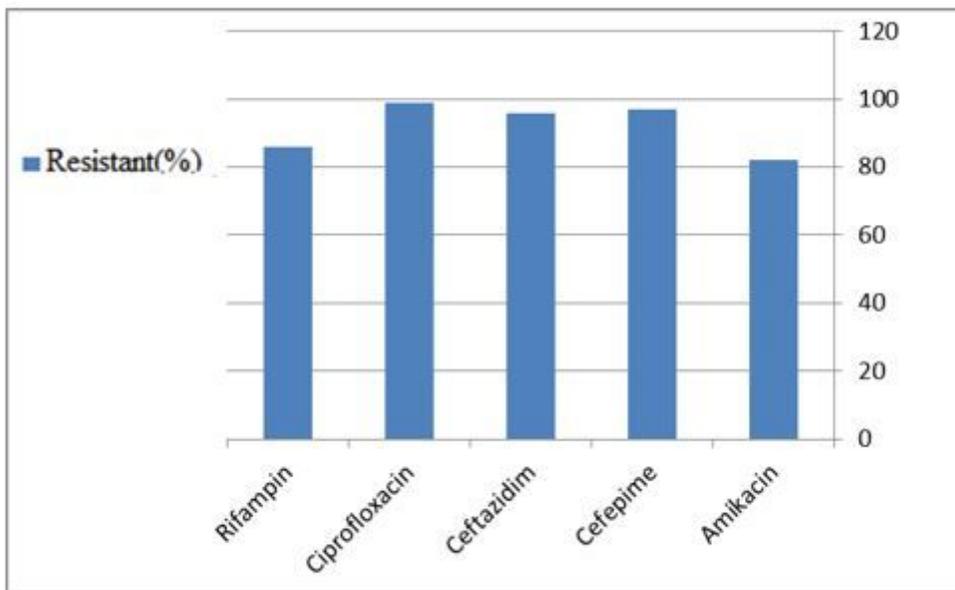


Figure 2

Antimicrobial resistance pattern of *Acinetobacter baumannii*

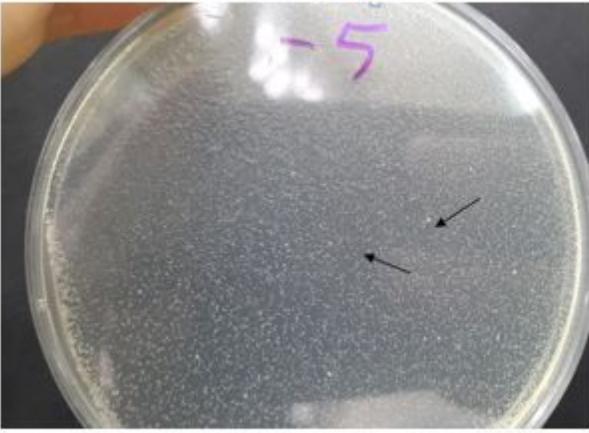


Figure 3

plaque assay with double-layer agar method

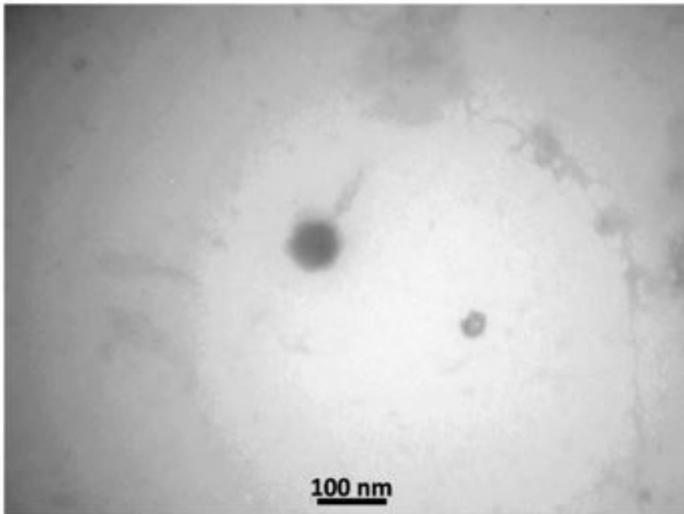


Figure 4

Transmission Electron Microscopy of plsf-AB2 phage

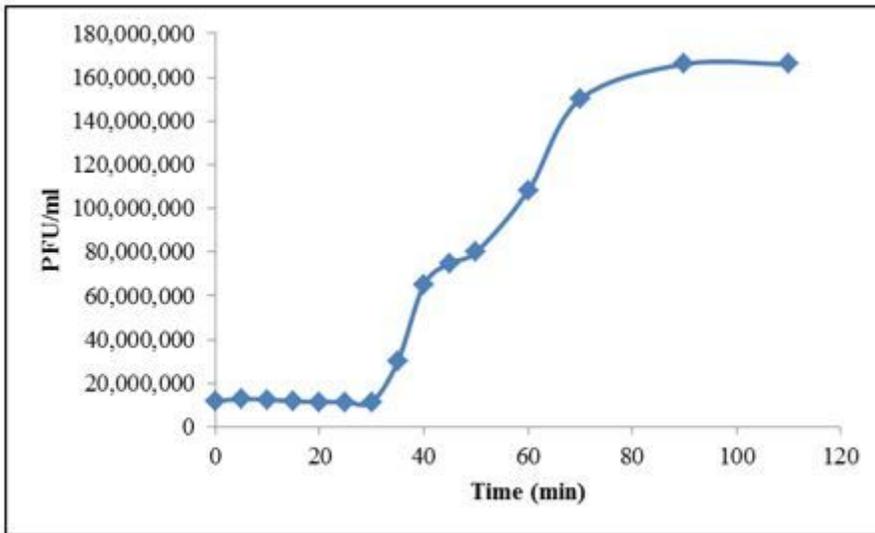


Figure 5

One step growth curve of plsf-AB2 phage

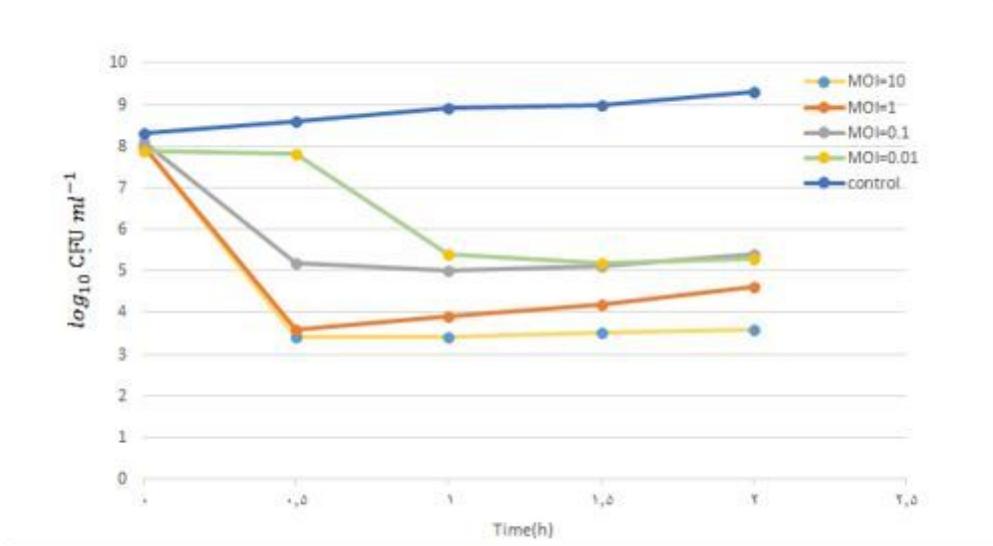


Figure 6

determination of optimal phage titer. The plsf-AB2 with MOI of 1 reduces MDR AB-2 from 108 CFU/ml to 103 CFU/ml in 30 min. Lower MOIs (0.1 and 0.01) decreased the virus titer to the same point in 1.5-2 h.

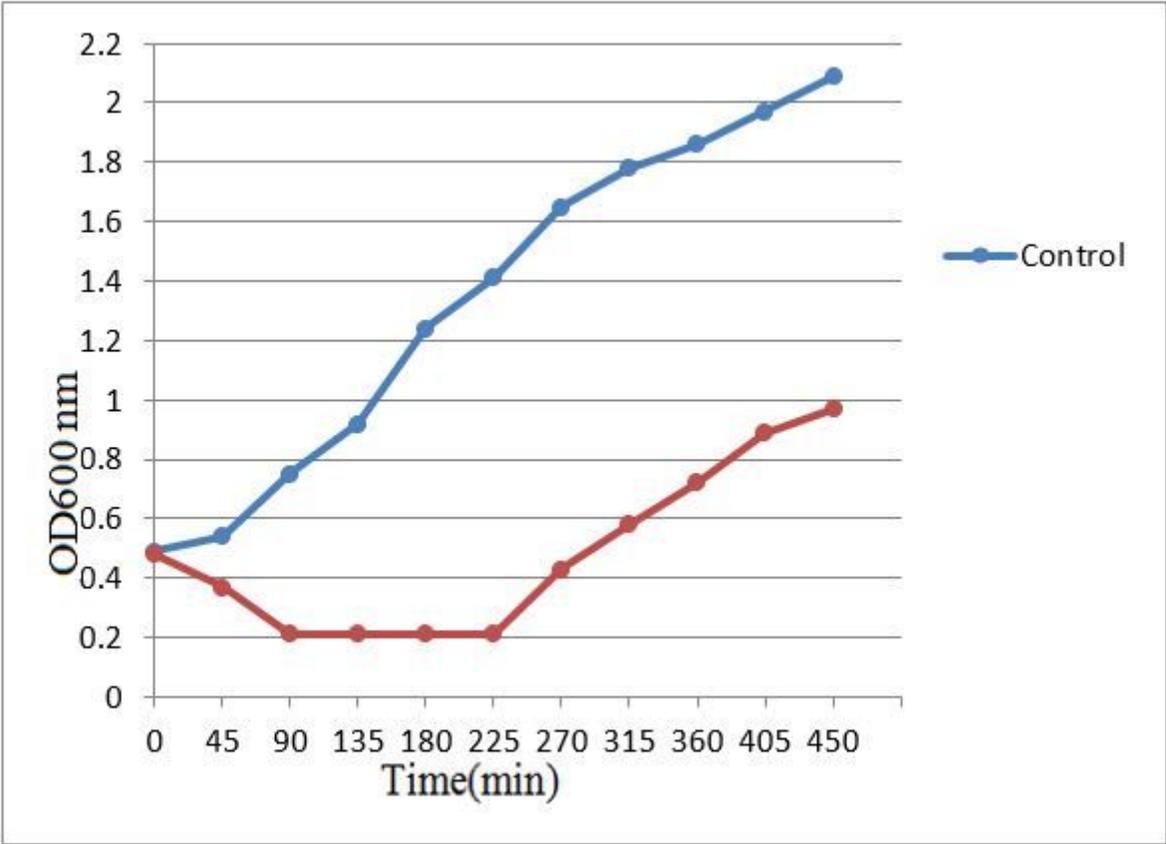


Figure 7

Reduction bacterial assay

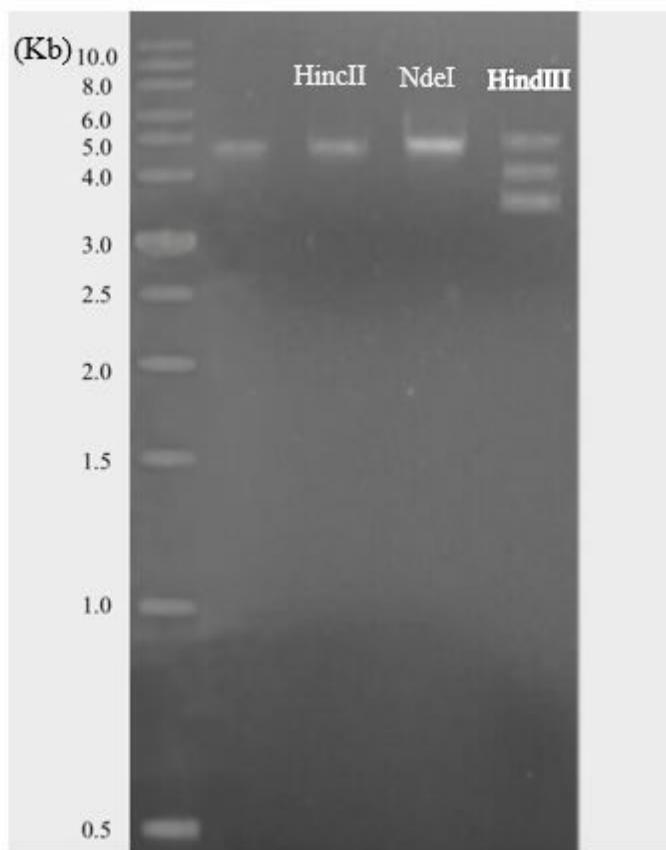


Figure 8

The plsf-AB2 phage genomic DNA restriction patterns and size determination

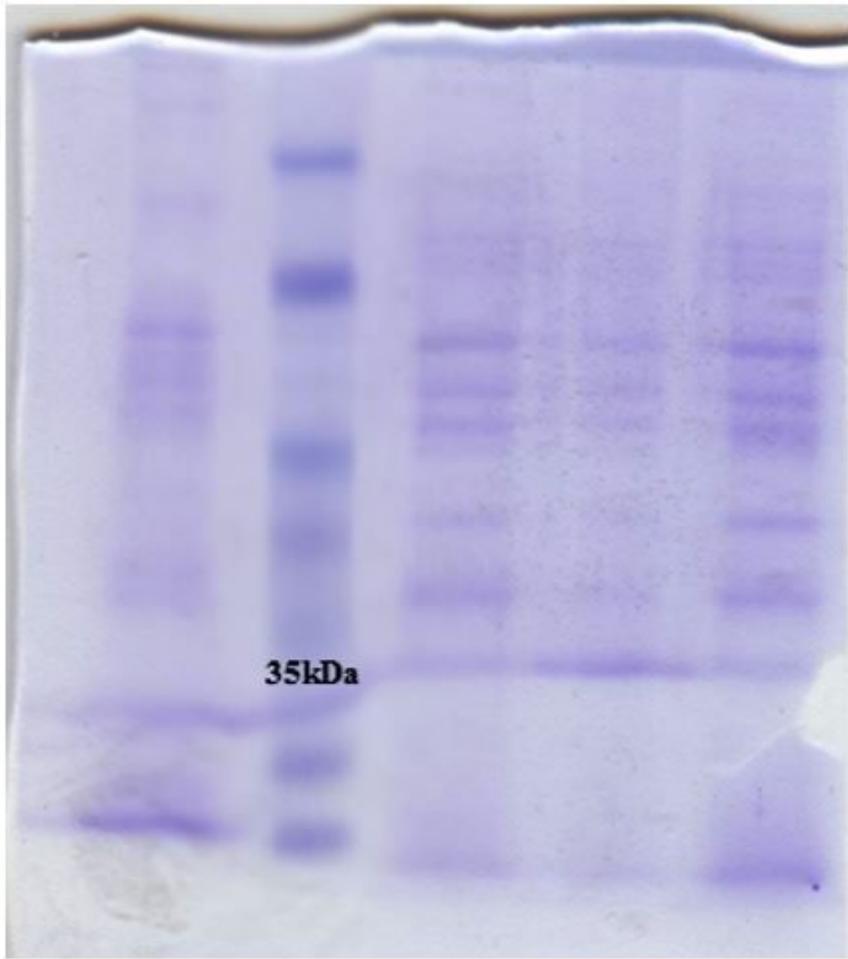


Figure 9

The plsF-AB2 phage proteins which separated by SDS PAGE

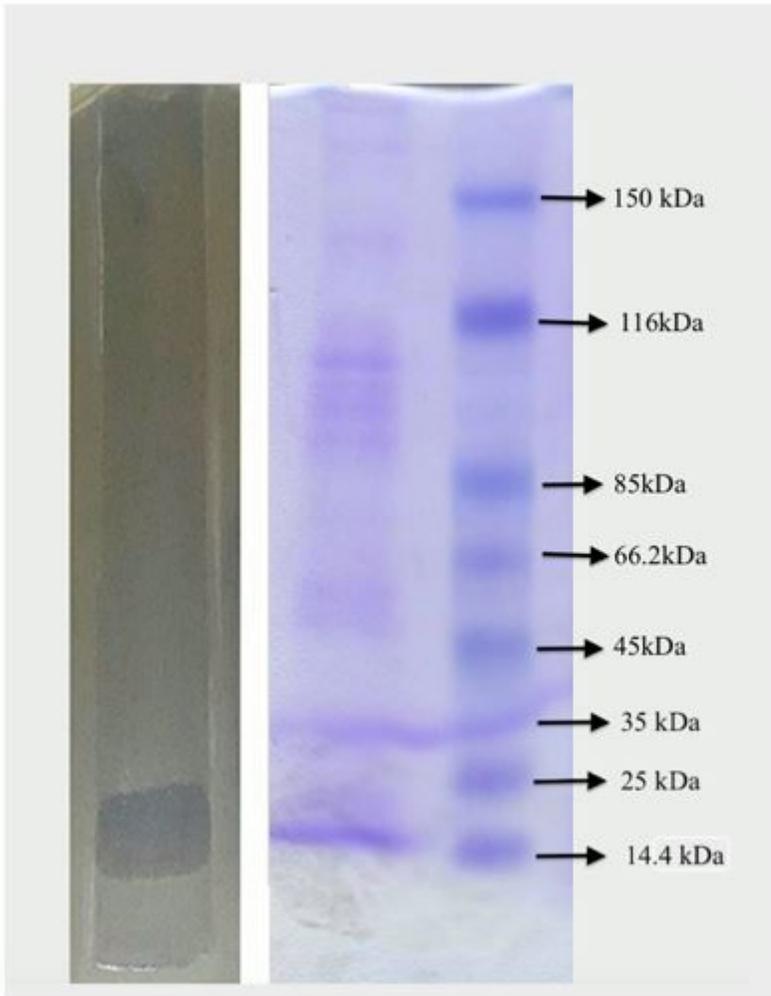


Figure 10

Phage endolysin activity in SDS-PAGE

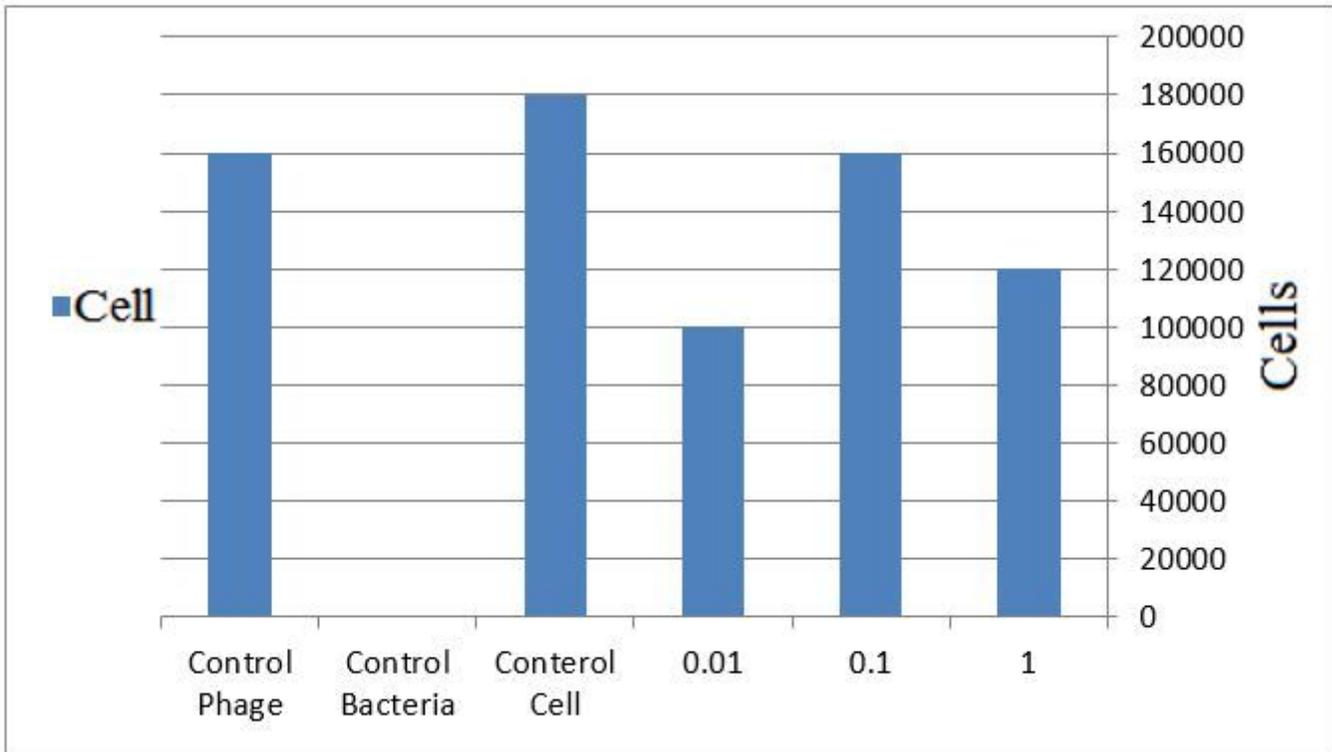


Figure 11

The cell survival rate

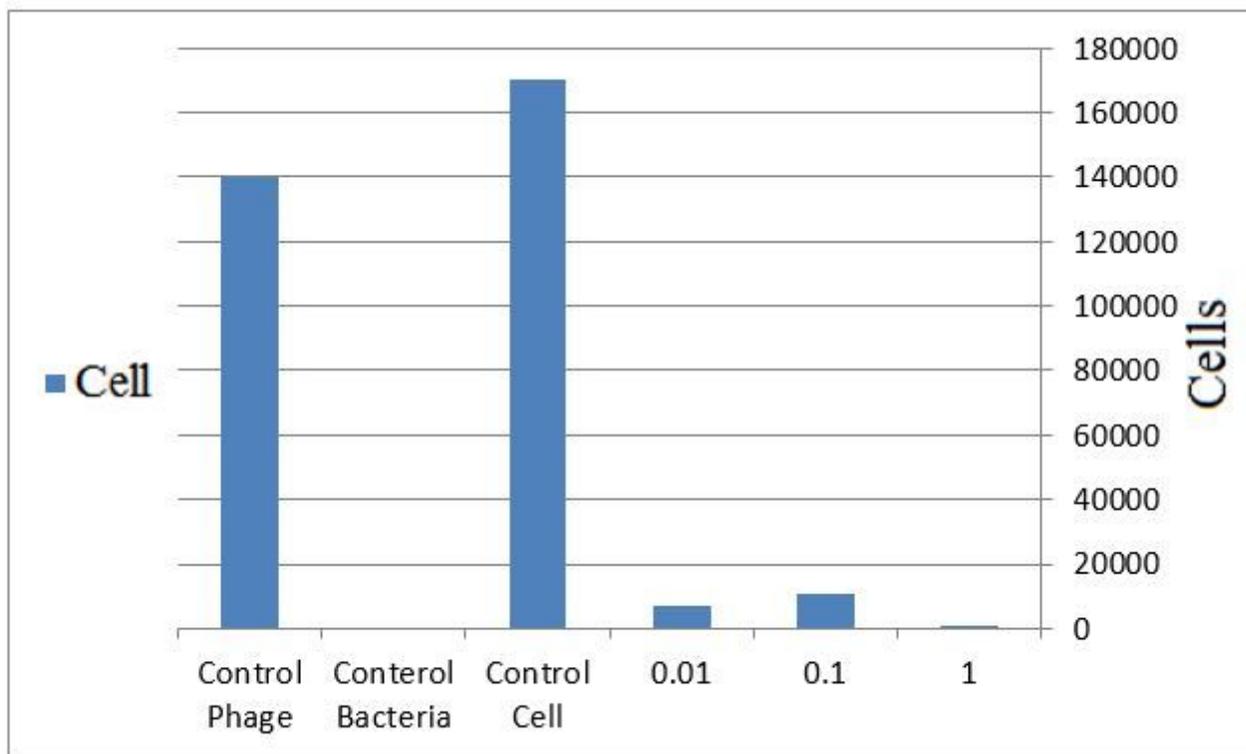


Figure 12

Cell culture results after 2hrs in the exposure of *A. baumannii*