

Original article

The impact of phage infection on the morphological characteristics of *Acinetobacter baumannii*

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Abstract

Background: *Acinetobacter baumannii*, as one of the most dangerous multidrug-resistant (MDR) pathogens has become a critical concern due to its ability to escape the modern health antibiotics. Phage therapy emerges as a promising alternative due to its specificity, abundancy, and effectiveness to address MDR pathogens.

Objectives: This study aimed to determine the killing profile of *A. baumannii* phages and characterize the phage-induced morphological change towards *A. baumannii* clinical isolates.

Methods: Phages were enriched using double layer agar method. Host range and efficiency of plating (EOP) were determined by performing a spot assay. Killing profile was identified with killing assay and morphological characteristics of infected cells were observed with fluorescence microscopy and analyzed by performing single cell analysis.

Results: The results indicate the broad host range of the six phages to clinical isolates. Phage phiAB014 was found to have a finer average of EOP value compared to the other phages and then selected for further characterization. Killing assay revealed that phiAB014 could inhibit the growth of bacteria in longer incubation time by 10-fold reduction compared to control. Furthermore, phiAB014 could induce several morphological changes of the infected cells indicating that phiAB014 possibly infect the host through several hijacking mechanisms.

Conclusion: This study revealed that phiAB014 with its killing profile could inhibit the bacteria growth favorably and induce the morphological change of the bacteria. All the properties of phiAB014 demonstrate the promising phage therapy as an alternative to antibiotics.

Keywords: *Acinetobacter baumannii*, characterization, morphological change, multidrug resistant, phage therapy.

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Bacterial infection is one of the most concerning global issues and is known to cause high mortality worldwide.⁽¹⁾ Antibiotics represent the best therapeutic solution as antimicrobial treatment.⁽²⁾ However, the overuse of antibiotics in human medicine and the misuse of last resort antimicrobial compounds such as colistin in agriculture, are contributing to the increasing number of antibiotic-resistant bacterial pathogens.⁽³⁾ In 2019, antimicrobial resistance was listed by the World Health Organization (WHO) as one of the top ten threats to global health. Hence, the rise of multidrug-resistant (MDR) bacteria has become a critical medical issue in human wellness and consistently associated with poor clinical outcomes.^(2,4) Recently, a gram-negative bacterium *Acinetobacter baumannii* is classified amongst the most dangerous MDR pathogens and has become a particular concern as it is an opportunistic human pathogen.^(5,6) Some strains of *A. baumannii* clinical isolate have developed resistance to antibiotics commonly used for treatment, such as β -lactams, aminoglycosides, tetracycline also colistin that considered as the last line defense.⁽⁷⁾ Thus, there is an urgent need to research and develop new antimicrobials as well as another alternative treatment.

Phage therapy has emerged as one of the most promising alternatives to antibiotics due to its abundance, diversity, and exceptional specificity for treating this drug-resistant pathogen in the upcoming 'post antibiotic era'.^(7,8) Bacteriophages or phages are prokaryotic bacterial viruses that specifically recognize, infect, replicate within and kill the specific host cell.⁽⁹⁾ In theory, phages have several advantages over antibiotics. Phages are highly specific, widely distributed, and efficient at lysing targeted pathogenic bacteria. Phages commonly enter a lytic or lysogenic life cycle. Considering one of the vital events throughout the lysogenic cycle is characterized by the insertion of phage genome and preserving its genetic material within the host without destroying the host cell DNA, lytic phages are highly preferred to use as therapeutic agents.⁽¹⁰⁾

In recent decades, phage therapy has been proven successful in many clinical cases in which patients were infected with MDR bacteria, including *Pseudomonas aeruginosa*, *Mycobacterium abscessus* and *Acinetobacter baumannii*.⁽²⁾ Despite its successful therapeutic application, the detailed mechanisms of phage infection, particularly the specific processes involved in hijacking by each phage,

remain largely unexplored. Mechanism of pre-killing (MOK) of phage is a mechanism by which phages compromise cellular pathway of the host prior to cell lysis. Recent studies demonstrated that morphological changes of bacteria upon treatment of antibacterial agents including phages could provide insight into the mechanism by which the antibacterial agents exert against the bacteria.^(7,11) A study conducted in *Vibrio* revealed that morphological changes revealed the MOK of vibrio phage against *V. parahaemolyticus*, suggesting that *Vibrio* phage interferes with host protein translation machinery at the early infection.⁽¹¹⁾ Hence, morphological change characterization is crucial for enhancing the efficacy of phage treatment against bacterial infections, being that it could provide significant clues about the MOK or the process by which cells are infected before exhibiting cell lysis.

Therefore, in this study, we aimed to characterize the killing profile of *A. baumannii* phages and characterize the phage-induced morphological change of *A. baumannii* through a single-cell infection assay and use the information to identify phage candidates with unique characteristics in phage therapy against *A. baumannii*.

Materials and methods

Phage enrichment

Six phages isolated from hospital wastewater together with six *A. baumannii* clinical isolates were obtained from Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. To enrich the phages, the double-layer agar method was performed. A hundred microliters of 10-fold diluted phage lysate (10⁻⁶ and 10⁻⁵ dilution) was prepared by mixing 20 μ L of phage lysate with 180 μ L of SM buffer and each dilution was added into 200 μ L of *A. baumannii* (OD 0.4), and 5 mL of molten 0.3% LB top agar into the plate. Then the plates were incubated at 30°C for 18 hours. The web-lysis plates with near confluent lysis were soaked with 5 mL of SM buffer and incubated at room temperature for 4 hours. The soaked SM buffer was taken and centrifuged at 8,000 rpm for 10 minutes. Then, the supernatant was filtered through a 0.45-micron filter to remove the remaining bacterial cells.

Host range and efficiency of plating (EOP) value determination

The host range and EOP value was determined by spot test assay by spotting 10 μ L of phage lysate

into the 1.6% LB plate with 200 μ L *A. baumannii* and 5 mL of molten 0.3% LB top agar. The EOP values were calculated as the ratio of the PFU value of phage with susceptible bacterial strain and the phage with indicator bacterial strain.

Killing assay and bacterial revival count

The log phase of *A. baumannii* S-AB8 was prepared by subculturing the overnight culture at 1:100 dilution. The day culture was then grown until OD600 reached 0.4. The culture of 100 μ L of *A. baumannii* clinical isolates was then added into 96-well plates and infected with all the six phages at various MOI as follows: 10, 1, 0.1 and 0. The final volume in each well was adjusted to 200 μ L using LB broth. The infected cultures were monitored by measuring the OD600 every 15 minutes for 18 hours. This experiment was carried out in triplicate. All infected cultures were then further monitored by counting the colony forming unit (CFU) every 24 hours for 5 days.

Single cell infection

Overnight cultures of *A. baumannii* clinical isolates were 100-fold diluted and incubated at 30°C until OD600 is in the log phase (0.4). Then, the bacterial cells were infected with the high-titer phage lysate at MOI 5. At desired time points: 15- and 30-minutes post infection (mpi), 500 μ L of infected cells were incubated with fluorescence dye DAPI (2 μ g/mL) and FM4-64 (1 μ g/mL) for 1 minute. The infected cells were collected with centrifugation at 10,000 rpm for 1 minute. Then, the stained infected cells were loaded onto an agarose pad (1.2% agarose with 20% LB) on concave glass slides and fluorescence microscopy was then performed to observe the morphologies of the bacterial cells after phage infection.

Single cell analysis

Raw images from the fluorescence microscope are pre-processed into an appropriate image format on ImageJ software. Then, cell features extraction is performed using CellProfiler 4.0 software. The morphological feature set is selected based on the previous study.⁽¹²⁾ The outlier, which summed about 10% of the cells, such as cells with unique morphology, incomplete cells along the image edge and abnormal image segmentation are removed with a 10% cutoff. Finally, the dimension of the data set is reduced and then visualized with an unsupervised data dimension reduction method, pairwise controlled manifold approximation (PaCMAP).

Results

***A. baumannii* phages infect vast host range with distinct EOP value**

To characterize potential phages candidates, a spot assay was performed determine the phages host range and EOP value. Results in **Table 1** (gray color in indicating no infection of the phage) suggest that all the six phages could infect clinical isolates which unlocked feasible phage candidates to address MDR *A. baumannii*. In addition, all the six phages have varying values of EOP. Efficiency of plating value implies the efficiency of the phage to produce the progeny on its host or other isolates and could be one of the characters that define the killing efficacy. Results in **Table 1** demonstrate that phage phiAB014 has finer EOP value on every *A. baumannii* clinical isolates compare to the other phages. Most of the phages, including phiAB014, phiAB016A, phiAB025A, phiAB027A displayed a small plaque size (**Figure 1A-D**) while phages phiAB030A displayed a big plaque size (**Figure 1E**). This suggests that phiAB014A that has a broad host range and small plaque size could be the superior candidate for further characterization.

Table 1. Host range and EOP value of six phages towards six *A. baumannii* clinical isolates

Bacterial strain (<i>A. baumannii</i>)	phiAB014	phiAB016A	phiAB025A	phiAB027A	phiAB030A	phiAB038
S-AB1		1.5 $\pm$ 0.3				
S-AB2						1
S-AB8	2.5 $\pm$ 0.4	<0.001	0.8 $\pm$ 0.1	0.6 $\pm$ 0.3	1	
SP2391	1	1.3 $\pm$ 0.3	1	1		
A2006	1.9 $\pm$ 0.2	1	<0.001	0.7 $\pm$ 0.2		
A2005	1.0 $\pm$ 0.1	0.7 $\pm$ 0.1	<0.001	0.25 $\pm$ 0.1		
Parental host	SP2391	A2006	SP2391	SP2391	S-AB8	S-AB2

Killing profile of phiAB014

Phage phiAB014 exhibits broad host range and finer EOP value compared to the other phages. Then killing assay and colony forming unit count were performed to further characterize the killing efficacy of phiAB014. **Figure 1F** suggests that phiAB014 able to inhibit the growth of *A. baumannii* S-AB8. However, the bacterial re-growth was observed after 14 hours in all MOI tested (10, 1, and 0.01). Nevertheless, in longer incubation, phiAB014 could inhibit the growth of *A. baumannii* S-AB8 inversely with the MOI (**Figure 1G**). Colony forming units (CFU) in the lowest MOI (0.1) in the first, third and fifth day, which were 4.6×10^7 , 2.6×10^8 and 4×10^8 CFU/ml, are lower than CFU in the highest MOI (10) which were 2.6×10^8 , 6.6×10^8 and 1.1×10^9 CFU/ml. Thus, suggest that the lowest MOI tested (0.1) could give better reduction of bacterial growth

compared to the highest MOI tested ($P < 0.05$), showing the significant reduction of *A. baumannii* S-AB8 growth. Also, this phage was found to have the highest EOP value towards *A. baumannii* S-AB8 compared to the other phages (**Table 1**).

Phages phiAB014 induce morphological change in *A. baumannii* S-AB8

Morphological characteristic of *A. baumannii* S-AB8 after infected by phiAB014 suggests that phiAB014 infection could induce DNA condensation in 15- and 30-minutes post infection (mpi) with several types of condensation compared with uninfected (**Figure 2A-2C**). Furthermore, single cell analysis (**Figure 2D**) demonstrates that bacterial cells infected with phiAB014 separately clustered from mock indicating that phiAB014 could induce significant morphology change in 15 and 30 mpi.

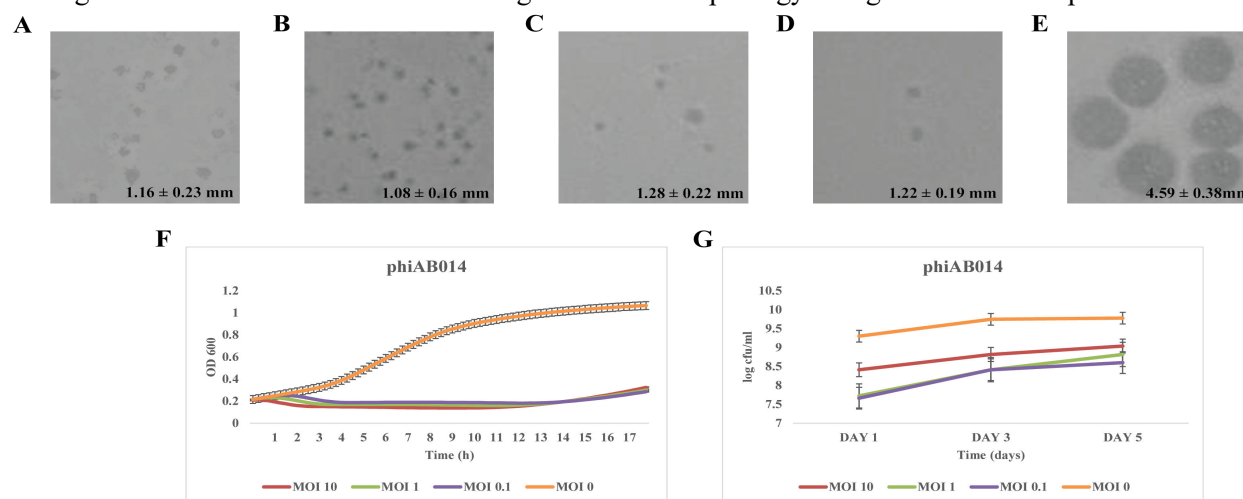


Figure 1. Plaque morphology of phiAB014 (A), phiAB016A (B), phiAB025A (C), phiAB027A (D), phiAB030A (E) with plaque size, killing assay of phiAB014 towards *A. baumannii* S-AB8 (F) and colony forming unit count (G).

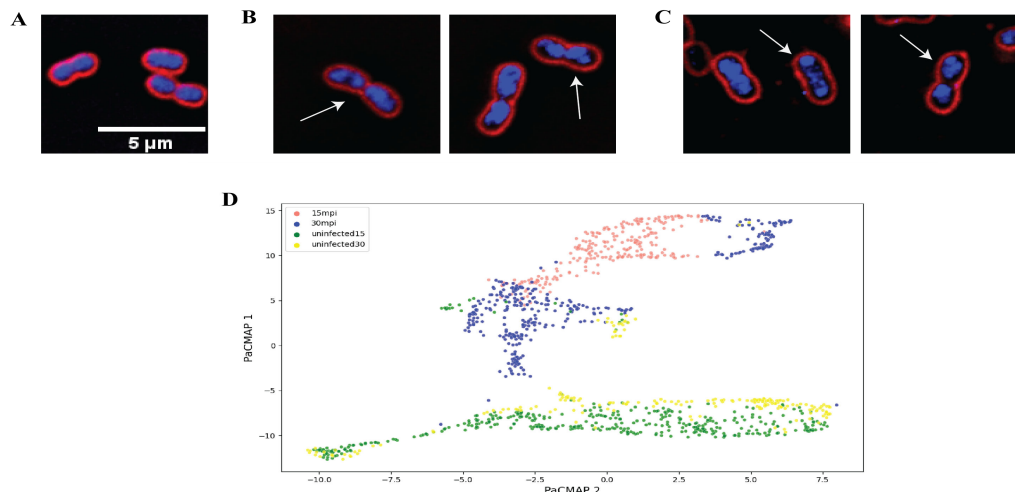


Figure 2. *A. baumannii* S-AB8 without phiAB014 infection (A), after phiAB014 infection in 15 mpi (B) and 30 mpi (C), single cell analysis of *A. baumannii* S-AB8 morphology (D) with approximately 300 cells of each uninfected and infected cell. White arrows point the DNA condensation in the infected cells.

Discussion

MDR pathogens have become one of the most concerning global issues that causes nosocomial infections with high mortality worldwide.^(1,5,6) One of the MDR pathogens, *A. baumannii*, has developed resistance to antibiotics commonly used for treatment.⁽⁷⁾ Therefore, there is an urgent need to research and develop new antimicrobials also another alternative treatment. Phage therapy is now emerging as a promising alternative due to its specificity, abundancy and efficiency in addressing the MDR pathogens.^(4,7,8)

In this study, some lytic *A. baumannii* phages were isolated from hospital wastewater. We investigate their killing efficacy and properties on the considered *A. baumannii* clinical isolates. Most of the phages display the broad host range towards the isolate with a variety of EOP values. Even so, one of the phages, phiAB014 shows better average of EOP value meaning that phiAB014 could produce the progeny more efficiently. In killing assay, phiAB014 display the significant inhibition in bacterial growth compared to control. However, bacterial re-growth was detected in 14-hour post infection suggesting the resistance emergence. This might be due to the ability of the bacteria to develop anti-phage mechanisms. Hence, this study on phage characterization still has room for another future study to gain more understanding about phage mechanism of infection to give the better formulation of phage cocktail in combating the emergence of bacteria anti-phage mechanisms. Interestingly, in longer incubation phiAB014 inhibits the growth of bacteria inversely with the MOI. We found that the lowest MOI tested (0.1) could give the best reduction in bacterial growth by inhibiting for 10-fold change compared to control. Those might be due to the high EOP value of phiAB014 towards the considered bacteria which is *A. baumannii* S-AB8 suggesting that the phage that have high EOP value, only need low MOI to give the optimal inhibition towards its target.

On top of that, we implemented morphological change to study the viral replicating or hijacking machinery, as we called it MOK. MOK based on morphological change could provide another perspective to investigate which metabolic pathway of *A. baumannii* S-AB8 is targeted during infection. A previous study conducted in *Vibrio* revealed that *Vibrio* phage infection could induce the formation of toroidal shape in the host DNA which likely indicates

that *Vibrio* phage interferes with the host protein translation.⁽¹¹⁾ Nevertheless, in this study, we found different morphological change induced by phiAB014 which potentially revealing another phage mechanism of pre-killing. We observed that phiAB014 could induce DNA condensation in 15 and 30-mpi with several different phenotypes of condensation. In 15-mpi, DNA of infected cells condensed in the middle of the cell while in 30-mpi the DNA condensed in the pole and middle of cell. This finding corroborated by previous study conducted on *Pseudomonas* phage revealed that different phages would induce different morphological changes, meaning that the phages possibly infect the host through different MOK.⁽¹³⁾ Additionally, the single cell analysis results demonstrate that the infected cells in 15 and 30-mpi are not only separated from uninfected cells but also from each other, suggesting the temporal effect of phage infection on bacterial morphological changes. Further research is needed to determine whether the temporal variations observed in this study are associated with different stages of the phage replication cycle.

Conclusion

To put it concisely, all the properties of phiAB014 demonstrate the promising phage therapy as an alternative to antibiotics and this finding could contribute to investigating more about phage-host interaction and the development of phage-cocktails in the future. Therefore, further research such as genome sequencing, TEM, and phage cocktail formulation will be needed.

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Conflict of interest statement

All the authors declare that they have no conflict of interest.

Data sharing statement

The authors confirm that the data supporting the findings of this study are available within the article. Raw data of this study are available from the corresponding author by request.

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