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Efficacy of a phage cocktail in controlling phage resistance development in multidrug resistant *Acinetobacter baumannii*

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ABSTRACT

The control and treatment of multidrug resistant pathogens infections has become a grand challenge for clinicians worldwide. Virulent phage has long been considered as an effective bactericidal agent, which may be a potentially alternative to antibiotics. However, the rapid development of phage resistance seriously hinders the wide and continuous application of virulent phages. In this study, *Acinetobacter baumannii* phage vB_AbaS_D0 was isolated, characterized and used to control the phage resistance development in bacterial strains. Transmission electron microscopy analysis of vB_AbaS_D0 indicated it belonged to the *Siphoviridae* family with an icosahedral head. Its whole genome was 43, 051 bp in size, with a GC content of 45.48% and 55 putative open reading frames. The data showed that vB_AbaS_D0 was a virulent phage. Although vB_AbaS_D0 had a very weak bactericidal activity, a wide range of *Acinetobacter baumannii* strains were sensitive to it. The results suggested that the cocktail of vB_AbaS_D0 and another *Acinetobacter baumannii* phage vB_AbaP_D2 could improve the therapeutic efficacy in vivo and in vitro. The resistance mutation frequency of *A. baumannii* cells infected with D0 or phage cocktail was significantly lower than cells treated with D2 ($P < 0.01$). Phage therapy in the murine bacteremia model results showed that the percentage of phage resistant mutant occurrence in the phage D0 or cocktail treatment group was significantly lower than in phage D2 treatment group ($P < 0.01$).

1. Introduction

The emergence of multidrug resistance in pathogenic bacteria has become a significant global health challenge (Zaman et al., 2017). Infections by multidrug resistant pathogens are difficult to treat, causing significant morbidity and mortality (Colomb-Cotinat et al., 2016). The worldwide prevalence of these pathogens is increasing rapidly and poses a global medical burden to health care institutions. *Acinetobacter baumannii* is one of the most clinically significant pathogens, having the ability to survive for long periods of time in hospital environments (Gonzalez-Villoria and Valverde-Garduno, 2016). Frequent reports of *Acinetobacter baumannii* strains being resistant to broad-spectrum antibiotics might predict future difficulties in treating multidrug resistant bacterial infections (Salehi et al., 2018).

Bacteriophages or phages are viruses that specifically target and infect their host bacteria. According to the type of life cycle (lytic or the lysogenic), phages are roughly divided into two groups (Dou et al., 2018). Virulent phages which undergo the lytic life cycle can lyse host bacteria to release progeny phage particles. Although temperate phages undergo a lysogenic cycle, they may choose to infect host strains through either lytic or lysogenic cycles. Recently, virulent phages have been reconsidered as a safe and effective therapeutic alternative to combat multidrug resistance (El-Shibiny and El-Sahhar, 2017). Unlike general broad-spectrum antibiotics, phages have no impact on normal flora due to their strict host specificity. Additionally, phages also have advantages over antibiotics, such as ability to remove biofilms, low toxicity and capacity to self-multiply (Drulis-Kawa et al., 2012). Virulent phages have been widely studied in human health, agricultural settings and food safety in order to control the spread of multidrug resistant bacteria. For example, in Poland, Georgia and the former Soviet Union, numerous cases of human bacterial infections were reported to of successfully been treated by phages (Miedzybrodzki et al., 2012; Reardon, 2014). In the USA, the first phage product, ListShield™, has been approved by the U.S. Food and Drug Administration (FDA) (Perez Pulido et al., 2016). However, there still are numerous challenges that impede the wide application of virulent phages (Hanlon, 2007; O'Flaherty et al., 2009). One of the main challenges is the rapid occurrence of phage resistant mutants that arise during treatment with

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lytic phages. These resistant mutants arise due to the fact that bacterial cells acquire different mechanisms to resist phage attacks, including preventing phage adsorption, inhibiting entry of phage DNA, cutting the phage genome and aborting phage infections (Labrie et al., 2010). Various approaches are currently being proposed to address the emergency of phage resistance. Phage cocktails or the combination of phages and antibiotics are always the first to be considered, but they have obvious drawbacks. It was previously proven that bacterial resistance to phage cocktails could eventually emerge, but inhibiting the appearance of phage resistant mutants can only be achieved when all phages in the cocktails maintain high titers throughout the treatment process (Atterbury et al., 2007; Carvalho et al., 2010; Nilsson, 2014; Yen et al., 2017). More importantly, treatments using phage cocktails containing too many phages can causes complex pharmacological and immune responses that may hinder the implementation of clinical trials (Nilsson, 2014). The combined use of phages and antibiotics can result in harmful effects on normal bacterial flora and with higher medical costs (Oechslin, 2018). Therefore, new approaches for phage applications are needed to delay or control the emergence of phage resistance.

In this study we isolated the virulent phage vB_AbaS_D0, which has weak bactericidal ability and low susceptibility of causing resistant mutants. Our goal was to test if it is possible to inhibit the appearance of resistant bacteria without large increases in phages. Our designed phage cocktails contained phage vB_AbaS_D0 and vB_AbaP_D2 in order to inhibit the appearance of phage resistant mutants.

2. Materials and methods

2.1. Bacterial strains and phages

List of bacterial strains used in this study can be found in Table S1. Gram-negative strains were cultivated in Luria Bertani (LB) broth at 37 °C while gram-positive strains of *Enterococcus* and *Staphylococcus* were cultivated in Brain-Heart Infusion (BHI) broth at 37 °C. The virulent *Acinetobacter* phage vB_AbaP_D2 (GenBank accession no. MH042230) was previously isolated from sewage at the Second Hospital of Dalian Medical University by our group (Yuan et al, unpublished results). The genome of phage D2 was composed of linear double-stranded DNA that is 39,964 bp in length with 39.23% GC content and 47 predicted open reading frames. Phage D2 has been classified as a member of the T7-like phages.

In this study, *A. baumannii* AB9 was used for phage propagation. Phages were isolated from hospital sewage sample as previously described (Yuan et al., 2019). In summary, 50 mL 2 × concentrated LB broth was mixed with 2 mL of an overnight incubation of *A. baumannii* AB9 and 50 mL of sewage sample. After overnight growth at 37℃ with shaking, this mixture was centrifuged (10,000 × g, 4℃, 5 min) to remove solid impurities and filtered through a 0.22-μm filter (EMD Millipore Co, Billerica, MA) to remove bacteria. Phage presence was confirmed using the spot-test method (Yuan et al., 2019). Spot-tests used 200 μL of host cells mixed with 10 mL of semi-solid LB agar and poured onto solid LB agar plates. A total of 10 μL of filtrate was spotted onto the surface of double layer agar plates. After incubation overnight, phages could form clearing zones on the double-layer agar plates. Purified phages were obtained by a modified double agar layer technique. This technique used 100 μL of phage lysate and 200 μL of *A. baumannii* AB9 mixed with semi-solid LB agar. This mixture was then poured onto the solid LB agar layer. After overnight incubation at 37 °C, single phage plaques had formed on the semi-solid agar layer. Phages were picked and purified by re-plating at last three times. Concentrated phage particles were obtained by precipitation with polyethylene glycol (PEG) 8000. Phage concentrations were suspended in SM buffer and stored at 4 °C.

2.2. Transmission electron microscopy (TEM)

The morphology of the phages were examined by TEM as previously described (Poduval et al., 2018). Purified phage suspensions ($\geq 10^9$ PFU/ml) were fixed to the carbon-coated copper acid grid and then stained with 0.5% (w/v) uranyl acetate. All phages were observed using a JEM-2000EX TEM (JEOL Co, Tokyo, Japan).

2.3. One-step growth

A one-step phage growth experiment was performed as described by Gong et al. (2016). In brief, purified phage suspensions were mixed with mid-log phase host cultures at a multiplicity of infection (MOI) of 100, adsorbed 5 min at 37 °C. Mixtures were centrifuged at 10,000 × g for 10 min and the pellet was suspended in fresh LB broth. All suspensions were cultured at 37 °C at 200 rpm. Samples were taken every 10 min for 120 min and the double-layer agar technique was performed to measure the titration of each sample. Each of the above experiments were repeated three times, each with triplicate samples.

2.4. Host range

All bacterial strains listed in Table S1 were used to study the host range of the phage. The double agar layer technique was used to test the sensitivity of bacterial strains to the phage and was carried out as previously described above. The degree of plaque clarity was divided into three categories: clear and highly susceptible (++), turbid and partially susceptible (+), and no resistant plaques (-).

2.5. Phage genome sequencing

Genomic phage DNA was extracted from prepared high titer phage particles ($\geq 10^{10}$ PFU/ml) using the Universal Phage Genomic DNA Extraction Kit (Knogen, Guangzhou, China) and stored at −20 °C for sequencing. Whole genome sequencing was performed on the Illumina Hiseq paired-end platform at the Beijing Genomics Institute (Bolger et al., 2014). Genome annotation and open reading frame (ORFs) identification was carried out using the RAST server (Aziz et al., 2008). Nucleotide sequence similarities was identified using Blast-N (NCBI). Putative functions of the ORFs were further identified using Blast-P based on amino acid sequences. A circular map of the phage was depicted using the CGView Server (Grant and Stothard, 2008). The prediction of bacterial virulence genes, antibiotic resistance genes, and tRNA genes were performed with the Virulence Factor Predictor, ResFinder server and tRNAscan-SE v. 2.0 program, respectively.

2.6. Bactericidal activity in vitro

In vitro lysis assays were performed in sterile 96-well plates using a microplate reader (Multiskan Go, Thermo Scientific). *Acinetobacter baumannii* AB9 were grown to mid-log phase before bacterial cells were centrifuged for (5 min, at 6000 × g, 4 °C). Bacterial cells were washed twice with PBS, resuspended in the fresh LB broth and adjusted to a final concentration of approximately 10^6 CFU/ml. Plate wells were filled with 200 μL of the bacterial suspension and 20 μL of phage stock dilutions added at different MOI's (MOI = 0.1, 1, 10 and 100 in triplicates). Three wells were treated with PBS and served as controls. Bacterial growth was monitored over 22 h by measuring the optical density at 600 nm (OD600) every 1 h. All assays were repeated in triplicates.

2.7. Mutation frequency assay

The mutation frequency of *A. baumannii* AB9 treated with phages were determined as previously described by Shen et al. (2018). Cultured of *A. baumannii* AB9 (mid-log phase) were washed twice and

resuspended in PBS. Bacterial suspensions were serially diluted 10-fold (10^0–10^{-7}) and the CFU of the original suspension was counted. Aliquots (0.1 mL) from the 10^0–10^{-5} dilutions were mixed with 10^8 PFU of phage and plated on LB agar plates. After incubating overnight at 37 °C, the mean CFU of surviving bacterial cells were divided by the mean CFU of the original suspension. All assays were repeated in triplicate.

2.8. Phage therapy in the murine bacteremia model

Six to eight week-old female KM mice (19 to 22 g), bred in Da Lian Medical University Laboratory Animal Center, were used for this study. *Acinetobacter baumannii* AB9 was cultured in LB broth overnight at 37°C with shaking, followed by washing of the bacterial cells and resuspending them in 100 μL sterile physiologic saline. Phage D2, phage D0 and the phage cocktail was adjusted to 10^9 PFU/ml using sterile physiologic saline (in a volume of 100 μL). For the murine bacteremia model, groups of five mice were inoculated intraperitoneally (i.p.) with different doses of *A. baumannii* AB9 (3×10^6 to 1×10^9 CFU/mice) to determine the 100% lethal dose (LD100). A total of 10 × LD100 was used in the following animal experiments. Briefly, mice were randomly divided into four treatment groups: (i) sterile physiologic saline (n = 10); (ii) phage D2 (n = 10); (iii) phage D0 (n = 10); (iv) the phage cocktail (n = 10). Mice from all groups were i.p. with 10 × LD100 of *A. baumannii* AB9 bacterial cells. Two hours post-infection, mice of each group were i.p. with an additional 100 μL of phage (10^9 PFU/ml) or sterile physiologic saline. Forty-eight hours after treatment, blood samples were collected from the caudal veins of all surviving mice and used for bacterial colony counts. All *A. baumannii* colonies of each mouse were tested for phage resistance as above described. Survival was tracked for seven days.

2.9. Statistics

Student's *t*-test was used to evaluate significance and considered values of $p < 0.05$ to be statistically significant.

3. Results

3.1. Phage isolation and characterization

Phages were isolated from a sewage sample taken from the second hospital of Dalian Medical University. We have assigned this phage the following name, vB_AbaS_D0. TEM images showed that phage D0 had a very long, non-contractile tail (240 × 15 nm) with about 20 transverse striations and an isometric head (75 nm) (Fig. 1A). TEM micrographs showed that phage D0 should be morphologically classified as a member of Siphoviridae. As shown in Table S2, all tested *A. baumannii* strains were sensitive to the infection of phage D0. Phage D0 formed turbid plaques on *A. baumannii* strains. Our one-step growth curve reflected two important parameters of phage growth cycle, the latent period and burst size (Fig. 1B). The burst size of the phage was determined as the ratio of the phage titer at the end of one cycle of growth to the number of infected bacterial cells. As shown in Fig. 1B, the latent period of D2 was 40 min and burst size was 39 PFU/cell.

3.2. Genome sequence analysis of phage vB_AbaS_D0

In general, analysis of a phage genome is an important approach to ascertain the safety of phage application. The whole genome sequence of *Acinetobacter* phage vB_AbaS_D0 has been deposited in the GenBank database under accession no. MK411820. Genomic sequencing of the complete genome indicated that phage D0 consists of circular, double-stranded DNA with a length of 43,051 bp and a G + C content of 45.48%. No genes suggesting potential virulence or antibiotic resistance were detected in the D0 genome. Prophage repressor and integrase genes were also not detected in the D0 genome. Additionaly, the D0 genome did not encode any tRNAs. Blast-N analysis revealed that only two phages, *Acinetobacter* phage vB_AbaS_Loki and IME_AB3, shared similarity with D0. D0 showed a 99% identity with 100% sequence coverage to IME_AB3 (GenBank: KF811200), and a 75% similarity with 60% sequence coverage to vB_AbaS_Loki (GenBank: LN890663).

The annotation of the D0 genome is summarized in Table S3. The circular genetic map of D0 can been seen in Fig. 2. A total of 55 putative ORFs were predicted in the complete D0 genome, with 27 ORFs predicted as putative functional proteins and the other 28 ORFs as hypothetical proteins. These ORFs could be modularly categorized into four modules: phage structure, host lysis, DNA packaging and replication, and those of other potential functions. Eleven proteins were predicted to be involved in phage structure, namely portal protein major capsid protein (ORF1), scaffold protein (ORF2), head protein (ORF3), tail protein (ORF42), capsid and scaffold protein (ORF45), distal tail protein (ORF46), tail tape measure protein (ORF47), tail completion protein (ORF48), tail chaperonin protein (ORF49, ORF50) and major tail tube protein (ORF51). Two host lysis proteins were predicted, endolysin (ORF7) and holin (ORF8). Three phage DNA packaging proteins were predicted, including portal protein (ORF4), terminase large subunit (ORF5) and terminase small subunit (ORF6). Nine phage DNA replication proteins were predicted, including endonuclease (ORF16), DNA primase/helicase (ORF31), MazG pyrophosphatase (ORF32), recombinase (ORF34), single-stranded DNA-binding protein (ORF35), exonuclease (ORF36), DNA helicase (ORF37), DNA polymerase subunit (ORF39) and DNA polymerase subunit (ORF40).

3.3. Inhibition of host cells growth by phage vB_AbaS_D0 and vB_AbaP_D2

The bacterial lysis assay was carried out to determine the effect of phage D0 and D2 on the growth of *A. baumannii* AB9 host cells. As shown in Fig. 3, the left indicated 1–3 h and the right indicated 1–22 h. The OD values increased in the first 1 h after phage treatment. D2 was also able to completely inhibit growth of the host cells up to 8 h at MOIs ranging from 0.01 to 100. In the bactericidal process of D2, the phage resistant cells occurred at about 10 h post-infection (Fig. 3A). Although the bactericidal ability of D0 was weak and dependent on the MOI, the bactericidal efficacy of D2 improved with D0 (Fig. 3B). In the presence of D0 (MOI = 0.01), D2 (MOI = 0.01) could completely suppress cell growth over 18 h (Fig. 3C).

3.4. The combination of phage vB_AbaS_D0 and vB_AbaP_D2 inhibits development of phage resistance

Phage resistance always occurred in the bactericidal phage process. The development of phage resistance was investigated from two aspects. First, we tested the treatment duration of phage D0 and D2, which is an important indicator for phage therapy. As shown in Fig. 4, almost all of the bacterial cells were killed in the phage cocktail (D0 + D2) treatment group at time points 12- and 24-h. Although the numbers of viable bacteria increased in the combination group at the 36-h time point, the bactericidal activity of the combination group was significantly stronger than the other treatment groups ($P < 0.01$). Secondly, we tested the mutation frequency towards phage resistance (Fig. 5). The frequency of phage resistance for D2 was 1651×10^{-7}, which are much higher than that of D0 or the phage cocktail ($P < 0.01$). These results imply that phage cocktail could control the development of phage resistance and extend the duration of phage therapy.

3.5. Therapeutic effectiveness of two phages against lethal bacteremia

The murine bacteremia model was established by i.p. injections with 10 × LD100 (2×10^7 CFU/mice) of *A. baumannii* AB9. Mortality was 100% after 24 h in the group of physiologic saline treatment.

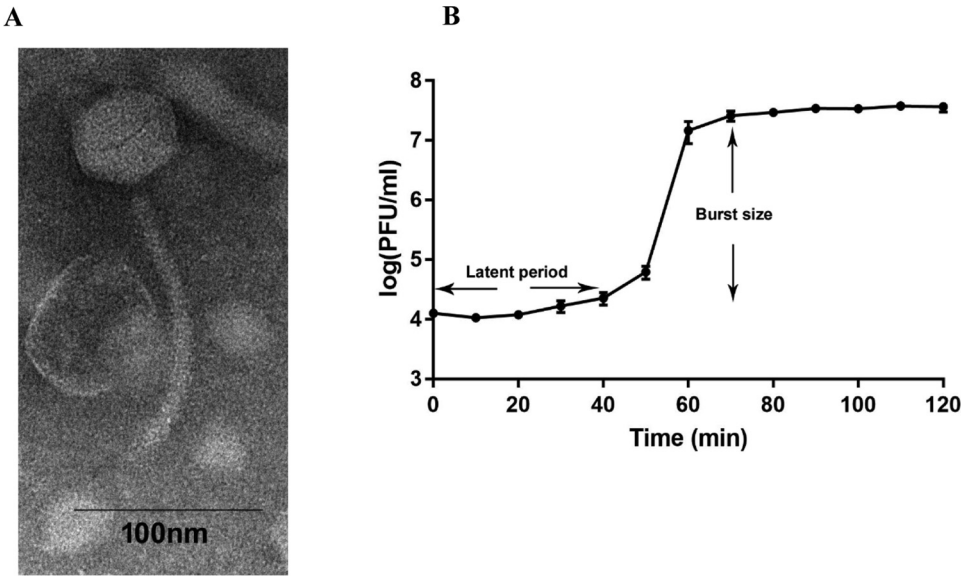

Fig. 1. (A) Transmission electron micrographs of phage vB_AbaS_D0. Scale bar represents 100 nm. (B) One-step growth curve of phage vB_AbaS_D0. Values represent the means ± SD (n = 3).

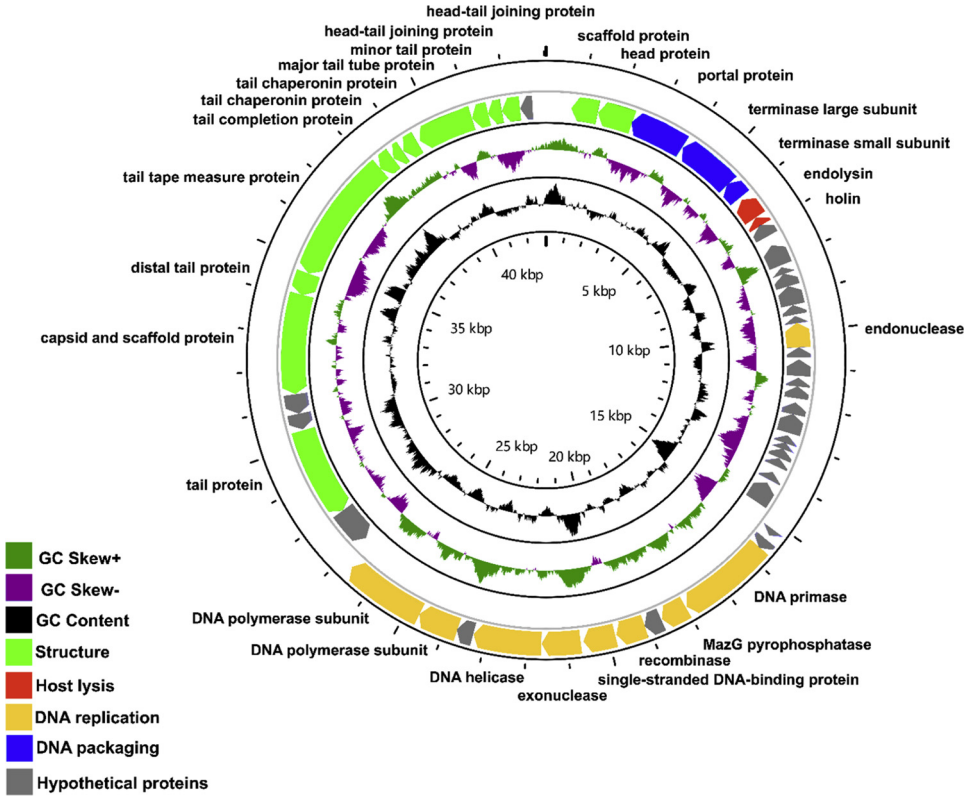

Fig. 2. Genetic map of phage vB_AbaS_D0 prepared using CGView (Circular Genome Viewer). The color of the ORFs refers to five modules: phage structure, light green; host lysis, red; DNA packaging, blue; DNA replication, yellow; and hypothetical proteins, gray. GC content, positive and negative GC skew is shown as black, green and purple, respectively.

Treatment with phage D0 yielded 50% survival after 36 h. In contrast, 90% or 100% of mice survived treatments with phage D2 or phage cocktail, respectively (Fig. 6). To determine development of phage resistance among the treatment groups, we tested phage resistance from colonies isolated from samples of mouse blood. As shown in Fig. 7 and Table S4, the percentage of phage resistant mutants occurring in the phage D2 treatment group was significantly higher than in the other two treatment groups ($P < 0.01$).

4. Discussion

Antibiotic resistance has become a major public health crisis, incurring both lives lost and economic costs (Magiorakos et al., 2012). For this reason, phages are now being reevaluated as a promising agent in the treatment of pathogenic infections worldwide (Nobrega et al., 2015). The advantages and challenges of phage use have already been extensively studied (Loc-Carrillo and Abedon, 2011). So far, the rapid emergence of phage resistant mutants has become a notable challenge for the effective treatment by phages (Dy et al., 2014). Phages and bacteria have coexisted for billions of years, and phage resistance is an

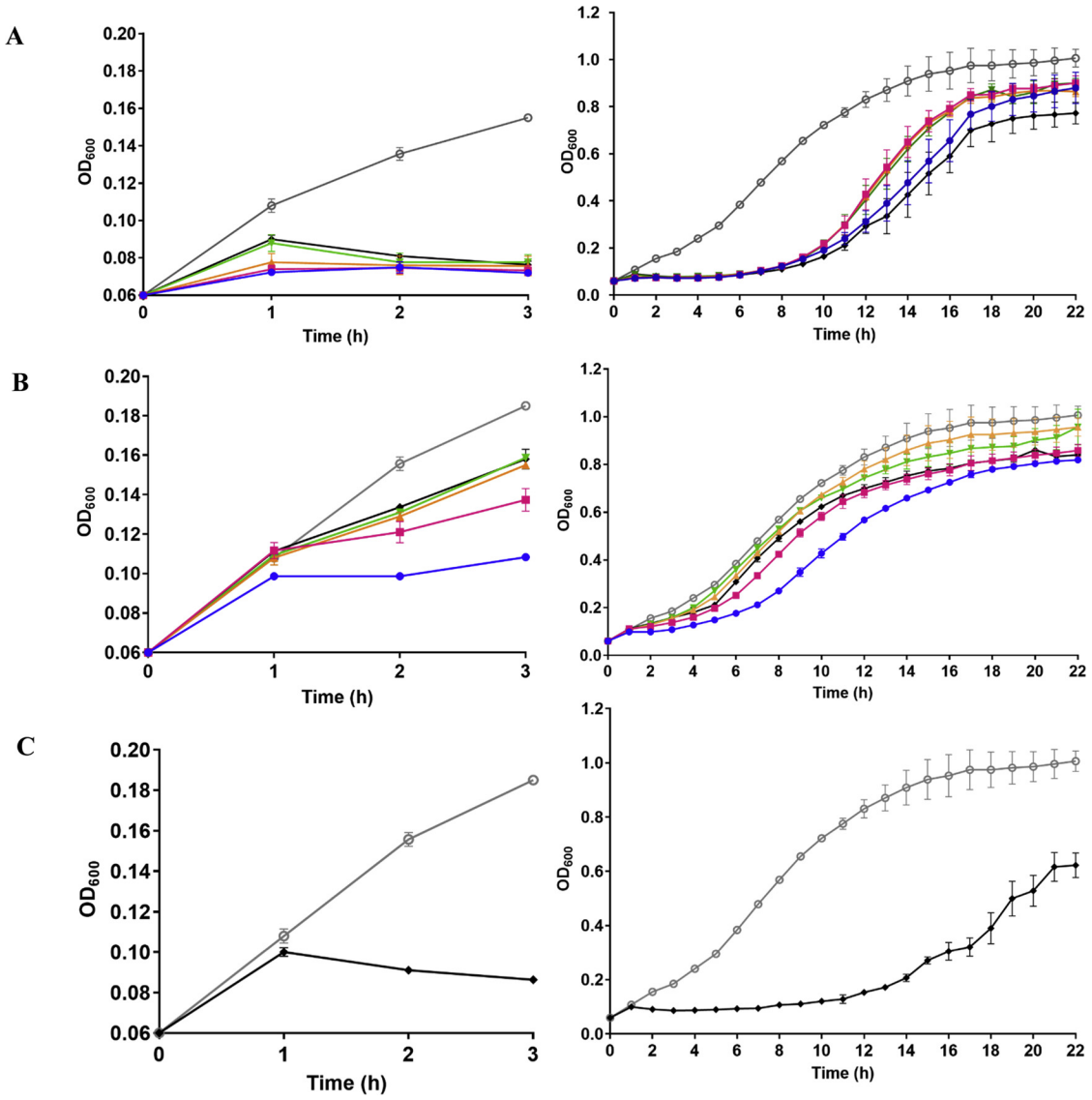

Fig. 3. The time course of host bacteria lysis by phages during 3 h and 24 h. *Acinetobacter baumannii* AB9 was infected with phage D0 (A), D2 (B) at different MOIs, respectively. (C) *Acinetobacter baumannii* AB9 was infected with phage D2 (MOI = 0.01) in the presence of D0 (MOI = 0.01). Optical density (OD) was measured at 600 nm. Error bars are standard deviations of three replicates. (○) Control, (●) MOI = 100, (■) MOI = 10, (▲) MOI = 1, (▼) MOI = 0.1, (◆) MOI = 0.01.

inevitable evolution of their interaction (Hatfull, 2008; Kortright et al., 2019). In a wide range of ecological niches, phage resistance is an important survival phenotype, both in the laboratory or in the natural environment (Chan et al., 2013). Therefore, the emergence of phage resistance is quite common in the process of phage therapy. Many approaches have been used to control the development of phage resistance. Among them, phage cocktails or the combination of phages and antibiotics is the first to be considered (Pereira et al., 2016; Valerio et al., 2017). Phage cocktails or the combination of phages and antibiotics can not only broaden host range, but also prolong the appearance of phage resistant mutants. However, there are unknown interaction mechanisms of phage and host in the use of phage cocktails or combinations of phages and antibiotics. Initially it was thought to increase the number of phages in the cocktail or increase the dose of antibiotics, however, this combination negatively effects the normal bacterial flora in raised animals and in humans. To remedy this Chan et al. (2013) suggested using less complex cocktails, consisting of two to ten distinct phages as a reasonable choice.

In this study, we used a phage that was difficult in causing resistance as a supplement to another virulent phage to control the appearance of phage resistant mutants and avoid the overuse of phages. In the process of phage isolation, we obtained *A. baumannii* phage D0. This phage not only exhibited a broad host range, but also had a low resistance potential.

The complete genome sequence and analysis of D0 showed no prophage repressor genes, integrases or other lysogeny related factors, and suggests that D0 is a virulent phage. To be on the safe side, phages that possess genes for virulence and antibiotic resistance should not be used as biocontrol agents. Phage-mediated transfer has been suggested as a major driving force of virulent bacteria evolution (Chen and Novick, 2009; Lindsay and Holden, 2004). In fact, several previous reports showed that phages were able to convert their host bacterial strains to new virulence phenotypes (Chen et al., 2015; Yamaguchi et al., 2000; Zhang et al., 2002). Thus, genomic analyses are also an essential tool in order to identify those phages of potential value and risk. In our study, the genomic analyses performed showed no lysogeny, virulence or antibiotic resistant genes were in phage D0. These results indicate that D0 is a virulent phage and is safe to be used as a therapeutic agent.

In our previous work, we had obtained the virulent phage D2, which had strong bactericidal abilities against multidrug resistant *A. baumannii*. However, the host bacterial cells could rapidly develop phage

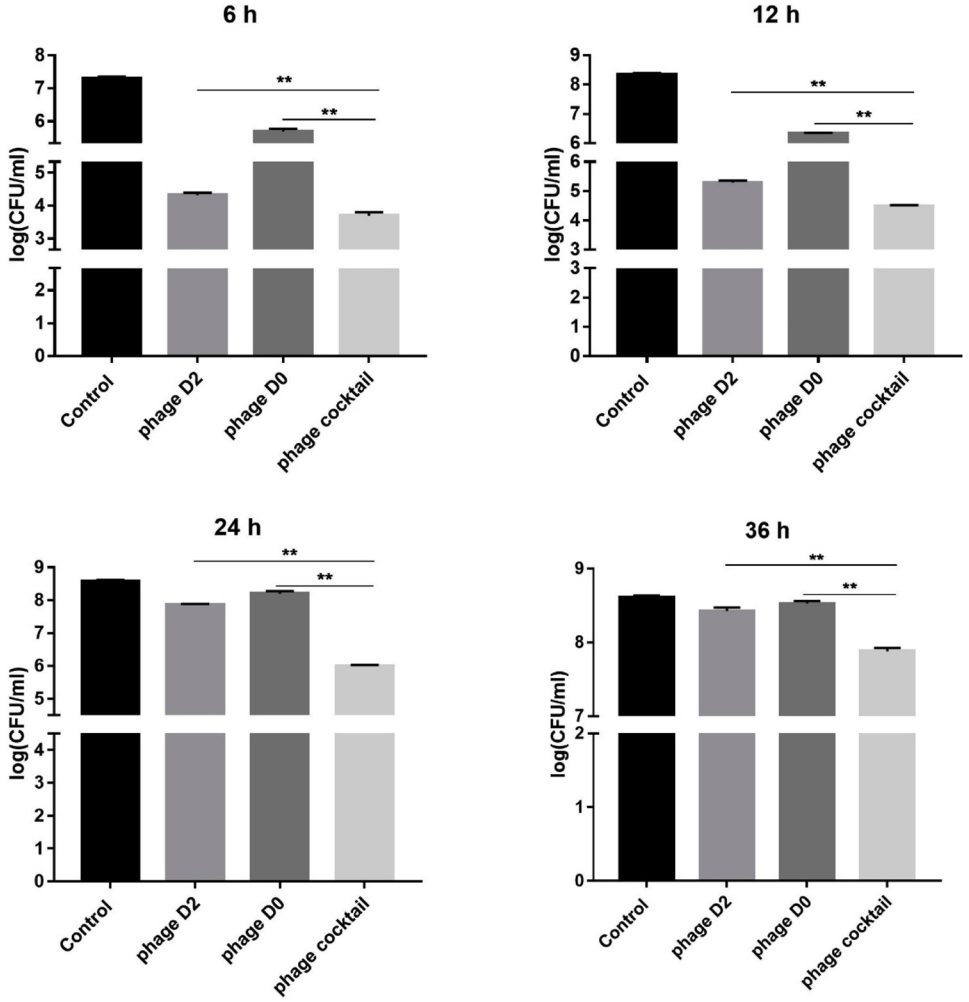

Fig. 4. Bactericidal activity of phages in vitro over a 36-h time course. The host bacteria of *Acinetobacter baumannii* AB9 was treated with phage D0, D2, the combination of D0 and D2 at an appropriate titer. The same volume of PBS was added as control. The viable host cell numbers (CFUs) were calculated by plate counting at different time points (6 h, 12 h, 24 h, 36 h). Error bars are standard deviations of three replicates. Significance was performed using the Student *t* test. ** $P < 0.01$.

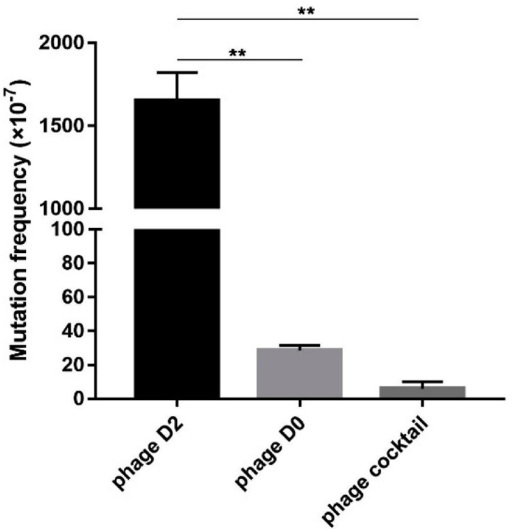

Fig. 5. The phage-resistant mutation frequency of the host bacteria. Error bars are standard deviations of three replicates. Significance was performed using the Student *t* test. ** $P < 0.01$.

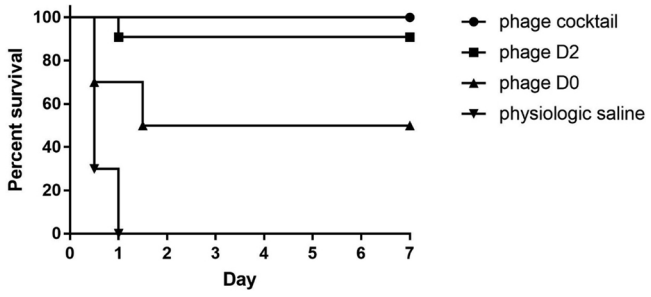

Fig. 6. Phages increased the survival rate of mice with a lethal infection of *Acinetobacter baumannii*.

resistance in the process of phage D2 infection. It is important to note that the rapid emergence of resistant mutants is a common feature in the phage treatment process (Maciejewska et al., 2018). To date, many approaches have been proposed to inhibit the growth of resistant mutants. For example, in one study, Gu and colleagues used both original bacterial strains and phage resistant mutants as hosts to isolate phages and develop phage cocktails (Gu et al., 2012). In another study, Filippov et al. used site-directed mutagenesis and trans-complementation, identifying six receptors for eight *Yersinia pestis* phages (Filippov et al., 2011). They also pooled phages in a phage cocktail to exploit different

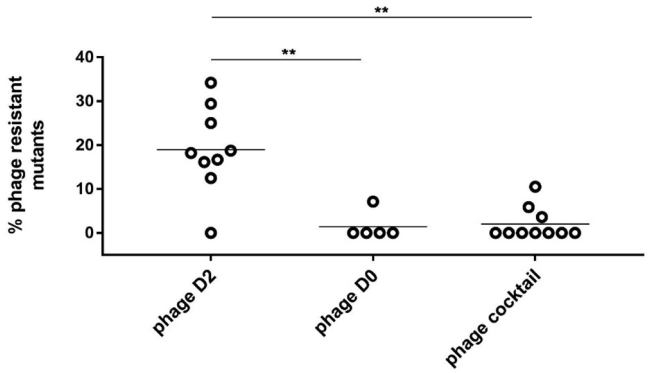

Fig. 7. In vivo phage resistant mutant percentage in the phage treatment groups. Forty-eight hours after treatment, blood samples were collected from the caudal veins of all surviving mice and used for bacterial colony counts. All colonies obtained from the surviving mice were tested for phage resistance. Each circle represents the phage resistant mutant percentage of one surviving mouse. Error bars are the mean of each treatment group. Significance was performed using the Student *t* test. ** $P < 0.01$.

receptors in order to overcome the emergence of phage resistant mutants. Similar to the above approaches, Kelly et al. developed a broad host range phage cocktail to eliminate phage resistance in *Staphylococcus aureus* (Kelly et al., 2011). In our study, we used the combination of only two phages (D2 and D0) to overcome their limitations. We also evaluated its therapeutic efficacy and the resistance potential in vivo and in vitro.

Our time course experiments showed that the phage cocktail was more efficient towards inhibiting the growth of host cells than using a single phage. The increase in the therapeutic efficacy by our phage cocktail was also observed and is due to the delayed emergence of phage resistant mutants. These results demonstrated that our phage cocktail had a stronger antibacterial activity and a longer usable time than single phages alone. This conclusion was also supported by counting active bacteria in the bactericidal activity assay. The host bacterial cells in the single phage group (phage D2) was shown to rapidly develop resistance over short periods (12 h). Relative to the use of a single phage (i.e., D2), our phage cocktail needed a longer period (36 h) to cause phage resistance in host cells. To further understand development of phage resistance during phage therapy, the mutation frequency of phage resistance was tested. Our results showed that the mutation frequency towards resistance of *A. baumannii* cells infected with phage cocktails or single phage D0 was significantly lower than cells treated with the single phage D2. We also evaluated the development of phage resistance over time. Use of phage cocktails or the single phage D0 was more effective in inhibiting development of phage resistance than the single use of phage D2. Such findings suggest that phage D0 might play an important role in controlling development of phage resistance in our phage cocktail.

To evaluate the therapeutic effect of our phages, we also performed in vivo experiments in mice. Treatment with the single phage D2 or the phage cocktail was very effective, reaching 90% and 100% survival rates, respectively. Nevertheless, bactericidal effect of the single phage D0 was very weak in vitro, yet still protected 50% of mice from the fatal *A. baumannii* infection. These results imply that our phages could aid in recovering mice from lethal infections of multidrug resistant *A. baumannii.* We also tested phage resistance in colonies isolated from smaples of mouse blood to evaluate development of phage resistance in vivo. The percentage of phage resistant mutants in mice treated with our phage cocktail or the single phage D0 was significantly lower than that of mice treated with the single phage D2.

Taken together, the cocktail of phage D0 and D2 could improve the therapeutic efficacy and overcome shortcomings of the single use of each phage. We propose that phage D0 can be used as a stable supplement to phage therapy of *A. baumannii* infections. Phage D0 can also be combined with another *A. baumannii* phage to form a therapeutic cocktail containing only two phages since phage D0 has a lower propensity to induce resistance. Moreover, phage D0 has a broad host spectrum, which enables it to combine with different *A. baumannii* phages. Phage D0 is an effective agent to delay and reduce the appearance of phage resistant mutants, thus to extend durability of other phage treatments.

Appendix A. Supplementary data

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