

ISOLATION OF LYTIC BACTERIOPHAGE AB72P AGAINST MULTI-DRUG RESISTANT *ACINETOBACTER BAUMANNII* ISOLATES OBTAINED FROM BURN INFECTION

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Abstract

Background & Aims: *Acinetobacter baumannii* is a gram-negative pathogen that causes a wide range of hospital-acquired infections. Due to its intrinsic traits and its remarkable abilities to quickly acquire resistance genes, it has become resistant to most antimicrobial agents and a major problem for hospitals. In recent years, application of lytic bacteriophages has been considered to eradicate multi-drug resistant (MDR) *A. baumannii* from the clinical setting. The purpose of this research was to isolate a lytic bacteriophage against MDR *A. baumannii* isolate obtained from an infected burn site.

Materials & Methods: Different samples including lake, river and seawater, soil and municipal wastewater were tested for phage isolation by spot test and agar overlay method.

Results: Lytic bacteriophage Ab72p against MDR *A. baumannii* isolate was isolated from municipal wastewater sample and classified as a member of *Myoviridae* family. The plaques formed by lytic phage Ab72p were completely clear plaque and had a diameter of 1-2 mm without halo. Phage Ab72p had an about 11 nm (in diameter) icosahedral head and a 17-22 nm (in length) contractile tail. The genome of phage Ab72p was double-stranded DNA with about 46 kb. Phage Ab72p was a very stable lytic phage in the high temperatures (up to 70 °C) and wide range of pH (3 to 11), with an appropriate adsorption rate (99% adsorbed within 10 minutes), and large burst size (200 PFU per infected cell).

Conclusion: Phage Ab72p could be appropriate choice to prepare phage cocktail against *A. baumannii* isolates.

Keywords: Bacteriophage; Ab72p; *Acinetobacter baumannii*; Multi-drug resistant; Wastewater

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Introduction

Acinetobacter baumannii is a gramnegative pathogen, cause of a wide range of hospital-acquired infections including skin and soft-tissue, urinary tract, wound, bloodstream and surgical site infections,

ventilator-associated pneumonia, secondary meningitis, endocarditis, intra-abdominal abscess, and osteomyelitis. It mainly affects patients hospitalized in intensive care units (ICUs) including trauma patients, burn patients and any debilitated and

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immunocompromised patient. Due to its intrinsic traits and its remarkable abilities to quickly acquire resistance genes, it has become resistant to most antimicrobial agents (almost all common antibiotics) and a major problem for hospitals (1-5). In recent years, new approaches such as application of lytic bacteriophages have been considered to eradicate multi-drug resistant (MDR) or pan-drug resistant (PDR) *A. baumannii* from the clinical setting. Up to now several lytic phages infecting MDR *A. baumannii* strains have been isolated and characterized that most of them had narrow host range and were strain or isolate specific phages (6-16). None of the isolated lytic phages were effective against all isolates of *A. baumannii* species. Thus, isolating different lytic phages against different MDR isolates of *A. baumannii* can be useful to prepare an effective phage cocktail for a broad range of *A. baumannii* strains. Considering the importance of confronting nosocomial *A. baumannii* infections, their increasing resistance to the most commonly used antibiotics, and the safety of phage therapy using highly specific lytic phages in humans; this research was aimed to isolate a lytic bacteriophage against MDR *A. baumannii* isolate obtained from an infected burn site.

Materials and Methods

Bacterial isolate

MDR *A. baumannii* ImdAb72 (resistant to amikacin, cefotaxime, ceftriaxone, ciprofloxacin, imipenem, and piperacillin) was isolated from burn site of a burn patient hospitalized in Isfahan, Iran (17) and it was selected for isolation of lytic bacteriophage. Identification of the bacterial isolate was performed using biochemical tests and sequencing of a part of the Zone 1 of *rpoB* gene.

Phage Isolation

Different samples including lake, fountain-head, river and seawater, soil of a recreational site, municipal

wastewater and sewage activated sludge were used for phage isolation. Fifteen milliliters of Brain-Heart broth (Merck, Germany) at double concentration was inoculated with 100 µl overnight culture of *A. baumannii* ImdAb72. After 2h of incubation with shaking (80 rpm) at 37 °C, 15 ml of the supernatant of a sample containing likely phage was added to it (to isolate phage from soil sample, an equal volume of water was added to soil. Then the mixture was shaken well. After sedimentation the supernatant was used). The mixtures were incubated with shaking (80 rpm) at 37 °C overnight. Then, the mixtures were centrifuged at 10000 g for 10 minutes at 4 °C and supernatants were filtered with a 0.45 µm filter (Orange Scientific Company, Belgium). The presence of lytic bacteriophage was investigated by plaque-forming after spotting 10 µl of the filtrate onto the top of doublelayer agar (soft Brain-Heart agar (0.7% agar) containing *A. baumannii* ImdAb72 overlaid on the surface of solidified Brain-Heart agar with 1.5% agar) (7).

Phage purification

To purify the lytic bacteriophage, serial dilutions (10^{-1} to 10^{-10}) of the filtrate in SM buffer (50 mM Tris-HCl PH 7.5, 10 mM MgSO₄ (7H₂O) and 100 mM NaCl) was prepared. One hundred microliters of each dilution was mixed with 100 µl overnight culture of *A. baumannii* ImdAb72 and 10 ml molten (45 °C) soft Brain-Heart agar (0.7% agar). The mixture was poured on the surface of solidified Brain-Heart agar with 1.5% agar. After incubation of the plate containing double-layer agar at 37 °C overnight, a single plaque was picked and placed in the Brain-Heart broth containing *A. baumannii* ImdAb72 to isolate single phage. To ensure the purity of the isolated phage, this process was repeated 3 times. The phage titer was determined by assaying serial dilutions of phage suspension with the agar overlay method, as described above.

Investigation of phage host range

Different isolates of *A. baumannii* (*A. baumannii* ImdAb04, ImdAb06, ImdAb10, ImdAb12, ImdAb35, ImdAb47, ImdAb48, ImdAb68, and ImdAb74) were identified using biochemical tests and sequencing of a part of the Zone 1 of *rpoB* gene. The obtained isolates from burn sites of burn patients (17) were used to determine the host range of isolated lytic phage by spot test and agar overlay method. Thus, 100 µl overnight broth culture of each *A. baumannii* isolate was mixed with molten 0.7% soft Brain-Heart agar (45 °C), and then this mixture was poured on the surface of solidified Brain-Heart agar with 1.5% agar. After hardening the top agar, 10µl phage stock solution was spotted onto the top agar layer. After overnight incubation at 37 °C, the observation of plaque was considered as an indication of susceptibility of *A. baumannii* isolate to the isolated phage.

Transmission electron microscopy

The phage suspension, at a concentration of 10^{11} PFU/ml, was placed on carbon-coated copper grids and after 3.5 minutes, negatively stained with 2% uranyl acetate. After drying, the phage particles were investigated by a transmission electron microscope (TEM CM10 PHILIPS, Netherlands).

Phage adsorption rate

Fifty microliters of phage suspension (10^8 PFU/ml) was mixed with 5 ml overnight broth culture of *A. baumannii* ImdAb72 ($OD_{600} = 0.15$) and incubated at 37 °C. One hundred microliters of the mixture was removed after 1, 2, 3, 4, 7, 10, and 20 minutes and diluted in 850 µl SM buffer and 50 µl chloroform. After centrifugation (8000 g at 4 °C for 2 minutes), the supernatants were removed and titrated for determination of unadsorbed phages by the double-layer method (7, 11).

One-step growth assay

In the one-step growth experiment to determine the latent period (the interval between adsorption of the phage to the bacterial cell and release of phage progeny) and burst size (the ratio of the final number of released phage particles to the number of infected bacterial cell during latent period) of the isolated phage, 20 ml overnight broth culture of *A. baumannii* ImdAb72 ($OD_{600} = 0.3$) was centrifuged (6000 g for 5 minutes). The pellet was resuspended in 0.5 ml sterilized and heated (37 °C) Brain-Heart broth. The phage suspension was added to the bacterial suspension at MOI of 0.01 and mixed. After incubation at 37 °C for 10 minutes to adsorb the phage, the mixture was centrifuged at 10000 g for 1 minute to remove unadsorbed phage particles. The pellet was resuspended in 10 ml of sterilized and heated Brain-Heart broth (37 °C) and incubated at 37 °C. The samples were taken at 10-minutes intervals up to 100 minutes and immediately titrated (11, 14).

Thermal stability

To determine the thermal stability of the isolated phage, 250 µl of phage suspension (10^8 PFU/ml) was incubated at different temperatures (50 °C, 60 °C, 70 °C, 80 °C, and 90 °C) for 1h and then titrated. Also, the stability of phage was determined after storage of the phage suspension at 4 °C for a month to 2 years and after lyophilizing at -50 °C.

pH stability

To measure the pH stability of the isolated phage, 10 µl of phage suspension (10^8 PFU/ml) was incubated in 990 µl SM buffer at different pH values of 3, 5, 7, 9 and 11 at 37 °C. Samples were taken after 1h and 2h and immediately titrated. One molar HCl or 1M NaOH was used to prepare SM buffers with specific pH values.

Isolation and restriction analysis of phage DNA

After incubation of purified and concentrated phage particles in lysis buffer (25mM Tris-HCl PH 8, 10mM Na_2EDTA ($2H_2O$) pH 8, 10mM NaCl and 1% SDS PH

7.2) and proteinase K (70 µg mL⁻¹; Fermentas)) at 37 °C for 15 minutes and then at 60 °C for 15 minutes, the phage DNA was extracted using phenol/chloroform/isoamyl alcohol (25/24/1) and precipitated with isopropanol. Endonucleases EcoR1 and Hind III were used to digest the phage DNA according to the instruction provided by the manufacturer. The phage genome and DNA fragments were investigated by loading on an agarose gel in TBE electrophoresis buffer. DNA ladder from 100 to 10000 bp and DNA of phage WP-2 with a certain size (about 19 kb) (18) were used as markers. The size of the phage genome was estimated by SequentiX-Gel Analyzer software (www.sequentix.de).

Results

Isolation of lytic bacteriophage against *A. baumannii* ImdAb72

Of different samples tested, lytic bacteriophage against *A. baumannii* ImdAb72 was isolated only from the municipal wastewater sample. Completely clear plaque without halo was detected by spot test and agar overlay method (Figure 1a). The sizes of plaques formed by individually purified bacteriophages were about 1-2 mm in diameter (Figure 1b). Since the isolated phage infected the *A. baumannii* ImdAb72, it was designated as Ab72p.

Phage host range determination

Phage Ab72p only infected *A. baumannii* ImdAb72 and did not infect other tested isolates of *A. baumannii*. Thus phage Ab72p had a high specificity for *A. baumannii* ImdAb72.

Phage morphology

According to transmission electron microscope results, phage Ab72p with an about 11 nm (in diameter)

icosahedral head and a 17-22 nm (in length) contractile tail, was classified as a member of *Myoviridae* family in the order *Caudovirales* (Figure 2).

Phage adsorption rate

Ninety-nine percent of the phage Ab72p particles adsorbed on the *A. baumannii* ImdAb72 within 10 minutes after incubation of the mixture of phage Ab72p and host bacterial cells at 37 °C (Figure 3).

One-step growth

Latent period and burst size are important traits of the phage infection process. To determine the latent period and burst size of phage Ab72p, one-step growth experiment was performed (Figure 4). The latent period of phage Ab72p was 50 minutes and the burst size was approximately 200 phage particles per infected cell.

Phage pH and thermal stability

By investigating phage Ab72p stability at different pH levels, phage stability of 100% was observed after 2 hours of incubation at pH values of 3 to 11.

The thermal stability test showed that phage Ab72p was extremely heat stable, so that 100% of the phages were stable and active after 1-hour of incubation at 50 °C, 60 °C, and 70 °C and none of the phage particles were detected at 80 °C after 1-hour incubation. Also, 100% of the phages were stable and active after 2 years storage at 4 °C. But only 10 percent of the lyophilized phages retained their activities after one-year storage at 4 °C.

Phage genome

As the DNA was digested by restriction endonucleases (EcoR1 and Hind III), the genome of phage Ab72p was double-stranded DNA (Figure 5). As shown in Figure 5, the phage genome size was estimated to be about 46 kbp.

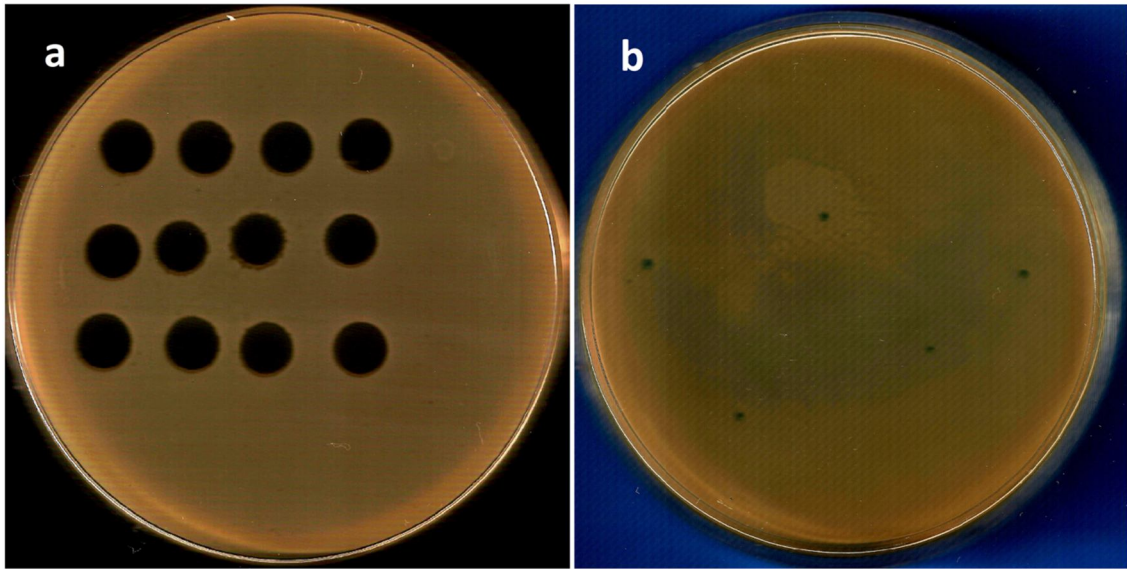


Figure 1. a) The clear plaques without halo formed by phage Ab72p after spotting 10 μ l of the phage onto the top of doublelayer agar containing *A. baumannii* ImdAb72, b) The sizes of plaques formed by individual purified bacteriophages.

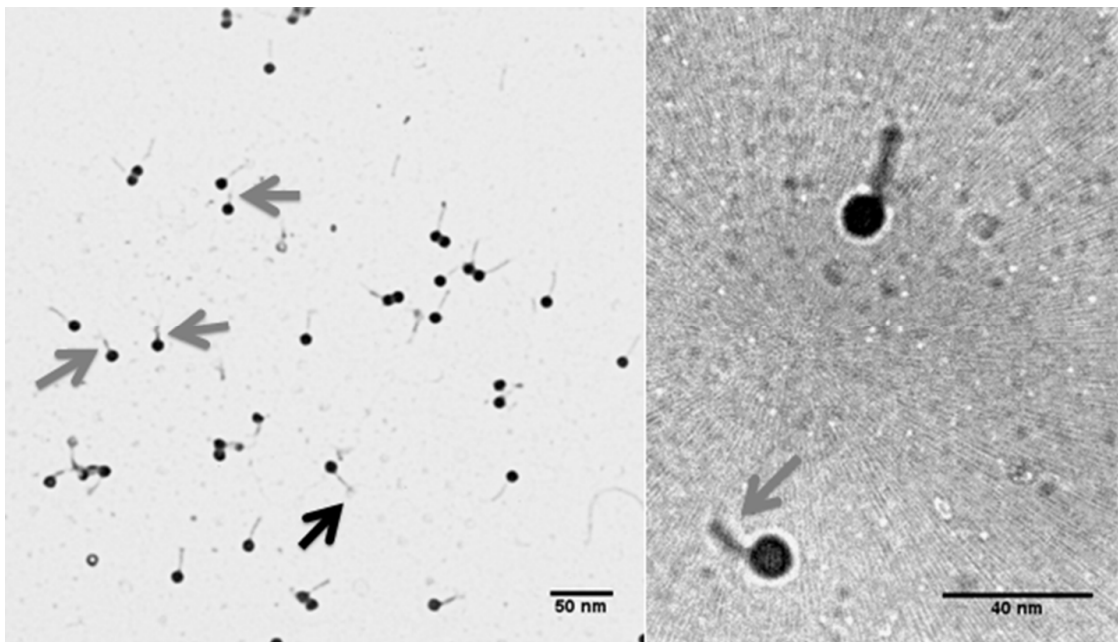


Figure 2. Transmission electron microscopy of phage Ab72p particles. Grey arrows: contracted tail; black arrow: baseplate with fibers.

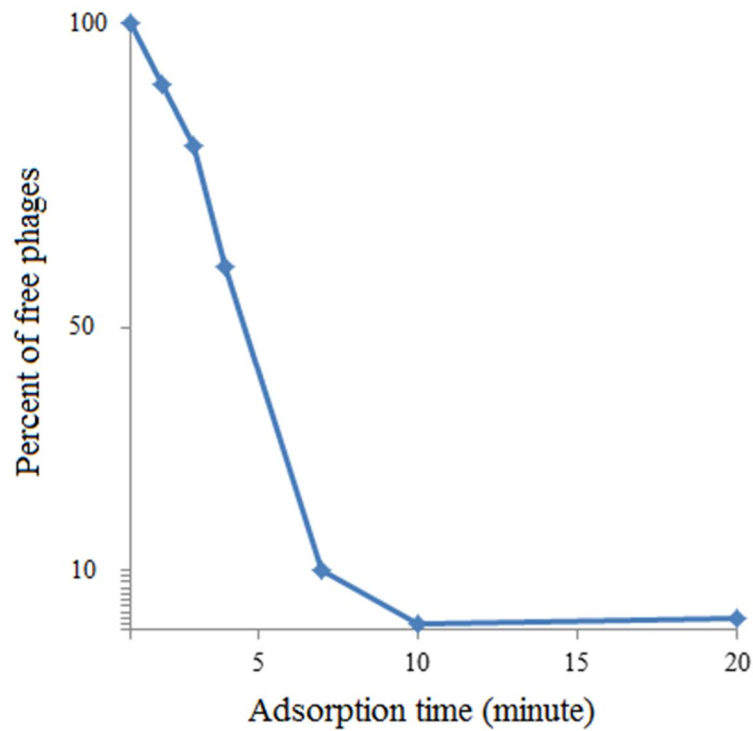


Figure 3. Adsorption curve of phage Ab72p.

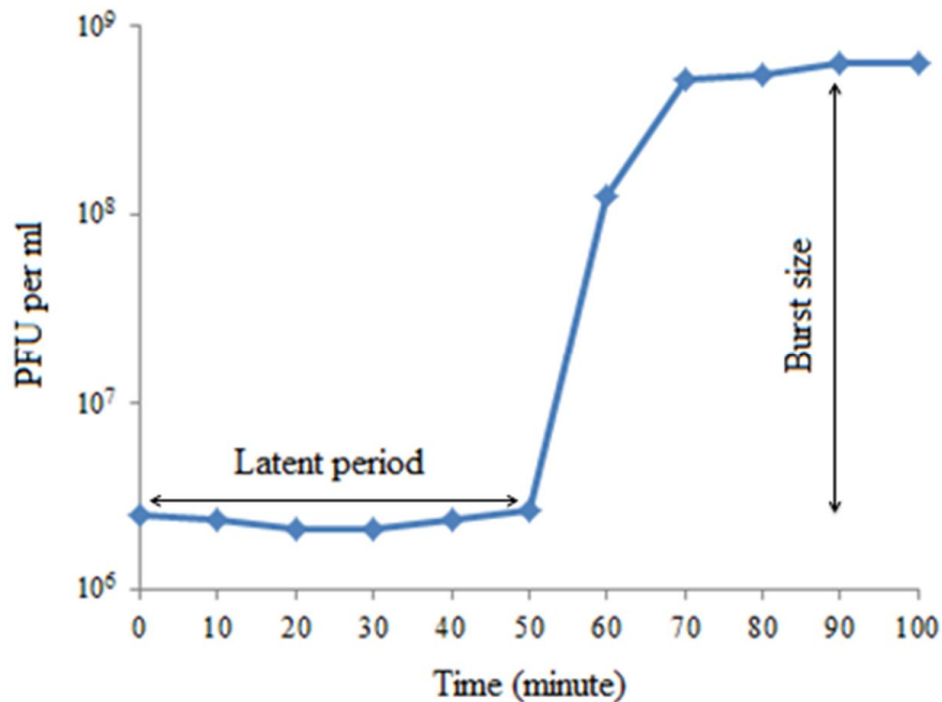


Figure 4. One-step growth curve of phage Ab72p. The latent period was approximately 50 minutes and the burst size was approximately 200 phage particles per infected cell.

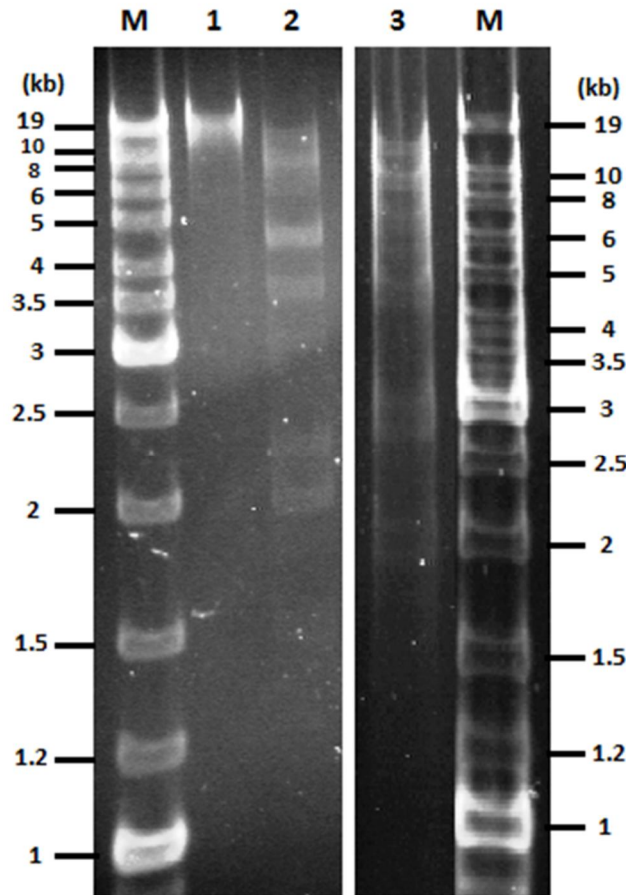


Figure 5. Restriction digestion of phage Ab72p genome. 1, phage Ab72p genome; 2, EcoRI; 3, HindIII; M, DNA ladder.

Discussion

MDR or PDR clinical strains of *A. baumannii* are the cause of a wide range of nosocomial infections. Infections caused by them sometimes life-threatening especially in debilitated and immunocompromised patients hospitalized in ICU. Due to resistance to antimicrobial agents such as detergents, disinfectants, and almost all common antibiotics, fighting against *A. baumannii* clinical strains has become an important challenge for clinicians and clinical settings (1-5, 11-14). In recent years, applications of lytic bacteriophages (phage therapy) has been considered to combat MDR bacteria and up to now lytic phages against important MDR bacteria have been isolated, characterized, and used to treat bacterial infections in various regions of the world such as phage therapy centers in Georgia and

Poland (19, 20). Also until now several lytic phages infecting MDR strains of *A. baumannii* have been isolated and characterized in different studies (6-16).

The major problem in the application of lytic phages against *A. baumannii* isolates for phage therapy is the narrow host range of isolated phages. Lytic phages including Ab72p (isolated in this study and belonging to *Myoviridae* family), AB1 (belonging to *Siphoviridae* family) (14), Abp1 (belonging to *Podoviridae* family) (6), IME-AB2 (belonging to *Myoviridae* family) (10), ZZ1 (belonging to *Myoviridae*) (7), Abp53 (belonging to *Myoviridae*) (8), AB7-IBB1 (belonging to *Siphoviridae*) (15) and AP22 (belonging to *Myoviridae*) (11) infected 1 of 10, 1 of 5, 2 of 80, 3 of 22, 3 of 23, 7 of 26, 23 of 39 and 89 of 130 clinical strains (or isolates) of *A. baumannii*, respectively. Of 8 lytic phages infecting *A. baumannii* strains isolated by Shen *et al.*

(2012), phage ϕ km18p belonging to *Corticoviridae* family, by lysing 15 of 34 *A. baumannii* isolates, had the widest host range (12). Among the above-mentioned lytic phages, only phages AP22 and AB7-IBB1 were capable of lysing more than 50% of *A. baumannii* isolates. Nonetheless these two phages were not able to infect all (or almost all) of *A. baumannii* isolates and up to now a phage lysing all isolates of *A. baumannii* (species-specific lytic phage) is not isolated. Thus, it seems that preparation of phage cocktail using appropriate lytic phages obtained from different studies is needed for phage therapy against infections caused by *A. baumannii* strains.

Different parameters such as type of plaque formed by phage, stability in different pH levels and temperatures, adsorption rate, latent period, and burst size are considered for selection of appropriate lytic phage for preparation of a phage cocktail.

Regarding stability at different pH values, phage Ab72p compared to other lytic phages infecting *A. baumannii* strains (such as phages ZZ1 (7), AP22 (11) and AB1 (14)) is stable in a wider range of pH values; so that the phage Ab72p completely retained its infectivity after 2 hours of incubation at pH levels between 3 and 11. While phage ZZ1 was active after 1-hour of incubation at pH levels between 4 and 9 and was completely inactivated at pH 10 or higher and pH 3 or lower. Also, phage AP22 was active after incubation at pH levels between 4 and 9 and phage AB1 was affected at higher and lower levels than pH 6.

In high temperatures, the thermal stability of phage Ab72p was higher than the thermal stability of other heat-stable lytic phages infecting *A. baumannii* strain such as phages ZZ1 (7) and AB1 (14). Phage Ab72p was completely active after 1-hour incubation at 70 °C, while only very few AB1 phage particles were alive after 1-hour incubation at 70 °C and phage ZZ1 was only

stable at 60 °C and was completely inactivated after 40 minutes of incubation at 70 °C. Phage Ab72p like phages ZZ1 (7) and Abp53 (8) was stable at 4 °C for months. It seems that phages are stable at 4 °C for a long time. In other studies, survival rates of isolated lytic phages against *A. baumannii* strains after lyophilization were not investigated. In this study only 10% of phage Ab72p particles were alive and active after lyophilizing at -50 °C after one-year storage at 4 °C. Overall, pH and thermal stability of phage Ab72p are more than the stability of other reported lytic phages lysing *A. baumannii* strains.

Adsorption is the first and important step of the phage infection cycle. Ninety-nine percent of phage Ab72p particles were adsorbed to the host bacterium within 10 minutes; similar to adsorption rate of phage Acibel007 (95% of the phage particles adsorbed within 10 minutes) (9), slower than adsorption of phages Abp53 (99% adsorbed at 6 minutes) (8), AP22 (> 99% adsorbed within 5 minutes) (11), AB7-IBB1 (> 99% adsorbed in 5 minutes) (15) and IME-AB2 (99% adsorbed within 9 minutes) (10) and faster than adsorption of phages Acibel004 (85% adsorbed within 15 minutes) (9) and AB1 (99% adsorbed in 20 minutes) (14). Phage Ab72p adsorbed to the bacterial host in a relatively short time and altogether has an appropriate adsorption rate.

In the one-step growth experiment, the latent period of phage Ab72p was 50 minutes. Up to now this time is the longest time among latent periods detected for lytic phages infecting *A. baumannii* strains such as AP22 (40 minutes) (11), AB7-IBB1 (30 minutes) (15), Acibel004 (27 minutes) (9), Acibel007 (21 minutes) (9), IME-AB2 (20 minutes) (10), AB1 (18 minutes) (14), Abp1 (10-15 minutes) (6), Abp53 (10 minutes) (8) and ZZ1 (9 minutes) (7). Also based on one-step growth experiment, phage Ab72p has a large burst size of 200

PFU per infected cell, similar to burst size reported for phage ZZ1 (7), smaller than those reported for phages AB1 (409 PFU/infected cell) (14), Abp1 (350 PFU/infected cell) (6) and AP22 (240 PFU/infected cell) (11), and larger than burst sizes of phages IME-AB2 (62 PFU/infected cell) (10), Acibel004 (125 PFU/infected cell) (9), AB7-IBB1 (125 PFU/infected cell) (15), Acibel007 (145 PFU/infected cell) (9), and Abp53 (150 PFU/infected cell) (8).

Accordingly, phage Ab72p that is a very stable lytic phage in the high temperatures (up to 70 °C), 4 °C, and wide range of pH with an appropriate adsorption rate and large burst size (although with a relatively long latent period compared to other lytic phages obtained from other studies against *A. baumannii* strains) could be appropriate choice to prepare phage cocktail against *A. baumannii* isolates.

The major problem in the application of lytic phages against *A. baumannii* isolates for phage therapy is narrow host range of isolated phages; thus it is suggested to collect all lytic phages infecting *A. baumannii* strains with appropriate stability and phenotypic characterization and also to store them as a phage bank of *A. baumannii* to use them in phage therapy.

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Conflict of interest

The authors declare that they have no conflict of interest.

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