Characterization and Sequencing of a Novel Phage Abp95 Against Multi-genotypes of Carbapenem-resistant *Acinetobacter Baumannii*

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Abstract

Acinetobacter baumannii has become a challenging conditional pathogen. *A. baumannii* can lead to different infections, such as wound or urinary tract infections and pneumonia. As an alternative strategy for antibiotic-resistant *A. baumannii* infections, phage therapy had been used and approved by several governments. Previously we had reported two potential phage therapy candidates named Abp1 and Abp9. In this study, a wide host range lytic phage Abp95 were isolated and sequenced. The biological characteristics of Abp95 are also stuied. Abp95 belongs to the *myoviridae* family, containing a G+C content of 38.07% with a genome of 43,176 bp. Abp95 genome encodes 77 hypothetical genes, without any known virulence genes. With a diabetic wound infection model, Abp95 could accelerate wound healing though clearing local infections of multidrug-resistant *A. baumannii*. In conclusion, wide host range lytic phage Abp95 shows the potential as phage therapy candidate against multi-genotypes of Carbapenem-Resistant Acinetobacter *baumannii.*

1. Background

Acinetobacter baumannii (A. baumannii) is a nonfermenting and gram-negative bacterium. *A. baumannii* exist in human body, soil, meat and vegetables. *A. baumannii* could bring different infections and about 10 percent of nosocomial infections arise from *A. baumannii* [1-3]. This is largely owing to the acquisition or up regulation of plasmids, integrons and transposons, which makes it one of the biggest obstacles to antibiotic therapy [4, 5]. Since 1980s, the number and types of drug-resistant strains of *A. baumannii* have increased year by year. Pan-drug-resistant *A. baumannii* strains has been reported[1]. The treatment of *A. baumannii* has become one of the thorny clinical problems. Recently, scientists started to resort to phage therapy which was reported 100 years ago. Phage has been used as natural antimicrobial in the treatment of various infections, including wound infections [6, 7], osteomyelitis [8], chronic prostatitis [9], pneumonia and so on [10]. As reported, phage has the following advantages: 1) Phage has high specificity and can cause minimal damage to normal flora. 2) Because of its self-replicating characteristics, the concentration of phage increased at the infected site, which reduced the initial dose requirement. 3) Phages can rapidly distribute in various organs throughout the body (such as prostate, bone, etc.) [11].

In previous studies, we have successfully isolated *podoviridae* phage Abp1 from hospital sewage, and its treatment effect was tested in a local and system mouse model of infection [12]. Using a drug-resistant *A. baumannii* strain ABzy9 separate from a patient's femoral venous catheter, and we also isolated a *myoviridae* phage Abp9 that cleaves ABzy9 [13]. Abp9 can effectively remove the biofilm produced by ABzy9 and reduce the mortality rate of rats infected with ABzy9. Unfortunately, Abp1 and Abp9 showed a narrow host range. In this study, we screen and isolate more than 20 phages from hospital sewage, and one of them showed a wide host range which was named Abp95. The biological characteristics, whole genome sequencing analysis and anti-infection potential of Abp95 have been studied.

2. Materials And Methods

2.1. Collection and Characterization of *A. baumannii* Strains

A total of 200 *A. baumannii* clinic strains were isolated and characterized in our previous study. All *A. baumannii* isolates were confirmed as *A. baumannii* by OXA-51 amplification and 16S rRNA gene sequencing[14]. Seven genes are sequenced for MLST analysis, including *gdhB, gltA, gyrB, cpn60, recA, gpi and rpoD*[15]. To obtain the locus number, we compared the sequencing result of each gene with the submitted sequence in the database. With seven locus numbers, the STs can be identified. When the seven loci have no matching submitted type, a new type is defined.

2.2. Transmission Electron Microscopy (TEM) and Bacteriophage Isolation

A total of 22 *A. baumannii* lytic phages were isolated using a combination of 30 *A. baumannii* isolates as the host. In short, use 1 liter of filtered hospital sewage and 150 ml of 30 A. Baumani(OD_{600} around 1.0, 5ml for each isolates) overnight incubation for isolation [14].Use different host incubated double-layer plate to collected and tested for phages from the supernatant. After incubation under 37°C overnight, transparent plaques can be observed on double-layer agar plate. For a primary selection, the 22 phages were dropped on double-layer plates with different hosts and Abp95 showed a wide host range compared to other phages. Using Cscl gradient ultracentrifugation to prepare Abp95 phage particles [16]. CsCl-purified Abp95, with a concentration of 10^{11} PFU/ml, allow 15 minutes for adsorption and deposited on a carbon-coated copper grid. Use 2% (w/v) potassium phosphate fertilizer to negatively stain the phage particles and observed with transmission electron microscope (TEM). Use ImageJ to measure its length and width, using phage genome kit (Norgen, Canada) to isolate Phage DNA.

2.3. One-Step Growth Assay and Thermal stability of Abp95

For one-step growth curve, Abp95 was added to the mid-exponential phase host bacteria and cultured at 25°C for 15 min. Next, in order to remove non-absorbed phages, the mixture was harvested by centrifugation (13,000 r 30 s) and discard the supernatant, then incubate with shaking at 25°C. 50 μL cluture are collected every 20 min, and use double-layer plate method measured the phage titer.

2.4. Host-Range Determination

With previously described method[14], mix 200 μL of 108 PFU/ml to-be-test host bacterial solution with 4 ml of melted 0.6% semi solid agar mediumr. To-be-test host bacterial include 200 clinical *A. baumannii* strains, *P. aeruginosa* strains PAO1 and *E. coli* strains DH5α, JM109. Then, pour the mixture onto the agar plate prepared in advance to double-layer agar plates. After cooling at room temperature for 5 min, Add 5 μl Abp95 to the middle of each double-layer plate. Incubate for 12 hours and observe whether there are plaques.

2.5. Genome Sequencing

The sequencing library is generated by the TruSeq DNA sample preparation kit (Illumina, USA) and the Template Prep Kit (Pacific Biosciences, USA). On both Pacific Biosciences and the Illumina Miseq platforms by the Personal Biotechnology Company (Shanghai, China) sequencing genome. Use SPAdes [17] and A5-miseq [18] to build scaffolding and continuum for data assembly. The data obtained from the sequencing of the PacBio platform was assembled using Canu software [19]. Then, integrating all the results to generate a complete sequence.

2.6. Bioinformatic analysis

Using GeneMark (version 4.32, http://topaz.gatech.edu/GeneMark/) prediction open reading frame (ORF). Using BLASTp database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) to predict each ORF encoded protein function. Glimmer 3.02 is used for performing Gene prediction [20]. tRNAscan-SE [21], RNAmmer [22] and Rfam are used for finding ribosomal RNA, transfer RNA [23] and other noncoding RNAs, separately. Submit the entire genome to https://www.ncbi.nlm.nih.gov/genbank/ (NCBI accession number: MZ618622.1). Use molecular evolutionary genetic analysis (MEGA version 7.0) package to construct a phylogenetic tree ground on the comparison results of the entire genome sequence

2.7. Phage therapy in diabetes wound infection model

36 six-week-old male C57 mice were subjected to abdominal injection of STZ (120 mg/kg body weight) after eight hours of fasting, blood glucose was measured at day 3 and 7, if the random blood glucose>16.8mmol/L, the diabetes mouse model established successfully. Then feed for another 1w and the locally infected mouse model can be established. Each mouse received cyclophosphamide (150 mg/kg body weight) through intraperitoneal injection 24 hours before establishing a local infection mouse model, mice were anesthetized with isoflurane and use depilatory paste to remove mouse back hips. Using a skin biopsy punch created a diameter of 1.0 cm round full-thickness skin defect on the back near the hip side of each mouse. Use 5 μL of PBS containing 1.0×10^6 $AB_{2013-95}$ cells infect each wound and the air-drying for 5min. 24h after infection, the mice were according to the principle of random allocation divided into two groups. In the experimental group, 5μL Abp95(1.0×10^9 PFU) was applied to the wounds of the mice, and the control group used the same volume of PBS as negative control. At day 3 after infection, repeating phage treatments. On days 1, 3, and 7, measuring and recording the wound sizes.

2.8. Ethics statement

The animal experiments are are authorized by the Laboratory Animal Welfare and Ethics Committee of Zunyi Medical University. The approval number for the animal experiment is 2019-2-032.

2.9. Statistical analysis

One-way analysis of log-rank test (Mantel-Cox) or variance (ANOVA) are used to data analyzing as suitable. when $P<0.05$ is considered to be significant.

3. Results

3.1. Abp95 is a lytic phage

As shown in Figure 1A, Abp95 can form plaques in a double-layer agar plate infected of $AB_{2013\text{-}95}$. Compared with the negative group, OD_{600} value of bacterial culture ($AB_{2013\text{-}95}$) with phage Abp95 drop from 1.0 to 0.3 after 240 minutes (Figure 1B). Meanwhile, after 3 hours of incubation, the bacterial culture can be lysed and clarified by Abp95 (Figure 1C and D).

3.2. One-Step Growth Curve and Thermal Stability

As shown in Figure 2A, it has a 20 min latent phase, and 40 min after infection Abp95 phage start to burst out. After 100 minutes, the phage produced the maximum number of progenies, and reaching its plateau phase. The burst size of Abp95 is 177 PFU per infected cells.

As shown in Figure 2B, Abp95 still maintained relative activity at 60 ℃, and there was no significant difference in phage titer at 20, 30, 40, 50 and 60 ℃. However, after incubation at 70 ℃ for 15 minutes, the phage titer decreased about 100 times. These results show that Abp95 can maintain good activity at 20-60 ℃, which make it more convenient to store and transport.

3.3. Electron Microscopy

As Figure 3 shows, Abp95 belongs to the *myoviridae* family member, and has an icosahedral head with a diameter of 60.502±2nm. the full length measuring (142.891±4.32) × (24.53±2.24) nm,with tail measuring 54.752±2.487 nm. Both contraction and relaxation states of Abp95 tail can be found under transmission election microscopy (TEM).

3.4. Major structural proteins and genome structure of Abp95

After protein SDS page, two major phage structure proteins can be found on the gel. Molecular weight of the big one is around 60kDa and the small one is around 45kDa (Figure 4A). To test whether the Abp95 genome is circular or linear, *Xho*I and *Nhe*I were used to cut the Abp95 genome. As shown in supplementary Figure S1, both *Xho*I and *Nhe*I have 3 cut sites among Abp95 genome. If the genome is linear, then genome digestion with these 2 enzymes will produce 4 fragments, if it is circular, then only 3 fragments will be produced. The patterns (Figure 4B) match with simulated electrophoretic patterns of *Xho*I and *Nhe*I digestion for linear genome.

3.5. General features and Sequencing results of the Abp95 Genome

Sequencing the whole genomes of abp95. After quality control, obtaining 8,539,910 reads and 29,484 genome coverage. At last genome assembly produced a 43,176b-bp dsDNA molecule with a G+C content of 38.07%. The ORF finder was used to identify for ORFs larger than 100 bp, and a total of 77 were found.

A gene's average length is close to 560bp. ORF density is approximately 1.78 genes per kb. As it shown at the complementary Table 1, the genome explanatory notes of Abp95 is listed. 10 genes were encoded on the negative strand and 67 genes on the positive strand. After nucleotide blast in NCBI, 69 of the 77 ORFs have similar ORFs and 14 ORFs are with predicted functions after bioinformatic analysis. The Abp95 genome is submitted to NCBI genome database under GenBank accession number MZ618622.1.

3.6. Host-Range Determination

For the host-range tests, Among the 200 clinical strains of A. Bowmani tested, 58 strains can be infected by Abp95. 55 strains of the 58 phages sensitive isolate are carbapenem-resistant while three are antibiotic sensitive strains. As shown in Figure 5 and Table 1, the 58 strains were isolated from wound (29), blood (10), sputum (8), catheter (5), purulent fluid (5) and tissue (1). The 58 strains belonged to 8 different STs, ST368 (25), ST195 (23), ST208 (5), ST369 (1), ST696 (1), ST1414 (1), ST1496(1) and a new type (1). For the cross-species host test, Abp95 was not infected with either *Pseudomonas aeruginosa* (PAO1) or *Escherichia coli* (JM109 and Dh5+).

Table 1
Host-range of Abp95

Isolate Number	Origin	ST type	Lysis Effect	Antibiotic Resistance
2013-003	bl	195	++++	CRAB
2013-004	ca	195	++	CRAB
2013-007	ca	195	+++	CRAB
2013-011	sp	195	++	CRAB
2013-020	ca	195	++	CRAB
2013-021	wd	208	++	CRAB
2013-026	wd	195	+++	CRAB
2013-034	wd	368	+	CRAB
2013-037	wd	195	++	CRAB
2013-038	bl	368	+++	CRAB
2013-041	ps	195	+	CRAB
2013-046	wd	368	+++	CRAB
2013-047	wd	208	+	CRAB
2013-051	wd	368	+	CRAB
2013-052	wd	368	+++	CRAB
2013-054	wd	195	++	CRAB
2013-057	wd	208	++	CRAB
2013-058	wd	1414	++	CRAB
2013-064	bl	208	++	CRAB
2013-065	wd	368	+++	CRAB
2013-069	sp	368	++++	CRAB
2013-072	sp	368	++++	CRAB
2013-082	wd	368	+++	CRAB
2013-083	wd	368	++++	CRAB
2013-088	bl	368	++++	CRAB
2013-089	wd	new	++++	

Isolate Number	Origin	ST type	Lysis Effect	Antibiotic Resistance
2013-094	ps	368	+	CRAB
2013-095	wd	696	++++	
2013-107	ps	195	++++	CRAB
2013-111	bl	195	+++	CRAB
2013-114	bl	368	+++	CRAB
2013-119	wd	1496	++++	
2013-125	wd	368	++++	CRAB
2013-129	sp	195	++++	CRAB
2013-131	bl	368	++	CRAB
2013-143	wd	195	+++	CRAB
2013-144	ti	368	+++	CRAB
2013-157	wd	368	++++	CRAB
2013-158	wd	368	+++	CRAB
2013-159	ps	195	++++	CRAB
2013-161	wd	368	+++	CRAB
2013-165	ps	195	++	CRAB
2013-166	wd	195	+++	CRAB
2013-167	wd	195	+++	CRAB
2013-169	ca	195	+++	CRAB
2013-171	wd	195	++	CRAB
2013-174	bl	195	+++	CRAB
2013-177	ca	195	+++	CRAB
2013-181	wd	195	+++	CRAB
2013-183	sp	368	++++	CRAB
2013-188	wd	195	+++	CRAB
2012-107	wd	208	++	CRAB
2012-108	sp	368	+	CRAB

Isolate Number	Origin	ST type	Lysis Effect	Antibiotic Resistance
2012-110	sp	369	++	CRAB
2012-085	bl	368	++++	CRAB
2012-090	sp	368	+++	CRAB
2012-094	wd	368	++++	CRAB
2012-096	bl	368	++++	CRAB

3.7. Phage therapy in diabetes wound infection model

To investigate the effects of Abp95 on infected diabetic wounds, a diabetic murine model for *A. baumannii* local infection was established. On days 3 and 7 after infection, the areaes of the phage-treated wounds were significantly smaller than that of wounds treated with control PBS (Figure 6). During a 2-week treatment, no mice died in either group. These results suggest that the Abp95 local therapy not only accelerated wound healing but also have the potential to treat local infections of multidrug-resistant *A. baumannii* in clinical application.

4. Discussion

Currently, polymyxin-based regimen is the most commonly used treatment for difficult-to-treat drug-resistant (DTR-AB) infections caused by *A. baumannii*. Two new antimicrobials have recently been approved: cifedorocol and Ilavacycline have been shown to be effective against DTR-AB. Recent clinical trials have shown that Cefiderocol may serve as a potential treatment option for patients with DTR-AB but Cefiderocol has a higher mortality rate [24–26]. Eravacycline is more commonly used for abdominal infections [27]. In contrast to the slow progress of antibiotics, bacteriophage therapy has made good progress. Recently, scientists discovered that the endolysins Ply6A3[28], LysSS and that of phage vB_AbaP_D2 all showed wide antibacterial capability [29, 30]. Studies have found that bacteriophage-resistant *A. baumannii* is due to the lack of GPI gene fragment, but loss of these receptors makes it re-sensitive to antibiotics agents, which provides a new direction for treating clinical drug-resistant bacteria [31].

Phage therapy is already being used in clinic treatment. Relevant literature reported that phages have been used in treating the infection of MDR *P. aeruginosa* or pandrug-resistant achromobacter xylosoxidans after lung transplantation[32, 33], the infection of *Klebsiella pneumoniae* after artificial knee replacement and *A. baumannii* infection in COVID-19 patients[10, 34]. Clinical trials of phage therapy for patients with urinary tract infection after transurethral resection of the prostate and phage therapy for *P. aeruginosa* infection in the burn wound has been carried out[35, 36]. In addition to using phage therapy

alone, scientists are also trying to combine phage-antibiotic treatments, this combination has been demonstrated that can inhibit the resistance in bacteria appear. In the same time, there is a synergy between the phage and antibiotic [37–40].

Abp95 is a lytic *myoviridae* phage,which is isolated from hospital sewage. Abp95 possess a relatively small genome of 42kb and it shows satisfactory thermal stability from 20 centigrade to 60 centigrade. Interestingly, Abp95 shows a relative wide host range. 58 of 200 clinical isolates are sensitive to Abp95. These 58 sensitive hosts are from 6 different resources and belong to 8 different STs. Although the primary host $AB_{2013-95}$ is an antibiotic-sensitive strain (Table 1), 55 of the 58 isolates are carbapenem-resistant *A. baumannii* strains.

About 6.3% of people with diabetes develop diabetic foot worldwidr, and at the infected diabetic foot ulcers, *Staphylococcus aureus* accounted for 42% [41], but *A. baumannii* is becoming one of the main bacteria in diabetic foot ulcers infections [42]. Diabetic patients suffer from shortage of tissue perfusion and limb necrosis due to microvascular disease, which obstructs the effect of active antibiotics against bacteria from chronic wound infection[41]. At the same time, due to the massive use of antibiotics, drug-resistant bacteria develop on such wounds. At present, the research and development rate of new antibiotics is significantly slower than the drug-resistant bacteria appear, and the hope of relying solely on antibiotics to treat infected wounds is becoming increasingly slim. Phage therapy has natural advantages, it is highly specific, and the dosage is small, moreover it can realize individualized design. In our diabetic wound models, Abp95 treatment did not reduce the survival rate of mice, proving that it has an acceptable safety profile. An acceleration of wound closure was observed by treating wounds topically with Abp95.

Nowadays, *A. baumannii* has undoubtedly become one of the most pan-drug resistant bacteria species. In the face of this situation, phage therapy is one of the most valuable treatment. Abp95 has a wide Host-Range, and 98% of them are CRAB, which has good resistance to drug-resistant *A. baumannii*, and we should conduct in-depth research on it in the future.

Declarations

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Conflicts of Interest

No competing interests.

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Figures

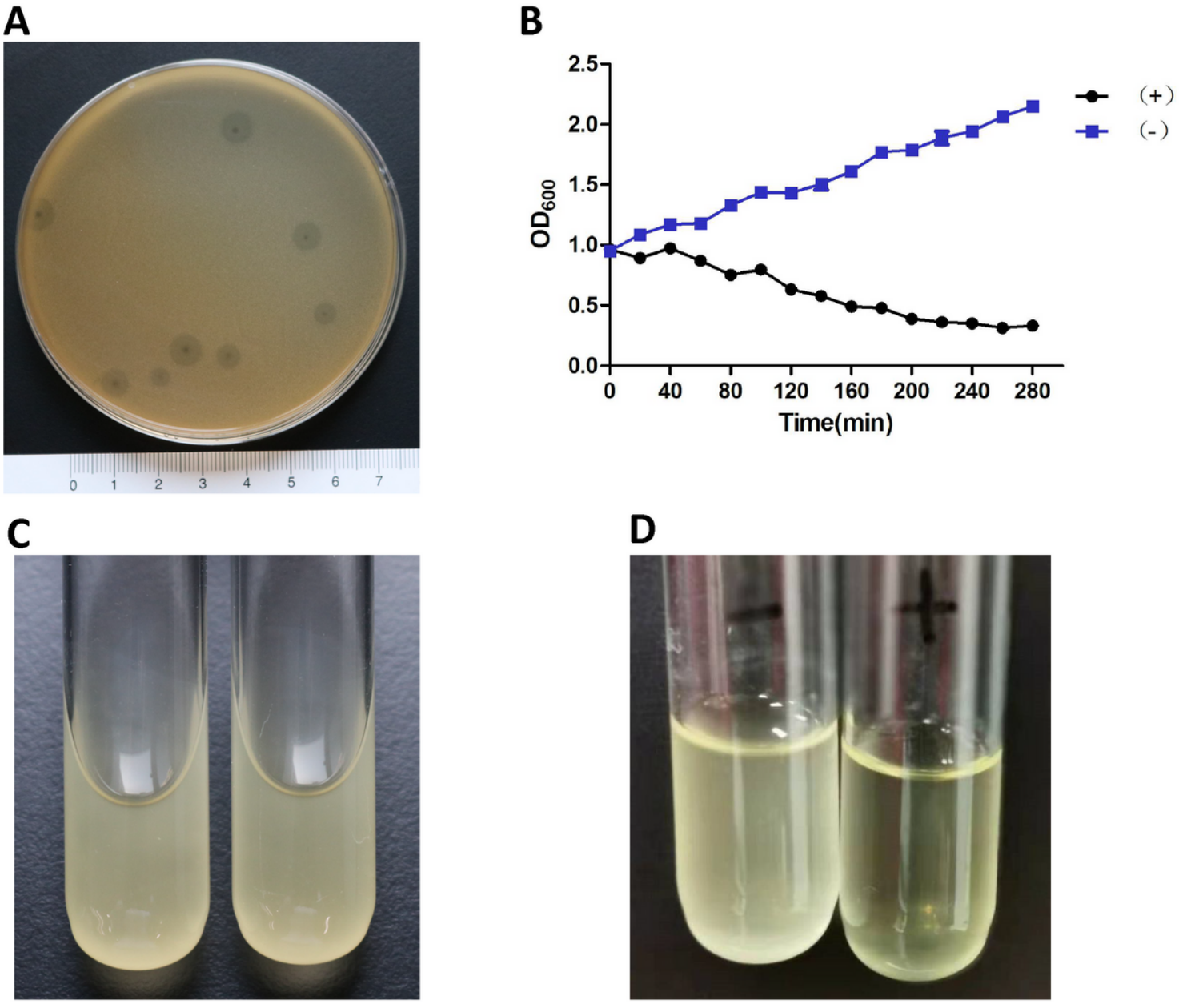

Figure 1

Abp95 is a lytic phage. A) Abp95 can form plaques in a double-layer agar plate infected of the AB2013-95, which is the host of Abp95; B) OD600 value of bacterial culture with or without Abp95; C) and D) Abp95 cause lysis in a bacterial culture after incubation. Abp95 can form plaques in a double-layer agar plate

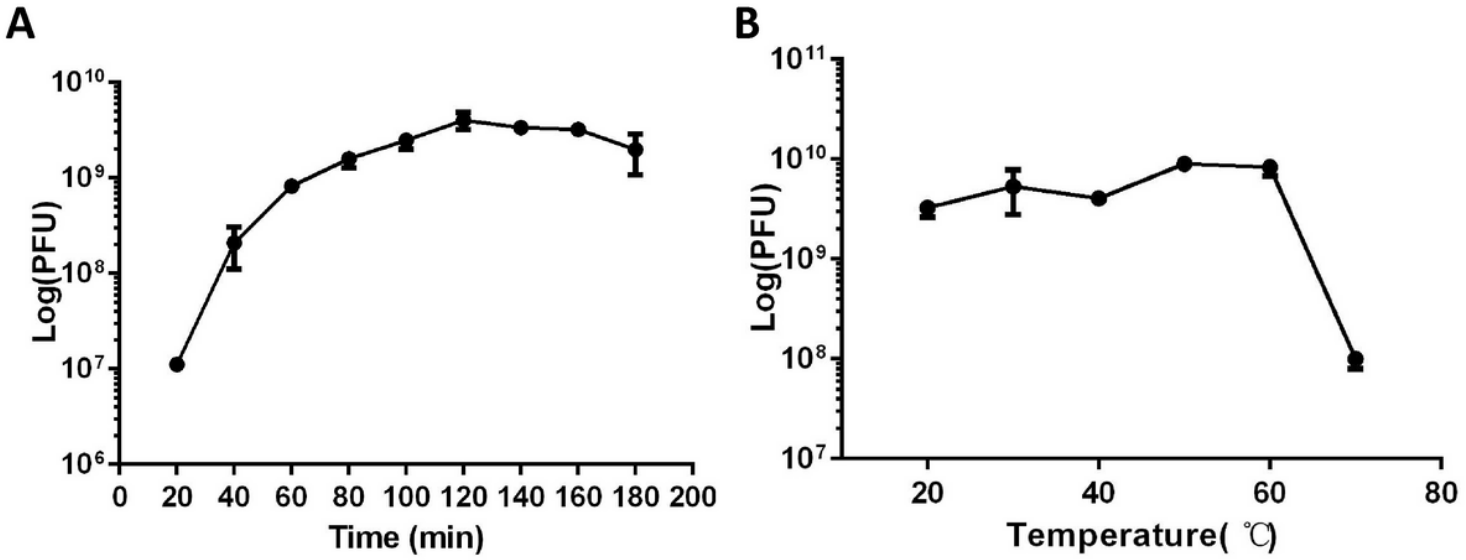

Figure 2

One-Step growth curve (A) and thermal stability of Abp95 (B).

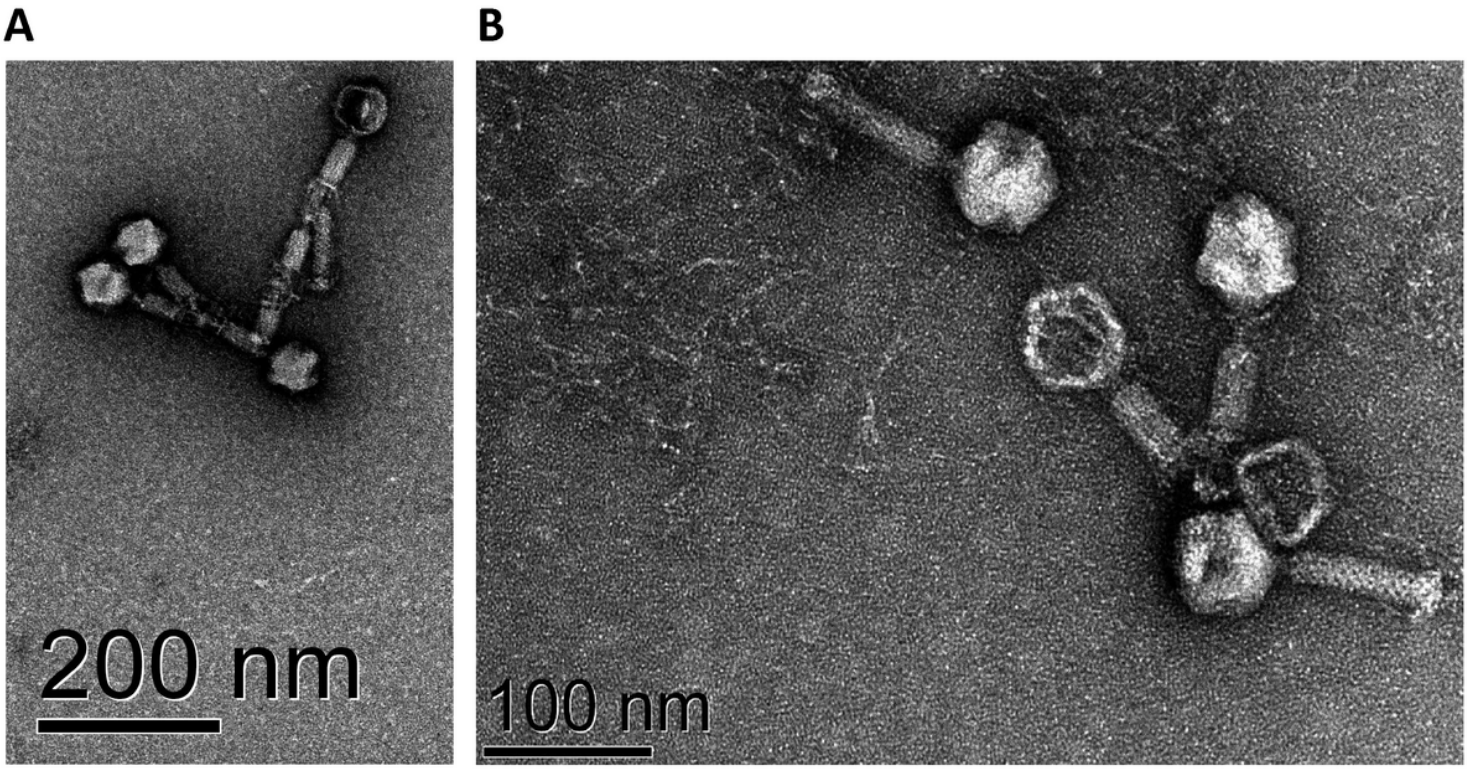

Figure 3

Electron microscopy of Abp95. A) The morphology of Abp95 under TEM magnified by 0.5 million times (A) and 1 million times (B).

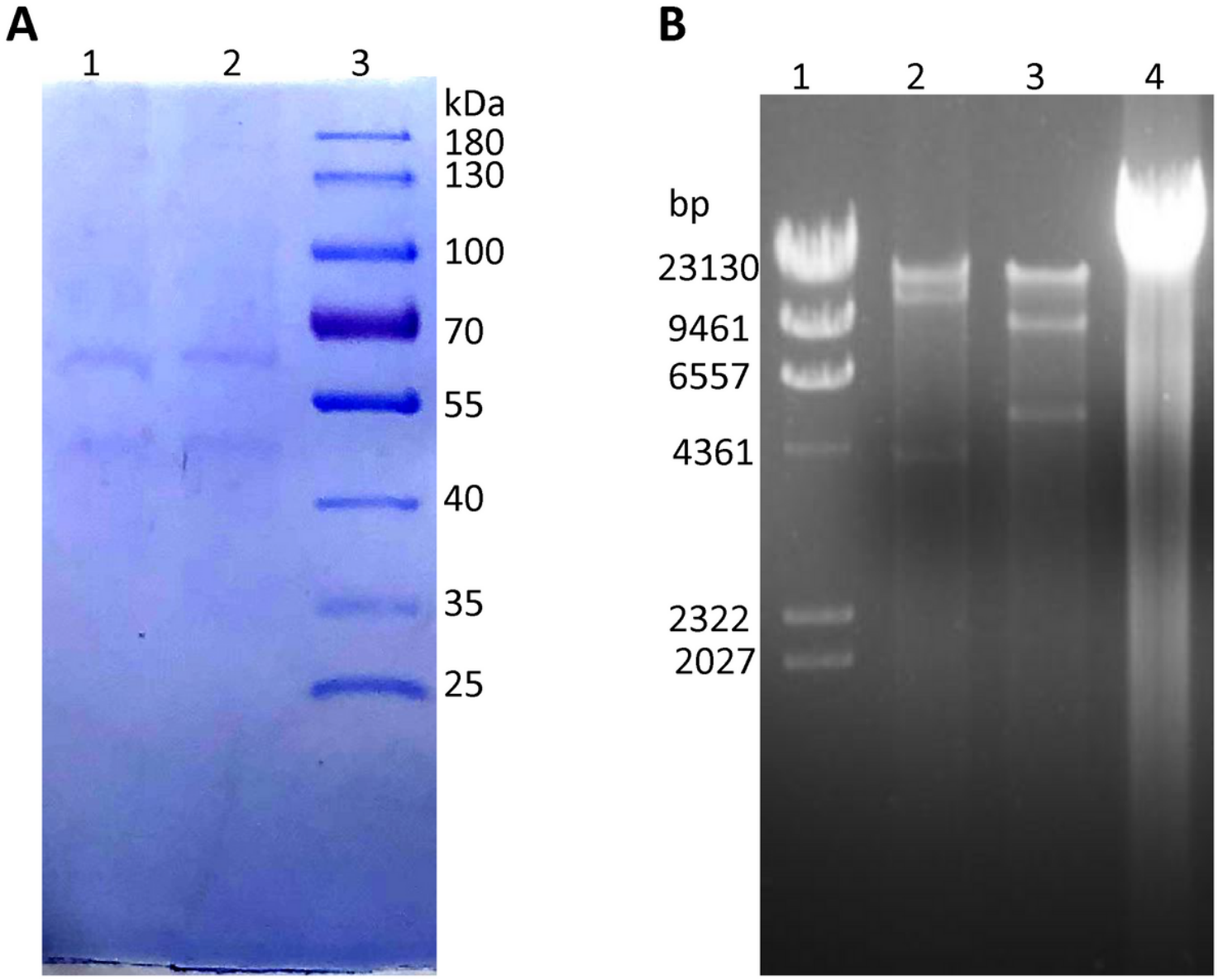

Figure 4

Major phage proteins and restriction endonuclease mapping of Abp95. A) lane 1 and lane 2, SDS-PAGE of purified phage particles; lane 2, protein molecular weight marker. B) Lane 1, DNA molecular weight markers; lane 2 and 3, Abp95 genome digested by XhoI and NheI; lane 4, Abp1 genome.

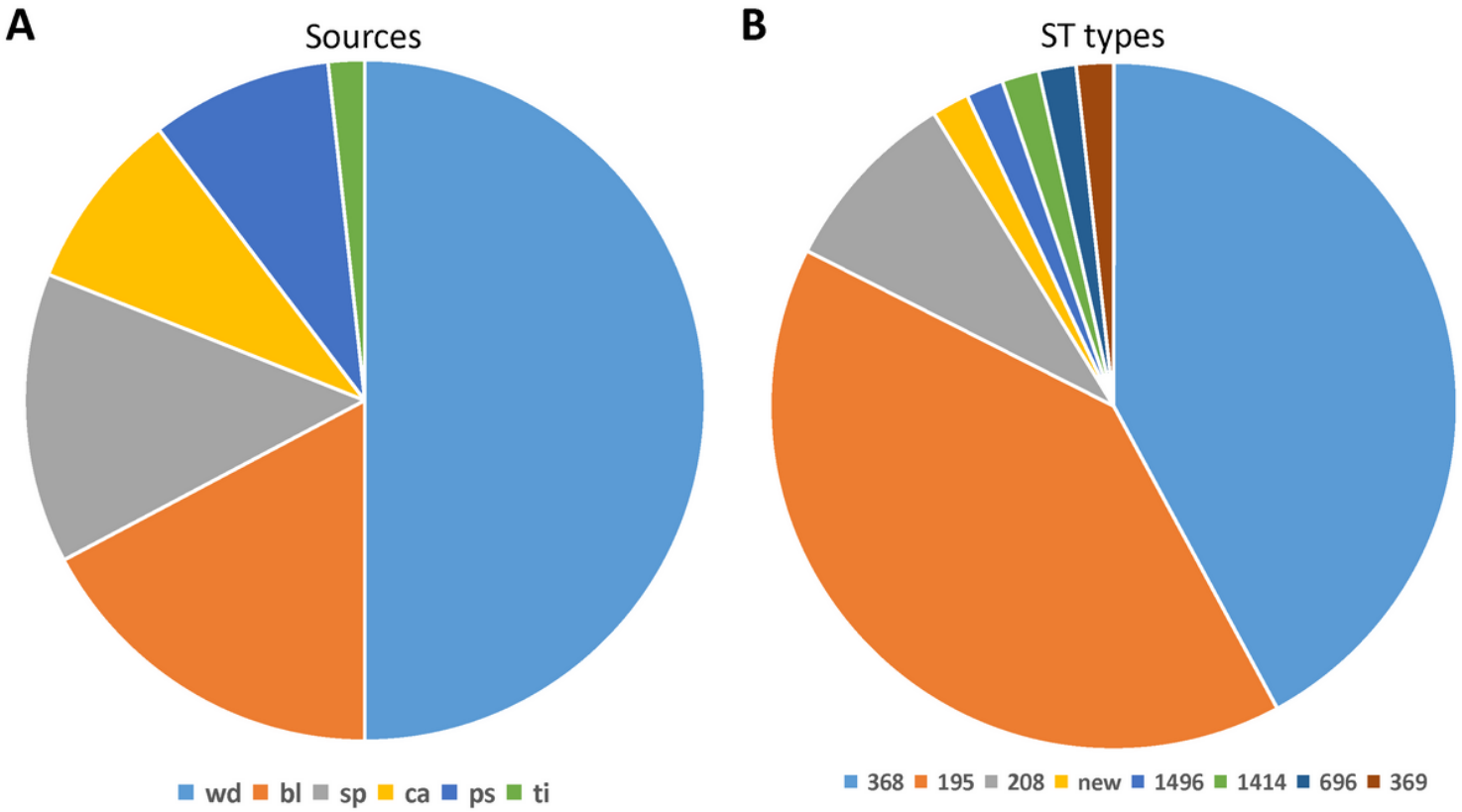

Figure 5

Characterization of 58 sensitive A. baumannii strains. A) sp (sputa), ca (catheter), wd (wound surface), ps (purulent secretion), bl (bloodstream) and ti (tissue); B) STs identified among 58 strains.

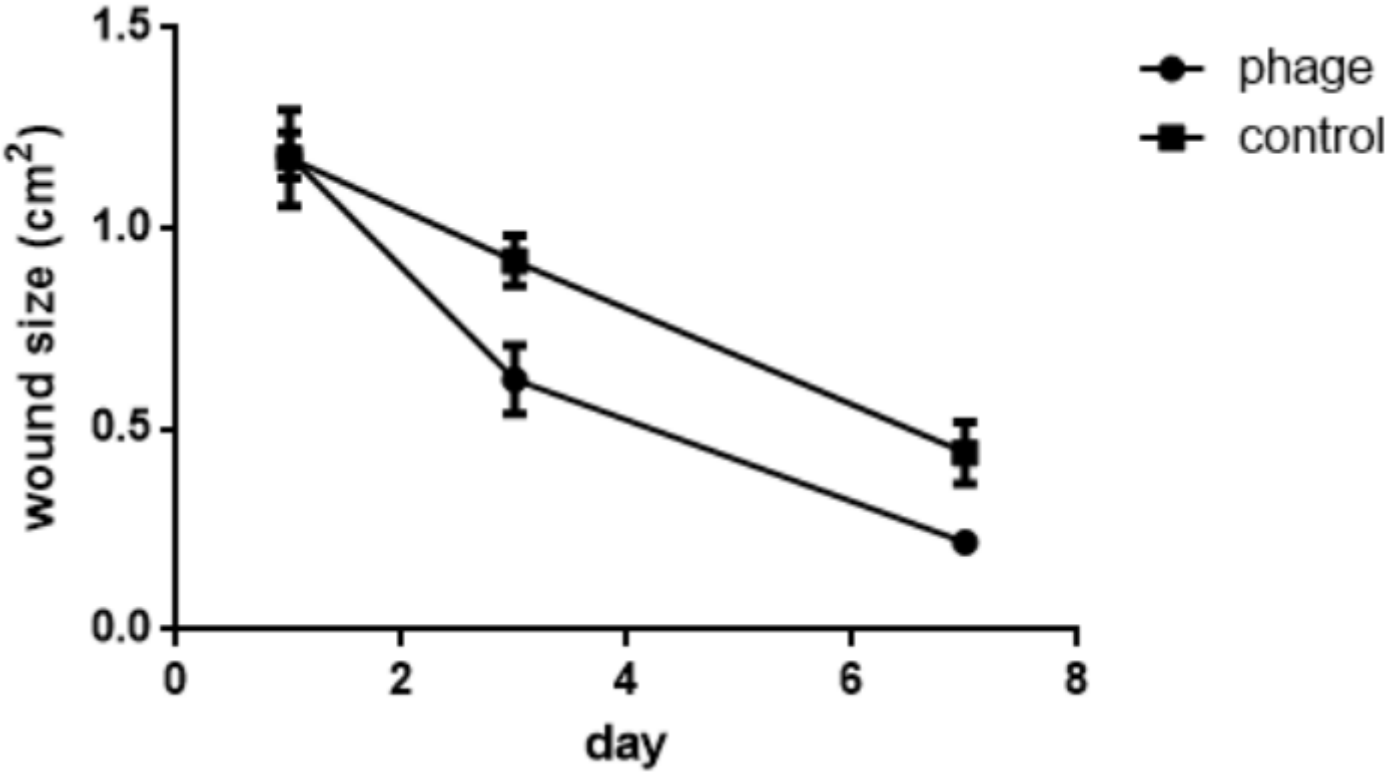

Figure 6

Wound size after Abp95 treatment At 1, 3, and 7 days after infection measure wound size and show as mean±standard deviation.

Supplementary Files

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