Peer-reviewed version available at Microorganisms 2019, 7, 556; doi:10.3390/microorganisms7110556

Article

Combined Use of the Ab105-2ϕΔCI Lytic Mutant Phage and Different Antibiotics in Clinical Isolates of Multiresistant *Acinetobacter Baumannii*

L. Blasco[1Ø], A. Ambroa[1 Ø], M. López[1], L. Fernández-Garcia[1], , I. Bleriot[1], R. Trastoy[1], J. Ramos-Vivas[2], T. Coenye[3], F. Fernández-Cuenca[4], J. Vila[5], L. Martínez-Martínez[6], J. Rodríguez-Baño[4], A. Pascual[4], JM. Cisneros[7], J. Pachón[7],G. Bou[1],M. Tomás[1] and GEMARA SEIMC/REIPI Bacterial Clinical Adaptation Study Group.

1Microbiology Department-Research Institute Biomedical A Coruña (INIBIC), Hospital A Coruña (CHUAC), University of A Coruña (UDC), A Coruña, Spain.
2Microbiology Department- Research Institute Biomedical Valdecilla (IDIVAL), Hospital Marques de Valdecilla, Santander, Spain.
3Laboratory of Pharmaceutical Microbiology, Ghent University, Gent, Belgium.
4 Clinical Unit for Infectious Diseases, Microbiology and Preventive Medicine, Hospital Universitario Virgen Macarena/Department of Microbiology and Medicine, University of Seville/ Biomedicine Institute of Seville (IBIS), Seville, Spain.
5Institute of Global Health of Barcelona (ISGlobal), Hospital Clínic-Universitat de Barcelona, Barcelona, Spain.
6Unit of Microbiology, University Hospital Reina Sofía, Department of Microbiology, University of Córdoba, Maimonides Biomedical Research Institute of Cordoba (IMIBIC), Cordoba, Spain.
7Clinical Unit for Infectious Diseases, Microbiology and Preventive Medicine, Hospital Universitario Virgen del Rocío/Department of Microbiology and Medicine, University of Seville/ Biomedicine Institute of Seville (IBIS), Seville, Spain.
Ø Correspondence:These authors equally contributed to this work.
* Correspondence:Correspondence:To whom correspondence should be addressed: María Tomás. Phone: +34 981176399; Fax: +34 981178273. Emails: MA.del.Mar.Tomas.Carmona@sergas.es

Abstract: Phage therapy is an abandoned antimicrobial therapy that has been resumed in recent years. In this study, we mutated a lysogenic phage from *Acinetobacter baumannii* into a lytic phage (Ab105-2phiΔCI) showing antimicrobial activity against *A.baumannii* clinical strains(such as Ab177_GEIH-2000 which showed MICs to meropenem and imipenem of 32 μg/ml and 16 μg/ml, respectively as well as belonging to GEIH-REIPI Spanish Multicenter *A. baumannii* Study II 2000/2010, Umbrella Genbank Bioproject PRJNA422585).We observed in vitro, an antimicrobial synergistic effect(from 4 log to 7 log CFU/ml) with meropenem plus lytic phage in all combinations analysed(0.1, 1 and 10 MOI of Ab105-2phiΔCI mutant as well as 1/4 and 1/8 MIC of meropenem).Moreover, we had a decrease in bacterial growth of 8 log CFU/ml for the combination of imipenem at 1/4 MIC plus lytic phage(Ab105-2phiΔCI mutant) and of 4 log CFU/ml for the combination of imipenem at 1/8 MIC plus lytic phage (Ab105-2phiΔCI mutant) in both MOI 1 and 10.These results were confirmed in in vivo(*G. mellonella*) obtaining a higher effectiveness in the combination of imipenem and Ab105-2phiΔCI mutant($P<0.05$).This approach could help to reduce the emergence of phage resistant bacteria and restore sensitivity to the antibiotics when used to combat multiresistant strains of *Acinetobacter baumannii.*

Keywords: *Acinetobacter baumannii*; multiresistant; mutant lytic phage; phage therapy; antibiotic-phage synergy

1. Introduction

Multi-drug resistant (MDR) bacteria such as *A. baumannii* are considered of major concern by the World Health Organization (WHO), because of their ability to acquire antimicrobial resistance via intrinsic mechanisms(e.g. presence of the outer membrane) or via mechanisms acquired by horizontal genetic transfer [1, 2]. This situation has led to an urgent need to develop new antimicrobial agents and to renewed interest in phage therapy. Phage therapy was first developed in the 1920s, but was abandoned in the Western world after the discovery of antibiotics. However, use of phage therapy was continued in Eastern countries, such as Poland and URSS, where bacteriophages are used for the prophylaxis and treatment of infections such as dysentery, ulcers and methicillin resistant *Staphylococcus aureus* (MRSA) infections [3, 4].

Phage therapy is now considered a real option for treating MDR bacteria. Phages are bacterial viruses, and like other viruses they are obligatory parasites that enter host cells via mechanisms based on receptor recognition. They then inject their genetic material into the infected bacteria and use the bacterial machinery to produce phage proteins [5, 6]. Phages generally undergo a lytic (virulent) or lysogenic (temperate) life cycle. Lytic phages infect and rapidly lyse and kill host cells, releasing phage progeny into the surrounding medium. Lysogenic phages infect the host cell and integrate their nucleic acid into the host genome to produce bacteriophages (prophages), which are transmitted to daughter cells and are only released when induced to enter the lytic cycle [7, 8]. The lysogenic/lytic cycle of temperate bacteriophages is controlled by Cro, CI and CII proteins; the Cro protein induces the lytic state and the CI repressor protein inhibits the Cro protein thus inducing the lysogenic state [14].

Only lytic phages are used in phage therapy as lysogenic phages can transfer resistance genes or virulence factors to the host [9].

The combined use of antibiotics and phages has been tested in several studies, demonstrating strong control of the bacteria and a reduction in the development of phage and/or antibiotic resistance [10]. Phages are good candidates for use in combination with antibiotics for various reasons: they have a different mechanism of action from antibiotics; they have narrow spectrum of activity, which protects the normal microbiota; they can multiply at the infection site, they are abundant in nature and can be easily isolated; and production costs are low [11-13].

In this study we produced a mutant lytic phage from a lysogenic phage incorporated in the genome of a clinical strain of *A. baumannii*, by deleting the CI repressor gene and thus preventing entry of the phage into the lysogenic cycle [14] [15]. We then tested the antimicrobial activity of the novel lytic phage, Ab105-2phiΔCI, in combination with carbapenem antibiotics (meropenem and imipenem) against a carbapenem-resistant strain of *A. baumannii*. The combined therapy enhanced the antimicrobial activity of both the phage and the antibiotic; the bacterium became sensitive to the antibiotics and the emergence rate of phage resistant bacteria was reduced.

2. Material and Methods

2.1. Bacterial strains and susceptibility to antimicrobials

In this study, we used 20 clinical strains isolated from Spanish hospitals during the GEIH-REIPI Spanish Multicenter *Acinetobacter baumannii* Study II 2000–2010, GenBank Umbrella project PRJNA422585 (https://www.ncbi.nlm.nih.gov/bioproject) (Table 1). From this collection, we selected *A. baumannii* strain Ab177_GEIH-2000 for which the highest MICs (minimum inhibitory concentration) were obtained (meropenem 32 μg/mL, imipenem 16 μg/mL and doxycycline 64 μg/mL) as well as phage susceptibility through EOP, efficiency of plating value (Table 1).

2.2. Obtaining the lytic phage mutant

The bacteriophage sequence Ab105-2phi (Genbank: KT5880759) detected in the clinical strain *A. baumannii* Ab105GEIH_2010 was analysed and the CI gene identified as ORF 17. The CI gene was deleted by double homologous recombination with the suicide vector pMo130TelR [16, 17]. Primers were first designed for amplification of the flanking regions (1000 bp) of the CI gene. Those regions were amplified by PCR and ligated and cloned into the pMo130telR vector (Table 2). This

construction was transformed in *Escherichia coli* DH5α to produce large numbers of the plasmid with the gene flanking regions. The plasmid was purified, transformed in the *A. baumannii* Ab105 clinical strain by electroporation and incubated for two hours at 37ºC without antibiotic to produce a recombinant wild type with the mutated gene integrated in its genome. Finally, the mutants were selected in the presence of kanamycin (50 μg/ml). The plasmid was lost in the presence of 15% sucrose, and a clone without the phage was obtained. In order to isolate the mutant Ab105-2phiΔCI when the clones with the correct insertion were selected, they were incubated in LB broth supplemented with mitomycin (10 ug/ml) to induce release of the phages. The supernatant was collected, treated with chloroform and filtered (20 μm). The filtered supernatant was used to infect the clone without the phage, and plaques were obtained by the agar overlay method [18]. A clear plaque was isolated and PCR testing was conducted to confirm correct deletion of the CI gene.

2.3. Host range and efficiency of plating analysis

The host range of the lytic mutant phage Ab105-2phiΔCI was established by applying the spot test [19] to the 20 clinical strains of *A. baumannii* under study. Efficiency of Plating (EOP) was established as the ratio between the test strain titre and the host strain titre [20].

2.4. Transmission electron microscopy (TEM) and live-cell imaging

Broth culture of strain Ab177_GEIH-2000 was infected with the lytic mutant phage Ab105-2phiΔCI. Lysates were centrifuged at 3400 × g for 10 min and the supernatant was filtered through a 0.22 nm filter (Millipore). NaCl was added to a final concentration of 0.5 M, and the suspensions were mixed thoroughly and left on ice for 1 h. The suspensions were centrifuged at 3400 × g for 40 min at 4 °C, and the supernatants were transferred to sterile tubes. PEG 6000 (10% wt/vol) was added and dissolved and incubated overnight at 4 °C. Bacteriophages were then precipitated at 3400 × g for 40 min at 4 °C and resuspended in SM buffer (0.1 M NaCl, 1 mM MgSO4, 0.2 M Tris-HCl, pH 7.5)[21]. The samples were negatively stained with 1% aqueous uranyl acetate before examination by electron microscopy.

Live-cell imaging was carried out by time-lapse microscopy after initial adsorption of the mutant lytic phage Ab105-2phiΔCI to the clinical strain Ab177_GEIH-2000 at 37ºC in agar slices, which were placed directly between stainless steel O-rings. Use of extracellular DNA markers enabled the lysis of more than 300 bacteria to be monitored in real time.

2.5. Adsorption curve, one step growth curve and infection curve

An overnight culture of *A. baumannii* clinical strain Ab177_GEIH-2000 was diluted 1:100 in LB broth and incubated at 37ºC at 180 rpm until an early logarithmic phase, which corresponded to an optical density of 0.2 (OD600nm). At this point the culture was infected with the lytic mutant phage Ab105-2phiΔCI at a multiplicity of infection (MOI) of 0.1. The adsorption curve and the one step growth curve were determined after growing the phage in LB supplemented with CaCl2, as previously described [18, 22]. In the one step growth curve the latent period was defined as the interval between adsorption of the phages to the bacterial cells and the release of phage progeny. The burst size of the phage was determined as the ratio of the final number of free phage particles to the number of infected bacterial cells during the latent period [20].

An early exponential culture of the strain Ab177_GEIH-2000 was infected with the lysogenic phage Ab105phi2 and the mutant lytic phage Ab105phi2ΔCI at different MOIs (0.1, 1 and 10), and the corresponding infection curves were constructed. The phage cultures were maintained at room temperature during the adsorption period and then incubated at 37ºC and 180 rpm for 6 hours. Optical density was measured at intervals of one hour during this period.

2.6. Frequency of occurrence of phage-resistant bacteria

Phage resistant mutants and phage-antibiotic resistant mutants were produced as previously described [23]. The same procedure was used to produce phage-antibiotic resistant mutants, except

the media was supplemented with doxycycline (MIC 1/4), meropenem (MIC 1/4) or imipenem (MIC 1/4). The frequency of phage resistant mutants and phage-antibiotic resistant mutants was calculated by dividing the number of resistant bacteria by the total number of sensitive bacteria.

2.7. Antimicrobial activity of the mutant lytic phage Ab105-2phiΔCI in biofilm

An overnight culture of the *A. baumannii* clinical strain Ab177_GEIH-2000 was diluted 1:100 and used to inoculate 100 µl of LB in some wells of a 96 multiwell plate. The plate was maintained at 37ºC in static conditions for 4 hours. The medium was then discarded and the wells were washed twice with PBS before 100 µl of fresh LB was added. After 24 hours at 37ºC, the medium was again discarded and the wells were washed with PBS and filled with 90 µl of SM buffer and 10 µl of phage Ab105-2phiΔCI at 10^7 PFU/ml were added. SM buffer (100 µl) was added to control wells. The plates were then incubated at 37ºC for 24 hours. Finally, the supernatant was discarded and the wells were washed with PBS. Half of the wells were used to quantify the CFU and the other half were used to quantify the biofilm. PBS (100 µl) was added to the wells used to quantify the CFU and the plates were agitated for 5 min and sonicated for another 5 min. The suspension was serially diluted and plated on LB plates. For quantification of the biofilm, 100 µl of methanol was added to each well and discarded after 10 min. Once the methanol had evaporated completely, 100 µl of crystal violet (0.1%) was added and discarded after 15 min. Finally, the wells were washed with PBS before the addition of 150 µl of acetic acid (30%), and the absorbance was measured at OD 595 nm.

2.8. Antimicrobial activity in combination with antibiotics

A time kill curve was constructed to determine the synergy of phage Ab105-2phiΔCI in combination with meropenem, imipenem and doxycycline at 1/8 and 1/4 of the respective minimum inhibitory concentrations (MICs) (meropenem 32 µg/mL, imipenem 16 µg/mL and doxycycline 64 µg/mL). An overnight culture of the tested strain was diluted at 1:100 in LB broth supplemented with 10uM $CaCl_2$ and incubated at 37ºC and 180 rpm until the culture reached an early exponential phase at 0.2 OD (600nm). At this point, antibiotic and the Ab105-2phiΔCI phage were added to the culture. The flasks were maintained at room temperature during the adsorption period before being incubated at 37ºC and 180rpm for 24 hours. Aliquots were removed at 6 hours and 24 hours, and serially diluted and plated in LB plates for subsequent counting of colony forming units (CFUs).

2.9. Galleria mellonella survival assay

The *Galleria mellonella* model used was an adapted version of a previously developed model also used to study bacteriophage therapy [24, 25]. The procedure was as follows: twelve *G. mellonella* larvae, acquired from TruLarvTM (Biosystems Technology, Exeter, Devon, UK), were each injected with 10 µl of a suspension of *A. baumannii* Ab177_GEIH-2000, diluted in sterile phosphate buffer saline (PBS) containing 1×10^5 CFU (± 0. 5 log). The injection was performed with a Hamilton syringe (volume 100 µl) (Hamilton, Shanghai, China). One hour after infection, the larvae were injected with 10 µl of the lytic mutant phage Ab105-2phiΔCI, of MOI 10, in combination with meropenem at ¼ MIC and imipenem at ¼ MIC. Controls included 10 µl of the lytic mutant phage Ab105-2phiΔCI at MOI 10, or meropenem at 1/4 MIC and imipenem at 1/4 MIC. The injected larvae were placed in Petri dishes and incubated in darkness at 37°C. The number of dead larvae was recorded after 72 hours. The larvae were considered dead when they showed no movement in response to touch [24].

The survival curves for the *in vivo G. mellonella* infection model were constructed using GraphPad Prism v.6, and the data were analysed using the Log-Rank (Mantel-Cox) test. The data were expressed as mean values, and differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Obtaining the lytic mutant of the phage Ab105phi-2ΔCI

After deleting the CI gene from the temperate phage Ab105-phi2, as previously reported in *Salmonella* [15], we obtained a lytic mutant, designated Ab105-phi2ΔCI, which produced characteristic clear lytic plaques - in contrast to the turbid plaques produced by the temperate Ab105-phi2 phage (Figure 1 A1). Deletion of the CI gene was confirmed by PCR of the DNA isolated from the Ab105-2phiΔCI phage and as expected no amplification was obtained.

Infection curves for the temperate phage Ab105-2phi and the lytic mutant phage Ab105-2phiΔCI were constructed and compared, showing that the lytic mutant killed the culture at all MOI levels tested, as reflected by a large decrease in the optical density. Although a reduction in growth was observed when the culture was infected with the lysogenic phage Ab105-2phi, the decrease was less than with the lytic mutant. In both cases, the reduction in growth was first observed at MOI 10, but regrowth was also first observed at this MOI, probably due to the emergence of resistance (Figure 1B).

3.2. Morphology and host range of the lytic mutant phage Ab105-phi2ΔCI

The lytic mutant Ab105-2phiΔCI was isolated and the virion morphology observed by TEM, which revealed that this phage has the typical structure of the Siphoviridae as the wild type phage Ab105-phi2 [26]. All plaques obtained were transparent and about 1mm of diameter (Figure 1 A2).

The lytic spectrum of activity of the mutant phage Ab105-2phiΔCI covered 25% of the clinical strains of *A. baumannii* tested. The strain Ab177_GEIH-2000 yielded the highest EOP (1.55) (Table 1).

3.3. Adsorption and one step growth curve

Both the adsorption and the one step growth curve were established using host strain Ab177_GEIH-2000, because the EOP of this strain was the most appropriate and also because this strain does not have complete prophages, as previously determined [26]. The adsorption time (12 min) was determined in order to establish the one step growth curve, which revealed a latent period of 30 min and a burst size of approximately 32±2 PFU per infected cell (Figure 1C).

3.4. Antimicrobial activity of the mutant lytic phage Ab105-2phiΔCI on biofilm

Biofilm was produced with the clinical strain of *A. baumannii* Ab177_GEIH-2000 susceptible to the mutant lytic phage Ab105-phi2ΔCI. Treatment of the biofilm with 10^7 PFU/ml of this lytic mutant phage caused a statistically significant reduction in the biofilm biomass. The antimicrobial activity against the biofilm forming bacteria was confirmed by a decrease in the CFU, quantified in the presence of the mutant lytic phage (Figure 1 D).

Finally, the lytic activity of the mutant phage can be observed in Videos 1 and 2.

3.5. Determination of the emergence rate of phage resistant mutants

As strain Ab177_GEIH-2000 was resistant to meropenem, imipenem and doxycycline, we determined the emergence rate of the phage resistant mutants in the presence of each of these antibiotics. In all cases the combination of the phage and antibiotic reduced the rate of emergence of phage-resistant mutants relative to the rate of resistant mutants in the presence of the phage alone (Table 3).

3.6. Effect of the combination of phage and antibiotic on the time kill curves

Time kill curves were constructed for *A. baumannii* clinical strain Ab177_GEIH-2000 in the presence of a combination of the lytic mutant phage Ab105-2phiΔCI at different MOIs (0.1, 1, and 10) and three antibiotics (at 1/4 and 1/8 MIC) to which Ab177_GEIH-2000 is resistant: meropenem, imipenem and doxycycline (Figure 2).

A reduction in the number of CFU was observed with the phage at both MOI 1 (4 log) and MOI 10 (1 log) after 6 hours, but no differences from the control were observed after 24 hours. The reduction was even higher when the phage was combined with meropenem or imipenem (both carbapenems)

For meropenem plus phage, a synergic effect was observed after 6 hours for all combinations (from 4 log to 7 log CFU/ml), but growth of the *A. baumanni* strain was similar to control levels after 24 hours for all concentrations of meropenem plus phage at MOI 1. The synergic effect was only maintained with the combination of meropenem at 1/4 MIC and phage Ab105-2phiΔCI at MOI10, yielding a difference in bacterial growth of 6 log CFU/ml relative to that corresponding to the meropenem control (Figure 2A1-A2).

As with meropenem, the combination of imipenem and the lytic mutant phage at different concentrations had a synergic effect after 6 hours in all cases, with a decrease in bacterial growth of 8 log CFU/ml for the combination of imipenem at 1/4 MIC plus phage and of 4 log CFU/ml for the combination of imipenem at 1/8 MIC plus phage. The synergetic effect was maintained for 24 h in the combinations of imipenen at 1/4 MIC with phage at MOI1 and MOI10, but not in the combinations of imipenem at 1/8 MIC and both phage concentrations (Figure 2B1-B2)

No synergic effects were observed with doxycycline, and the combination had no more effect than the phage alone at MOI 1. However, when the combinations included the phage at MOI 10, a slight decrease in the CFU count was observed (less than 1 log CFU/ml), independently of the antibiotic concentration (Figure 2C1-C2).

The curves obtained for the lytic mutant phage controls showed that Ab177_GEIH-2000 grew at the control rates after 24h due to the acquisition of phage resistance. However, growth was higher at MOI 10 than at MOI 1 after 6 hours, probably because resistance emerges faster at this MOI than at lower MOI.

3.7. Galleria mellonella survival assays in the presence of meropenem and imipenem in combination with the lytic mutant phage Ab105-phi2ΔCI

The combinations of antibiotic and the phage Ab105-2phiΔCI that resulted in a reduction in the CFU of Ab177_GEIH-2000 at 24h in vitro were assayed in a *G. mellonella* (wax moth) larvae survival model (Figure 3). Survival of the infected larvae treated with imipenem in combination with mutant lytic phage Ab105-2phiΔCI was statistically significantly higher than that of the larvae treated with the antibiotic or the phage alone and of untreated larvae ($P<0.05$). Similar results were obtained for meropenem but in this case, although larval survival was higher after the combinatory treatment than after phage only or no treatment, the difference relative to meropenem alone was not statistically significant ($P=0.2183$). This was probably due to the higher MIC of meropenem than of imipenem (32 μg/ml *versus* 16 μg/ml) for the Ab177_GEIH-2000 strain, indicating the need to administer greater amounts of mutant lytic phage Ab105-2phiΔCI.

4. Discussion

Lytic phages are widely used in phage therapy, but temperate or lysogenic phages have not generally been considered suitable for the purpose because they can enhance host competence and survival. However, temperate phages are present in almost half of bacteria that have been sequenced, and phages that are specific to the pathogens that cause infections can be easily identified. In addition, the problems caused by horizontal genetic transfer can also now be avoided thanks to next generation sequencing, which enables selection of phages that do not pose a risk of transferring undesirable genes such as endotoxins [12]. Temperate phages can also be easily engineered in their lysogenic state for use in phage therapy, by different means: by modifying genes of interest as phage receptors to extend the host range; by inhibiting the lytic ability of phages without release of endotoxins; by modifying genes to enhance the killing effect of bacteriophages; by increasing the life time of phages in the circulatory system of mammalians; and also by transforming lysogenic phages into lytic phages [15, 27-31].

In this study, we selected a temperate phage, Ab105-2phi, which did not have any toxic or virulence genes in its genome (Figure 1 A1). This phage was selected with the objective of converting it into a lytic phage with potential use in phage therapy. The technique was previously described in *Salmonella enterica* bacteriophage SPN9CC and in the mycobacteriophage BPs33ΔHTH_HRM10 recently used in a phage cocktail to treat a patient with a disseminated drug-resistant *Mycobacterium*

abscessus [15, 32]. The technique is based on deletion of the CI repressor gene, which encodes the CI protein that binds to two operators to repress the Cro gene required for lytic development. Deletion of the CI gene thus maintains the phage in a lysogenic state [14, 15].

Conversion of the lysogenic into a lytic phage was confirmed by the presence of clear plaques and by the infection curves for both phages: the lysogenic Ab105-phi2 and the lytic mutant Ab105-phi2ΔCI. The differences in the optical density detected in both cases confirmed production of a lytic mutant, and the emergence of phage resistant mutants for both phages was observed. At MOI 10, inhibition of growth was greater and occurred earlier than at other MOI, but resistant bacteria emerged earlier than at lower MOI. The mutant lytic phage also presented a latent period of 30 minutes, and a moderate burst size of 32±2 PFU per cell was obtained with the mutant lytic phage including phages from *A. baumannii* [33-36]. The burst size is inversely related to the risk of emergence of phage resistant bacteria [37], which is one of the main objectives of phage therapy research, commonly addressed by the use of phage cocktails. Although the antimicrobial activity of this mutant lytic phage was well established by its ability to reduce the absorbance in a bacterial culture and also reduce the biofilm biomass, any reduction in the development of phage resistance will increase its potential use as a therapeutic phage. In this case, the strategy we used to enhance the potential of the Ab105-2phiΔCI phage as a therapeutic phage was to combine the phage with antibiotics, as we observed an almost 1 log reduction in the emergence of phage resistant mutants in the presence of meropenem or imipenem. The synergetic effect resulting from the combination of the lytic mutant phage Ab105-2phiΔCI and meropenem or imipenem enhanced the bactericidal effect of both the antibiotic and the phage. A strong antimicrobial effect was obtained by combining the phage at high MOI and the antibiotic at concentrations much lower than the MIC, thereby restoring the sensitivity of the strain to imipenem and meropenem. As the host strain does not possess beta-lactamases, the resistance is probably due to the action of a Resistance-Nodulation-Division (RND) efflux pump containing proteins that can act as phage receptor proteins, so that the phage would block the efflux pump and the antibiotic sensitivity of the strain would thus be increased [38]. The synergism between the carbapenem antibiotics and the mutant lytic phage was also confirmed in the survival assays with *G. mellonella*, as survival was higher in larvae that received the combined treatments. However, when the combination included meropenem (MIC, 32 μg/ml), survival was not statistically significantly higher, indicating that administration of a larger number of mutant lytic phage Ab105-2phiΔCI would be necessary (*in vivo*).

In conclusion, to our knowledge, this is the first in vitro and in vivo study in which a mutant lytic phage has been used in combination with carbapenem antibiotics (imipenem and meropenem) to reduce the emergence of resistance to the phages and restore the sensitivity to antibiotics, thereby increasing the therapeutic potential of the phage. Conversion of temperate phages (with a known genomic profile) into lytic phages may provide a new source of phages for use in phage therapy.

Acknowledgments: This study was funded by grant PI16/01163 awarded to M. Tomás within the State Plan for R+D+I 2013-2016 (National Plan for Scientific Research, Technological Development and Innovation 2008-2011) and co-financed by the ISCIII-Deputy General Directorate for Evaluation and Promotion of Research-European Regional Development Fund "A way of Making Europe" and Instituto de Salud Carlos III FEDER, Spanish Network for the Research in Infectious Diseases (REIPI, RD16/0016/0001, RD16/0016/0006, RD16/0016/0007, RD16/0016/0008, RD16/0016/0009 and RD16/0016/0010) and by the Study Group on Mechanisms of Action and Resistance to Antimicrobials, GEMARA (SEIMC, http://www.seimc.org/). M. Tomás was financially supported by SERGAS (ACIS, Axencia de Conecemento en Saude, Xunta de Galicia). R. Trastoy and L. Fernández-García were financially supported by respectively an SEIMC grant and a fellowship from the Deputacion Provincial da Coruña , Xunta de Galicia.

Conflicts of Interest: The authors declare that have no conflict of interest.

Table 1. Bacterial strains used in this study. Host range and efficiency of plating (EOP).

Strain	ST	Spot	EOP	Spanish Hospital where the strain was isolated
Ab105 GEIH-2010	2	+/-	1	Hospital Universitario Virgen del Rocío (Seville, Spain)
Ab192 GEIH-2000	2	+/-	0.22	Hospital Universitario Virgen del Rocío (Seville, Spain)
Ab404 GEIH-2010	80	+	0.0002	Hospital Dr. Molines (Valencia, Spain)
Ab166 GEIH-2000	2	+/-	-	Hospital Universitario Virgen del Rocío (Seville, Spain)

Ab177_GEIH-2000	2	+	1.55	Hospital Universitario Virgen del Rocío (Seville, Spain)
Ab13_GEIH-2010	79	-	-	Hospital Santiago de Compostela (Santiago de Compostela, Spain)
Ab09_GEIH-2010	297	-	-	Hospital Santiago de Compostela (Santiago de Compostela, Spain)
Ab160_GEIH-2000	2	-	-	Hospital Universitario Virgen del Rocío (Seville, Spain)
Ab155_GEIH-2000	2	-	-	Hospital Universitario Virgen del Rocío (Seville, Spain)
Ab05_GEIH-2010	186	-	-	Hospital A Coruña (A Coruña, Spain)
Ab22_GEIH-2010	52	-	-	Hospital Pontevedra (Pontevedra, Spain)
Ab421_GEIH-2010	2	-	-	Hospital Insular (Gran Canaria, Spain)
Ab77_GEIH-2000	2	-	-	Hospital Universitario Ramon y Cajal (Madrid, Spain)
Ab141_GEIH-2000	179	-	-	Complejo Hospitalario Toledo (Toledo, Spain)
Ab217_GEIH-2010	2	-	-	Hospital Reina Sofía (Cordoba, Spain)
Ab235_GEIH-2010	2	-	-	Hospital Marqués de Valdecilla (Santander, Spain
Ab37_GEIH-2010	2	-	-	Hospital Virgen del Rocío (Seville, Spain)
Ab222_GEIH-2000	181	-	-	Hospital Bellvitge (Barcelona)
Ab461_GEIH-2010	2	-	-	Hospital del Mar (Barcelona, Spain)
Ab173_GEIH-2010	88	-	-	Hospital San Agustín (Avilés, Spain)

Table 2. Primers used to delete the CI gene.

Primer	Sequence	Strain/plasmid
UPCI(NotI)Fw	GGG*GCGGCCGC*TGAAGAATTCATCACTTG	Ab105_GEIH-2010
UPCI(BamHI)Rev	GGG*GGATCC*CGTTACTTCTATCGGAAT	Ab105_GEIH-2010
DWCI(BamHI)Fw	GGG*GGATCC*ATTAAGGTTTTAGGTGAT	Ab105_GEIH-2010
DWCI(SphI)Rev	GGG*GCATGC*TAAATCATCCAAATCGAC	Ab105_GEIH-2010
CIFw	ATGGACAAATTTATGGCTAC	Ab105_GEIH-2010
CIRev	TAACTTTTTCTAACACGCT	Ab105_GEIH-2010
IntCIFw	AAAGCGCTGCCAACTTTT	Ab105_GEIH-2010
IntCIRev	CAACAGATTCATCCTCAT	Ab105_GEIH-2010
pMo130TelRFw	ATTCATGACCGTGCTGAC	pMo130TelR
pMo130TelRRev	CTTGTCTGTAAGCGGATG	pMo130TelR
Plasmid	**Description**	**Origin**
pMo130TelR	Suicide plasmid, *xylE*$^{+}$, *sac*B^{+}, km^{R}, Tel^{R}	[17]

Restriction enzyme sites are shown in italics.

Table 3. Frequency of phage resistant mutants. Phage resistant mutant frequency in the presence of the combination of doxycycline, meropenem and imipenem at ¼ MIC in combination with lytic mutant phage Ab105-2phiΔCI was calculated.

Sample	Frequency of phage resistant mutants
Ab105-2phiΔCI	1.70E-06
Ab105-2phiΔCI+Doxycycline	1.31E-07
Ab105-2phiΔCI+Meropenem	2.10E-07
Ab105-2phiΔCI+Imipenem	1.90E-07

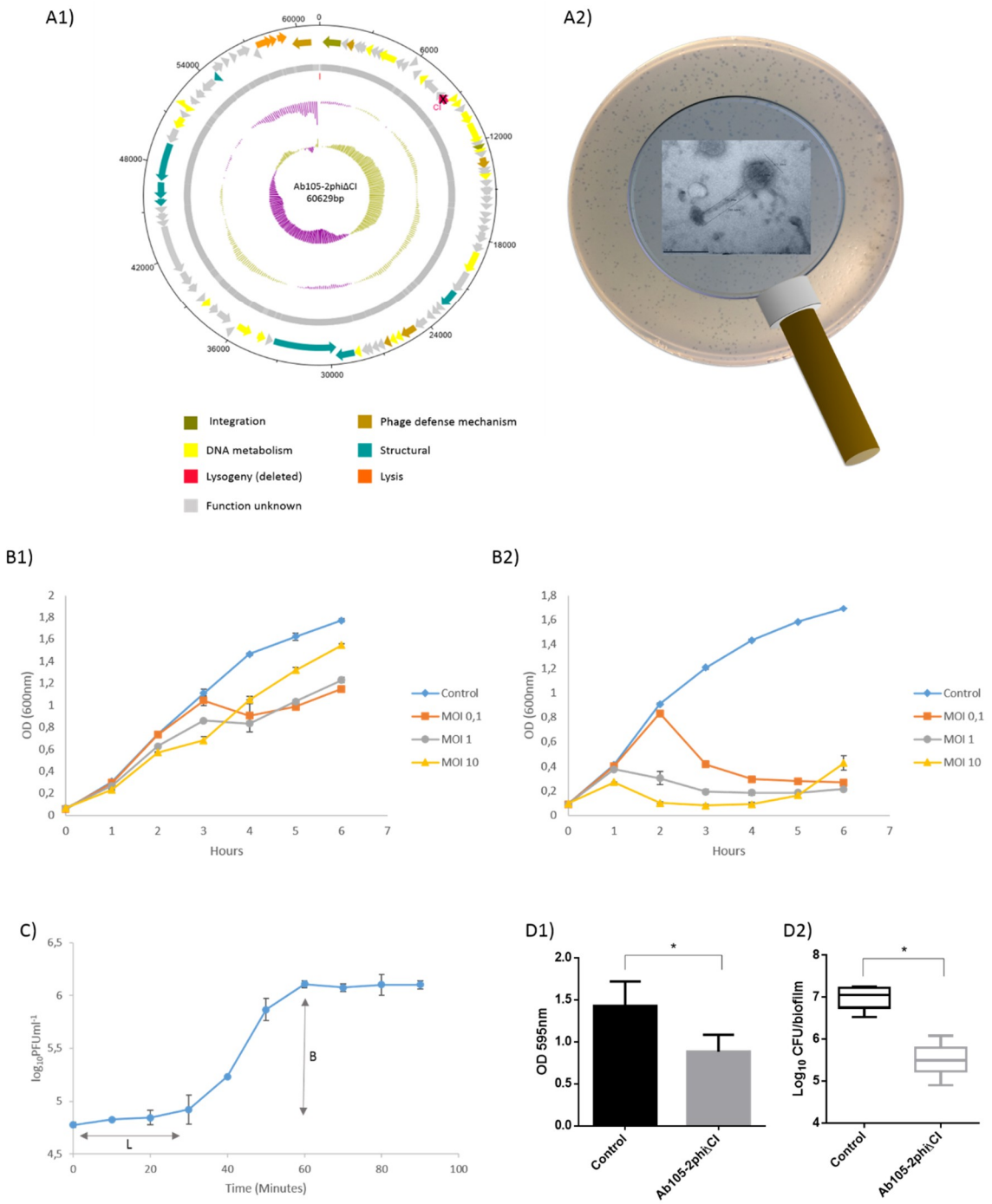

Figure 1. Graphical representation of the Ab105-2phiΔCI phage. The ORF and direction of transcription are indicated by arrows. (A1) The protein functions are indicated in different colours, and the GC content and GC skew are shown as pink and green circles respectively. (A2) Lytic plaques and TEM image of the mutant lytic phage Ab105-phi2ΔCI. (B1) Infection curves for the lysogenic phage Ab105-2phi and (B2) the mutant lytic phage Ab105-2phiΔCI. (C) One step growth curve of the mutant lytic phage Ab105-phi2ΔCI (L: Latent period; B: burst size). Mutant lytic phage Ab105-phi2ΔCI antibiofilm activity on the biofilm produced by the clinical strain of *A. baumannii* Ab177_GEIH-2000. (D1) Reduction in the biofilm and reduction in the number of CFUs present in the biofilm after treatment with the mutant lytic phage Ab105-phi2ΔCI.

Video 1. Initial adsorption of phages to bacteria at 37ºC in agar slices placed directly between stainless steel O-rings for live-cell imaging. Use of extracellular DNA markers enabled lysis of more than 300 bacteria to be followed in real time.

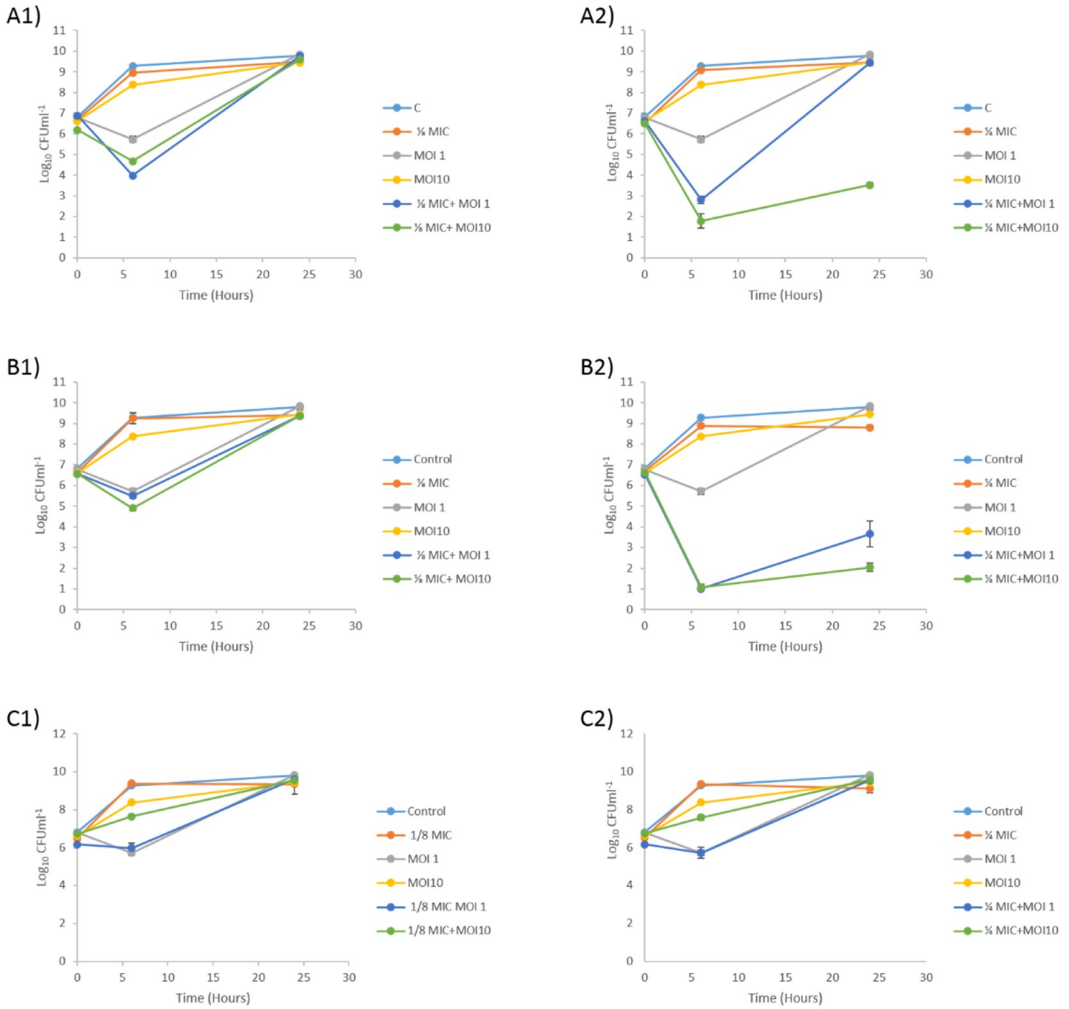

Figure 2. Kill curves in of *A. baumannii* clinical strain Ab177_GEIH-2000 using the mutant lytic phage Ab105-2phiΔCI at MOI 1 and MOI10 in combination with meropenem at (A1) 1/8 MIC and (A2) 1/4 MIC;(B1) imipenem at 1/8 MIC and (B2)1/4 MIC, and (C1) doxycycline at 1/8 MIC and (C2)1/4 MIC.

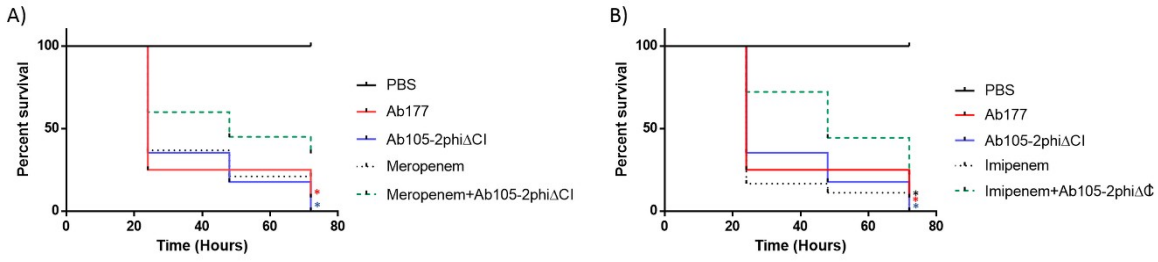

Figure 3. *G. mellonella* survival 96 h after an infection with Ab177_GEIH-2000 and treatment with mutant lytic phage Ab105-2phiΔCI at MOI 10 and the antibiotics meropenem at (A) ¼ MIC and imipenem at (B)1/4 MIC. The Log-Rank (Mantel-Cox) test, */* (P<0.05) was used to compare the combination of imipenem and meropenem plus phage (line green) with each antibiotic alone (*) or the phage alone (*); *(P<0.05) for comparison of the combination of the phage (line green) and antibiotics (imipenem or meropenem) and untreated infection (*).

References

[1] Santajit S, Indrawattana N. Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens. Biomed Res Int. 2016;2016:2475067.

[2] Oliveira H, Vilas Boas D, Mesnage S, Kluskens LD, Lavigne R, Sillankorva S, et al. Structural and Enzymatic Characterization of ABgp46, a Novel Phage Endolysin with Broad Anti-Gram-Negative Bacterial Activity. Front Microbiol. 2016;7:208.

[3] Kutter E, De Vos D, Gvasalia G, Alavidze Z, Gogokhia L, Kuhl S, et al. Phage therapy in clinical practice: treatment of human infections. Curr Pharm Biotechnol. 2010;11:69-86.

[4] Advantages and Disadvantages in the Use of Antibiotics or Phages as Therapeutic Agents. Enzybiotics. Antibiotic Enzymes as Drugs and Therapeutics. 2009.

[5] Ofir G, Sorek R. Contemporary Phage Biology: From Classic Models to New Insights. Cell. 2018;172:1260-70.

[6] Torres-Barcelo C. The disparate effects of bacteriophages on antibiotic-resistant bacteria. Emerg Microbes Infect. 2018;7:168.

[7] Clokie MR, Millard AD, Letarov AV, Heaphy S. Phages in nature. Bacteriophage. 2011;1:31-45.

[8] Rohde C, Resch G, Pirnay JP, Blasdel BG, Debarbieux L, Gelman D, et al. Expert Opinion on Three Phage Therapy Related Topics: Bacterial Phage Resistance, Phage Training and Prophages in Bacterial Production Strains. Viruses. 2018;10.

[9] Howard-Varona C, Hargreaves KR, Abedon ST, Sullivan MB. Lysogeny in nature: mechanisms, impact and ecology of temperate phages. ISME J. 2017;11:1511-20.

[10] Tagliaferri TL, Jansen M, Horz HP. Fighting Pathogenic Bacteria on Two Fronts: Phages and Antibiotics as Combined Strategy. Front Cell Infect Microbiol. 2019;9:22.

[11] Casey E, van Sinderen D, Mahony J. In Vitro Characteristics of Phages to Guide 'Real Life' Phage Therapy Suitability. Viruses. 2018;10.

[12] Monteiro R, Pires DP, Costa AR, Azeredo J. Phage Therapy: Going Temperate? Trends Microbiol. 2019;27:368-78.

[13] Torres-Barcelo C, Hochberg ME. Evolutionary Rationale for Phages as Complements of Antibiotics. Trends Microbiol. 2016;24:249-56.

[14] Schubert RA, Dodd IB, Egan JB, Shearwin KE. Cro's role in the CI Cro bistable switch is critical for lambda's transition from lysogeny to lytic development. Genes Dev. 2007;21:2461-72.

[15] Shin H, Lee JH, Yoon H, Kang DH, Ryu S. Genomic investigation of lysogen formation and host lysis systems of the *Salmonella* temperate bacteriophage SPN9CC. Appl Environ Microbiol. 2014;80:374-84.

[16] Hamad MA, Zajdowicz SL, Holmes RK, Voskuil MI. An allelic exchange system for compliant genetic manipulation of the select agents *Burkholderia pseudomallei* and *Burkholderia mallei*. Gene. 2009;430:123-31.

[17] Amin IM, Richmond GE, Sen P, Koh TH, Piddock LJ, Chua KL. A method for generating marker-less gene deletions in multidrug-resistant *Acinetobacter baumannii*. BMC Microbiol. 2013;13:158.

[18] Kropinski AM, Mazzocco A, Waddell TE, Lingohr E, Johnson RP. Enumeration of bacteriophages by double agar overlay plaque assay. Methods Mol Biol. 2009;501:69-76.

[19] Raya RR, H'Bert E M. Isolation of Phage via Induction of Lysogens. Methods Mol Biol. 2009;501:23-32.

[20] Merabishvili M, Vandenheuvel D, Kropinski AM, Mast J, De Vos D, Verbeken G, et al. Characterization of newly isolated lytic bacteriophages active against *Acinetobacter baumannii*. PLoS One. 2014;9:e104853.

[21] Hargreaves KR, Colvin HV, Patel KV, Clokie JJ, Clokie MR. Genetically diverse *Clostridium difficile* strains harboring abundant prophages in an estuarine environment. Appl Environ Microbiol. 2013;79:6236-43.

[22] Hyman P, Abedon ST. Practical methods for determining phage growth parameters. Methods Mol Biol. 2009;501:175-202.

[23] Lopes A, Pereira C, Almeida A. Sequential Combined Effect of Phages and Antibiotics on the Inactivation of *Escherichia coli*. Microorganisms. 2018;6.

[24] Peleg AY, Jara S, Monga D, Eliopoulos GM, Moellering RC, Mylonakis E. Galleria mellonella as a model system to study *Acinetobacter baumannii* pathogenesis and therapeutics. Antimicrob Agents Chemother. 2009;53:2605-9.

[25] Thiry D, Passet V, Danis-Wlodarczyk K, Lood C, Wagemans J, De Sordi L, van Noort V, Dufour N, Debarbieux L, Mainil JG, Brisse S, Lavigne R. New Bacteriophages against Emerging Lineages ST23 and ST258 of *Klebsiella pneumoniae* and Efficacy Assessment in Galleria mellonella Larvae. Viruses. 2019 May 3;11(5).

[26] Lopez M, Rueda A, Florido JP, Blasco L, Fernandez-Garcia L, Trastoy R, et al. Evolution of the Quorum network and the mobilome (plasmids and bacteriophages) in clinical strains of *Acinetobacter baumannii* during a decade. Sci Rep. 2018;8:2523.

[27] Matsuda T, Freeman TA, Hilbert DW, Duff M, Fuortes M, Stapleton PP, et al. Lysis-deficient bacteriophage therapy decreases endotoxin and inflammatory mediator release and improves survival in a murine peritonitis model. Surgery. 2005;137:639-46.

[28] Lu TK, Collins JJ. Engineered bacteriophage targeting gene networks as adjuvants for antibiotic therapy. Proc Natl Acad Sci U S A. 2009;106:4629-34.

[29] Pouillot F, Blois H, Iris F. Genetically engineered virulent phage banks in the detection and control of emergent pathogenic bacteria. Biosecur Bioterror. 2010;8:155-69.

[30] Borysowski J, Międzybrodzki R, Gorski A. Phage therapy : current research and applications. Wymondham?: Caister Academic Press; 2014.

[31] Nobrega FL, Costa AR, Kluskens LD, Azeredo J. Revisiting phage therapy: new applications for old resources. Trends Microbiol. 2015;23:185-91.

[32] Broussard GW, Oldfield LM, Villanueva VM, Lunt BL, Shine EE, Hatfull GF. Integration-dependent bacteriophage immunity provides insights into the evolution of genetic switches. Mol Cell. 2013;49:237-48.

[33] Popova AV, Zhilenkov EL, Myakinina VP, Krasilnikova VM, Volozhantsev NV. Isolation and characterization of wide host range lytic bacteriophage AP22 infecting *Acinetobacter baumannii.* FEMS Microbiol Lett. 2012;332:40-6.

[34] Jin J, Li ZJ, Wang SW, Wang SM, Huang DH, Li YH, et al. Isolation and characterization of ZZ1, a novel lytic phage that infects *Acinetobacter baumannii* clinical isolates. BMC Microbiol. 2012;12:156.

[35] Dalmasso M, Strain R, Neve H, Franz CM, Cousin FJ, Ross RP, et al. Three New *Escherichia coli* Phages from the Human Gut Show Promising Potential for Phage Therapy. PLoS One. 2016;11:e0156773.

[36] Gonzalez-Menendez E, Arroyo-Lopez FN, Martinez B, Garcia P, Garrido-Fernandez A, Rodriguez A. Optimizing Propagation of *Staphylococcus aureus* Infecting Bacteriophage vB_SauM-phiIPLA-RODI on *Staphylococcus xylosus* Using Response Surface Methodology. Viruses. 2018;10.

[37] Torres-Barcelo C. Phage Therapy Faces Evolutionary Challenges. Viruses. 2018;10.

[38] Oechslin F. Resistance Development to Bacteriophages Occurring during Bacteriophage Therapy. Viruses. 2018;10.