Interaction of Phage and Gentamicin Combinations in *Pseudomonas aeruginosa* and *Staphylococcus aureus* Polymicrobial Biofilms

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Introduction

Pseudomonas aeruginosa and *Staphylococcus aureus* are opportunistic pathogens and commonly found in polymicrobial biofilm-associated diseases, namely chronic wounds. Their co-existence in a biofilm contributes to an increased tolerance to antibiotics. Combined treatments of bacteriophages and antibiotics have shown a promising antibiofilm activity, since their mechanisms of bacteria-killing differ profoundly. In this study, 48 hours old mono and dual-species biofilms were treated with a newly isolated *P. aeruginosa* phage (**vB_PaM_EPA1**) and gentamicin (**GEN**) alone and in simultaneous or sequential combinations. After 24 hours treatment, the number of viable cells were enumerated by CFU counting and observed under confocal laser scanning microscopy (CLSM) with fluorescence probes. Probes were designed to specifically target the bacterial species and consists of a recombinant tail fiber protein (*P. aeruginosa*-specific) fused to mCherry (EPA1_TFP+mCherry) and a cell wall binding domain of a phage endolysin (*S. aureus* specific) fused to GFP (LM12_AMI-SH3+GFP).

Goal of the study

We aimed at assessing the antibiofilm activity of combinations of gentamicin and a *P. aeruginosa* specific phage vB_PaM_EPA1 in order to infer if combination of antimicrobials can potentiate biofilm killing in a synergistic effect.

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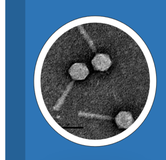
Methods

Bacteriophage

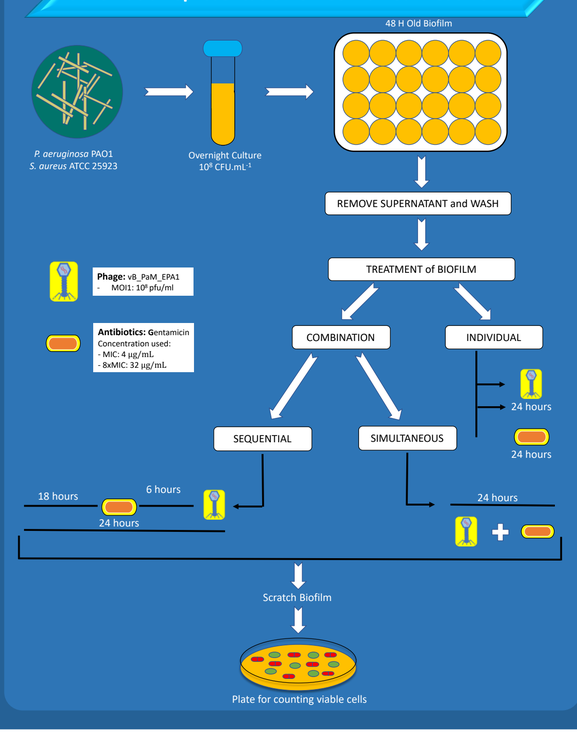


- Isolation of **Phage vB_PaM_EPA1**
- Host range
- Efficiency of plating (EOP)
- One-step Growth Curve
- Morphological analysis (TEM)
- Genome *in silico a*nalysis

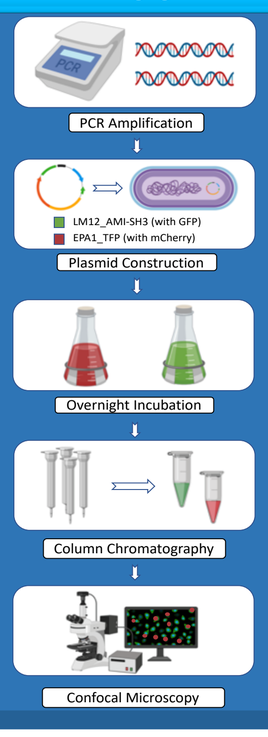
Bacterial Strains

- 17 *P. aeruginosa* clinical strains and 3 collection strains were used for host range.
- Antibiogram profiles (Vitek2)
- *P. aeruginosa* PAO1 and *S. aureus* ATCC 25923 were used to perform 48 h old biofilms.

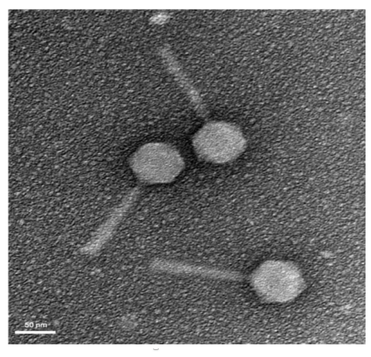
Schematic Representation of Combined Treatments



Probes for Biofilm Imaging and CLSM Analysis



Results



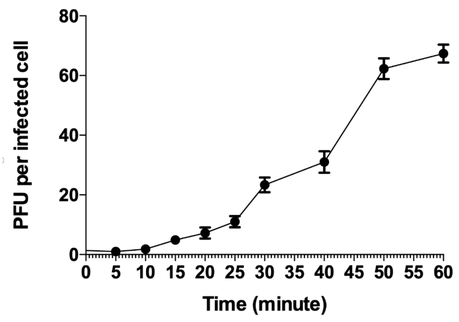


Figure 1. Phage EPA1 analysis. (A) TEM image. Scale bar = 50 nm. (B) One step growth curve.

EPA1 has an icosahedral head with 69 nm in diameter and a contractile tail of 145 x 24 nm. According to Ackermann's classification, EPA1 belongs to the *Caudovirales* order and ***Myoviridae*** family.

The latent period of EPA1 was around 10 minutes, and the burst size was approximately **34 progeny** phages per infected cell.

Table 1. Host range analysis of EPA1

Clinical Strains	Collection Strain	Infectivity
17	3	14

EPA1 possesses a **broad lytic spectrum** on strain tested. Furthermore, the phage had a high EOP in 14 out of 20 *P. aeruginosa* strains.

Table 2. General overview of the efficacy of combined treatments in 48 hours *P. aeruginosa* mono-species biofilms. Synergistic – the biofilm reduction using phage-antibiotic combinations is greater than the sum of their individual treatments. Additive – the biofilm reduction using phage-antibiotic combination is similar to the sum of their individual treatments.

Treatment	Gentamicin
Simultaneously MIC	Synergistic
Simultaneously 8 MIC	Additive
Sequentially MIC	Synergistic
Sequentially 8 MIC	Synergistic

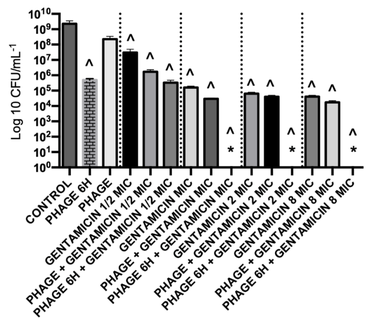


Figure 2. Treatment of *P. aeruginosa* PAO1 48 hours biofilms with gentamicin at different concentrations. A prefix PHAGE indicates EPA1 in MOI 1, 1/2 MIC indicates the dose of antibiotics with 1/2x MIC value, MIC indicates the dose of antibiotics with 1x MIC value of *P. aeruginosa*, 2 MIC indicates the dose of antibiotics with 2x MIC value of *P. aeruginosa*, 8 MIC indicates the dose of antibiotics with 8x MIC value of *P. aeruginosa*, PHAGE + antibiotic indicates simultaneous treatment and PHAGE 6 H + antibiotics indicates phage was added first then antibiotic was added with 6 hours delay. * Under detection limit ($<10^2$). (^) Statistical differences between the control and treated biofilms were determined by two-way repeated-measures analysis of variance (ANOVA) with a Tukey's multiple comparison test.

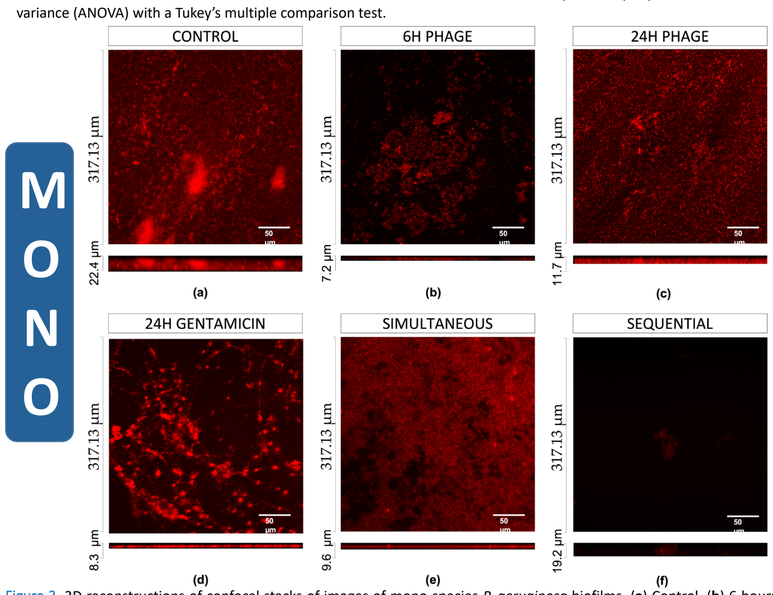


Figure 3. 3D reconstructions of confocal stacks of images of mono-species *P. aeruginosa* biofilms. (**a**) Control, (**b**) 6 hours phage treatment, (**c**) 24 hours phage treatment, (**d**) 24 hours Gentamicin treatment, (**e**) 24 hours simultaneous treatment, (**f**) 24 hours sequential treatment. All biofilms were stained with EPA1_TFP (with mCherry) recombinant protein. Scale bar represents 50 µm.

EPA1 or GEN alone had a modest effect in reducing biofilm bacteria. However, when applied simultaneously, a profound improvement in the killing effect was observed. Moreover, an impressive biofilm eradication was observed when gentamicin was added **sequentially** after 6 hours of phage treatment.

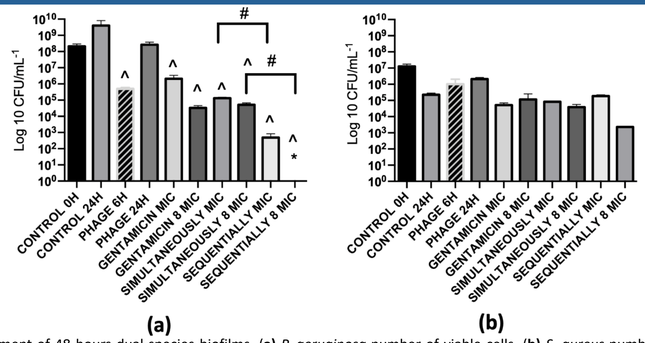


Figure 4. Treatment of 48 hours dual-species biofilms. (**a**) *P. aeruginosa* number of viable cells. (**b**) *S. aureus* number of viable cells. A prefix PHAGE indicates EPA1 in MOI 1, 6 H and 24 H indicates treatment time period for 6 and 24 hours, MIC indicates the dose of antibiotics with 1x MIC value of *P. aeruginosa*, 8 MIC indicates the dose of antibiotics with 8xMIC value of *P. aeruginosa,* PHAGE + antibiotic indicates simultaneous treatment and PHAGE 6 H + antibiotics indicates phage was added first then antibiotic was added with 6 hours delay. * Under detection limit ($<10^2$). (^) Statistical differences between the control and treated biofilms. (#) Statistical differences between the simultaneously and sequentially treated biofilms. Statistical differences were determined by two-way repeated-measures analysis of variance (ANOVA) with a Tukey's multiple comparison test.

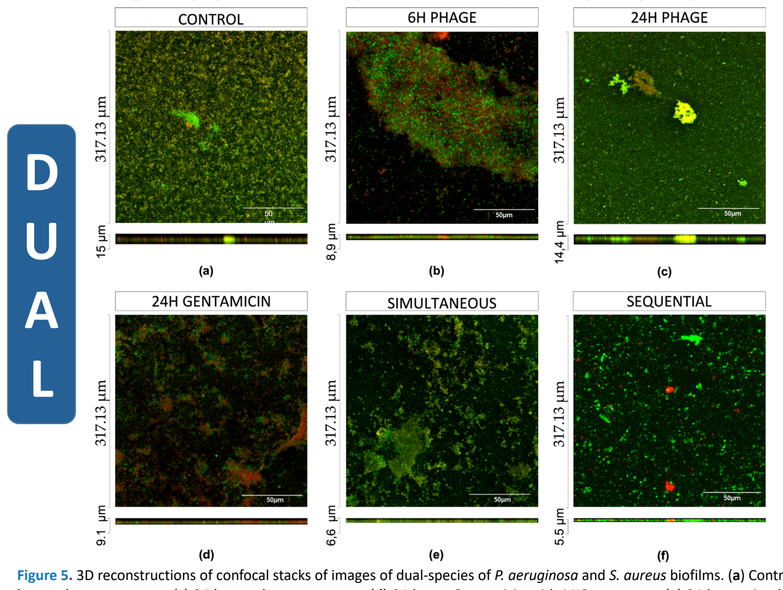


Figure 5. 3D reconstructions of confocal stacks of images of dual-species of *P. aeruginosa* and *S. aureus* biofilms. (**a**) Control, (**b**) 6 hours phage treatment, (**c**) 24 hours phage treatment, (**d**) 24 hours Gentamicin with MIC treatment, (**e**) 24 hours simultaneous treatment, (**f**) 24 hours sequential treatment. 48 hours old intact biofilms were stained by using LM12_AMI-SH3 (with GFP) and EPA1_TFP (with mCherry) recombinant proteins. Scale bar represents 50 µm.

Dual-species biofilms were **more tolerant** to all treatments than mono-species *P. aeruginosa* biofilms. Nevertheless, the sequential treatment at 8xMIC almost eradicated *P. aeruginosa* biofilm cells, but it did not increase the antimicrobial effect on *S. aureus*. CLSM images indicate that both species were randomly distributed throughout the biofilm 3D structures.

Main conclusions

Overall, from this study, two main conclusions emerge. First, the combined treatment with a sequential application of phages and then antibiotic is the most promising approach to combat infectious biofilms when compared with their individual and simultaneous treatments. Second, the majority of the studies of antibiofilm approaches are conducted in mono-species biofilms, and as demonstrated herein, the treatment outcomes are completely different when a second species is added. So, to achieve success, phages should be tested prior to antibiotic addition.

References

1. Akturk, E.; Oliveira, H.; Santos, S.B.; Costa, S.; Kuyumcu, S.; Melo, L.D.R.; Azeredo, J. (2019). Synergistic Action of Phage and Antibiotics: Parameters to Enhance the Killing Efficacy Against Mono and Dual-Species Biofilms. Antibiotics 8, 103.
2. Pires, D. P., Vilas Boas, D., Sillankorva, S., & Azeredo, J. (2015). Phage Therapy: a Step Forward in the Treatment of *Pseudomonas aeruginosa* Infections. Journal of Virology, 89(15), 7449–7456.
3. Chaudhry, W. N., Concepción-Acevedo, J., Park, T., Andleeb, S., Bull, J. J., Levin, B. R., ... Gupta, S. (2017). Synergy and Order Effects of Antibiotics and Phages in Killing *Pseudomonas aeruginosa* Biofilms. Plos One, 12(1), e0168615.

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