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Microbiology

The Action of Bacteriophages and β-Lactam Antibiotic on *P. aeruginosa* Biofilm Formation

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ABSTRACT: We observed the effects of the combined action of bacteriophages and beta-lactam antibiotic on *P. aeruginosa* bacteria. The *P. aeruginosa* PAO1 wild-type strain, β-lactam antibiotic (Imipenem) and the commercial bacteriophage preparation (Pyobacteriophage) were used in this study. The results have shown that the phage preparation which contains few different types of *P. aeruginosa* phages effectively decreased *P. aeruginosa* biofilm formation whereas the action of single *P. aeruginosa* phages alone, which were isolated from phage preparation, did not have an important action on biofilm dispersal. Moreover, the combined use of phage preparation and antibiotic (Imipenem) has shown sinergistic action on biofilms. Our results prove that bacteriophages have the ability to penetrate and cause significant dispersal of biofilms formed by *P. aeruginosa* microorganisms, which in turn could increase the accessibility of the antibiotic to enable elimination of the infection. This phenomenon lends considerable importance to phage therapy used both independently and in combination with antibiotic therapy for treatment of infections caused by mucoid bacterial strains. *© 2016 Bull. Georg. Natl. Acad. Sci.*

Key words: *P. aeruginosa*, bacteriophage, β-lactam antibiotic, biofilm formation, synergistic effect

The growth of pathogen microbes including the drug-resistant forms makes it more difficult to fight against infectious diseases. Antibiotics still remain effective therapeutics against bacterial infections, but the growth of drug resistant forms of microbes requires alternatives and the new treatment solutions to be worked out [1].

P. aeruginosa is a facultative pathogenic microorganism and it is a highly relevant opportunistic pathogen. *P. aeruginosa* plays a leading role in nosocomial pneumonia, urological diseases and purulent-surgical infections. It is the most common cause of infections of burn injuries and is a frequent colonizer of medical devices [2, 3]. Cystic fibrosis patients are also predisposed to *P. aeruginosa* colonisation and infection of the lungs [2, 4].

P. aeruginosa is naturally resistant to a large range of antibiotics, making one of its most worrisome characteristics its low antibiotic susceptibility. The ability of *P. aeruginosa* mucoidal strains to produce biofilms makes them inaccessible to therapeutic concentrations of antibiotics - another important mechanism of multi-resistance to antimicrobial agents of these strains [5]. The ability of bacteriophages to penetrate into *P. aeruginosa* biofilms makes them very relevant in the treatment of *P. aeruginosa* infec-

tions.

Phage therapy is one potential solution to fight against resistant pathogenic bacterial strains using bacteriophages as the therapeutic agent. Phage therapy is generally regarded as safe for human beings, and no serious side effects have been identified. Due to their specificity of infection, phages do not suppress the natural flora. Phage therapy is effective against bacterial infections of different etiologies [6]. Bacteriophages can cause dispersal of biofilm surfaces and the combination of bacteriophage and antibiotic application has been suggested as a valuable approach for biofilm control. More of, combined treatment of antibiotics and phages may reduce the incidence of phage resistance or antibiotic resistance. Phages can act as an antibiotic adjuvant and could enhance antibiotic antimicrobial efficacy [7].

In the presented work we aim to determine if the combined use of beta-lactam antibiotics and bacteriophages of a therapeutic phage cocktail would reduce the biofilm formation of *P. aeruginosa* cells. This work shows the potential effectiveness of the use of the combination of phages and antibiotics, as well as phages alone to treat acute infections caused by mucoid bacteria.

Materials and Methods

Investigated materials. The PAO1 wild type strain, the beta-lactam antibiotic Imipenem and commercially available therapeutic bacteriophage preparation Pyobacteriophage were used in the study. Pyobacteriophage is a phage cocktail - mixture of sterile filters of phage lysates of *Streptococcus*, *Staphylococcus, P. aeruginosa, E. coli* and *Proteus*. The single *P. aeruginosa* bacteriophage - phage N1 was isolated and purified from chosen bacteriophage cocktail according to plaque purification method [6].

Determination of lysis activity and spectrum of bacteriophages. One drop of indicator strain ($5x10^8$ p/ml) on a sterile loop was used to draw parallel lines on 1.5% agar Petri plate. After the drying of bacterial lines (15-20 min), 0.05ml of test phage was spotted on the bacterial lines (modified spot test) [6]. After drying the plates were then incubated at 37^0C for 18-24 hours. Phage presence is identified as areas of lysis on the bacterial lawn (modified method of phage host range spectrum) [8]. Designations for evaluation the lysis rate of bacteriophage activity on bacterial strains are: 4+ (Absolute lysis - Clear plaques, absolutely transparent), 3+ and 2+ (Secondary growth - spots show evenly dispersed secondary bacterial growth), 1+ (Incomplete growth - areas of lysis are presented as the small plaques), 0 (Non reaction - strain is resistant to phage).

Biofilm production. A single colony from each strain was inoculated into 5ml LB (37°C at 200rpm). After 12 hours of growth the overnight culture diluted into 1/100 in 3ml. The wells are then inoculated with 100 µl of the 1/100 dilution of night culture, or night culture + bacteriophage stock. The plates are then incubated 24 hours at 37°C with no shaking. After incubation the media was discarded into a waste container. The plates were then immersed in distilled water by inserting from one side and allowing all the wells to fill with water. The water was discarded into the waste container and plate shaken thoroughly. The plate was then tapped on an absorbent surface to remove excess water. 150 µl of 0.1 % (v/v) Crystal violet was added to each of the test wells and leave for 10 min at room temperature. The crystal violet was then discarded into the waste container and the plates were washed twice in two different wash containers, containing distilled water. Finally, the plates were placed face down firmly on an absorbent surface and left air dry. Once the plates were dry, 200 µl of 95% Ethanol was added to each well and incubate at room temp. for 15 mins. The plates were then read at 595 nm [9].

Results

Lysis activity of bacteriophages on PAO1 bacteria

We have isolated and purified a single phage (*P. aeruginosa* phage N1) from the commercial pyophage cocktail using on *P. aeruginosa* strain PAO1. The

Table 1. Lysis activity of phages

P. aeruginosa strain	Bacteriophage	Lysis activity
PAO1	Pyobacteriophage (phage cocktail)	3+
	Phage N1 (single phage)	4+

isolated phage, as well as Pyobacteriophage itself was tested for their lysis activity on PAO1 strain. The tested strain was sensitive to both phage cocktail and the monophage – *P. aeruginosa* phage N1. Table 1 shows the lysis activity of phages on PAO1 bacteria.

Action of Phages on Biofilm Formation

We studied the action of bacteriophages, both the phage cocktail and purified monophage, on biofilm formation. Biofilms were formed by PAO1, or PAO1 + phage during 24 hours. Studies show that the phage cocktail effectively decreases biofilm formation, whereas the monophage does not have significant action on biofilm dispersal. The results obtained are given in Fig.1.

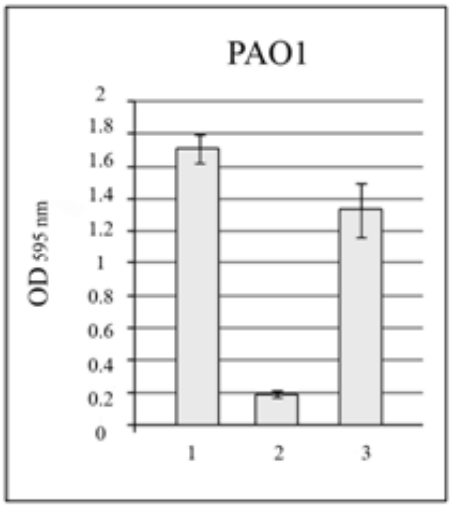

Fig. 1. Action of phage cocktail and single phage on biofilm formation (mean of three independent experiments) - Measured after 24 hours of incubation. 1 - Biofilm Control formed by PAO1. 2 - effect of Pyobacteriophage (~$3x10^6$pfu/ml). 3 - effect of single phage (phage 1~$3x10^6$pfu/ml) isolated from Pyobacteriophage.

Synergistic Effect of Phages and Antibiotic on Biofilm Formation

The combined use of beta lactam antibiotic Imipenem (concentrations close to MIC~1.5μg/ml) and the phage cocktail on biofilm formation has given a synergistic effect as shown in Fig.2.

Biofilms were formed during 24 hours with no shaking on microplate wells and the phage, antibiotic, or both in combination were added to the wells and incubated for 24 hours. The results show that combined use of antibiotic and bacteriophages were much more effective for biofilm dispersal, than phages alone.

Discussion

P. aeruginosa is one of the most resistant bacterial pathogens. These bacteria can easily get colonized on medical devices and rapidly form biofilms thus posing a significant threat for hospitals. [10]

In this study a monophage was isolated and purified from the commercially available phage cocktail Pyobacteriophage, which had a high lysis activity on strain PAO1. Our studies show that the monophage did not have a significant effect on biofilm dispersal. However, phage cocktail containing a few different types of *P. aeruginosa* bacteriophages caused an important decrease of biofilm formation after 24 hours of incubation with the PAO1 strain.

Our experiments showed that a phage cocktail has a much stronger effect on biofilm dispersal in comparison to the action of a single phage, which did not cause significant decrease of biofilm formation. Despite of high lysis activity on against the bacterial strain the isolated bacteriophage was not able to cause a significant effect on biofilm reduction. One possible explanation is that there is an emergence of phage resistant mutants within the biofilm which protect it from phage infection, this can be explained due to the emergence of phage resistant strains in bacterial culture which can go on to produce biofilms effectively. But in case of phage cocktail, the development of phage resistance to all differ-

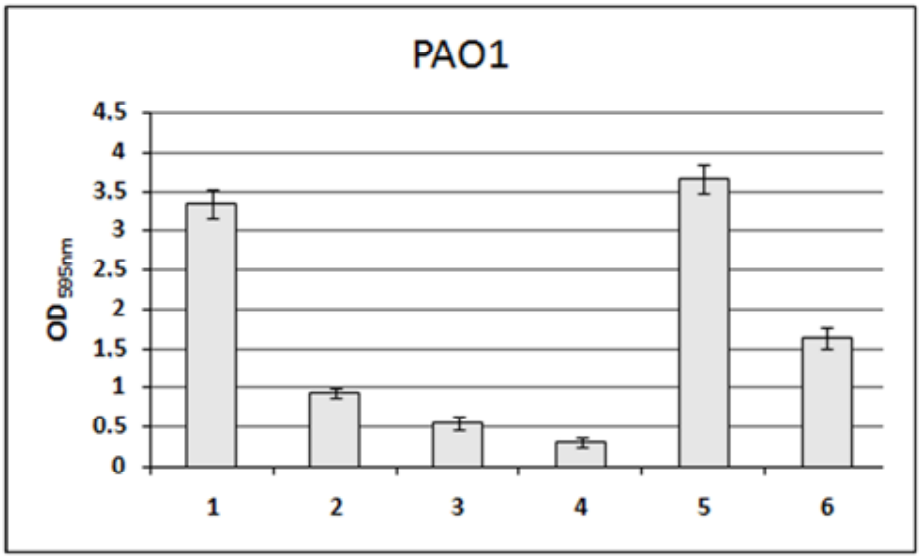

Fig. 2. Synergistic effect of phage cocktail (Pyobacteriophage) and antibiotic (Imipenem) on biofilm formation. The mean of three independent experiments: 1 - control of biofilm after 48h of incubation. 2 - effect of phagesti added at 24h biofilms and measured after 24 h of action. 3 - effect phage ($3x10^6$pfu/ml) and Imipenem (0,9µg/ml<MIC). 4 - effect of phage ($3x10^6$pfu/ml) and Imipenem (3,5µg/ml>MIC). 5 - effect of Imipenem(0,9µg/ml<MIC). 6 - effect of Imipenem (3,5µg/ml>MIC).

ent types of phages would take longer time as a result we get a larger decrease of biofilm formation compare to the action of single phage.

We have determined the MIC value for antibiotics and some concentrations close to MIC were selected to study the action on biofilm formation in combination with phages. Studies showed the synergistic effect of these agents. The use of a phage cocktail causes significant decrease of biofilm formation, but the combined use of phage cocktail and antibiotics decrease biofilm formation even more, whereas the antibiotic alone does not have an effect. Combinations of those agents showed the synergy of phage and antibiotics against biofilm formation.

The phage-antibiotic synergism is described by other scientists as well. The amikacin-phage combination is shown to have potentially more benefits on *P. aeruginosa* biofilms than using phages or antibiotics alone [11]. Ryan et al. reported that combinations of T4 bacteriophage and cefotaxime significantly enhanced the eradication of bacterial biofilms when compared to treatment with cefotaxime alone [12].

Our research suggests that the phage therapy has importance in treatment of infections caused by mucoid strains. Combined use of phages and antibiotics could have even better effect than phages or antibiotics alone to treat acute infections associated with biofilm formation.

მიკრობიოლოგია

ბაქტერიოფაგებისა და ბეტა-ლაქტამ ანტიბიოტიკის მოქმედება *P. aeruginosa*-ს ბიოფილმების წარმოქმნაზე

ი. პაპუკაშვილი, ე. ლომაძე, თ. მძინარაშვილი

ი. ჯავახიშვილის სახ. თბილისის სახელმწიფო უნივერსიტეტი, ზუსტ და საბუნებისმეტყველო მეცნიერებათა ფაკულტეტი, თბილისი

(წარმოდგენილია აკადემიის წევრის დ. მიქელაძის მიერ)

ჩვენ მიერ შესწავლილ იქნა ბაქტერიოფაგებისა და ბეტა-ლაქტამ ანტიბიოტიკის კომბინირებული მოქმედების ეფექტები *P. aeruginosa* ბაქტერიებზე. კვლევებში გამოვიყენეთ *P. aeruginosa* PAO1 ველური ტიპის შტამი, ბეტა-ლაქტამ ანტიბიოტიკი (იმიპენემი) და კომერციული ფაგის პრეპარატი (პიობაქტერიოფაგი). შედეგებმა აჩვენა, რომ ფაგის პრეპარატი, რომელიც შეიცავს რამდენიმე განსხვავებული ტიპის *P. aeruginosa* ფაგს, ეფექტურად ამცირებს *P. aeruginosa* ბიოფილმების ფორმირებას, მაშინ როცა პრეპარატიდან გამოყოფილი ერთი ტიპის *P. aeruginosa* ფაგი ბიოფილმების დაშლაზე მნიშვნელოვან ზემოქმედებას ვერ ახდენს. ფაგის პრეპარატისა და ანტიბიოტიკის (იმიპენემი) კომბინირებულმა მოქმედებამ კი აჩვენა სინერგიული ზემოქმედება ბიოფილმების ფორმირებაზე. ჩვენი შედეგები ადასტურებს, რომ ბაქტერიოფაგებს გააჩნია უნარი შეიჭრას და გამოიწვიოს *P. aeruginosa* ბაქტერიების მიერ წარმოქმნილი ბიოფილმების დაშლა, რაც თავის მხრივ ხელს შეუწყობს ანტიბიოტიკების მიერ ინფექციის ელიმინაციას. ეს ფენომენი განსაკუთრებულ მნიშვნელობას აძლევს ფაგოთერაპიას გამოყენებული იყოს როგორც დამოუკიდებლად, ასევე ანტიბიოტიკურ თერაპიასთან ერთად მუკოიდური ბაქტერიული შტამებით გამოწვეული ინფექციების მკურნალობისთვის.

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