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Research Article

INHIBITION OF DIFFERENT STAGES OF BIOFILM OF *PSEUDOMONAS AERUGINOSA* PA01 BY ISOLATED BACTERIOPHAGE P2

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ABSTRACT

Biofilm mediated protection of pathogens from environmental stresses are recognized as one of the major threat worldwide, which encourage the researcher to search for other alternative approach. Bacteriophage therapy is one such effective and controlled strategy that undertakes the utilization of tailored phage mediated biofilm inhibition. Hence, in this study, P2 phage, specific to *Pseudomonas aeruginosa* PA01 was isolated and its potential to inhibit biofilm formation was studied. Important findings are summarized in the manuscript.

Keywords: *Pseudomonas aeruginosa,* Biofilm, Bacteriophage and Transmission electron microscopy, Antibiotic resistance.

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative, opportunistic pathogen, which is an inhabitant in broad host range and diverse environment due to its vast genome size [1,2]. *P. aeruginosa* cause diseases such as septicemia, myocarditis, pneumonia, chronic lung infection, endocarditis, dermatitis and osteochondritis. Irregular use of anti-biotics against such type of pathogens causes emergence of multiple drug resistance. One common survival strategy employed by pathogenic bacteria is to form biofilm, which being amorphous and dynamic in nature not only provides resistance against antimicrobial compound, but also protects it from host resistance clearance. Biofilm is complex communities of microbes encased in synthesized hydrated matrix, which is composed of exopolymeric substances [3,4]. These microbial structures can also be formed on indwelling medical devices and subcutaneous biomedical implants such as cardiac pacemakers, heart valve, urinary tract prosthesis, peritoneal membrane and peritoneal dialysis catheters, vascular graft and stent, joint prostheses and cerebrospinal fluid stunts [5-7]. Biofilm forming on indwelling prosthetic medical devices are the major source of infection. Such microorganisms embedded in biofilm are reportedly 1000 fold more resistant than planktonic cells for several classes of commercial antibiotics [8]. Several protective mechanisms have been proposed to explain the biofilm resistance, including impaired penetration of antibiotics into the biofilm matrix, reduced growth rates of the bacteria within the biofilm, and an induced expression of specific resistance genes [3,9,10]. The failure of anti-biotics in the treatment of biofilm forming pathogenic bacteria has encouraged the search for alternative methods of biofilm control.

Lytic phage has drawn the attention of the researcher to control such type of pathogenic bacteria [11,12]. Phages are most abundant and obligate bacterial parasite with an estimate of about 10^{31} phage over 10^{30} bacteria [13,14]. Phage and their associated enzymes have been considered as potentially valuable approach for control of sessile as well as free living bacterial growth [15]. Nonmedical uses of phages include the selective removal of pathogenic or problematic species from contaminated surfaces. Hence, phage might provide possibly natural, highly specific, nontoxic ways for control of biofilm of pathogenic bacteria.

In the present study, P2 phage was isolated and applied on the different stages of biofilm of *P. aeruginosa* and it was seen that phage P2 has efficiency to inhibit biofilm at any stage (at formation stage, 24 hrs established biofilm and biofilm formed by biofilm associated dispersed planktonic cells [BADPC]).

METHODS

Bacterial strain and growth media

P. aeruginosa PA01 was taken as host for bacteriophage isolation, provided by Dr. Shilpa Kaistha, Microbiology Department, C. S. J. M. University, Kanpur (India) and was maintained on tryptone soy Agar or tryptone soy Broth (TSB) medium (Hi Media, India) at 37°C.

Isolation of bacteriophage

The enrichment method, as per Cerveny *et al.* [16] was adopted for the isolation of phages specific to *P. aeruginosa* PA01 from Ganges River at the Kanpur city. The reason behind the selection of this source was; this river water is known to harbor many different bacteria and hence the likelihood of prevalence of phages against them (different bacteria) is more. Sample was collected and carried to the laboratory within 6 hrs for the phage isolation.

In brief, river water sample was collected; centrifuged (10,000 rpm for 10 minutes at 4°C) and supernatant was filter sterilized using 0.45 µm pore size Millipore filter. A volume of 5 ml filtered sewage sample and 5 ml sterile SM buffer were mixed with 5.0 ml overnight culture of *P. aeruginosa* PA01 and incubated at 37°C overnight. The bacteria were removed by centrifugation, supernatant was filter sterilized and checked for the presence of phage.

Phage propagation and purification

Isolated phage was purified by successive single-plaque isolation until homogenous plaques were obtained by the standard procedure described by Sambrook *et al.* [17]. Briefly, one well separated phage was picked with sterile micro tip with the surrounding cell mass and inoculated into 5.0 ml TSB, in which overnight culture of 10^9 host cells was added and incubated at 37°C with agitation at 140 rpm. After complete lysis, the mixture was centrifuged at 10,000 rpm for 10 minutes at 4°C, filter sterilized and treated with 1% v/v chloroform to remove any bacterial contamination. Purified phage was stored in 60% glycerol at −20°C for long term storage. Short term stock preparations were maintained at 4°C.

Prevention of biofilm formation

Culture of *P. aeruginosa* PA01 grown in TSB (optical density of 0.1 at 620 nm) having cell count of 10^8 cfu/ml was used. The culture

(190 µl) was dispensed in 96 wells of sterile polystyrene microtiter plate. Afterward, 10 µl phage lysate (MOI = 0.01) was added to above wells. Wells with 200 µl culture and no phage lysate were kept as a control. Samples were withdrawn after 24 hrs and crystal violet biofilm (CVB) assay was performed as per O'Toole and Kolter [18].

Eradication of biofilm formation with bacteriophages

P. aeruginosa PA01 was allowed to form biofilm on the microtiter plate under static conditions for 18 hrs at 37°C. Thereafter, the biofilm were washed twice in fresh and sterile phosphate buffered saline (PBS) aseptically. Biofilm were treated with 100 µl phage suspension (10^7 pfu/ml) and 100 µl of broth. The assembly was incubated at 37°C for 24 hrs. After incubation, planktonic optical density was taken at 620 nm and subsequent adherent biofilms measured using the CVB Assay.

Biofilm formation with BADPC

Dispersed planktonic cells of 18 hrs old biofilm of *P. aeruginosa* PA01 were taken and allowed to form biofilm on the microtiter plate under static conditions for 24 hrs at 37°C. Biofilm associated cells (190 µl) were dispensed in the wells of sterile polystyrene microtiter plate. Afterward, 10 µl phage lysate with (MOI = 0.01) was added to the above wells. Wells with 200 µl culture and no phage lysate was kept as a control. Samples were withdrawn after 24 hrs and CVB assay was performed as described previously.

Viability test of biofilm cells

Viability test of biofilm was done by MTT assay. Biofilm on the microtiter plate was stained with 0.3% MTT dye for 2 hrs at reverse transcription, after incubation stained biofilm was dissolved with dimethyl sulfoxide (DMSO) and absorption of DMSO was taken at 540 nm.

Electron microscopy

To observe the phage morphology, transmission electron microscopy of *P. aeruginosa* PA01 specific phage was performed as described by Goodridge *et al.* [19] with some modification. A drop of the ultracentrifuge phage sample was dropped on copper coated grids (diameter, 3 mm; 300 meshes). After 5 minutes, the phage particles were stained with 2% (w/v) phosphotungstic acid (PTA) for 10 seconds. The grids were allowed to dry for 20 minutes and examined under a transmission electron microscope (FEI Tecnai S Twin) at 200 Kv.

Biofilm development on glass cover slip surfaces was visualized by scanning electron microscopy (SEM) as per Soboh *et al.* [20] with some modification. Cover slip was rinsed gently in sterile PBS and then fixed in 5% (v/v) glutaraldehyde in PBS buffer (pH 6.2) for 2 hrs and then fixed with post fixative 1% osmium tetroxide for 1 hr, dehydrated through a graded series of 10-min ethanol immersions (30, 50, 70, 90 and 100%). Specimens were mounted on aluminum stubs, coated with gold ((JFC 1600, Auto Fine Coater, JEOL, Japan), and observed on SEM (JEOL-JSM-6490 LV, Japan). The entire cover slip surface was examined, and images were chosen that represented the typical field of view.

RESULTS

Bacteriophage isolation, purification and morphological characterization

There were 5 phages isolated from the Ganges river water, out of them only one phage (P2) had high lytic activity. The plaque size of this phage was approximately 2 mm in diameter on *P. aeruginosa* lawn. *P. aeruginosa* PA01 was used to isolate, propagate and characterized P2 phage. P2 phage was isolated and purified by a single plaque picking, dilution and titration method. Morphological characterization was done by negative staining of bacteriophage sample examined under the electron microscope. According to electron microscopy, P2 phage has a hexagonal head of approximately 100 nm with 150 nm long tail as shown in Fig. 1. In the electron micrograph, head was separated from the tail sheath by a collar. According to observed morphology, P2 phage tentatively belongs to Myoviridae family, (according to Ackermann classification of phage, [21]).

Impact of bacteriophage on different stages of *P. aeruginosa* PA01 biofilm

Bacteria in the biofilm exist in stationary phase, i.e. their multiplication and metabolic activity slow down and are a big reason for failure of many antimicrobials against biofilm cells. Biofilm forming cells exhibit three stages-initial stage is biofilm formation, second is the establishment of the matrix around the biofilm and maturation of biofilm. The microbes residing in the biofilm behave in different manner at every stage of biofilm formation and are difficult to control using a single antimicrobial compound. Therefore, an alternate strategy of using P2 phage was applied to check the inhibition activity at different stages of biofilm of *P. aeruginosa* PA01. 1 × 10^6 pfu/ml of phage P2 with an MOI of 0.01 at 18 h killed 91% biofilm while at 24 hrs 92% killing efficiency was achieved as shown in Fig. 2. Above analysis of killing of biofilm cells was done by MTT viability test. MTT assay was done by staining of biofilm cells with MTT dye and absorbance of dissolved biofilm cell was taken at 540 nm, shown in Fig. 2.

Eventually, a new approach was used in the present study wherein BADPC were used to form biofilm for 6 hrs (Fig. 3) and this biofilm was exposed to the phage P2 for 18 hrs, where phage killed 91% of the biofilm as shown in Fig. 2.

SEM of biofilm treated with bacteriophage

Herein, we want to know whether P2 phage is able to kill cells at the initial stage of biofilm formation or not, for that SEM analysis of

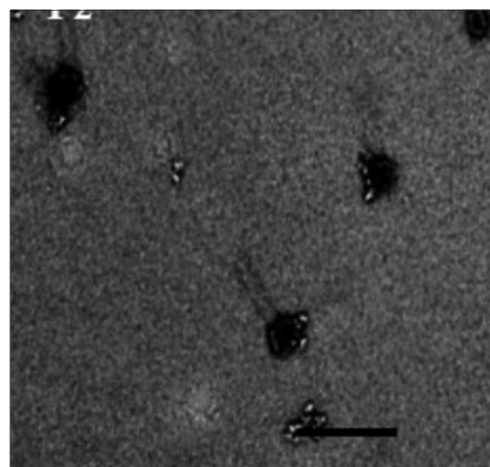

Fig. 1: Electron microscopy of isolated bacteriophage

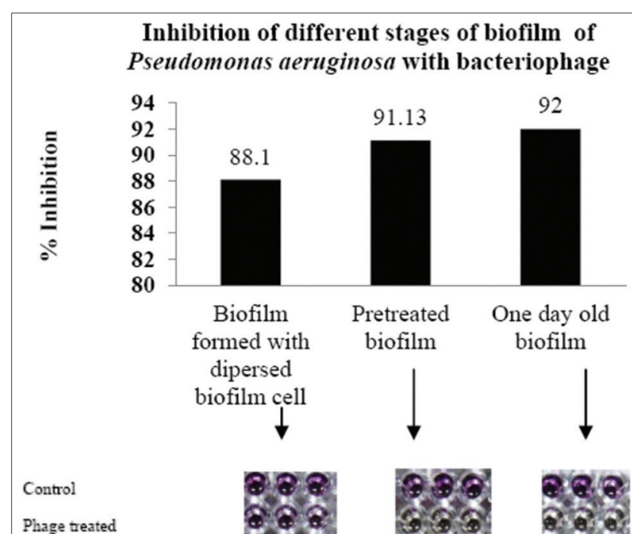

Fig. 2: Application of bacteriophages at different stages of biofilm

biofilm treated with phage P2 was performed and it was seen that P2 phage has potential to kill cells at the initial stage of biofilm formation as shown in Fig. 4. From the comparative results of microtiter plate's biofilm formation data and SEM images, it is clear that Phage P2 has high affinity to inhibit *P. aeruginosa* cells to form biofilm.

DISCUSSION

Bacterial biofilm is responsible for several infectious diseases and the biofilm forming pathogens are associated with medical devices, which are used for external and internal uses. Biofilm also displays unique properties, such as multiple drug resistance, resistance to opsonization and phagocytosis enables them to survive in the hostile environmental conditions and to resist selective pressure [22]. Control of such virulent biofilm is a great challenge in the scientific community. Several studies have been proposed for the potential use of phages to treat infectious diseases in animals [23-26] and humans [27], even those diseases caused by multiple drug resistant bacteria [28]. Bacteriophages arose as a good alternative of antibacterial agents to cure drug resistant pathogens.

P2 phage is a lytic bacteriophage isolated from Ganges river water at Kanpur, North India, is used in this study. Phage has ability to increase in number during the infection process, makes phage excellent potential diagnostic and therapeutic agent for fighting against bacterial diseases. Electron microscopy of isolated bacteriophage was performed for the morphological analysis and further characteristic features determine that phage belongs to myoviridae family.

P2 phage was applied on different stages of the biofilm of *P. aeruginosa* - at the initial stage of biofilm formation, on 24 hrs established biofilm and on biofilm formed by BADPC. P2 gave biofilm inhibition up to 91% when applied initially on the biofilm, as shown in Figs. 2 and 4. In our study, P2 phage proficiently inhibited biofilm forming cells up to 91% while Pires *et al.* [29] reported 50% biofilm inhibition of *P. aeruginosa* PA01 with phage, while Balakshina *et al*. [30] reported 65-91% removal of biofilm of *Pseudomonas fluorescent,* but not for *P. aeruginosa* as we have done in this study. Knezevic *et al*. [31] reported 95% killing of *P. aeruginosa* biofilm as well as planktonic cells with 10 MOI of isolated bacteriophage. Conversely, in this study killing of biofilm cell was achieved by 0.01 MOI of phage, which is very low concentration than the previously reported studies.

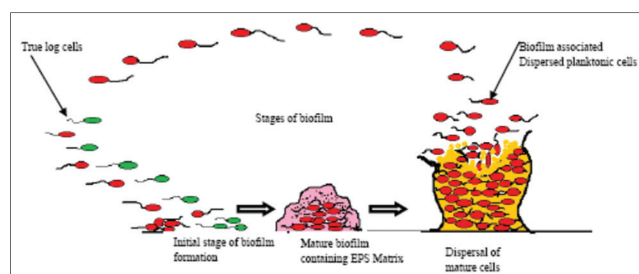

Fig. 3: Biofilm associated dispersed planktonic cells

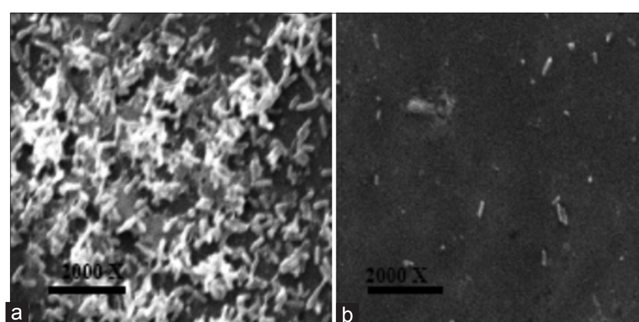

Fig. 4: Scanning electron microscopy of biofilm treated with bacteriophage: (a) SEM of untreated biofilm of *Pseudomonas aeruginosa* (PA01) (magnification ×2000) formed on glass cover slip, (b) SEM of biofilm *P. aeruginosa* (PA01) 24 hrs established biofilm treated with P2 phage (magnification ×2000)

24 hrs established biofilm cells are embedded in the exopolymeric matrix, and they cannot be removed by commercial antibiotics because the matrix of the biofilm imparts protection to the biofilm cell. Hence, phage and phage associated enzyme like depolymerase can be helpful in dispersing and killing of established biofilm cells. Therefore, the potential of phage P2 against 24 hrs established biofilm was examined as shown in Fig. 2. Amusingly, it was found that P2 causes 92% killing of established biofilm cells. The most interesting thing about this study is that entire study was done with 0.01 MOI, whereas Hosseinidoust, [32] reported the killing of biofilm of *P. aeruginosa* using 2 MOI of bacteriophage.

In this study, a novel approach was employed to form the biofilm, herein BADPC were used for the formation of biofilm in place of true planktonic cells. BADPC are those cells which have dispersed after maturation of biofilm, and dispersed biofilm associated planktonic cells may have some kind of different working mechanism than the true planktonic cells and can form stronger biofilm and may inhibit the adsorption or penetration of phage, were analyzed in this experiment. Biofilm formed by biofilm associated planktonic cells was usually less accessible for phage than the biofilm formed by fresh log culture. This is a novel mechanism to form biofilm, which has not been still reported in the literature.

CONCLUSION

Cells at different stages of biofilm have different working mechanism, and commercial anti-biotics are unable to control them at these stages. Hence for their control, isolated P2 phage was applied at the different stages of biofilm, and it gave 92% inhibition activity with very low concentration. P2 phage and its associated enzyme proficiently dispersed the mature biofilm which led to killing of dispersed cells by phage. Hence, use of phage in controlling biofilm of *P. aeruginosa* at any stages can be a good alternative. This phage therapy is effective, ecofriendly and economical technique for controlling the pathogen.

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