Isolation, Characterization, and Comparison of Efficiencies of Bacteriophages to Reduce Planktonic and Biofilm-Associated *Staphylococcus aureus*

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Abstract

Introduction In the present era, wherein occurrence of antimicrobial resistance compounded with biofilms in disease conditions has rendered present antibiotic therapy ineffective, the need for alternative strategies to treat bacterial infections has brought bacteriophages to the forefront. The antimicrobial activity of phages is often determined by a viable cell reduction assay which focuses only on planktonic forms. The physiology of an organism in biofilm differs from those that are planktonic; hence, there is a need to evaluate the activity of phages both on planktonic forms, as well as on biofilms, to select candidate therapeutic phages.

Materials and Methods Bacteriophages for *Staphylococcus aureus* were isolated from environmental samples and characterized based on growth kinetics and DNA fingerprint patterns. Activity of isolated phages on planktonic forms was determined by viable count reduction assay. Phage ability to prevent biofilm formation and ability to disperse formed biofilms were performed in 96-well microtiter plates and biofilm estimated by crystal violet assay.

Results Four bacteriophages designated, that is, P3, PD1, PE1, and PE2, were isolated and characterized. Planktonic cells of *S. aureus* were found to be sensitive to phages PD1, PE1, and PE2. Phages PD1 and PE2 were efficient in preventing biofilm formation and phages PD1, PE1, and P3 were efficient in dispersing formed biofilms.

Conclusion The ability of some phages to disperse biofilms effectively, while unable to show the same efficiency on planktonic cells, indicates that viable count reduction assay alone may not be a sufficient tool to imply bactericidal activity of bacteriophages, especially while trying to eradicate biofilms.

Keywords
- bacteriophages
- biofilm
- *Staphylococcus aureus*

Introduction

Emergence of multiple drug resistance in strains of *Staphylococcus aureus* with the ability to form biofilms has led to listing this organism as one of the top five major clinical and epidemiological problem in hospitals and a high-priority pathogen for the research and development of new antibiotics.[1,2] Antimicrobial therapy against this organism, especially in cases of nonhealing wounds is a challenge and hence the search for alternative strategies has come of prime importance. Bacteriophages, viruses that specifically lyse bacterial cells, have the potential to control emerging antimicrobial-resistant bacterial strains due to their ability to continually evolve with bacterial populations.[3] They are more

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specific in their activity and hence can be used for targeting individual species.

Increasing reports on the prospective use of bacteriophages against *S. aureus* in various clinical manifestations has come to light in recent years.[4-6] Phage activity is often determined by routine test dilution method, plaque assay, or culture lysis method.[7] All these methods are well established and use actively growing cells that are often freely dispersed in the culture media. The ability of phages and phage-derived proteins to remove *S. aureus* in clinical models has been lower compared with planktonic forms as tested in vitro.[8-14] More than 80% human infections caused by bacteria, infections are manifested by growth of the organism in biofilms.[15] The physiology of an organism in biofilm differs from those that are planktonic,[16] hence there is a need to evaluate the activity of phages both on planktonic forms, as well as on biofilms to select candidate therapeutic phages. Little has been done to establish methods to determine the effective use of bacteriophages on staphylococcal biofilms. Isolated reports of simple biofilm treatment methods have been studied[17] and more complex methods of three-dimensional–printed porous titanium scaffolds have been used to determine the antibacterial activity of *S. aureus* phages.[18] A reduction in the viable count of a growing culture by the lytic activity of a phage is frequently used to select candidate phages.[19] Our study objective was to determine if viable cell count reduction alone was sufficient to determine ability of phages to disrupt biofilms.

Materials and Methods

Bacterial Culture

S. aureus ATCC 29213 procured from Himedia Products, India, and was used as the test organism. Culture was grown in nutrient broth/agar at 37°C unless otherwise mentioned in the text.

Bacteriophages

Growing culture of ATCC 29213 was used as bait to isolate bacteriophages by the soft agar overlay technique. Phages were isolated from waste water samples. Isolated plaques were purified by three consecutive rounds of single plaque purification. Purified phages showing uniform plaque morphology in agar overlays were characterized based on plaque morphology, one step growth curve,[20] and restriction fragment length polymorphism (RFLP).[21] For studying the growth kinetics, one-step growth curve was determined. Briefly, the host bacterium was infected with the phage at a multiplicity of infection (MOI) of less than 0.01. The number of adsorbed phages was determined by overlay of an aliquot of the infected culture 5-minute postinfection with and without chloroform treatment. The number of phages adsorbed is the difference between the number of plaque-forming units (PFUs) with and without chloroform treatment. Free phages were determined at regular time points of 20 minutes up to 3 hours by soft agar overlay technique with the supernatant of the infected culture obtained after centrifugation at 8,000 rpm for 10 minutes The burst size was calculated as ratio of the phage titers at plateau phase by the initial no. of adsorbed phages and latent period was calculated as the time between each rise in the growth curve. Restriction fragment length polymorphism was used as a molecular method of characterization. The phage DNA was extracted by the method described by Su et al.[22] Phage DNA (quantified using Nanodrop) of µg was treated with 1 unit of restriction enzyme HaeIII in the reaction buffer provided by the manufacturer (New England Biolabs) and incubated at 37°C for 4 hours. The digested product was resolved on 1% agarose gel stained with ethidium bromide. The gel was analyzed using a gel documentation system (Biorad). Difference in the restriction pattern between the phage DNA indicated a difference in the phages at the genomic level.

Activity of phages on planktonic cells: midlog culture of *S. aureus* ATCC 29213 was diluted to approximately 1×10^{-5} cells ml in 5-mL sterile physiological saline and infected with phages from MOI 10 to 0.01 in 10-fold dilutions.[13] The tubes were incubated at 37°C with gentle shaking. At every hour, up to 8 hours, viable count was enumerated by spread plate method.

Efficacy of isolated phages in preventing biofilm formation: the wells of a microtiter plate were loaded with phage lysate of decimal dilutions and incubated at room temperature for an hour to let the phages adhere. The solutions were aspirated and plates were air dried for 20 minutes. *S. aureus* culture of 150 µL that grown overnight was loaded into the wells. Plates were incubated at 37°C at 20 rpm for 48 hours. The biofilm was measured by the crystal violet biofilm assay.[23] In this assay, contents of the plates were first aspirated out, the plate was rinsed gently by immersing in sterile physiological saline to remove any planktonic cells. The remnant biofilm was air dried for 10 minutes and then each well treated with 150 µL of 1% aqueous crystal violet for 15 minutes. The excess stain was discarded and the stain absorbed by the biofilm was released by treatment with 150 µL of 30% acetic acid. The intensity of the stain which is proportional to the biofilm formed was then estimated at 450 nm in a spectrophotometer.

Effectiveness of phages in disrupting biofilms: 150 µL of *S. aureus* culture that was grown overnight was loaded into 96 well microtiter plate. Plates were incubated at 37°C at 20 rpm for 48 hours. Solution in the plate was aspirated out to remove planktonic cell and air dried for 20 minutes. 150 µL of phage lysate in decimal dilutions was added. Plates were incubated at 37°C overnight. The biofilm was measured by the crystal violet biofilm assay as mentioned in the previous section.

Analysis of variance (ANOVA) was determined to compare the effect of the phages compared with control/lower dilutions of the phage. $p < 0.05$ was considered as statistically significant.

Detection of Depolymerases in Phage DNA

The extracted phage DNA was tested for the presence of genes encoding for two depolymerases, Dpo7,[24] and N-acetyl glucoronidase (NAG) by polymerase chain reaction (PCR)

using the following primers (Dpo7 primers: 5′-TCAGAAA GATTCCACGAAGG-3′ and 5′-TAATGGCCATGTGAGCATC-3′; NAG primers: 5′-ATGACAGATTCAATAAATGCC-3′ and 5′-TCA CACCTGACGCTG-3′). Reaction mixture (30 µL) was set up with 3 µL of 10Xbuffer (100 mM Tris-HCl and 1.5m M of $MgCl_2$), 2.5 nM each of deoxyribonucleotide triphosphates (dATP, dTTP, dGTP, and dCTP), 10 picomoles each primer and 1U of Taq DNA polymerase (Himedia Laboratories Pvt. Ltd., India), approximately 100 ng of template DNA and volume made up to 30 µL with nuclease free water. The amplifications were performed in a thermocycler (Eppendorf NexusGX2). The annealing temperature for Dpo7 was 48°C while that for NAG was 53°C. PCR products were resolved by electrophoresis on 2% agarose gel, stained with SYBR safe, and bands were visualized using a gel documentation system.

Results

Bacteriophage Isolation

Four phages designated P3, PD1, PE1, and PE2 were isolated from water samples and the phages were differentiated based on their plaque morphology, growth curve, and RFLP. Phages PE1 and PE2 each had a unique plaque morphology of being pin point and large 3-mm circular plaques, respectively, and hence were considered as different phages. Phages P3 and PD1 were similar in plaque morphology (►**Fig. 1**) but could be differentiated based on their latent period (30 and 50 minutes, respectively) and burst size (6 and 47, respectively) as determined by the one-step growth curve represented in ►**Fig. 2** (►**Supplementary Table S1**, available in the online version). Though PD1 and PE1 were similar in their growth kinetics (latent period of 50–60 minutes and burst size of 40), the marked difference in plaque morphology on the same host could be used as a differentiation tool. RFLP aided in the further differentiation between PD1 and PE1. A comprehensive account of phage characterization is depicted in ►**Table 1**.

Activity of Phages on Planktonic Cells

Viable cell reduction assay was performed to determine activity of phages on planktonic cells. Colony counts in phage treated groups were compared with those of the cell control (►**Fig. 3** and ►**Supplementary Table S2**, available in the online version). Phages PD1 and PE1 were active on the culture at all concentrations (MOI = 10, 1, 0.1, and 0.01) compared with the cell controls (statistical significance $p = 3 \times 10^{-5}$ and $p = 5 \times 10^{-5}$ respectively). In case of phage PE2, 10 MOI, and 1 MOI were highly effective in decreasing cell counts within 1 hour and retaining low counts up to 8 hours ($p = 4.9 \times 10^{-7}$); however, bactericidal activity at MOI < 1 was not statistically significant ($p = 0.15$).

There was no significant difference in the colony-forming units (CFU) recovered in cell control compared with phage treatments for phage P3.

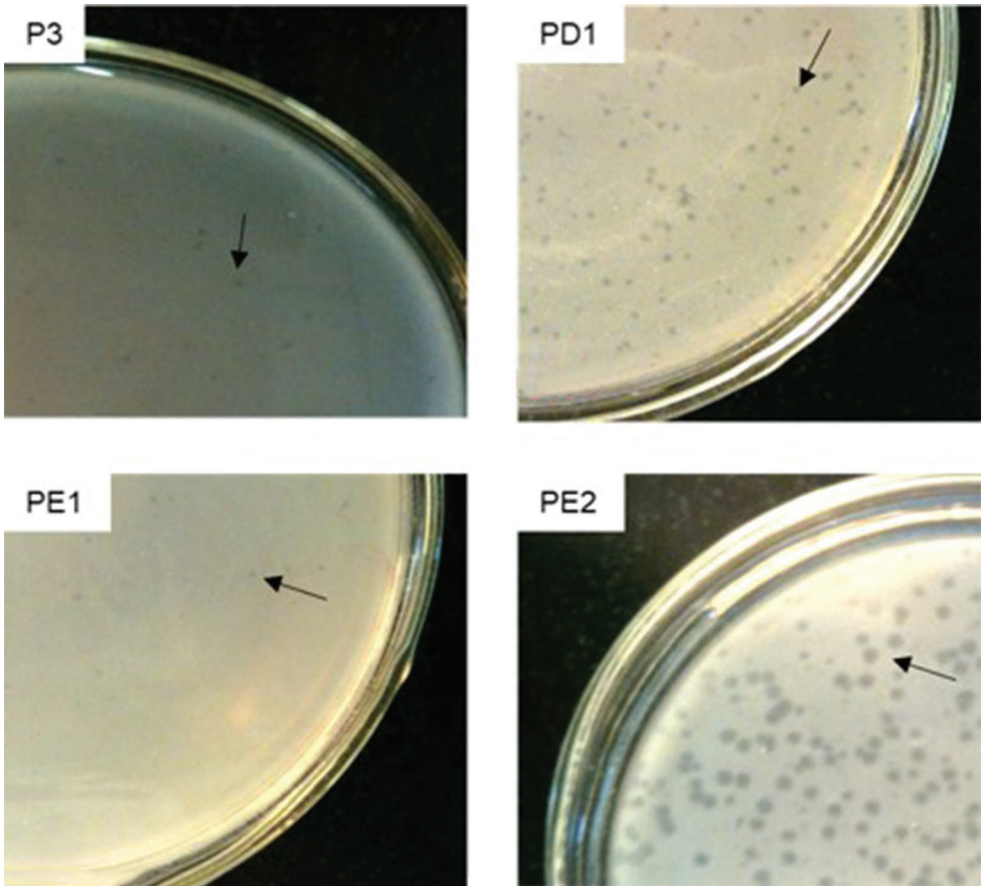

Fig. 1 Plaque morphology observed for the different phages.1 mm circular plaques observed for phages P3 and PD1, pin point plaques of PE1, and larger clear plaques of PE2,

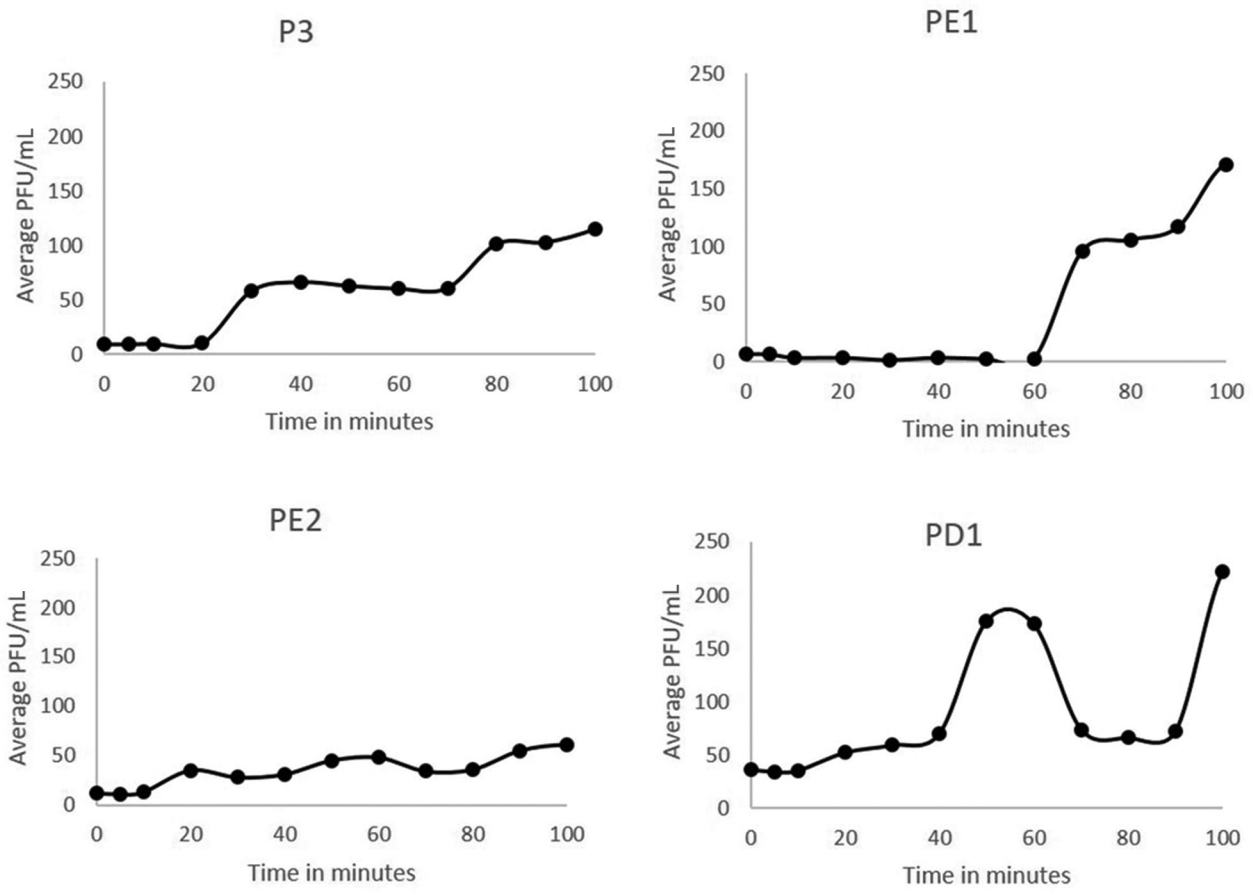

Fig. 2 One-step growth curve of the phages P3, PD1, PE1, and PE2.

Table 1 Characterization of the four phages

Phage	Plaque morphology	Latent period (min)	Burst size (PFU)	Digested by enzyme HaeIII
P3	1-mm clear circular plaque	30	~6	Not done[a]
PD1	1-mm clear circular plaque	50	47	Yes
PE1	Pinpoint clear plaque	60	40	No
PE2	3-mm circular clear plaque	20	5	No

Abbreviation: PFU, plaque-forming units.
[a]No quantifiable DNA due to low titer.

Efficacy of Isolated Phages in Preventing Biofilm Formation

To determine efficacy of the isolated phages in preventing biofilm formation, the wells of a 96-well microtiter plate were pretreated with phage and then inoculated with culture.

A significant reduction in the biofilm formation was observed when the wells of the microtiter plate were pretreated with phages PD1 and PE2 (p = 0.034 and 5 × 10^{-6}, respectively). However, phages P3 and PE1 were not efficient in preventing biofilm formation (p = 0.26 and 0.75, respectively; ►**Fig. 4**; ►**Supplementary Table S3**, available in the online version).

Effectiveness of Phages in Disrupting Biofilms

Therapeutic use of phages would require that the phages be able to eradicate biofilms. To test the ability of the isolated phages to disperse biofilms, biofilms of *S. aureus* ATCC 29213 were developed in the wells of a 96-well micro titer plate, and later treated with phages. A significant dispersion of the biofilm on treatment for 24 hours was observed with phages PD1 at 10 MOI (p = 0.03) and phage P3 at all MOI (p = 7 × 10^{-5}). Phages PE2 and phage PE1 were not efficient in disrupting biofilm (p = 0.62 and 0.78, respectively; ►**Fig. 5**; ►**Supplementary Table S4**, available in the online version).

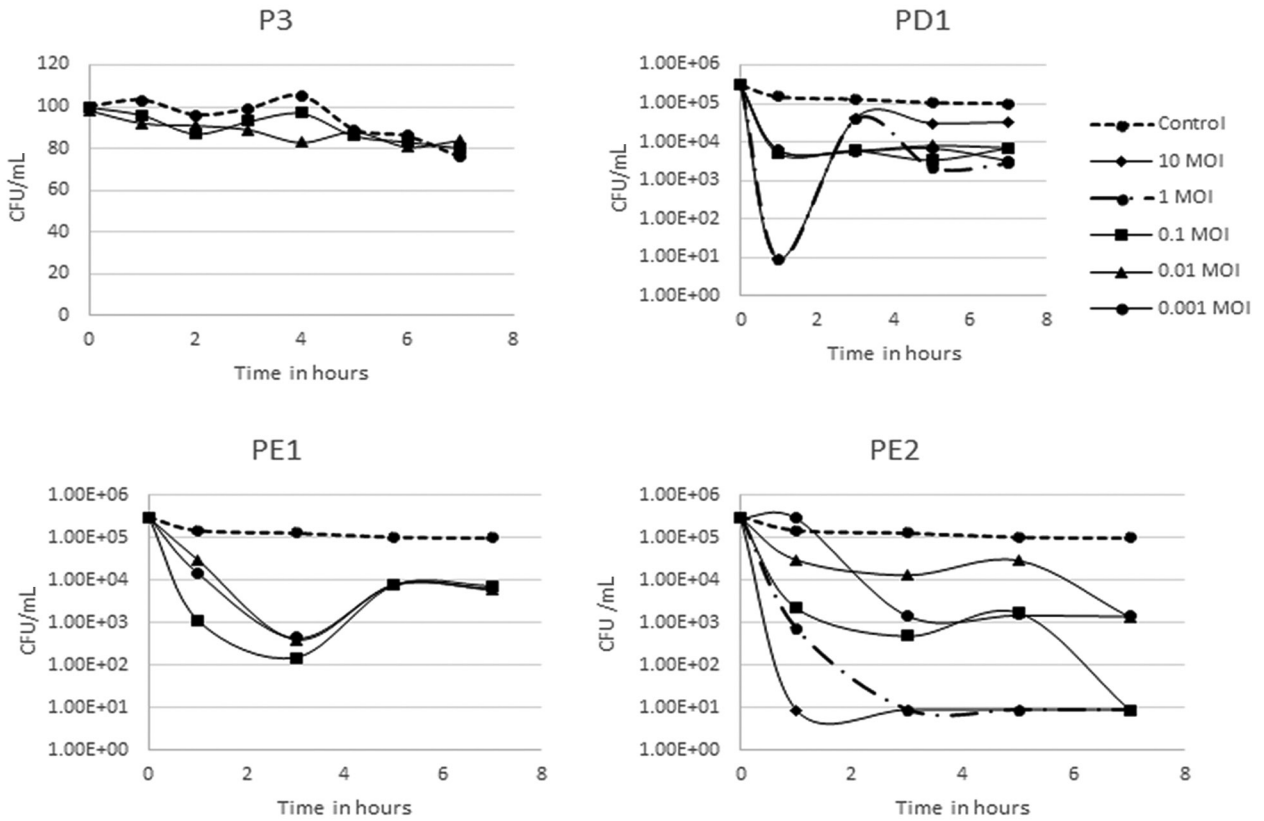

Fig. 3 Viable cell reduction assay on planktonic cells of *Staphylococcus. aureus* ATCC 29213. The colony-forming units (CFU) were enumerated at different time points after treating the culture at different concentrations of phage. A decrease in CFU indicated lytic activity of the phage.

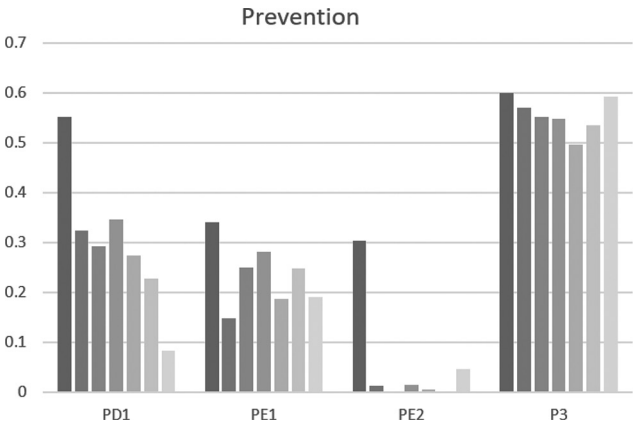

Fig. 4 Ability of the phages in preventing biofilm formation. The absorbance is indicated on the Y axis. Decrease absorbance on treatment with phage indicates ability to prevent biofilm.

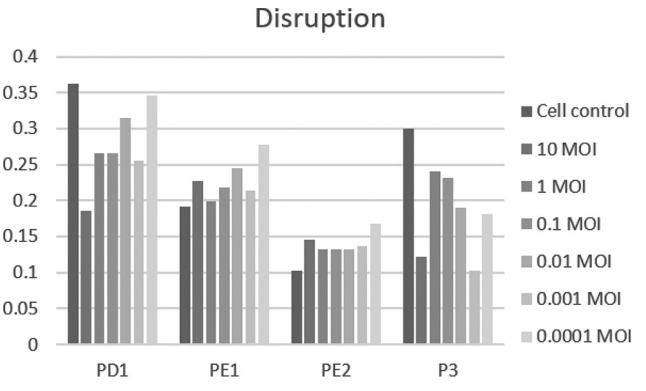

Fig. 5 Ability of the phages in dispersing biofilms. The absorbance is indicated on the Y axis. Decrease absorbance on treatment with phage indicates ability to disperse biofilm. MOI, multiplicity of infection.

Detection of Depolymerases in Phage DNA

Dpo7 has been described as depolymerase of *S. aureus* biofilms.[24] A major constituent of the *S. aureus* biofilm matrix is polymeric NAG (PNAG).[25] Hence the phage DNA was tested for the tail spike protein NAG and Dpo7 by PCR. None of the four phages tested in this study were positive for the genes encoding for these enzymes as no bands were observed on resolving the PCR products by electrophoresis.

Discussion

Failure of antibiotics to cure antimicrobial resistant infections has favored phage therapy as a possible alternative. In this study, four *Staphylococcus* phages were isolated and characterized based on culture dependent methods of plaque morphology and growth kinetics. Plaque morphology is the first basis for differentiating phages. Size, shape, clarity of lysis, and presence or absence of a halo help in differentiating

phages.[26] Based on plaque morphology, phages P3, and PD1 appeared to be the same while PE1 and PE2 were very different. The one-step growth curve has been a primary method to study the growth kinetics of phage as it describes the number of progeny phage obtained from single phage (burst size) and the time required for the phage to complete one lytic cycle (latent period). The latent period has been identified as a response to resource availability including media composition and number of host cells.[26] In this study, though phages P3 and PD1 appeared similar in plaque morphology, the burst size (6 for P3 and 40 for PD1) and latent period (30 minutes for P3 and 50 minutes for PD1) differed significantly indicating that they were different phages. Phages PD1 and PE1 though different in plaque morphology were similar in growth kinetics. Molecular fingerprinting technique of RFLP was used for the molecular characterization, and it confirmed that the phages PD1 and PE1 were different from each other since PD1 was digested by the restriction enzyme HaeIII while PE1 was resistant to the restriction activity of this enzyme. Phages can evade restriction enzymes by the production of methyltransferases or modifying host methylase activity to protect their nucleic acid. Additionally the phage nucleic acid itself can have modified bases thereby avoiding restriction.[27] Thus, a combination of culture-dependent methods and use of molecular techniques together aide in efficient characterization of phages.

The lytic activity of the phages on planktonic cells was tested by the viable cell reduction assay. Three of the four phages isolated were effective in reducing planktonic cell counts. Phage P3 did not reduce the viable count of the host at the MOI tested. We infer that this inefficacy may be due to the low efficiency of plating of the phage on the tested host. The highest obtainable titer for phage P3 was 10^6 PFU per mL. Hence, the highest achievable MOI for testing viable cell reduction was 0.1.

Antibiotics have been reported to have reduced permeability in biofilm and the concentration of these agents against biofilms are estimated to be 1,000 times more than that required for planktonic cells.[28] Our present study indicated that the same concentration of phage would suffice for efficient activity on planktonic cells, as well as on biofilms.

The potential use of *S. aureus* phages against hospital isolates in culture has been reported for more than four decades. However, the ability of a phage to lyse planktonic cells, as well as those embedded in biofilms, is essential for its therapeutic use. Recent studies on use of *S. aureus* phages have focused on the need for the ability of phages to disperse biofilms.[7-14] Studies that have looked at deciphering the reasons for phage therapy failure in experimental animals, wherein in vitro results were very promising and have suggested that absence of depolymerase may result in inability of phages to disrupt biofilms.[29] In the present study, planktonic cells of *S. aureus* were sensitive to phages PD1, PE1, and PE2. A comparison in activity of phages in biofilm prevention and in the disruption of formed biofilms of *S. aureus* by phages showed that phage PD1 was active against planktonic cells, in preventing biofilm formation and disrupting formed biofilms; PE2 was efficient in preventing biofilm formation but could not disperse formed biofilm. The most interesting activity was observed in phage P3 which did not show sufficient CFU reduction while testing with planktonic cell nor was is efficient in preventing biofilm formation, but its activity in dispersing biofilms was highly efficient. Hence, a comparison of bactericidal activity of phages on planktonic forms complemented by data in its ability in both preventing and dispersing biofilms will be decisive on selecting candidate phages for therapeutic purpose.

The *S. aureus* biofilm matrix is thought to be composed of host factors, secreted and lysis-derived proteins, polysaccharide, and eDNA which varies based on strain and environmental conditions.[30] The *S. aureus* biofilm matrix is often reported to be composed of β-1,6-linked NAG known as PNAG.[25] Hence, the phages were tested for the presence of tail spike NAG. Dpo7 is a preneck appendage protein isolated from a *S. aureus* phage with a pectin lyase domain. This protein was reported to be effective in the dispersal of *S. aureus* biofilms[24] and similar conditions for gene amplification were used in our study. However, the phages did not show any amplification for Dpo7 or NAG gene.

Conclusion

The success of therapy is based both on the effectiveness of the antimicrobial and the susceptibility of the pathogen to the antimicrobial.[31] It is essential that a phage have the ability to disperse biofilms when it is being used as a therapeutic since biofilms are often associated with persistent infections. The effectiveness of many dispersal mechanisms is dependent on the matrix composition.[32] Phages are known to evolve with the host and have acquired mechanisms for dispersing biofilms. Though the composition of biofilm may be complex, phages do have mechanisms to disrupt these. The mechanisms studied so far are do not suffice to explain the activity of phages on biofilms as seen in our study. High throughput phage genome sequencing, adequate annotation, and prediction tools would aide in the identification of novel phage enzymes which can be efficiently used for biofilm dispersal.

Note

The study was approved by the Institutional Research Advisory Committee of Nitte University Centre for Science Education and Research via document INST.RAC/2016–17/18 dated September 1, 2017.

Conflict of Interest

None declared.

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