Characterization of vB_Sau_S90, and vB_Sau_S165, a Broad Host Range Phages, and Multiple Phage Doses as a Potential Therapeutic Strategy to Eliminate Transient Phage Resistant Mutants in *Staphylococcus Aureus*

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Research Article

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Abstract

Methicillin-Resistant *Staphylococcus aureus* is a human pathogen. MRSA has acquired resistance to major antibiotics; thus, phage therapy has become a potential alternative treatment. In this work, two broad host range *Staphylococcus* phages were characterized for lifecycle, physio-chemical parameters and bacterial killing kinetics, and the *in vitro* behavior of phage insensitive bacterial cells to alternative serial passage and multiple phage doses were assessed by reduction in the bacterial turbidity and spot assay. Phage vB_Sau_S90 showed an absorption efficiency of $91 \pm 0.6\%$ with an adsorption time of 17 ± 1 min and vB_Sau_S165 of $95 \pm 0.5\%$ adsorption efficiency and 15 ± 2 min adsorption time. Both the phages were stable over a wide range of temperature (20 to 50 °C) and pH (3 to 11). vB_Sau_S90 phage belonging to the family *Siphoviridae* [order *Caudovirals*] showed killing efficiency against 88% (181/205) of *S. aureus* isolates, and vB_Sau_S165 belonging to family *Podoviridae* [order *Caudovirals*] showed killing efficiency against 94% (192/205) of *S. aureus* isolates. The sensitive and transient phage-resistant cells that remained uninfected during the single dose of phage treatment were eliminated upon a minimum of five alternative serial passage and multiple phage doses. This study concludes that both the phages showed promising activity against methicillin-resistant *Staphylococcus aureus*. Our study revealed that despite phage auto-dosing and high therapeutic efficiency, both phages did not produce a complete bacterial clearance at a single phage dose; hence indicated that multiple phage doses were required to attain a successful and complete bacterial eradication.

Introduction

Staphylococcus aureus is an important opportunistic nosocomial pathogen (Kwiecinski and Horswill 2020), causes infection in immunocompromised and high-risk groups. The World Health Organization (WHO) has identified *S. aureus* as one of the high-priority pathogens that requires immediate attention (2017). Methicillin-resistant *Staphylococcus aureus* (MRSA) is distributed globally and β -lactams are the treatment of choice for early MRSA infections (Baek et al. 2014). However, with the clones developing resistance to the β -lactam antibiotics, higher-order antibiotics such as linezolid, vancomycin, and daptomycin are being practiced recently (Choo and Chambers 2016). However, recent findings on growing resistance to higher-order antibiotics have created a trepidation and prompted a quest for an alternate treatment method. Nanoparticles (Hamida et al. 2020), vaccines (Miller et al. 2020), bacteriophage therapy (Kornienko et al. 2020), and other innovative techniques are proposed to enhance treatment choices. Although a plethora of alternative therapies have emerged in recent years, the therapeutical evidence from clinics has shown that phage therapy is a superior alternative to antibiotics. A recent survey shows that phage therapy has more than 80 to 90% of success rate in treating antibiotic resistance infections (Sulakvelidze et al. 2001).

Bacteriophages (Phage) are bacterial viruses that prey and kill the bacteria during the multiplication process. The use of phages in therapy is an age-old concept and the first phage application can be dated back to 1919 (Sulakvelidze et al. 2001). With the recent search for antibiotic alternatives, the phage therapy has been reintroduced into many clinics and laboratories

S. aureus is highly composed of wall teichoic acid (WTA) which serves a major receptor for phage binding (Ingmer et al. 2019). It is known that the *Staphylococcus* phages exhibit broad host range activity against diverse clones or even species (Moller et al. 2021). At present, phage therapy is regarded as one of the alternative treatments, as the bacterial evolution can lead to phage resistance. Phage resistance is generally observed during treatment and strongly reported in the recent studies. Accordingly, many research groups are working on designing a potential method of phage dosing to improve the therapeutical index of phage therapy and methods to overcome phage resistance during treatment (replaced infection) (Ferry et al. 2020). The rate of appearance of phage-resistant mutants is governed by the interaction between the phages with their host and other environmental conditions. Recent research on phage-resistant mutants has found a trade-off cost in resistance cells, which can lose their virulence or resistance during the evolution (Gordillo Altamirano et al. 2021).

Here, we report the characteristic feature of two lytic bacteriophages, vB_Sau_S90 and vB_Sau_S165, with the broad host range activity against the clinical isolates of *Staphylococcus aureus*. This research mainly focuses on examining the phenotypic characteristics of phage resistant bacterial cells during *in vitro* phage-bacterial interaction and highlights the importance of serial passage, and multiple phage doses in eliminating the transient phage-resistant mutants.

Methods

Bacterial strains

A total of 206 non-duplicate clinical isolates of *Staphylococcus aureus* were used in the study for *in vitro* characterization of phages. All bacterial isolates used in this study was collected from the diagnostic laboratories located in Tamil Nadu and Mizoram, India. The antibiotic resistance profiling and the clonal analysis of the bacterial isolates was performed at Antibiotic Resistance and Phage Therapy Lab, VIT, Vellore. Out of 206 isolates, 106 isolates were conformed as MRSA by antibiotic resistance profiling. This prospective phage study on *Staphylococcus aureus* has been approved by the Institutional Ethical Committee (IEC) of Vellore Institute of Technology, Vellore (VIT/IECH/004/January 28, 2017). Antibiotic resistance profiling experiments were conducted following the guidelines of the Indian Council for Medical Research (ICMR) with Biosafety Level II and regulations.

Phage isolation, purification, and propagation

The phages specific to *Staphylococcus aureus* was screened in various water and soil samples such as sewage waters collected from hospital outlets, soil from veterinary hospitals, water samples from the lake, sewage from water treatment plants, sewage sample from schools, hostels located in and around Vellore, Tamil Nadu. The collected water and soil samples were transported to Antibiotic Resistance and Phage Therapy Laboratory, VIT, Vellore, and the phage was isolated by the enrichment technique. Briefly, 2 ml of the log phase culture and

10 ml of sewage water was added to the 10 ml of the double strength BHI/CaCl₂ broth and incubated at 37 °C overnight. The mixture was centrifuged at 5000 x g for 15 to 20 min, and the supernatant was filtered using a 0.22-micron syringe filter.

The filtrate obtained was tested for the presence of phage by spot test and double agar overlay method. Briefly, for the spot test, the bacterial lawn was prepared in BHI/CaCl₂ agar plates, and 5 µl of phage filtrate was spotted on the top of the bacterial lawn. In the double agar overlay method (DAOL), 200 µl of host bacteria was mixed with 10 µl of the phage filtrate. The mixture was incubated at 37 °C for 5 min, and 2 mL of molten soft agar (BHI/CaCl₂) was added, and overlaid onto the hard agar (BHI/CaCl₂). The plates were incubated at 37 °C overnight. The appearance of a clear zone in the spot test and the plaques in the DAOL represents the presence of bacteriophage. Throughout the study, 1% hard agar and 0.5% soft agar were used to improve the transparency and enhance the plaque visibility. The phage was purified by picking a single, well-isolated, transparent plaque in SM buffer (Sodium-Magnesium buffer) (1 L: 5.8g, NaCl; 50 mL, 1M Tris-HCl [pH 7.5]; 2g, MgSO₄.7H₂O; 5 mL, 2% gelatin and 1 mM of CaCl₂) and purified for three successive rounds using the double agar overlay method. Purified phage was propagated by multiplying with the host bacterium by web pattern DAOL (plate with an excessive number of plaques that are hazily defined, neither entirely clear nor distinct plaques) (Liu et al. 2020). For web pattern DAOL, 200 µl of host bacteria was mixed with 10⁴ to 10⁵ PFU/mL of phage (concentration varies upon the plaque size), and a double agar overlay was performed as described previously. Following incubation at 37 °C overnight, SM buffer of 3 mL was added onto the plate and kept at 4 °C for 4 hrs to remove the phage from the DAOL plate. After incubation, the buffer collected was filtered sterilized and stored at 4 °C for further analysis.

Lifecycle analysis – Adsorption assay

The phage adsorption onto the host bacteria was determined by analyzing the number of non-adsorbed phages at a standard time interval. Briefly, adsorption assay was performed by mixing bacterial host with phage at a multiplicity of infection (MOI) 0.001 and incubated at 37 °C. At every 2 min interval, an aliquot of 100 µl was drawn from the interaction tube, diluted in BHI broth (4.4 mL) and chloroform (0.5 mL). The dilution was incubated at room temperature for 30 minutes, and a double agar overlay technique was performed to assess the number of non-absorbed phages.

Lifecycle analysis – latency time and burst size

A one-step growth curve experiment was carried out to determine the latency period, and burst size of the phages studied. Briefly, phage was mixed with the bacterial host to obtain an MOI 0.001, and the mixture was incubated at 37 °C for phage adsorption (duration as determined from adsorption assay). After phage adsorption, the mixture was centrifuged at 13,000 x g for 2 min, and BHI/CaCl₂ broth was added to the pellet. The aliquots were removed at 2 min (vB_Sau_S90) and 5 min (vB_Sau_S165) intervals, and a double agar overlay was performed. The absorption efficiency was calculated by ((Control – Test) / Control) x 100, and the values are expressed as percentage (%).

Stability analysis

The viability of the phages at varying temperature and pH ranges was analyzed using the phages at 10^9 PFU/mL. The prepared phages were subjected to a varying temperature range of 20, 30, 40, 50, 60, 70, 80 °C for 60 min, and the activity was determined using the double agar overlay method. For pH stability studies, phage particles were prepared in pH-controlled buffer ranging from pH 1-14 and incubated at 37°C for 60 min, and the activity was assessed using the double agar overlay method.

Transmission Electron Microscopy

The morphology of the phage particles was determined by negative staining with 2% uranyl acetate and visualized under Transmission Electron Microscopy (TEM). Each phage of 10 µl volume (10^9 PFU/mL) was spotted on top of the carbon-coated copper grid, and the phage was allowed to absorb to the copper grid for 2 to 3 min. After incubation, the grid was carefully stained with 2% uranyl acetate for 2 min, and the excess stain was washed. The grid was carefully dried in a desiccator, and the phage morphology was viewed under TEM (Transmission Electron Microscope) (FEI-TECNAI G2-20 TWIN, VIT, Vellore).

Phage host range and efficiency of plating

The host range of the bacteriophages against 205 non-duplicate clinical strains of *S. aureus* was determined using a spot test. The phage bacterial host (SA-90 and SA-165) was employed as control strains for the respective phage, and the remaining 205 isolates were taken as a test strain. The host range of the phage was validated by spotting a phage lysate of 5 µl (10^6 PFU/mL) onto the bacterial lawn, and the phage activity was confirmed as sensitive if a complete zone of clearance was observed, intermediately sensitive if discrete plaques were seen, and resistant for no spot. To further analyze lysis efficiency (plaque formation), phage from 10^{10} to 10^6 PFU/mL was spotted onto the test strains that showed a zone of clearance in the host range analysis. The efficiency of plating (EOP) was interpreted by a range of scoring system in comparison to the controls following the methods of Kutter et al. (Kutter 2009). High EOP - ≥ 4 spots of complete clearance and produced individual plaques as phage concentration decreased, Moderate EOP - < 4 and ≥ 2 spots of complete clearance and individual plaques in the least phage concentration, Low EOP - < 2 spots of complete clearance and individual plaques in the least phage concentration and No EOP - clearance throughout but with faintly hazy background till 10^1 PFU/mL with no observable plaques.

In vitro time killing assay

Time kill analysis was performed to assess the antibacterial efficacy of phages and to determine the phage dosing (MOI) as described previously (Liu et al. 2020). Briefly, *S. aureus* bacterial culture (10^8 CFU/mL) was mixed with phage at different MOIs of 0.01, 0.1, 1 and, 10 and incubated at 37 °C. The optical density (OD₆₀₀) was measured at every 2 hrs intervals for 22 hrs. The assay was performed using the host bacterium (only). The results are expressed as log fold reduction. (Log-fold reduction was calculated using the formula - log (OD before treatment / OD after treatment). All data analysis was performed using the GraphPad Prism version 8.0 software package.

Screening and elimination of phage insensitive bacterial mutants by multiple phage dose and serial passage

The behavioral response of phage-insensitive bacterial mutants to multiple phage doses and serial passage was assessed. The work of Denes et al. has been modified here to understand the behavior of phage insensitive mutants (Denes et al. 2015). This experiment was performed separately for both the phage reported in this study. Briefly, five independent tubes (A, B, C, D, E) containing the same proportion of phage (MOI 10) and their respective host bacteria (10^8 CFU/mL) (SA-90 with vB_Sau_S90 and SA-165 with vB_Sau_S165) was incubated at 37 °C for 24 hrs. After incubation for 24 hrs (period for bacterial evolution), multiple phage treatments and serial passage were performed independently on each tube at 37 °C.

The bacterial cells that regrew (Phage insensitive cells) after the phage bacterial interaction was interpreted based on their response to subsequent phage infection and scored as– **Sensitive type** – bacterial cells that were susceptible to phage without any requirement of serial passage, **Transient resistant type** – bacterial cells that become phage susceptible only after the serial passage, **Resistant type** – bacteria cells that showed phage resistance even after several serial passages or multiple phage doses.

The multiple dosage and serial passage were conducted in each tube as follows - In tube A, the mixture was centrifuged at 10,000 X g for 5 min, and the pellet was washed twice with SM buffer and resuspended in 200 µl of BHI broth. The spread plate was performed in a bacteriophage-free agar plate and incubated at 37 °C for 24 hrs. The rate of phage resistance was determined after incubation, and a spot test was performed on random colonies to check for the bacterial cell type that evolved after single phage treatment in liquid media. In tube B, the mixture was centrifuged at 10,000 X g for 5 min, and the pellet was resuspended in 200 µl of BHI broth. The resuspended pellet was mixed with the second dose of phage (10^9 PFU/mL), and a double agar overlay was performed to eliminate any growing phage-sensitive cells and the rate of resistance after removing the sensitive cells was determined. The plates were incubated at 37 °C for 24 hrs to determine the appearance of phage-insensitive bacterial colonies. The individual colonies obtained in DAOL was picked and inoculated in 1 ml of BHI broth. For each colony, five alternative serial passage (day - 1, 3, 5, 7, 9) along with five alternative phage doses (10^9 PFU/mL) (day – 2, 4, 6, 8, 10) was administered to check the effect of bacterial clearance by multiple phage doses and serial passage. Simultaneously, spot test was performed after each serial passage to check, if serial passage aided in the reversion (from transient phage resistance to phage sensitive cells). In tube C, the phage insensitive cells were isolated similar to tube B and the colonies from DAOL were treated only by multiple phage treatment (10^9 PFU/mL) for five subsequent days. Here, serial passage was eliminated to check the effect of bacterial clearance only by multiple phage doses. Simultaneously, spot test was performed after each phage treatment to check, if reversion (from transient phage resistance to phage sensitive cells) occurred without serial passage. In tube D, the bacterial cells obtained from single phage treatment in liquid media was treated with five alternative days of serial passage and five alternative days

of phage treatment (10^9 PFU/mL). The tube D experiment analyzes the combinatorial effect of serial passage and phage multiple doses in liquid media without eliminating uninfected phage sensitive cells. In tube E, the bacterial cells obtained from single phage treatment in liquid media was treated with only multiple phage doses (10^9 PFU/mL) for five subsequent days without serial passage. Tube E aided in determining the significance of the serial passage in eliminating phage insensitive and phage sensitive cells in liquid media. The experimental setup described above are depicted in **supplementary figure 1**. The rate of resistance was calculated using the formula: Phage resistant rate (CFU/mL) = (Number of bacterial colonies after phage treatment / Initial number of the colony).

Statistical analysis

The plaque size was analyzed manually using a ruler, and the values are provided as mean $\pm$ SD. All data were expressed as mean $\pm$ standard deviation (SD) and analyzed using the GraphPad Prism version 8.0 software package.

Results

Phage isolation, identification, and titration

A total of seven *Staphylococcus* phage was isolated. Among seven phages, two were considered for the therapeutical characterization as they showed a broad host range activity against the clinical strains of *S. aureus*. The phages were designated as vB_Sau_S90 and vB_Sau_S165 in compliance with recent phage nomenclature guidelines (Adriaenssens and Brister 2017). The plaque size of both the phages showed clear, small plaques, ranging from 0.75 to 2.2 mm in diameter (Fig. 1b). Here, in this study, the enrichment process was modified to increase the frequency of isolating *Staphylococcus* phage. Notably, adding CaCl_2 of 0.5 mM to 1 mM enhanced phage enumeration from the sewage sample, while the absence of CaCl_2 didn't give successful phages from the same sample examined. However, the addition of CaCl_2 showed no visible change in plaque morphology. The phage titer obtained from agar media by web pattern yielded high phage titer (10^{14} PFU/mL) than in liquid media (10^{10} PFU/mL) (Note – Phage yield and concentration used in web pattern may vary upon the phage plaque size, bacterial growth rate, and multiplication cycle). The web pattern DAOL is shown in Fig. 1a.

One-step growth kinetics

The adsorption efficiency showed 50% of the phages attached to the bacterial host within 8 ± 1 min for phage vB_Sau_S90 and 5 ± 1 min for phage vB_Sau_S165. The maximum adsorption efficiency for phage vB_Sau_S90 was $91 \pm 0.6\%$ with a duration of 17 ± 1 min with its host strain SA-90 and $95 \pm 0.5\%$ in 15 ± 2 min for phage vB_Sau_S165 with its bacterial host SA-165. This observation shows that both the phages reached their maximal adsorption in a very shorter duration of time, reflecting a super affinity between the bacterial host and the phage. On adsorption, the phage vB_Sau_S90 showed a latency period of approximately 9 ± 1 min, releasing 101 ± 5 phage progeny per cycle. The phage vB_Sau_S165 showed a latency period of approximately 7 ± 1 min, releasing 90 ± 4 phage progeny per cycle (Fig. 2).

Physicochemical characteristic

The stability to temperature and pH was evaluated, and the phages were considered stable upon showing $\geq 50\%$ viability. Exposing phage to higher temperatures (20 to 80 °C) revealed that phage vB_Sau_S90 were stable over 20 to 50 °C and vB_Sau_S165 stable from 20 to 40 °C. In pH, vB_Sau_S90 were stable from 5 to 9 and vB_Sau_S165 from 7 to 8. The highest stability (greater than 90%) was attained at pH 7 and 8, respectively (Fig. 3).

Morphological analysis

The morphological analysis using TEM showed that phage vB_Sau_S90 had an icosahedral head of 92 ± 0.48 nm and a short non-contractile tail of 19 ± 0.68 nm in length, showing that the phage belongs to the *Podoviridae* family. Phage vB_Sau_S165 showed an icosahedral head of 71 ± 0.99 nm and a long non-contractile tail of 230 ± 0.59 nm showing that the phage belongs to the *Siphoviridae* family (Fig. 1c).

Host spectrum and phage sensitivity to methicillin-resistant *Staphylococcus aureus*

The host spectrum by spot test showed that phage vB_Sau_S90 was able to kill 88% (181/205) of the tested isolates, of which 56% (115/205) were sensitive, 32% (66/205) were intermediate sensitive, and 12% (24/205) were resistant to the phage infection. The phage vB_Sau_S165 had activity against 94% (192/205) of the tested isolates, of which 80% (165/205) were sensitive, 13% (27/205) were intermediate sensitive, and 6% (13/205) were resistant to the phage infection. The EOP test revealed that vB_Sau_S90 had activity against 98% (177/181) of the isolates tested positive in spot test, with 61% (111/181) exhibiting high EOP, 32% (58/181) with moderate EOP, and 4% (8/181) with low EOP, and 2% (4/181) with no plaques. The phage vB_Sau_S165 had activity against 97% (187/192) of the isolates tested positive in spot test, with 83% (160/192) exhibiting high EOP, 12% (22/192) with moderate EOP, and 3% (5/192) with low EOP, and 3% (5/192) with no plaques were observed. The infectivity of the phages using spot test against MRSA showed that vB_Sau_S90 was able to kill 83% (88/106) of MRSA. Similarly, vB_Sau_S165 had activity against 92% (98/106) of MRSA. In EOP, the phage vB_Sau_S90 killed 99% (87/88) of MRSA, whereas vB_Sau_S165 phage killed 97% (95/98) of MRSA. (Table 1). (**Note** – Both SA–90 and SA–165 are MRSA)

Table 1: Host activity of phages by spot test showing resistant (red), intermediate (yellow) and sensitive (green) with their respective efficiency of plating scored from high, moderate, low and no EOP for phage vB_Sau_S90 (blue) and vB_Sau_S165 (orange), alone with phage activity and EOP in MRSA and MSSA strains

Phage	Antibiotic pattern	Analysed by	Isolates resistant				Isolates intermediate				Isolates sensitive				Total activity*
			High	Moderate	Low	No	High	Moderate	Low	No	High	Moderate	Low	No†	
vB Sau S90	MRSA	Host range	18				30				56				86 (83%)
		EOP	0	0	0	18	0	28	2	0	55	0	0	1	85 (82%)
	MSSA	Host range	6				36				58				94 (94%)
		EOP	0	0	0	6	0	30	6	0	55	0	0	3	91 (91%)
vB Sau S165	MRSA	Host range	8				13				83				96 (92%)
		EOP	0	0	0	8	0	12	1	0	80	0	0	3	93 (89%)
	MSSA	Host range	5				14				81				95 (95%)
		EOP	0	0	0	5	0	10	4	0	79	0	0	2	89 (89%)

MRSA: Methicillin Resistant *Staphylococcus aureus*; MSSA: Methicillin Susceptible *Staphylococcus aureus*; DAOL: Double agar overlay

* Total activity for host range = (Isolates intermediate + Isolates sensitive), Total activity for EOP = ((Isolates intermediate + Isolates sensitive) – (no EOP in Isolates intermediate + no EOP in Isolates sensitive))

† No – Represent the EOP analysis showing clearance with faintly hazy background in the spotted phage dilutions till 10^1 PFU/mL with no observable plaques

Time kill analysis

The killing curve for phage vB_Sau_S90 at MOI 10 showed 0.4 log fold reduction, MOI 1 showed 0.3 log fold reduction, MOI 0.1 showed 0.06 log fold reduction and no log fold reduction was observed in MOI 0.01, 0.001. Notably, the phage killing curve for vB_Sau_S90 showed that MOI 10 and 1 were highly effective to other MOIs. However, no complete clearance was seen in any of the MOI. (Fig. 4a).

The killing curve for phage vB_Sau_S165 showed a 0.3 log fold reduction at MOI 10 and 1, and no log fold reduction in other MOIs. Notably, the phage killing curve for vB_Sau_S165 showed that MOI 10 and 1 were highly effective to other MOIs; however, none of the MOI could clear the bacterial cells completely (Fig. 4b).

Effect of multiple phage doses and serial passage on phage insensitive bacterial cells

The spread plate of bacterial cells from tube A showed a mixture of phage sensitive and phage insensitive bacterial cells as confirmed by spot test (Fig. 5). The rate of uncleared bacteria observed from liquid media (Tube A - phage-sensitive cells and phage insensitive cells) for phage vB_Sau_S90 and vB_Sau_S165 was 2.6×10^{-8} and 1.3×10^{-8} CFU/mL. The rate of uncleared bacteria after eliminating phage-sensitive cells from tube B by DAOL showed 2.6×10^{-12} CFU/mL for phage vB_Sau_S90 and 3.6×10^{-13} CFU/mL for vB_Sau_S165. The spot test of the serial passage colony from tube B showed significant reversion. Transient mutants isolated against vB_Sau_S90 showed that 85% (17/20) of the colonies became phage sensitive after the first serial passage. The remaining 15% (3/20) became susceptible after two serial passages. For phage vB_Sau_S165, all the colonies 100% (27/27) become sensitive after two serial passages. All the transient phage-resistant mutants were eradicated on five alternative serial passages along with alternate days of phage treatment, as observed by the reducing bacterial turbidity; however, the bacteria reappeared when treatment was stopped. The effect of multiple phage dosage alone in tube C showed no significant effect on transient phage-resistant cells. The effect of alternate serial passage and multiple phage treatment on the bacterial cells in liquid media of tube D (phage sensitive and transient phage resistant cells) showed a bacterial clearance after five alternative serial passage and phage treatment; however, re-emerging bacterial cells were seen emerging after 24 hrs when the treatment was stopped. Similarly, the effect of phage alone treatment in the liquid media of tube E (phage sensitive and transient phage resistant cells) showed no bacterial clearance at any point of phage treatment.

Discussion

The lytic bacteriophages are emerging as an alternative therapy for killing antibiotic-resistant bacteria. However, very little information is available on the therapeutic characteristics of the *Staphylococcus* phage and its therapeutic efficacy on different MRSA and MSSA clones circulating in India. This work highlights that multiple phage doses were required to eliminate relapsing bacteria growth during phage-bacterial interaction.

Two *Staphylococcus* phages reported in this study were isolated from the sewage water. Among the various sample sources used for isolating *Staphylococcus* phage, hospital sewage showed the highest frequency of *Staphylococcus* phage followed by soil samples from hospitals. Even though most *Staphylococcus* phages are known to exhibit broad host range activity, the difficulty in isolating *Staphylococcus* phages is reported frequently (Adriaenssens and Brister 2017, Mattila et al. 2015).

This study confirms the extremely broad host range of the phages to *Staphylococcus aureus*. *Siphoviridae* and *Podoviridae* reported in this study showed higher infectivity to diverse *S. aureus* in the spot test, which includes both MRSA and MSSA. The four isolates infected with vB_Sau_S90 and five isolates infected with vB_Sau_S165 exhibited a zone of clearance in the spot test; however, they did not show plaques in EOP analysis, showing that they might have resulted from lysis from without phenotype.

This necessitates the importance of EOP in determining phage infectivity. A similar finding was already reported earlier by Manohar et al (Sinha et al. 2018). This difference in spot test and EOP for a few isolates can be attributed to several factors, as explained by Stephen T. Abedon (Abedon 2011b). Firstly, lysis from without, and secondly, higher phage threshold that breaks the cell without adsorption of the phage.

The lifecycle of the phages showed a short multiplication time with moderate phage yield per infection cycle. This result shows that both the phages could be more effective in killing bacterial cells even at lesser phage concentrations. However, on the other hand, the time-kill analysis of planktonic culture revealed that both phages studied did not completely eradicate the bacterial cells even at higher MOIs of a single dose of phage treatment. We hypothesize that these variations in bacterial clearance even at higher MOI could be due to shorter adsorption time of the phages, which resulted in incomplete adsorption. In co-relation with the above hypothesis, our study found a convincing result in the phage-bacterial interaction study, in which we observed a large number of sensitive cells that remained uninfected by phage in liquid media during a single dose of phage treatment (Tube A). This revealed that the phages were not effective against the sensitive cells in a single phage dose. To address this question, we investigated the regrowing bacteria during phage-bacterial interaction, which showed a range of clones that re-emerged after 8 ± 1 hrs.

Interestingly, we found that no stable phage-resistant mutants appeared for both the phages studied as regrowing bacterial cells lost resistance to phage with a maximum of two serial passages, thus showing the presence of transient phage resistant mutants. Similar observations were noted in *Salmonella enterica* by Fong et al., in which most of the transient phage-resistant mutants isolated become susceptible to serial passage (Fong et al. 2020).

The experimental findings by Whittard et al. observed the appearance of Pacman-like colonies (described as half-lysis colony type in this study) during phage-bacterial interaction, and they also found that most of them were susceptible to phage although they remained resistant to phage in the liquid medium (Whittard et al. 2021). A study by Głowacka-Rutkowska et al. demonstrated the equilibration of phage bacteria in a mixture in which the authors outlined that phage carrier state cells and phage resistant coexist in a mixture, and the resistant phenotype can be reverted upon removing the phage by subculturing (Głowacka-Rutkowska et al. 2018). This similar phenotype of reverting to sensitivity upon subculturing was also observed in our study. In addition to sensitivity by serial passage, our study also revealed that multiple phage dosing significantly reduced the bacterial cells; however, a similar effect could not be attained without intermediate serial passage. The requirement of multiple dosing might be due to the lack of 100% phage adsorption onto the bacterial cells. According to Abedon et al., not even a single phage that can be isolated can undergo 100% adsorption onto the bacterial host and complete bacterial clearance without continuous or multiple phage doses (Abedon 2011a). The unadsorbed bacterial cells tend to adapt or escape the phage infection and multiply despite phage auto-dosing. This phenotype exhibited by our phage concludes that auto-dosing was not sufficient to attain complete bacterial eradication despite being phage sensitive. Thus, the requirement of multiple phage doses was

essential in eliminating the bacterial cells. As observed from this study, we found that intermediate serial passage aided in the conversion of phage transient resistant mutants to phage sensitive cells, and the addition of multiple phage doses aided in the elimination of existing phage sensitive cells as well as the transient phage resistant mutants that were reverted on intermediate serial passage. Our study also revealed that a minimum of five serial passage was essential to eradicate the bacterial cells; however, the appearance of bacterial cells on halting the phage treatment were notable. We predict that this discrepancy could be overcome by reducing the phage dosing duration or increasing days of phage treatment.

Our study also highlights the difference in phage evading ability in liquid (BHI/CaCl₂ broth) and solid media (BHI/CaCl₂ agar). This could partly be explained by the variation in growth kinetics of bacteria or the point of phage bacteria saturation in two different states of media. These variations observed between liquid and solid media were significantly supported by observing a higher phage titer in web pattern DAOL to liquid media after 24 hrs of multiplication. Also, the examination of colonies from liquid media showed a large proportion of sensitive cells that remained unaffected by the phages, showing that they could be the probable reason for lower phage yield in liquid media (Fig. 5). This shows that the high rate of uncleared bacterial cells observed in liquid media was due to the high number of phage-sensitive cells that remained unabsorbed by phages. On the other hand, the colonies on the double agar overlay plate did not show any sensitive cells. Thus, higher phage adsorption was attained in agar media resulting in high phage yield.

Despite significant findings in this study, few limitations were notable, especially with regards to the duration of phage-bacterial interaction. As this study evaluated phage-insensitive bacteria at the 24th hour, it might be possible that the duration is too short to achieve full resistance in bacterial cells. However, numerous studies have reported that bacterium could evolve complete resistance even at a single step (Borin et al. 2021). We also found that other *Staphylococcus* phages from our phage library developed phage resistance in less than 24 hrs (data not shown as they are out of scope for the study). We also found a serious limitation in studying the growth kinetic difference in solid bacteriological medium to liquid bacteriological medium due to the requirement of sophisticated instruments. The exact genomic relationship, receptor modification, or bacteria immunity are yet to be studied and not reported here as they are beyond the scope of this study. However, our study has detailed a fundamental finding on transient phage-resistant mutants, and this study has opened up a new avenue of research in phage biology to be explored. It is essential that the limitations of the study are considered in future studies.

Conclusion

In this study, two *Staphylococcus* phages that were stable at varying physical conditions were identified, and their ability to infect bacteria was determined *in vitro*. Both the phages had a broad host range against *S. aureus*, and the strong lytic activity of the phages can be utilized as a therapeutic drug. Though the appearance of transient phage-resistant mutants possessed a significant concern, we also found that repeated phage dosing eliminates the bacteria. As a result, emphasizing the need for multiple

or continuous phage doses may be a promising strategy for developing phage therapy for *Staphylococcus aureus* infections.

Declarations

Statements and declarations

Competing Interests

The authors have no financial and non-financial conflicts of interest to declare.

Availability of data and materials

All data are represented as figures, tables, and supplementary files. Any further inquiries may be referred to the corresponding author.

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Submission declaration

The authors declare that the work submitted is not under consideration for publication elsewhere or has not been published previously.

Ethical approval and consent to participate

Ethical approval for the study was received from Institutional Ethical Committee for Human Studies from VIT University (certificate of approval no. VIT/IECH/004/January 28, 2017).

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Authors Contribution:

AL and RN were involved in the conceptualization of the study. AL performed the formal analysis and wrote the initial manuscript draft. RN obtained the funds, evaluated and finalized the manuscript. All the authors agreed on the final version of the manuscript.

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Figures

Figure 1

a) Illustrative image showing web pattern agar overlay of *Staphylococcus* phage, b) Double agar overlay plate showing plaque morphology of phage vB_Sau_S90 against bacteria SA90 (left) and vB_Sau_S165 against bacteria SA165 (right), c) Transmission Electron Microscopy image of phage vB_Sau_S90 showing Podoviridae morphology (left), and vB_Sau_S165 showing Siphoviridae morphology (right)

Figure 2

The lifecycle of two lytic phages, a) Adsorption kinetics of phage vB_Sau_S90 (left), and vB_Sau_S165 (right), b) One-step growth curve showing latency period (L) and burst size (B) of phage vB_Sau_S90, (left), and vB_Sau_S165 (right)

Figure 3

Stability of phages at various physical conditions, a) Temperature stability of the phage vB_Sau_S90 (left), vB_Sau_S165 (right), b) pH stability of the phage vB_Sau_S90 (left), and vB_Sau_S165 (right)

Figure 4

In vitro time kill analysis of phage. a) vB_Sau_S90, and b) vB_Sau_S165

Figure 5

Illustration showing the heterogenous colony morphology observed on spread plate of bacterial pellet from tube A during screening for phage-insensitive mutants and the spot test with phage vB_Sau_S90 (left) and vB_Sau_S165 (right) on different colony type after serial passages

Supplementary Files

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