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# Isolation and characterization of bacteriophage SA-19 and investigation of antibiofilm effect against clinical strains

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**ABSTRACT**: Because the emergence of antimicrobial resistance worldwide and alternative treatment strategies against Staphylococcus aureus infections are still being sought, we investigated a new bacteriophage with a lytic effect against S. aureus strains in this study. Then, we isolated potentially therapeutic lytic S. aureus phage from seawater and determined the biological characterization and in vitro lytic effect against various clinical bacterial strains. As a result, our study provides evidence of phage application for S. aureus strains, combination studies with conventional antibiotics as an alternative treatment option in clinical.

**KEYWORDS**: Phage-antibiotic combination; Staphylococcus aureus; bacteriophage; biofilm.

## 1. INTRODUCTION

*Staphylococcus aureus* is a pathogenic gram-positive bacteria that causes various diseases in humans and can form biofilm and grow on human skin [1]. The diseases caused by *S. aureus* mostly comprise pericarditis, bacteremia, meningitis and pneumonia in humans [2,3]. They are also life-threatening bacteria because of their virulence factors and antibiotic resistance. Treatment of *S. aureus* infections becomes challenging due to antibiotic resistance. The inadequate treatment of *S. aureus* infections has led to economic loss, and it has been difficult to treat infectious diseases [1-4]. Hence, developing novel therapeutic strategies to control infections that can replace or supplement antibiotics is very important. Bacteriophages are bacterial viruses and promise significant alternative strategies for bacterial infections. Bacteriophage therapy could be a significant alternative treatment method for infectious diseases caused by these bacteria.

Bacteriophages are bacterial viruses and the most prevalent biological entities which target their specific bacterial hosts. Firstly, bacteriophages were discovered by a British bacteriologist, M. Ernest Hankin in 1896. He observed that water samples from the Yamuna and Ganges rivers had antimicrobial activity. In 1917, French microbiologist Felix d'Herelle named "invisible microbe toward dysentery bacillus" as bacteriophage and declared the antibacterial effect of phages [5,6]. Phages were used to treat diseases such as burn wounds, abscesses, upper respiratory tract infections in USA, Poland, and Georgia until the 1940s [7,8]. After the antibiotics were discovered in 1941, phages fell into the background; however, in the Soviet countries, phage therapy was used in the treatment like the soldiers' wounds caused by *Pseudomonas aeruginosa*, *S. aureus* and *Escherichia coli* in the 1990s. Phages kill their specific bacterial host; this situation is related to the function and structure of bacteria and bacterial diversity [9]. Bacteriophages have lysogenic and lytic life cycles. Lytic phages can reproduce, replicate, produce lysine and have lytic activity against bacterial hosts [1,10]. Lytic phages are defined as virulent. Besides, it is important that phages have a lytic activity against bacterial cells and a strong bacterial host specificity when used in the treatment. Phages have been known as safe, natural and significant alternatives to conventional antibiotics in treating or preventing infections caused by *S. aureus* [11].

This study aims to isolate a potentially therapeutic lytic *S. aureus* phage from seawater, determine its biological characterization, and investigate its in vitro lytic effect on various clinical *S. aureus* bacterial strains. For this reason, we isolated and characterized phage SA-19 and investigated its biological

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characteristics for its potential as an alternative agent. The isolation studies were performed using the spot test method. Susceptibility, one-step growth curve, and ph/thermal stability of phage were determined. Besides, the antibiofilm effects of isolated phage were investigated alone or with a combination of ciprofloxacin and meropenem.

## 2. RESULTS

### 2.1. Isolation and purification studies of phage

Phage SA-19 was isolated from seawater of the Marmara Sea in Istanbul/Turkey. *S. aureus* 3019 was the host strain of phage SA-19 and was used to determine its plaque morphology. As shown in Figure 1, the plaque of phage SA-19 formed about 1,0 mm in diameter and produced small clear plaques on the bacterial host. As shown in Table 1, the highest phage concentration of SA-19 was $4\times10^{10}$ pfu/mL.

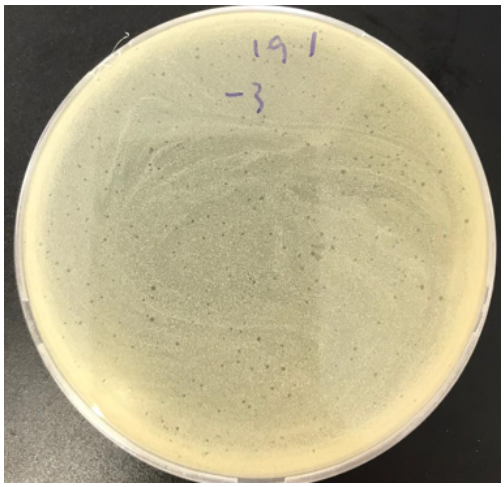


**Figure 1.** Appearance of clear plague formed by SA-19 phage with its host with the double layer agar method.

**Table 1.** General features of plaques of SA-19 phage.

| Phage | Source | Plaque appearance | Plaque size (mm) | Host | Stock concentration |
|---|---|---|---|---|---|
| SA-19 | Seawater | Small and clear plaques | 1,0 mm | *S. aureus* | $4\times10^{10}$ |

### 2.2. The susceptibility of phage

The susceptibility of phage was assessed on 75 various clinical bacterial strains, including 25 *P. aeruginosa*, 25 *S. aureus*, and 25 *E. coli* strains. As shown in Table 2, the lytic effects of isolated phage SA-19 were categorized as confluent lysis (+++), semi-confluent lysis (++), individual plaques (+) and no lysis (-). As shown in Table 3, among the 25 *S. aureus* strains 24% were susceptible to phage SA-19 and showed confluent lysis, however 40% of *S. aureus* strains were resistant to isolated phage. Besides, 4% *E. coli* clinical isolates were found susceptible to SA-19 phage and showed no effect on *P. aeruginosa* clinical strains.

**Table 2.** Susceptibilites of phage SA-19 against various clinical strains.

| *P. aeruginosa* strains | SA-19 | *E. coli* strains | SA-19 | *S. aureus* strains | SA-19 |
|---|---|---|---|---|---|
| PA 1 | - | EC 1 | - | SA 1 | +++ |
| PA 2 | - | EC 2 | + | SA 2 | ++ |
| PA 3 | - | EC 3 | ++ | SA 3 | - |
| PA 4 | - | EC 4 | - | SA 4 | + |
| PA 5 | - | EC 5 | + | SA 5 | ++ |
| PA 6 | - | EC 6 | - | SA 6 | ++ |
| PA 7 | - | EC 7 | - | SA 7 | ++ |
| PA 8 | - | EC 8 | - | SA 8 | +++ |
| PA 9 | - | EC 9 | + | SA 9 | +++ |
| PA 10 | - | EC 10 | - | SA 10 | +++ |
| PA 11 | - | EC 11 | - | SA 11 | +++ |
| PA 12 | - | EC 12 | - | SA 12 | + |
| PA 13 | - | EC 13 | - | SA 13 | ++ |
| PA 14 | - | EC 14 | - | SA 14 | - |
| PA 15 | - | EC 15 | +++ | SA 15 | ++ |
| PA 16 | - | EC 16 | + | SA 16 | - |
| PA 17 | - | EC 17 | ++ | SA 17 | - |
| PA 18 | - | EC 18 | - | SA 18 | - |
| PA 19 | - | EC 19 | - | SA 19 | +++ |
| PA 20 | - | EC 20 | ++ | SA 20 | - |
| PA 21 | - | EC 21 | ++ | SA 21 | - |
| PA 22 | - | EC 22 | - | SA 22 | - |
| PA 23 | - | EC 23 | - | SA 23 | - |
| PA 24 | - | EC 24 | ++ | SA 24 | ++ |
| PA 25 | - | EC 25 | - | SA 25 | - |

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**Table 3.** Results of spot tests of phage SA-19 against clinical strains.

| Phage | Bacterial strains | Results of Spot Test (%) | | | |
|---|---|---|---|---|---|
| | | R | + | ++ | +++ |
| **SA-19** | All strains (n=75) | 67% | 8% | 16% | 9% |
| | *E. coli* strains (n=25) | 60% | 16% | 20% | 4% |
| | *P. aeruginosa* strains (n=25) | 100% | - | - | - |
| | *S. aureus* strains (n=25) | 40% | 8% | 28% | 24% |

## 2.3. pH/thermal stability

Ph and thermal stability of phage SA-19 were assessed under various conditions. For pH stability, phage SA-19 was kept at pH 2, pH 7 and pH 11 for 48 hours at 4 ◦C. Phage titer was unaffected at pH 7. However, the activity of phage decreased nearly 2 logs at pH 11 at the end of the second day. As shown in Figure 2, phage titer was inactivated entirely after 48 hours at pH 2.

Thermal stability tests show that SA-19 phage titre decreased by about 5 logs, and about 6 logs at 65◦C and 90◦C, respectively. However, as shown in Figure 2, the phage titer did not decrease at 4◦C, 25◦C and 37◦C.

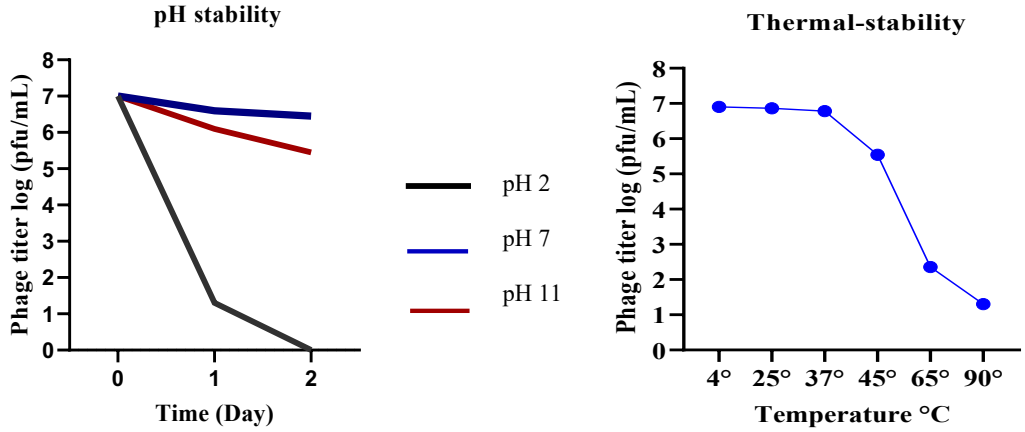


**Figure 2.** pH and thermal stability on phage SA-19.

## 2.4. One-step growth curve

As shown in Figure 3, the latent period was 30 minutes and burst size of SA-19 phage was 100 pfu/cfu, respectively. This result showed the effective and rapid growth of phage SA-19 for adsorption in its host.

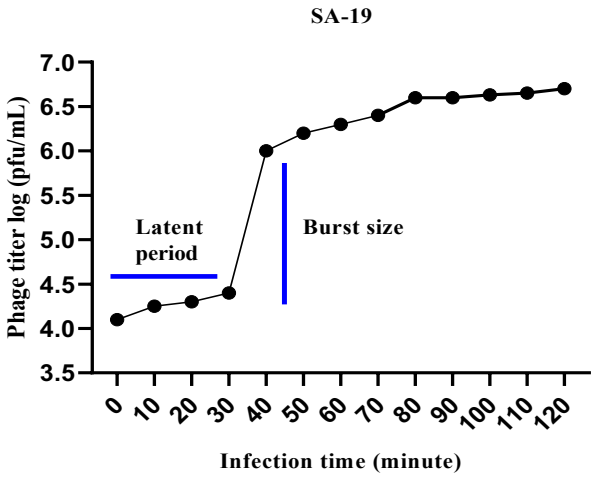


**Figure 3.** One-step growth curve of SA-19 phage.

### 2.5. Inhibition of biofilm formation

Firstly, the biofilm-forming abilities of 10 tested *S. aureus* strains were assessed. As indicated in Table 4, seven strains of S. aureus exhibited strong biofilm production, two clinical strains displayed moderate biofilm formation, and one clinical strain showed weak biofilm production.

Then, MICs of tested antibiotics were determined against two strong biofilm-producer *S. aureus*. As shown in Table 5, MICs of ciprofloxacin were 0.06 and 0.5 µg/mL for *S. aureus*-1 and *S. aureus*-2, respectively. MICs of meropenem were 0.06 µg/mL for both *S. aureus* strains. *E. coli* 25922 ATCC was used as a reference strain for susceptibility testing.

The inhibition of the biofilm formation effect of phage SA-19 was investigated alone or in combination with meropenem or ciprofloxacin against two strong biofilm-producer bacteria.

Results showed that 1×MIC, 1/10×MIC and 1/100×MIC of meropenem and ciprofloxacin prevented *S. aureus*-1 biofilm formation at 64,92%, 61.87% and 52%, and 71.01%, 65.86% and 14.04% alone, respectively. Phage SA-19 showed a 61.7% inhibitory effect alone as antibiotics did alone. As shown in Figure 4, when evaluated, ciprofloxacin in combination with phage SA-19 at 1/100×MIC of antibiotics concentrations was effective and remarkably increased the biofilm inhibition.

For *S. aureus*-2 strain, 1×MIC, 1/10×MIC and 1/100×MIC of meropenem and ciprofloxacin alone prevented *S. aureus*-2 biofilm formation at 24.42%, 10% and 5%, and 60.51%, 31.34% and 1% respectively. Phage SA-19 inhibited biofilm formation alone by 64.83%. When evaluated, meropenem, in combination with phage SA-19, increased inhibition of biofilm formation remarkably at all concentrations. As shown in Figure 4, besides, at 1×MIC concentration, phage SA-19 and ciprofloxacin combination prevented biofilm formation by 60.75% compared with ciprofloxacin alone.

**Table 4.** Ability of biofilm formation clinical *S. aureus* isolates.

| Strains | Non-biofilm producer | Weak biofilm producer | Moderate biofilm producer | Strong biofilm producer |
|---|---|---|---|---|
| *S. aureus (n=10)* | - | %10 (1) | %20 (2) | %70 (7) |

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**Table 5.** The MICs of antibiotics against *S. aureus* strains which form strong biofilm producers

| | MIC (μg/mL) | |
|---|---|---|
| **Bacteria** | **Meropenem** | **Ciprofloxacin** |
| *S. aureus*-1 | 0.06 | 0.06 |
| *S. aureus*-2 | 0.06 | 0.5 |
| *E. coli* 25922 ATCC | 0.008 | 0.008 |

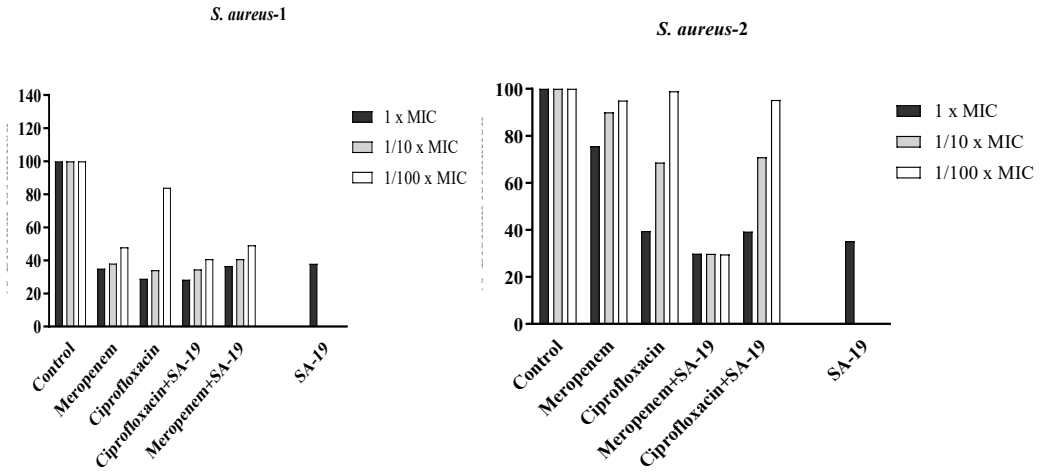


**Figure 4**. Inhibition of biofilm formation of *S. aureus* strains by antibiotics and phage SA-19 alone or in combination.

### 2.6. Quantification of biofilm biomass

As shown in Figure 5, *S. aureus*-1 mature biofilm mass was reduced by 44.67% using a combination with phage SA-19 and meropenem only at 5×MIC compared with meropenem alone. Phage SA-19 could reduce 36.54% and 45% of the *S. aureus*-1 and *S. aureus*-2 biofilm mass, respectively. Besides, combination treatment of phage and antibiotics could not stimulate the antimicrobial effect against the biofilm mass of the mature *S. aureus*-2 strain.

## 3. DISCUSSION

The antimicrobial resistance is a severe global challenge worldwide. Alternative treatment methods are urgently needed to struggle with antimicrobial resistance. Some of them are immune stimulation, antibodies, probiotics and bacteriophages. This particular situation in antibiotic resistance has drawn attention to phages. Phages used as alternative antimicrobial agents to struggle with antimicrobial resistance are an efficient solution against microorganisms.

In the present study, we investigated lytic *S. aureus* phage from seawater samples. Host bacteria in the environment affect the distribution of bacteriophages. Animal or faecal, plant, mud, soil and water samples are places used to isolate phages. *S. aureus* is a ubiquitous and free-living bacteria in the biosphere and exists widely in the environment [12]. Similar studies showed that *Staphylococcus* phages were isolated from various water samples in different locations [13-15].

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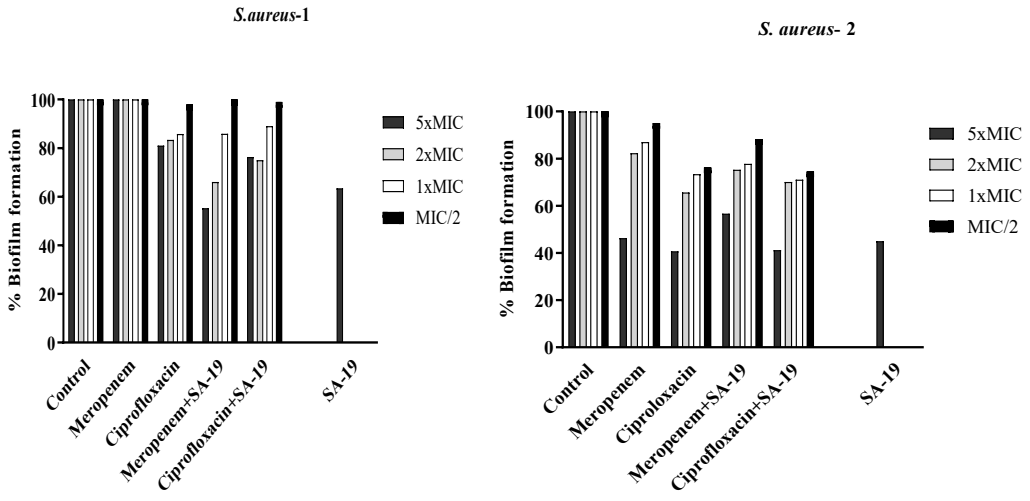


**Figure 5**. Eradication of mature biofilm biomass of *S. aureus* strains by antibiotics ( 5×MIC, 2×MIC, 1×MIC, MIC/2) and phages alone or in combination.

Phage plaques are formed with different appearances on host cells when co-plated with host bacteria and its phage. Plaque with a halo, turbid edges, and clear plaques are standard. Characterization of isolated phages is essential to show their potential therapeutic effect. Besides, non-characterized phages against infections are a critical reason for failure in phage therapy [16]. In industrial areas or treatment, lytic phage candidates are preferred instead of lysogenic phages. Lysogenic phages can transfer resistance genes to bacteria. Besides, stability to physicochemical factors, lytic spectrum, and one-step growth analyses are critical factors to characterize potential phage candidates [17].

Lytic spectrum assays showed that phage SA-19 lysed 24% of *S. aureus* strains and 4% of *E. coli* strains with clear plaques. However, phage SA-19 could not lyse any *P. aeruginosa* strains. Besides phage, SA-19 has lytic activity against 20% of *E.coli* and 28% of *S. aureus* strains with opaque plaques (++).

According to previous studies, the phage isolated by Wang et al. lysed 23% of *S. aureus* strains [18]; also, Jiang et al. displaced that *S. aureus* phage isolated from a pig slaughterhouse had potent lytic spectrum against several resistant *S. aureus* strains [19]. These results have indicated the importance of phages in treatment.

The stability of phage under stress conditions is critical to evaluate for industrial and therapeutic uses. Phage as a therapeutic agent should be stable in a wide pH range [20]. In this study, we tested the pH/thermal stability of SA-19 phage. The results displayed that SA-19 was consistent at pH 7 for 48 hours. It was entirely inactivated at pH 2 on the second day. Besides phage titer reduced 2 log at pH 11 after 48 hours. The thermal stability of phage SA-19 was consistent between 4◦C and 45◦C. Phage SA-19 was inactivated at 65◦C and 90◦C after 120 min.

In the study of Jiang. et al., the isolated *S. aureus* phage was completely inactive at 90°C, and there was a remarkable decline at 60°C. Besides isolated phage plaques level decreased at pH 3-4 however at pH 6-7 there was the most plaques [19]. Esmaeilzadeh et al. isolated a phage that had the highest stability at 4°C and could survive at 37 °C and 25 °C, but the titre of the phage decreased at a temperature of 50°C -70°C. Besides at pH 6 to 8 there was the highest lytic activity [21].

The latent period defines as is the period between the phage adsorption and release. Burst size is the number of phages per host single bacteria. One-step growth curve is essential to assess the proliferation of phage under different conditions (in vitro/in vivo) [22-24]. The curve showed that latent period of phage SA-19 was 30 minute and burst size was burst size was 100 pfu/cfu. Compared with the one-step growth analyses of *S. aureus* phages in studies, Wang et al. isolated an *S. aureus* phage which a latent period of 10 min and burst size of 293 pfu/cfu [18]. Shimamori et al. found a latent period of 40 min and a 117 pfu/cfu burst size [25].

Biofilm production of *S. aureus* causes the resistance to antimicrobials. Microorganisms compose structures such as biofilm synthesis or spore formation to protect themselves in crucial conditions [26]. Recent studies have indicated that bacteria that colonize humans can form biofilms and thus become resistant to the immune system and antimicrobials. Antibiotics are used to destroy biofilms; however, biofilm formation is encountered in medical devices such as shunts, pacemakers, or catheters, and treatments are unsuccessful because of the prevention of drug penetration [27]. Because of this reason,

searching for new alternative treatment options such as photodynamic effect, antibiofilm-effective antibiotics, or phages has continued all over the world. In this study, the eradication of mature biofilm layer and biofilm inhibition effects of tested antibiotics or phage SA-19 alone and in combination were assessed against two clinical *S. aureus* strains.

According to our results, 1×MIC, 1/10×MIC, and 1/100×MIC of meropenem and ciprofloxacin prevented *S. aureus*-1 biofilm formation at 64.92%, 61.87%, 52%, and 71.01%, 65.86%, 14.04%, respectively. Phage SA-19 inhibited biofilm formation by 61.7% as antibiotics did. When antibiotics were evaluated in combination with phage SA-19, at 1/100 MIC of antibiotics concentrations, the biofilm inhibition increased remarkably.

For *S. aureus*-2 strain, 1×MIC, 1/10×MIC and 1/100×MIC of meropenem and ciprofloxacin prevented biofilm formation at 24.42%, 10% and 5% and 60.51%, 31.34%, 1%, respectively. Phage SA-19 inhibited biofilm formation by 64.83%. When our tests were evaluated, meropenem and phage SA-19 combinations remarkably increased the biofilm formation inhibition effect at all concentrations.

Akturk et al. showed synergistic effects of meropenem and phage combination against *P. aeruginosa*–*S. aureus* mix biofilm [24]. Kumaran et al. showed a synergistic effect of the *S. aureus* phage and antibiotic combinations, which inhibited cell-wall synthesis on *S. aureus* biofilms [28]. Besides, Dickey et al. showed the synergistic effects of various antibiotics and PYO phage combinations against *S. aureus* biofilms [29]. It is essential to discover phage-antibiotic combinations for antimicrobial-resistant bacteria. Phage-antibiotic combinations can prevent the antibiotic resistance, enhance the effects of antibiotics, decrease the side effects and the dose of antibiotics and can be used as adjunctive agents to treat infectious diseases.

On the other hand, *S. aureus*-1 mature biofilm could be eradicated at 44.67% at 5×MIC meropenem in combination with SA-19. Phage SA-19 could reduce %36.54 and %55 of the *S. aureus*-1 and *S. aureus*-2 biofilm mass. Besides combination treatment of phage and antibiotics could not stimulate the antimicrobial effect against *S. aureus*-2. This finding aligns with studies that investigated the efficacy of phages PEV20, NP1/NP3 and PB-1 phages against mature biofilms. (30, 31, 11).

## 4. CONCLUSION

In conclusion, the isolated SA-19 phage can be use as a promising agent. Besides further studies should be conduct for its in vivo efficacy of SA-19 phage.

## 5. MATERIALS AND METHODS

### 5.1. Bacterial isolates

The clinical *S. aureus* 3019 strain was used as a bacterial host strain. A total of 75 clinical strains (25 *S. aureus* strains, 25 *P. aeruginosa* strains, and 25 *E. coli* strains) were used to determine the antimicrobial susceptibility of newly isolated phage. All clinical strains were obtained from Istanbul University, Faculty of Pharmacy, Pharmaceutical Microbiology Laboratory, Turkey, and identification of them has been done by API20 E, API20 NE and API STAPH (Biomérieux, France) identification kits. Luria Bertani (Difco) (LB) broth was used to culture the bacteria at 37◦C overnight. LB agar and LB-soft agar (Merck) (0.7% w/v) were used for phage isolation studies.

### 5.2. Phage isolation and enrichment

*S. aureus* 3019 was used as host cell. The spot test method was carried out for phage isolation studies. 10 mL of seawater sample was centrifuged for 10 min 5000 × g for removing cell debris and then the supernatant was filtered using 0.22-µm membrane filter. The 10 µL of filtrate was spotted onto tested *S. aureus* strains to observe the possible presence of phage. After overnight incubation, the presence of lytic zones was selected for the further studies. Additionally for enrichment of phage, 10 mL of the phage filtrate and 100 µL of bacterial host were mixed and incubated at 37◦C. The enrichment phage filtrate was centrifuged and then filtered by 0.22 µm pore size filter.

### 5.3. Purification of phage

The purification of isolated phage was performed using the double-layer agar method described in Yuan et al [32]. Firstly, serial dilutions of lysate were conducted in Saline Magnesium (SM) buffer. Then 200 µL of dilution and 200 µL of bacterial host cell were mixed in soft LB agar and poured over the LB agar plates. After overnight incubation, single plaques were taken from the agar plates. Then, the plaques were mixed with LB broth and its bacterial host. After incubation, phage filtrate was centrifuged for 10 min 5000 × g and then the supernatant of this filtrate was filtered using a 0.22-µm pore size filter (Sartorious). Plaque

picking was performed 5-6 times to purify the phage suspension. The double layer agar method was performed for the phage titer (pfu/mL).

### 5.4. The susceptibility of phage

The phage susceptibility ($2\times10^9$ pfu/ml) performed against 75 clinical bacterial isolates by spot test method as described above. After incubation for 24 hours at 37 °C, the absence or presence of lytic zones was determined [33].

### 5.5. pH/thermal stability of phage

For the pH stability, phage ($10^{10}$ pfu/mL) was mixed with SM buffer solution (Himedia) at pH 2-7-11 values and incubated at 4◦C for 48 hours. Besides, phage was incubated at different temperatures for 120 min (4◦C, 25◦C, 37◦C, 45◦C, 65◦C and 90◦C) to determine thermal stability, and phage titer was calculated. [34].

### 5.6. One-step growth curve

A one-step growth curve assay was performed to determine biological characteristics of isolated phage such as burst time and latent period. The host cell was first transferred to 5 mL LB media and incubated at 37◦C overnight. 0.1 mL of phage ($10^8$ pfu/mL) was transferred to 0.9 mL bacterial culture ($1\times10^8$ cfu/mL) and incubated at 37◦C for 10 min. Then, the culture (host cell and phage) was centrifuged and the pellet was mixed with LB broth to remove non-adsorbed phage. 0.1 mL samples were taken at every 10-minute for 90 minutes and phage titre was determined [18,35].

### 5.7. Biofilm formation

Ten clinical strains of *S. aureus* were determined for their ability to form biofilm. *S aureus* strains were cultured in 5 ml tryptic soy broth (Difco) (TSB) supplemented with 1% glucose and incubated at 37◦C for 24 hours. These strains were diluted in TSB media to adjust approximately $1x10^7$ cfu/mL bacterial concentration. 200 µl of bacterial suspensions were transferred to the 96 wells plates and then incubated for 24 hours at 37◦C. The wells were washed with phosphate buffer saline (PBS) to removed bacteria and dried. Biofilms in each well were fixed with 99% methanol for 15 minutes and stained for 5 minutes with 1% crystal violet. The crystal violet was solubilized for 30 min by 95% ethanol for 30 min and quantified at OD595 nm (BioTek) [36].

### 5.8. Minimum inhibitory concentration (MIC)

MICs of meropenem and ciprofloxacin were studied using broth microdilution method against two strong biofilm producer bacteria. Serial two-fold dilutions of antibiotics (meropenem and ciprofloxacin) (Astra Zeneca and Bayer) were made in cation-adjusted Mueller Hinton Broth (CAMHB). The turbidity of bacteria was adjusted to $1\times10^8$ cfu/mL concentration. The bacterial inoculum was diluted in CAMHB for approximately $5\times10^5$ cfu/mL. In 96 wells plates, 50 µL of antibiotics and 50 µL *S. aureus* strains were mixed and then incubated for 18-24 hours at 37°C [37]. MIC is the concentration of antibiotics which inhibited growth of tested bacteria in each wells.

### 5.9. Inhibition of biofilm formation

Two clinical *S. aureus* formed strong biofilms and were tested. The strains were grown in TSB media with meropenem or ciprofloxacin (1×MIC, 1/10×MIC and 1/100×MIC), phages ($10^{10}$ pfu/mL) and a combination of them in 96 wells plate at 37◦C for 24 hours. Then, biofilms in each well were washed with PBS and measured at OD595 nm [36].

### 5.10. Quantification of biofilm biomass

For this experiment, two clinical strains were grown in TSB for 24 hours in 96 wells plate. After 24 hours, biofilms were swilled using PBS for 3 times. Then, biofilms treated with meropenem or ciprofloxacin at 5×MIC, 2×MIC, 1×MIC and 1/100×MIC and phages ($10^{10}$ pfu/mL) alone or in combination with phage SA-19 and antimicrobials and incubated for 24 hours. After 24h incubation, biofilms were washed with PBS and stained with crystal violet. Biofilm mass was measured at OD595 nm by microplate reader [11].

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**Conflict of interest statement:** The authors declared no conflict of interest

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