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RESEARCH ARTICLE

Four Temperate Bacteriophages from Methicillin-resistant *Staphylococcus aureus* Show Broad Bactericidal and Biofilm Removal Activities [1]

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Abstract

The emergence of multi-drug resistance among many bacteria including zoonotic pathogens in the food chain poses a growing public health threat to humans, animals, and the environment worldwide. The inefficiency of current antibiotics to control these pathogens necessitated the development of alternative approaches, such as phage therapy, for the prevention and treatment of human and animal infections, food safety, and wastewater treatment. In this study, four temperate bacteriophages, designated as Trsa205, Trsa207, Trsa220, and Trsa222 were isolated by mitomycin C induction from methicillin-resistant *Staphylococcus aureus* (MRSA) strains. The phages were characterized based on their electron microscope morphology, burst size, host range, and biofilm removal potential. Based on their morphology, all four phages with isometric heads and long non-contractile tails belong to *Siphoviridae* family. The one-step growth curves of phages revealed that Trsa205 and Trsa207 have latent periods of about 20 min that results in a burst size of 30 and 45 virions/host cell, respectively, while Trsa220 and Trsa222 showed 25 min of latent period and produced 20 virus particles/cell. The agar-spot assay was used for phage host range determination, and biofilm removal activities were measured spectrophotometrically after crystal violet staining. It was found that at least two-thirds of 56 *S. aureus* strains (66%) could be lysed by phages when used in combination, and 20-38% by one of the phages. The four phages in combination were able to remove the *S. aureus* biofilms by 65%. Our results indicated that the newly identified bacteriophages have the potential to be used in phage therapy against multi-drug resistant *S. aureus* including MRSA and removal of biofilms.

Keywords: *Bacteriophage, Siphoviridae, Staphylococcus, Biofilm, Host range*

Geniş Bakterisidal ve Biyofilm Giderme Etkisi Gösteren Metisiline Dirençli *Staphylococcus aureus* Kaynaklı Dört Yeni Ilıman Bakteriyofajın Tanımlanması

Öz

Çoklu antibiyotik direncinin besin zincirindeki zoonotik patojenler dahil tüm dünyada birçok bakteride ortaya çıkması insanlar, hayvanlar ve çevre için artan bir halk sağlığı tehdidi oluşturmaktadır. Bu patojenleri kontrol etmek için mevcut antibiyotiklerin yetersizliği, insan ve hayvan enfeksiyonlarının tedavisi, gıda güvenliği ve atık su arıtımı için faj terapisi gibi alternatif yaklaşımların geliştirilmesini gerektirmiştir. Bu çalışmada, metisiline dirençli *Staphylococcus aureus* (MRSA) suşlarından mitomisin C indüksiyonu ile Trsa205, Trsa207, Trsa220 ve Trsa222 olarak adlandırılan dört ılıman bakteriyofaj izole edilerek tanımlanmıştır. Fajlar, elektron mikroskop morfolojisi, konak hücre başına oluşan faj sayısı, konak genişliği ve biyofilm giderme potansiyellerine göre karakterize edildi. Morfolojilerine göre, izometrik başlı ve uzun kasılmayan kuyruklu dört fajın *Siphoviridae* ailesine ait oldukları belirlendi. Fajların tek aşamalı büyüme eğrilerine göre, Trsa205 ve Trsa207'nin 20 dakikalık latent periyotlara sahip olduğu ve sırasıyla hücre başına 30 ve 45 faj partikülü oluşturduğu, Trsa220 ve Trsa222'nin ise 25 dakikalık latent periyotu takiben hücre başına 20 virüs partikülü oluşturduğu saptandı. Faj konak genişliği tayini için agar-damlatma yöntemi kullanıldı ve kristal viyole boyamadan sonra biyofilm giderme aktiviteleri spektrofotometrik olarak ölçüldü. Fajların tek başlarına kullanıldıklarında 56 *S. aureus* suşunun %20-38'unu, dört faj birlikte kullanıldığında ise tüm suşların üçte ikisini (%66) enfekte ederek lize ettiği saptandı. Dört fajın kombine kullanıldığında *S. aureus* biyofilmini %65 oranında giderebildiği gösterildi. Sonuçlarımız, yeni tanımlanan bakteriyofajların, MRSA dahil çoklu ilaca dirençli *S. aureus* suşlarına karşı ve biyofilm giderme amacıyla faj tedavisinde kullanılma potansiyeline sahip olduğunu göstermiştir.

Anahtar sözcükler: *Bakteriyofaj, Siphoviridae, Staphylococcus, Biyofilm, Konak spektrumu*

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Introduction

Antimicrobial resistance (AR) in bacteria is a growing problem worldwide today and will remain a major threat to humans and animals as well as sustainable economic growth. In a recent report by the World Health Organization (WHO), 12 bacterial species were listed as global priority pathogens (GPP) including *Staphylococcus aureus,* emphasizing the immediate need for new antimicrobial alternatives [1]. This report discusses the current antibacterial therapy options, such as phage and phage endolysin therapy against infections caused by methicillin-resistant *S. aureus* (MRSA). *Staphylococcus* species are one of the most common bacteria of healthcare-associated infections and mainly responsible for infections related to catheters, implants, and medical devices [2]. Many of the healthcare-associated infections, particularly those that are caused by *S. aureus* including MRSA, are mainly transmitted from person to person through contaminated medical devices or via direct contact with colonized healthcare workers or patients. *S. aureus* as a member of ESKAPE *(Enterococcus, S. aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Escherichia coli)* organisms result in prolonged hospital stay, hospital-associated mortality, and substantial economic burden [3]. Contamination and infection of *S. aureus* is also a major problem in the agri-food sector, particularly bovine mastitis, which adversely affects animal health and the quality of the milk [4].

Staphylococcal strains participating in the biofilm structure exhibit more resistance to conventional antibacterial agents than their free-living counterparts [2]. Due to the intensive use and the emergence of antibiotic resistance globally, phage therapy has become one of the promising alternatives to treat these persistent infections [5]. The phage therapy has been performed by simply adding naturally existing bacteriophages at the infection site to kill the pathogenic bacteria. Moreover, the recent advancement of biotechnology expanded the potential applications of phage therapy and phage derived lytic proteins, such as endolysins [6]. The use of the lytic phages in the reduction of pathogenic bacteria and their biofilms either alone or in combination have been reviewed elsewhere [7,8]. However, compared to the estimated global phage population size, which is the most numerous and diverse viruses on the planet, the reported phages are very limited in numbers [9]. Phages can readily be isolated from any environmental samples that can support bacterial growth such as aquatic habitats [9], soil [10], wastewater [11] or induction of phages from lysogenized host strains [12]. Therefore, there is still potential for novel phages to be explored. The induction of prophages from various bacterial strains including *S. aureus* by treatment with mitomycin C has previously been described [13,14]. In this study, we aimed to isolate and characterize bacteriophages of lysogenic origin infecting *S. aureus* with broad host range and biofilm removal potential.

Material and Methods

Bacterial Strains, Media and Culture Conditions

A total of 56 *S. aureus* including methicillin-resistant strains isolated previously from various samples and maintained at our culture collection were included in this study. Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) (Lab M. UK) were used for culturing of staphylococci at 37°C if indicated otherwise.

Induction and Purification of Phages

Prophages were isolated by mitomycin C (Sigma-Aldrich, USA) induction as described elsewhere [14]. Briefly, overnight cultures of staphylococci were subcultured in fresh media containing 10 mM $CaCl_2$ and grown at OD_{600nm} = 0.5). The mid-log cultures were induced with 0.5 µg/mL mitomycin C for 4 to 6 h or until the clearance of the turbid cultures was observed. The induced cultures were centrifuged at 10.000 xg for 20 min to remove cell debris and unlysed cells. The supernatant containing the page particles filtered through a 0.2 µm filter (Sartorius Stedim Biotech GmbH, Germany) and maintained at 4°C until use. Five microliters of phage lysate were dropped onto lawns of the staphylococcal host strains. Observation of clear zone or single phage plaques on the agar surface indicated the presence of lysogenic phages. Several single phage plaques were picked from the agar plates and transferred into a microcentrifuge tube containing 100 µL SM buffer (50 mM Tris-HCl, pH 7.5; 8 mM $MgSO_4.H_2O$; 100 mM NaCl) for further enrichment using plaque assay with some modification as described elsewhere [15]. Briefly, 100 µL of phage lysate was diluted 10-fold and mixed with 100 µL of logarithmic phase *S. aureus* cultures in the presence of 1 mM $CaCl_2$ in a microcentrifuge tube and incubated for 30 min to facilitate phage attachment to their hosts. A 4 mL of soft agar (0.6%) at 49°C was added to the phage infected cells, mixed and poured over agar plates, and incubated for 12-24 h to allow phage plaques to form. The phage particles were collected from the soft agar by scraping in a centrifuge tube containing 4 mL of SM buffer. Following the centrifugation, the supernatant was filtered, treated with 1 µg/mL DNase at room temperature, and phage particles were precipitated with 10% (w/v) polyethylene glycol 8000 in 0.5 M NaCl solution at 4°C overnight [16]. The phage particles were collected by centrifugation at 10.000 x g for 15 min and resuspended in SM buffer, filtered and stored at 4°C for short term and -80°C for long term use.

Transmission Electron Microscopy

Purified and concentrated phage particles were stained with 2% uranyl acetate (pH 4.0), and examined for their morphologies under Transmission Electron Microscopy (TEM) as described elsewhere with some modifications [17].

Ten microliters of phage lysate containing 10^{10} plaque-forming unit per milliliter (PFU/mL) was added to the

formvar carbon-coated grid (Electron Microscopy Science, USA) and allowed to absorb for 4 min. The girds were then stained for 3 min by adding 10 µL of 2% uranyl acetate. Excess stain was removed and the girds were air dried for overnight. The phages were viewed at 75 kV using Jeol JEM-1010 Transmission Electron Microscope.

Host Range

The host ranges of the phages were determined by the agar- spot assay using various staphylococcal strains as previously described [17]. Briefly, 100 µL exponential phase cultures were mixed with 4 mL of 0.6% soft agar at 49°C and poured on TSA plates. After the agar solidified for about 10 min, 5 µL from each phage lysate was spotted on the agar surface and incubated for 12-24 h. The clear or turbid phage plaques were examined visually or under a stereomicroscope.

One-step Growth Curve

The one-step growth curve of each phage was determined as described by Li and Zhang [4] with slight modification. The phage lysate and early-mid culture of S. aureus TRSA 201 were mixed at a multiplicity of infection (MOI) of 0.01. Following the phage adsorption at 37°C for 30 min, infected cells were collected by centrifugation at 10,000 xg for 1 min. The infected cells were resuspended in 3 mL fresh pre-warmed TSB medium and incubated at 37°C with shaking at 160 rpm to allow the life cycles of the phages. A 100 µL of the samples were collected at every 10 min intervals (up to 2 h), diluted, and plated by a double agar overlay method and incubated for 12 to 24 h to allow the formation of single phage plaques. The latent-period (the interval between phage adsorption by the host and the beginning of lysis) and the burst size (the ratio of the final average number of phage particles liberated) of each phage were by enumerating the plaque-forming units per milliliter (PFU/mL).

Biofilm Removal Assay

A microtiter plate biofilm assay was carried out essentially by the procedure described by Soni and Nannapaneni [18]. Briefly, an overnight culture of *S. aureus* TRSA 201, a common host strain for all four phages, was adjusted to 10^6 CFU/mL in fresh TSB broth containing 0.25% w/v glucose and 200 µL was transferred into 96 wells flat-bottomed microtiter plate for biofilm formation at 37°C without agitation for 48 h. The media from the wells were removed and biofilm was rinsed two times with sterile phosphate-buffered saline (PBS) and treated with 200 µL phage particles (10^{10} PFU/mL) in SM buffer for 12 h by incubating at 37°C. The contents of the wells were removed and the biofilm was washed with PBS as described. The biofilm was stained with 200 µL of 1% (w/v) crystal violet solution for 5 min, and unbound stain was washed away. The crystal violet was solubilized and removed from the biofilm with 200 µL ethanol-acetone (80:20). The remaining biofilm after phage treatment was quantified by measuring the OD of the crystal violet in ethanol-acetone solution at 595 nm, and the percentage reduction in biofilm biomass was estimated as compared to untreated control biofilm. For each phage and combination of phages, four biofilm wells were used and experiments repeated at least three times.

Statistical Analysis

Analysis of Variance (ANVO) and Student's t-test of the SPSS software version 22 were used for the analysis of the data. P-values of <0.05 were considered statistically significant.

Results

Characterization of Phages

Four phages, Trsa205, Trsa207, Trsa220, and Trsa222 were confirmed for their lytic functions by spot and plaque assay methods *(Fig. 1-A,B)* on the lawn of *S. aureus* TRSA 201 host strain. TEM analysis of phages revealed that all four phages morphologically were similar with isometric heads measuring about 40 to 62 nm and long non-contractile tails of approximately 90 to 210 nm in length *(Table 1)*. Based on the morphology, they belong to

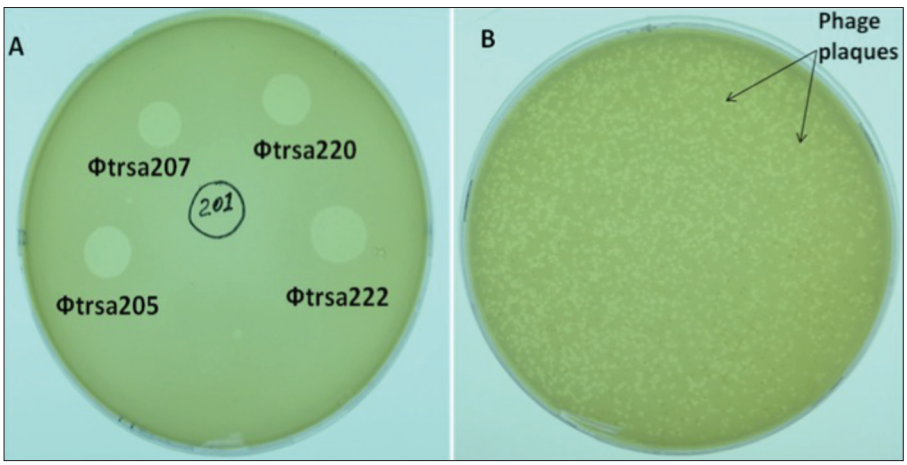

Fig 1. Lytic activity of phages (Trsa205, Trsa207, Trsa220, and Trsa222) against *S. aureus* TRSA 201 indicator strain using the agar-spot (A), and single plaque assay method for Trsa205 (B)

Table 1. Morphological characteristics of phages

Phage	Capsids Diameter (nm)	Tail Width (nm)	Tail Length (nm)
Trsa205	62.3±1.3	14.2±0.3	143.4±1.3
Trsa207	46.4±2.0	10.7±2.1	165.0±2.8
Trsa220	48.6±1.0	10.3±0.8	128.3±0.3
Trsa222	41.8±0.8	12.5±1.3	96.2±2.0

Trsa207 were about 20 min which is followed by a raise period of 30 min that results in a burst size of 45 and 30 PFU/infected cells, respectively *(Fig. 3-A)*. Phages Trsa220 and Trsa222 had a latent period of about 25 min and a raise period of 30 min that resulted in a burst size of 20 PFU/infected cell *(Fig. 3-B)*.

Antibacterial and Anti-biofilm Potential of Phages

Spot assay on a lawn of host bacteria was applied for the determination of the lytic spectrum of the phages. A total of 56 *S. aureus* isolates were infected by each phage. The phage Trsa205 showed lytic infection against 21 (37.5%) while Trsa207, Trsa220, and Trsa222 were effective against 15 (26.7%), 12 (21.4%), 11 (19.6%) of the strains tested, respectively. The combination of four phages lysed 37 (66%) of the strains *(Table 2)*.

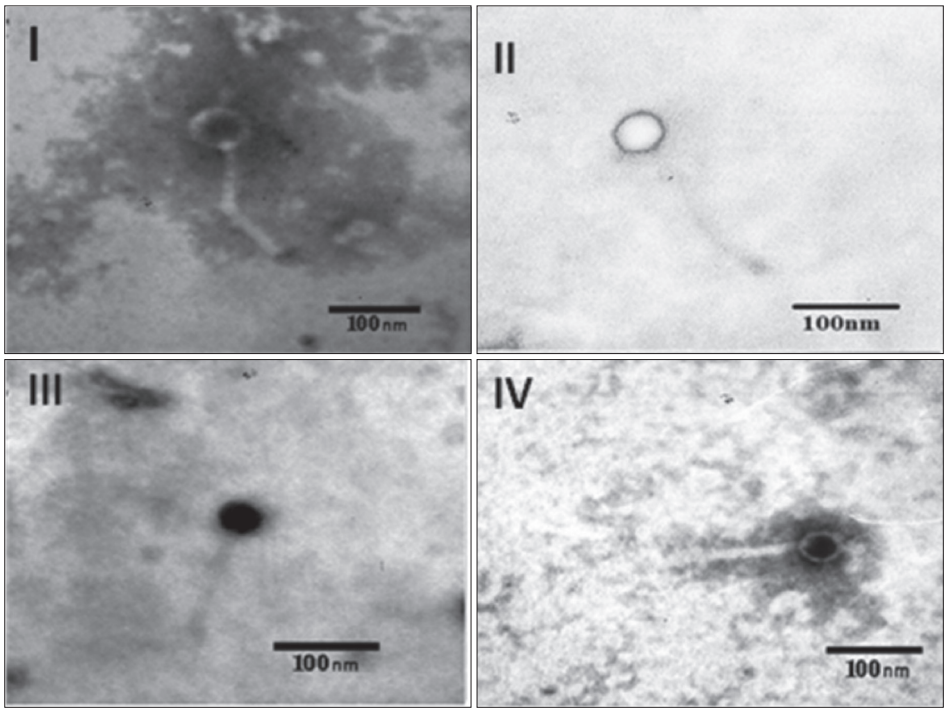

Fig 2. Transmission Electron Microscopic images of phages: I, Trsa205; II, Trsa207; III, Trsa220; IV, Trsa222; the scale bar denotes 100 nm

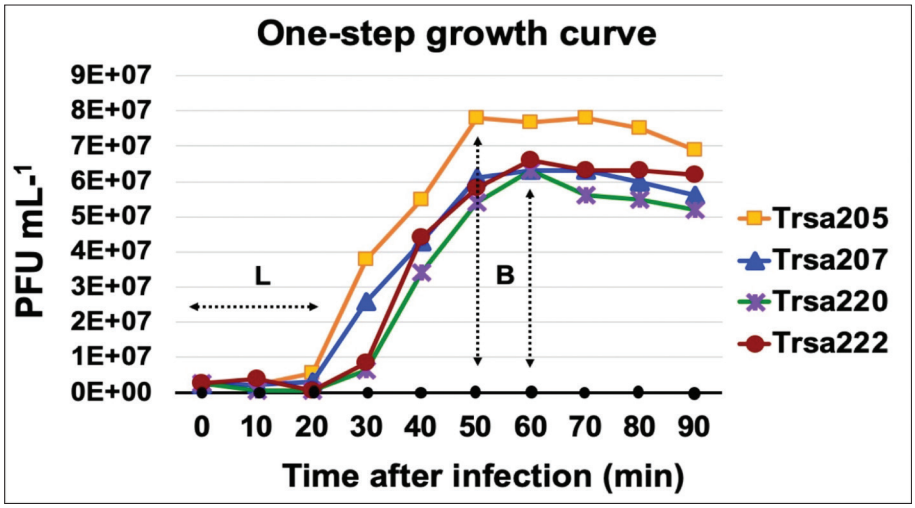

Fig 3. One-step growth curves of phages; The curves shown here is the PFU/infected cell at several time points over 90 min. L, latent periods; B, burst size

the *Siphoviridae* phage family, which is characterized by phages with a non-contractile tail *(Fig. 2)*.

One-step Growth Curve

The results obtained from the one-step growth curve experiments reviled that the latent periods for Trsa205 and

Table 2. *Host range of four phages and phage cocktail against different S. aureus strains*

No	Strains	Origin	Phages				
			Trsa 205	Trsa 207	Trsa 220	Trsa 222	Cocktail
1	*S. aureus* TRSA 1	Pus	-	-	-	-	-
2	*S. aureus* TRSA 2	Wound	+	+	+	+	+
3	*S. aureus* TRSA 3	Blood	+	-	-	-	+
4	*S. aureus* TRSA 4	Tracheal aspirate	-	-	-	-	-
5	*S. aureus* TRSA 5	Tracheal aspirate	-	-	-	-	-
6	*S. aureus* TRSA 6	Tracheal aspirate	-	-	-	-	-
7	*S. aureus* TRSA 8	Tracheal aspirate	+	+	-	-	+
8	*S. aureus* TRSA 9	Ear	-	-	+	-	+
9	*S. aureus* TRSA 10	Wound	+	-	-	-	+
10	*S. aureus* TRSA 11	Ear	-	-	-	-	-
11	*S. aureus* TRSA 12	Wound	-	-	-	-	-
12	*S. aureus* TRSA 13	Tracheal aspirate	-	-	+	-	+
13	*S. aureus* TRSA 14	Wound	-	-	-	-	-
14	*S. aureus* TRSA 15	Tracheal aspirate	+	-	-	+	+
15	*S. aureus* TRSA 16	Wound	-	-	-	-	-
16	*S. aureus* TRSA 17	Sputum	-	-	-	-	-
17	*S. aureus* TRSA 18	Urine	-	-	-	-	-
18	*S. aureus* TRSA 19	Tracheal aspirate	+	+			+
19	*S. aureus* TRSA 20	Wound	-	-	+	-	+
20	*S. aureus* TRSA 22	Wound	+	-	-	-	+
21	*S. aureus* TRSA 23	Wound	-	+	-	-	+
22	*S. aureus* TRSA 24	Tracheal aspirate	+	-	-	-	+
23	*S. aureus* TRSA 25	Tracheal aspirate	-	+	-	-	+
24	*S. aureus* TRSA 26	Wound	+	-	-	-	+
25	*S. aureus* TRSA 27	Nose swab	-	-	-	-	-
26	*S. aureus* TRSA 28	Wound	-	-	+	-	+
27	*S. aureus* TRSA 29	Wound	-	-	-	-	-
28	*S. aureus* TRSA 30	Pus	-	-	-	+	+
29	*S. aureus* TRSA 31	Tracheal aspirate	-	-	-	-	-
30	*S. aureus* TRSA 32	Wound	+	+	+	-	+
31	*S. aureus* TRSA 33	Tracheal aspirate	-	-	-	-	-
32	*S. aureus* TRSA 35	Wound	+	+	-	-	+
33	*S. aureus* TRSA 36	Blood	+	+	-	-	+
34	*S. aureus* TRSA 37	Tissue	-	-	-	+	+
35	*S. aureus* TRSA 38	Blood	-	+	-	-	+
36	*S. aureus* TRSA 39	Wound	-	-	+		+
37	*S. aureus* TRSA 40	Wound	-	-	-	-	-
38	*S. aureus* TRSA 41	Tracheal aspirate	+	-	-	-	+
39	*S. aureus* TRSA 42	Wound	-	-	-	-	-
40	*S. aureus* TRSA 43	Pus	-	-	+	-	+
41	*S. aureus* TRSA 44	Blood	-	-	-	-	-
42	*S. aureus* TRSA 196	Wound	+	+	+	+	+
43	*S. aureus* TRSA 197	Wound	-	-	-	-	-

+ clear lysis, - no lysis

Table 2. *Host range of four phages and phage cocktail against different S. aureus strains (continued)*

No	Strains	Origin	Phages				
			Trsa 205	Trsa 207	Trsa 220	Trsa 222	Cocktail
44	*S. aureus* TRSA 198	Wound	+	-	-	-	+
45	*S. aureus* TRSA 199	Tracheal aspirate	-	-	-	-	-
46	*S. aureus* TRSA 200	Tracheal aspirate	+	+	-	-	+
47	*S. aureus* TRSA 201	Sputum	+	+	+	+	+
48	*S. aureus* TRSA 202	Pus	+	-	+	-	+
49	*S. aureus* TRSA 203	Wound	-	+	-	-	+
50	*S. aureus* TRSA 204	Tracheal aspirate	+	-	-	-	+
51	*S. aureus* TRSA 205	Wound	-	-	+	-	+
52	*S. aureus* TRSA 207	Catheter	+	-	-	+	+
53	*S. aureus* TRSA 216	Blood	+	-	-	+	+
54	*S. aureus* TRSA 220	Blood	-	+	-	-	+
55	*S. aureus* TRSA 221	Blodd	-	-	-	+	+
56	*S. aureus* TRSA 222	Tracheal aspirate	-	-	-	-	-
Total number of sensitive (%) strains			**21 (37.5)**	**15 (26.7)**	**12 (21.4)**	**11 (19.6)**	**37 (66.0)**

+ clear lysis, - no lysis

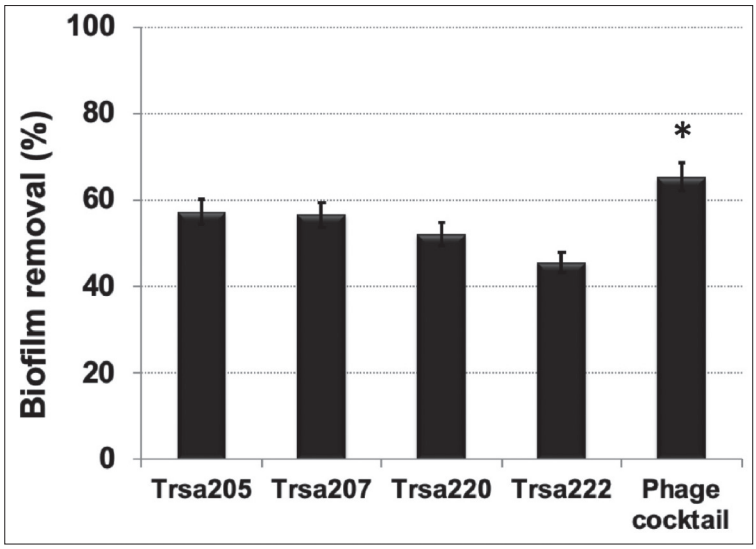

Fig 4. Biofilm removal capacity of phages as determined by 96-well microtiter plate assay. Error bars represent the SD of three independent experiments. Significant differences by two-tailed t-test (P<0.05) compared with control are marked by an asterisk (*)

To assess the biofilms removal potential of the four phages *in vitro,* 48 hour-old *S. aureus* TRSA 201 biofilms were established in 96-well polystyrene microtiter plates. Following the treatment of established biofilms with each of the four phages and phage cocktail for 12 h, remaining biofilm mass was stained with crystal violet, and the remaining biofilm was estimated by spectrophotometric measurement of the solubilized biofilm-bound crystal violet concentration. The data demonstrated that phage treatment reduced the amount of biofilm significantly relative to the control when they were applied as a cocktail *(Fig. 4)*. The amount of *S. aureus* TRSA 201 biofilm was removed for about 65% by the phage cocktail and 57%, 56%, 52%, and 45% by Trsa205, Trsa207, Trsa220, and Trsa222, respectively.

Discussion

Among coagulase-positive staphylococci *S. aureus* is one of the most commonly isolated pathogens responsible for local and systemic infections in both humans and animals. They cause healthcare-, community- and live-stock-associated infections via transferring from humans to animals and vice versa resulting in outbreaks and major economic burden [19,20].

With the increasing problem of antibiotic resistance in foodborne pathogens, alternative therapeutic approaches, such as phage and phage endolysin therapies, have attracted the interest of scientists for the treatment of these pathogenic bacteria [21,22].

Therefore, in this study, we aimed to identify and characterize new temperate phages and assess their potential to lyse *S. aureus* strains and reduce staphylococcal biofilms. Using the induction technique, four functional temperate phages were isolated and confirmed by the agar-spot and plaque assays *(Fig. 1-A,B)* since clear zones can also be produced by bacteriocins [23].

The transmission electron microscopy analysis revealed that all four phages have long tails with isometric and icosahedral heads *(Fig. 2)*. The tails of Trsa205, Trsa207, Trsa220, and Trsa222 were measured to be 143.4±1.3, 165.0 ±2.8, 128.3±0.3, and 96.2±2.0 nm, respectively *(Table 1)*. The icosahedral heads of phages were very similar in size. According to their morphological characteristics, which is one of the most frequently used methods for the classification of bacteriophages, all four phages belong to *the Siphoviridae* family in the order of *Caudovirales (Fig. 2)*. Furthermore, as they have long tails, they belong to type B phages in Bradley's classification [23]. More than 95% of the reported phages so far were assigned to the family of *Siphoviridae* in the order of *Caudovirales* and about 60% of these phages have long and flexible tails [24].

The one-step growth curve experiments were performed as the estimation of the burst size and burst period in the life cycle of phages has great importance in phage therapy [8]. The scheduling of host cell lysis by phages is crucially controlled by holin protein and any mutation in this protein can modify the lysis timing or the latent period [25]. The burst size of phage Trsa207 was relatively higher (45 PFU/mL) than those of others, which is the more preferable features of the lytic phages that can be used as an antibacterial agent. The burst size plays a crucial role in phage propagation [26] and this can increase the initial dose of the infective phages several folds in a short period of time [8,27].

Among four phages, phage Trsa205 had a broader host range with lytic activities against 37.5% of the strains tested. While phage Trsa207, Trsa220, and Trsa222, were effective against 27%, 21%, and 20% strains, respectively. The low lytic activity may be associated with lysogenicity, which required induction for the lytic activity or with superinfection immunity by resident prophages [14], as this was also confirmed in the present study, in which, the addition of Trsa205, Trsa207, Trsa220, and Trsa222 did not infect their respective host in which they were originated. The potency against *S. aureus* isolates increased when the combinations of the four phages applied. From a total of 56 staphylococcal strains tested, 37 (66%) strains were sensitive to at least one of the four phages. This highlights the use of phage cocktails that are applicable to broad staphylococcal targets including biofilms. The biofilm removal capacity of the newly identified phages was significant (66%) when used as a cocktail as compared to the individual phages and control *(Fig. 4)*.

In conclusion, due to limited therapeutic options to combat life-threatening infections, the phages of staphylococci could be used for local infections, such as wound infections caused by multidrug-resistant *S. aureus* strains including MRSA. Also, they could be used as a sanitizer for surfaces or topical agents for eliminating *S. aureus* from human and animal skin, where they serve as a reservoir for infections. Due to their inherent capacity to mediate gene transfer via transduction between bacteria, historically the lysogenic phages have not often considered for phage therapy. However, the advanced technologies including sequencing, recombination, and gene editing enable the use of temperate phages for the prevention and treatment of bacterial infections [28]. Therefore, these bacteriophages have the potential to be used not only in phage therapy but also serve as a source for endolysins which could be produced recombinantly and used as anti-staphylococcal and anti-biofilm agents.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

A.O. KILIÇ, M.A. ABDURAHMAN, and I. TOSUN, planned and designed the research. M.A. ABDURAHMAN, I. DURUKAN and M. KHORSHIDTALAB carried out experiments and helped analysis of the results. All authors contributed to writing of the final manuscript.

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