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Characterization of novel *Staphylococcus aureus* lytic phage and defining their combinatorial virulence using the OmniLog® system

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ABSTRACT

Skin and soft tissue infections (SSTI) caused by methicillin resistant *Staphylococcus aureus* (MRSA) are difficult to treat. Bacteriophage (phage) represent a potential alternate treatment for antibiotic resistant bacterial infections. In this study, 7 novel phage with broad lytic activity for *S. aureus* were isolated and identified. Screening of a diverse collection of 170 clinical isolates by efficiency of plating (EOP) assays shows that the novel phage are virulent and effectively prevent growth of 70–91% of MRSA and methicillin sensitive *S. aureus* (MSSA) isolates. Phage K, which was previously identified as having lytic activity on *S. aureus* was tested on the *S. aureus* collection and shown to prevent growth of 82% of the isolates. These novel phage group were examined by electron microscopy, the results of which indicate that the phage belong to the *Myoviridae* family of viruses. The novel phage group requires β-N-acetyl glucosamine (GlcNac) moieties on cell wall teichoic acids for infection. The phage were distinct from, but closely related to, phage K as characterized by restriction endonuclease analysis. Furthermore, growth rate analysis via OmniLog® microplate assay indicates that a combination of phage K, with phage SA0420$^{\Phi}$1, SA0456$^{\Phi}$1 or SA0482$^{\Phi}$1 have a synergistic phage-mediated lytic effect on MRSA and suppress formation of phage resistance. These results indicate that a broad spectrum lytic phage mixture can suppress the emergence of resistant bacterial populations and hence have great potential for combating *S. aureus* wound infections.

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Introduction

The rise in number of multi-drug resistant (MDR) microorganisms is a major threat to human health. Currently, it is estimated that at least 2 million infections are caused by MDR organisms every year in the United States leading to approximately 23,000 deaths.[1,2,3] Methicillin resistant *Staphylococcus aureus* is an MDR organism of great concern in the clinical setting. MRSA is responsible for over 80,000 invasive infections and 11,285 related deaths and is the primary cause of hospital acquired infections.[2] This organism has been identified in several congressional reports as threat to public health and safety.[1] Additionally, the World Health Organization (WHO) has identified MRSA as organisms of international concern.[4] In response to this threat, the US government devised a national strategy to combat the spread of multi-drug resistant organisms and promote the development of alternate therapies.[1,2]

S. aureus are gram positive cocci that can cause skin and soft tissue infections (SSTI), pneumonia, necrotizing fasciitis, and blood stream infections.[5-11] *S. aureus* has developed a mechanism to evade the antimicrobial activity of β-lactam antibiotics by expressing an alternate form of penicillin binding protein (PBP2a) encoded by *mecA*.[12,13] PBP2a has a reduced affinity for β-lactam antibiotics, permitting cell wall synthesis.[14] On the basis of susceptibility to β-lactam antibiotics, *S. aureus* strains are either methicillin sensitive *S. aureus* (MSSA) or methicillin resistant *S. aureus* (MRSA). MRSA infections are divided into either health care associated (HA-MRSA) with infection originating within 4 days of hospitalization, or community acquired (CA-MRSA) infection that begins within the

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first day of admission.[15] The USA300 pulse field type is associated with CA-MRSA and is responsible for up to 70% of *S. aureus* SSTIs in the US, this makes USA300 a prime target for broad spectrum phages.[16,17]

A potential treatment for MDR infections involves the use of phage. Lytic phage are viruses that infect bacteria, multiply and subsequently lyse the host cell.[18,19] The successful therapeutic use of phage depends on the capacity to infect a broad range of pathogenic strains and a low frequency of resistance. Like bacterial resistance to antibiotic treatment, bacterial resistance to phage can develop. However, this can be reduced by utilizing a mixture of virulent phages that target diverse cell surface receptors.[20,21] A candidate for combating *S. aureus* MDR infections is the polyvalent bacteriophage K, a well characterized member of the *Myoviridae* virus family.[22-25] Phage K requires cell wall teichoic acid (WTA) moieties for efficient absorption into the host cell.[26] WTAs are glycopolymers involved in cell wall formation and resistance to cationic antimicrobial agents.[27-31] In *S. aureus*, most strains WTAs contain polyribitol phosphate (RboP) substituted by D-alanine and N-acetylglucosamine (GlcNAc) linked via α and β glycosidic bonds.[32-34] In *S. aureus*, biosinthesis of WTAs commences by linking a GlcNAc moiety to undecaprenol lipids a reaction mediated by the N-acetyl glucosamine transferase, *tarO*. The nascent glucopolymer is modified by addition of α and β linked GlcNAc moieties a reaction catalized by *tarM* and *tarS* respectively.[29,35] Upon completion, WTAs are exported to the extracellular environment where it is linked to peptidoglycan (PG).

In search for alternate treatments for MRSA infections, 7 lytic phage were isolated from sewage samples. These phage are polyvalent in nature individually affecting 70% to 91% of 170 *S. aureus* clinical isolates. Additionally, we demonstrate that phage K was virulent in 82% of the isolates. The combination of phage K and the novel phage have a synergistic effect on bacterial growth inhibition. These results indicate that a phage mixture can enhance growth inhibition effects of phage in *S. aureus* and reduce the formation of phage-resistance. Electron microscopy studies show that all novel phage are members of the *Myoviridae* virus family. Also, the β-N-acetyl glucosamine covalent modification of teichoic acids was identified as required for lytic activity of phage SA0414$^{\Phi}$1, SA0414$^{\Phi}$2, SA0420$^{\Phi}$1, SA0456$^{\Phi}$1, SA0470$^{\Phi}$1, SA0482$^{\Phi}$1 and SA11987$^{\Phi}$1, suggesting teichoic acids are the main receptor.

Materials and methods

Bacterial strains and growth methods

The *S. aureus* clinical isolate collection was available at NMRC-Silver Spring. MRSA strains were isolated from hospital staff, outpatients, and inpatients over a 5-year period. Strains with the prefix NSC or NSI were collected at the Naval Medical Research Unit-6 (NAMRU-6) in Lima and Iquitos, Peru, respectively. Community acquired *S. aureus* isolates were collected from the community hospital at Fort Benning, GA. Strains were grown at 37°C in Tryptic soy broth (TSB). Solid media contained 1.5% (wt/vol) bacteriological agar (BD).

Bacteriophage isolation

Phage K samples were purchased from the American Type Culture Collection (ATCC19685-B1) and propagated in *Staphylococcus hyicus*. The novel phage group were isolated from untreated sewage obtained from the municipal water treatment plant at Frederick, MD. Three 500 ml batches of influent sewage water were collected and fortified with 15 g of TS, 1 mL of each MRSA strain (NSC0414, NSC0420, NSC0456, NSC0470, NSC0482, NSC0409 and NSC0419) and incubated for 24 h at 37°C. Following incubation, 1 ml was centrifuged for 5 min at 8000 g and the the supernatant sterilized by 1 min of centrifugation at 8000 g with a 0.22 μm microfuge filter (Costar® Cat#8160). A total of 10 μl of filtrate were screened for lytic phage via plaque assays using the soft agar overlay technique.[36] Plates were incubated at 37°C for 24 h before scoring for the presence of plaques. Plaques were purified 3 times by removing plugs and overnight elution in SM buffer (50 mM Tris pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$ and 0.01% gelatin) followed by amplification in the host strain. Phage titer was assessed by plating 10-fold serial dilutions and calculating the plaque forming units per ml (PFU).

Host range analysis and phage comparison

The host range of the novel phage group and phage K was examined via an efficiency of plating assay (EOP). . 3.5 μl of a 10-fold dilution series starting at 1×10^8 PFU/ml were spotted on TSA seeded with 800 μl of bacteria at 0.6 OD_{600}.[37] Plaque formation at any dilution is considered an indication of phage virulence. Phage species specificity was tested by spotting 10μl of 1×10^8 PFU/ml on

bacterial lawns of *S. cohnii, S. epidermitis, S. haemoliticus, S. saprophiticus, S. sciurii, S. xylosus* and *S. hyicus*. A clear area was considered indicative of virulence.

Phage purification

The corresponding *S. aureus* host strain was grown to 0.1 OD_{600} at 37°C. Cells were infected with phage at a multiplicity of infection (MOI) of 0.5 and incubated at 37°C until the culture was clear. The lysate was cleared via centrifugation at 10,000 g for 10 min and 360 g (10% w/v) of polyethylene glycol 8000 (PEG) was added to the supernatant and precipitated overnight at 4°C. The solution was centrifuged at 5,000 g for 1 h, the supernatant decanted, and the pellet resuspended in 5 ml of SM buffer. Next, 0.75 g of Cesium chloride per ml of precipitate was added and mixed by inversion. The sample was centrifuged on a 90 Ti rotor at 58,000 g at 4°C for 24 h. The resulting band was retrieved and dialyzed by using a 10,000 Da MWC Slide-A-Lyzer® dialysis cassette (Pierce, cat#66373) in 4 L of dialysis buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl). After 24 h the dialysis buffer was exchanged and dialyzed for 4 h. Phage was collected from the dialysis cassette and titered.

Phage DNA purification

Purified phage (150 μl) was mixed with 6 μl of 0.5 M EDTA pH 8, 10 μl of Proteinase K (20 mg/ml) and 7.5 μl of 10% SDS and incubated for 1 h at 56°C. Once cooled to room temperature, 3 extractions with 150 μl of Tris-EDTA saturated phenol pH 7.0 and 3 extractions with 150 μl of Chloroform: Isoamyl alcohol 24:1. The supernatant was mixed with 10 μl of 3 M Sodium acetate and 1 ml of 100% ice cold ethanol overnight at −20°C. The DNA sample was centrifuged at 10,000 g for 5 min and the pellet washed 3 times with 750 μl of 70% isopropanol. The pellet was air dried for 5 min and resuspended in 50 μl of distilled water. The DNA concentration was determined by reading OD_{260} on a spectrophotometer.

Pulse-field gel electrophoresis (PFGE)

Purified phage DNA (20 μg) was digested with *EcoR1* restriction endonuclease for 3 h at 37°C. The digested samples were loaded into a 1% pulse field agarose gel prepared with 0.5X TBE and resolved for 11 h at 14°C with a pulse rate of 30 V/s and a switch time of 1–6 seconds under constant buffer recirculation. Upon completion, the gel was incubated for 10 min in 0.5X TBE containing 0.1 μg/ml ethidium bromide followed by three, 10 min washes with 0.5X TBE and imaged on a BioRad® gel documentation system.

Electron microscopy

Standard methods of sample preparation were employed for transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Briefly, Cesium chloride purified bacteriophages (1×10^5-5×10^7 total phage) were fixed in 4% paraformaldehyde with 1% glutaraldehyde in 0.1 M Sodium cacodylate buffer for 2 h. After fixation, a portion of each sample was spread onto carbon coated copper grids, washed with water to remove fixative, and negatively stained with 1% Uranyl acetate. The grids were imaged with an FEI Tecnai T12 TEM at 100 kV. The remaining sample was processed for SEM analysis. The fixed samples were washed 3 times with 0.1 M Sodium cacodylate buffer, post fixed for 1 h with 1% Osmium tetroxide buffer, washed, and then immersed in a 0.5% Uranyl acetate solution for 1 h. The samples were subsequently dehydrated through an ethanol series, critically point dried, and coated with gold-palladium. The coated SEM samples were imaged in an FEI Quanta 200 FEG SEM at 5 kV.

Preparation of microtiter plates for OmniLog® assay

Microtiter plates (96 well) were prepared as follows. 90μl of TS broth with 0.1% v/v tetrazolium dye was added to each well. 10μl of 1×10^8 PFU/ml of each phage were added to the first well and diluted 10-fold down to 10 PFU per well. 10μl of 0.4 OD_{600} of bacteria (4×10^6 cells) were added to each well for a final volume of 100μl corresponding to a multiplicity of infection (MOI) range of 2.5 to 2.5×10^{-5}. Media and phage only controls were added. The 96 well plates were incubated in the OmniLog® system (Biolog®) at 37°C for 48 h. Phage mixtures were prepared at equal volumes of phage for a final titer of 1×10^8 PFU/ml.

Results

Isolation of novel S. aureus phage

In an effort to isolate broad spectrum lytic phage against MRSA strains, 7 MRSA clinical isolates were used as hosts to isolate phage from environmental sources, primarily raw sewage; a rich source of environmental phage.[23] Seven plaque-forming phage were

identified in their corresponding *S. aureus* host strains SA0414$^{\Phi}$1, SA0414$^{\Phi}$2, SA0420$^{\Phi}$1, SA0456$^{\Phi}$1, SA0470$^{\Phi}$1, SA0482$^{\Phi}$1, and SA11987$^{\Phi}$1 henceforth referred to as "novel phage group." Phage SA0414$^{\Phi}$1 and SA0414$^{\Phi}$2 are independent phage that were isolated against strain NSC0414 (data not shown).

The S. aureus *phage are polyvalent in nature*

To determine the spectrum of the novel phage group, a set of 170 *S. aureus* clinical isolates was obtained from different geographical regions to enrich strain diversity. This collection includes MSSA and MRSA from both, community and health care settings. The *S. aureus* isolates were screened for lytic activity by efficiency of plating (EOP) assays. The EOP assay determines the relative virulence of each phage against a *S. aureus* strain, thereby allowing side-by-side comparisons of phage virulence.[37] In addition, spotting phages on strain-specific *S. aureus* lawns creates individualized phage typing profiles. The novel phage were virulent against 70% to 91% of the strains tested (Fig. 1A and 1B). Individually, phage SA0414$^{\Phi}$1 and SA0414$^{\Phi}$2 were virulent against 80% and 74% of the isolates, respectively. This establishes a distinction between these independent isolates of strain NSC0414. Phage SA0470$^{\Phi}$1 affected 85%, phage SA0420$^{\Phi}$1 affected 91%, phage SA0456$^{\Phi}$1, SA0482$^{\Phi}$1 and SA11987$^{\Phi}$1

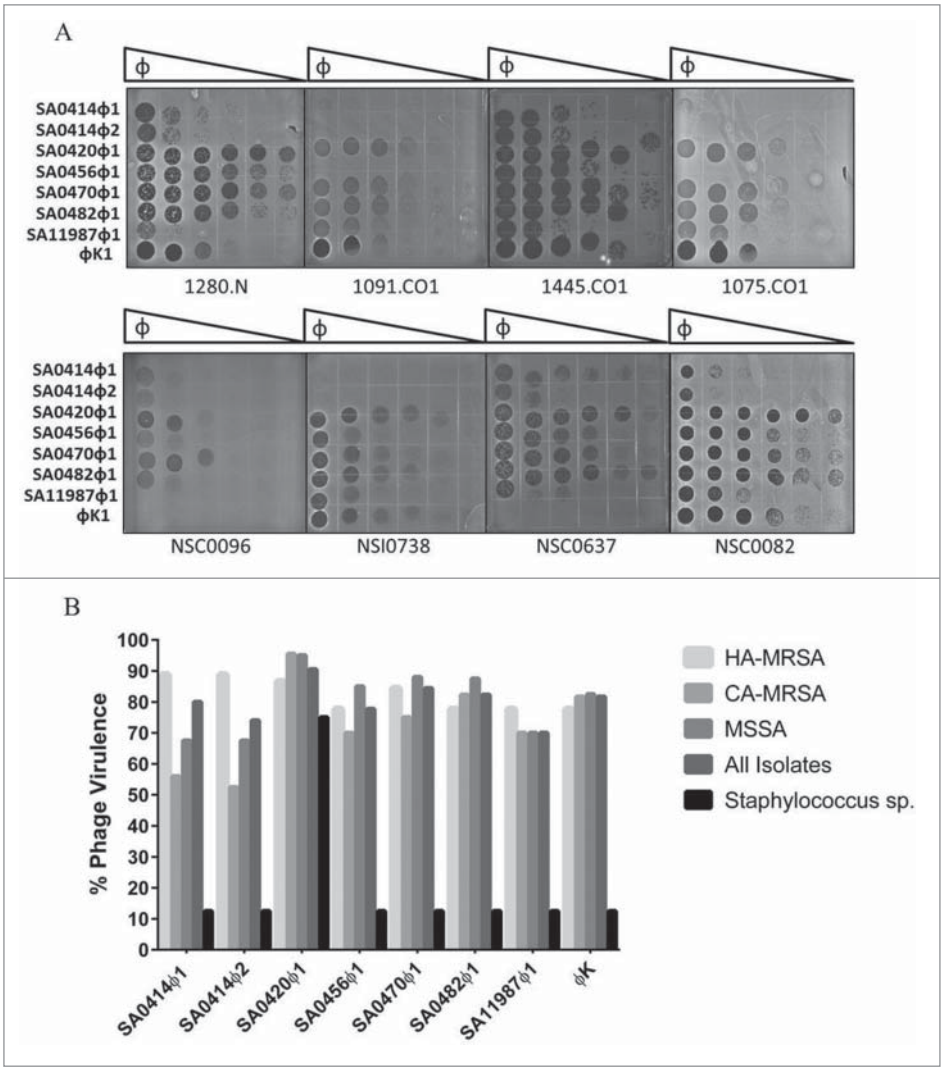

Figure 1. Efficiency of platting (EOP) assay for *S. aureus* clinical isolates. (A) 10-fold serial dilution series of phage SA0414$^{\Phi}$1, SA0414$^{\Phi}$2, SA0420$^{\Phi}$1, SA0456$^{\Phi}$1, SA0470$^{\Phi}$1, SA0482$^{\Phi}$1, SA11987$^{\Phi}$1 and phage K were spotted on several clinical isolates. A total of 1×10^5 phage particles were loaded in the first row. Plates were incubated overnight at 37°C and scored for the ability to inhibit bacterial growth. (B) Virulence spectra of all *S. aureus* phage as a percentage of strains infected. The *Staphylococcus* species used in this study include *S. cohnii, S. epidermidis, S. hyicus, S. haemoliticus, S. saprophyticus, S. sciuri,* and *S. xylosus*.

each affected 78%, 84% and 82% of isolates (Fig. 1A and 1B). For HA-MRSA isolates, virulence ranged from 78% to 89% whereas for CA-MRSA strains, virulence range is 43% to 93% of the isolates (Fig. 1A and 1B). In the case of methicillin sensitive (MSSA) strains; the phage were proven virulent against 85% to 93% of the strains tested.

To assess species specificity, the *S. aureus* phage were tested against *S. cohnii, S. epidermitis, S. haemolyticus, S. saprophiticus, S. sciurii, S. xylosus* and *S. hyicus*. The results demonstrate that phage SA0414$^{\Phi}$1 and SA0414$^{\Phi}$2, SA0456$^{\Phi}$1, SA0470$^{\Phi}$1, SA0482$^{\Phi}$1, and SA11987$^{\Phi}$1 can only form plaques on *S. aureus* and *S. hyicus*. In contrast, SA0420$^{\Phi}$1 was capable of infecting all *Staphylococcus* species tested with the exception of *S. xylosus* (Fig. 1B). In addition, the phage did not infect gram negative bacterial species to include *Escherichia coli, Enterococcus faecium, Acinetobacter baumannii, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (data not shown). The results of these experiments indicate that the novel phage are polyvalent phage and specific to *S. aureus* and *S. hyicus*.

Previous studies demonstrated that phage K is a polyvalent *S. aureus* phage. The virulence of phage K was tested in this collection of clinical isolates and results show phage K is virulent against 82% of the isolates. For HA-MRSA strains, virulence was 78% compared to 87.5% of the CA-MRSA and 82.5% MSSA strains (Fig. 1A and 1B). It is noteworthy that phage K was not virulent on strains NSC0637 and NSC0096; however the novel phage infected these strains (Fig. 1A and B). This establishes a phenotypic distinction between the novel phage and phage K. In addition, phage K is virulent on a geographically independent set of strains underscoring the polyvalent nature of phage K.

Electron microscopy analysis of S. aureus *phage*

S. aureus phage are members of the *Myoviridae, Syphoviridae* and *Podoviridae* family of viruses.[38] Due to the similarities in virulence spectrum of the novel phage and phage K, structural similarities among the phage are possible. To assess the morphology of the *S. aureus* phage, electron microscopy studies were performed on the novel phage group and contrasted to phage K. Consistent with the structural features of phage K, the novel phage group has a polyhedral shaped capsids and long contractile tails (Fig. 2A-H). Furthermore, the phage genomic DNA size is greater than 140 Kb (data not shown). These results classify the novel phage group as members of the *Myoviridae* family. In addition, we identified the novel phage in both the relaxed and contracted conformations (Fig. S1). The binding of the phage to *S. aureus* strain ATCC11987 was monitored by electron microscopy.

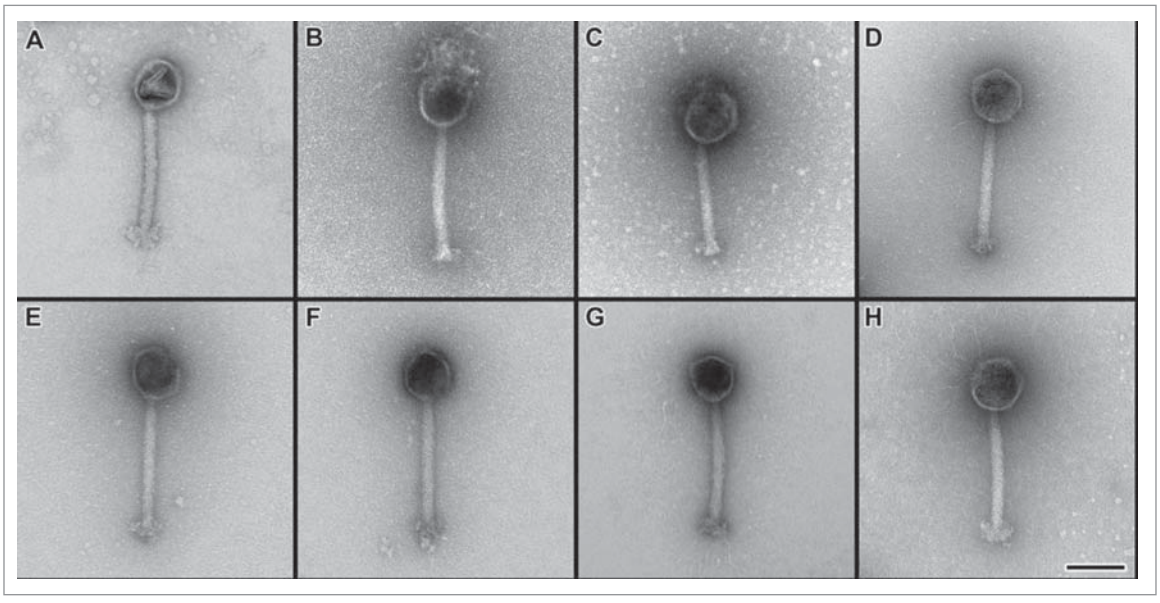

Figure 2. Morphological analysis of novel *S. aureus* phage. Transmission electron micrographs of negative stained bacteriophages (A) Bacteriophage K, (B) Bacteriophage SA0414$^{\Phi}$1, (C) Bacteriophage SA0414$^{\Phi}$2, (D) Bacteriophage SA0420$^{\Phi}$1, (E) Bacteriophage SA0456$^{\Phi}$1, (F) Bacteriophage SA0470$^{\Phi}$1, (G) Bacteriophage SA0482$^{\Phi}$1 (H) Bacteriophage SA11987$^{\Phi}$1. Scale bar equals 100 nm.

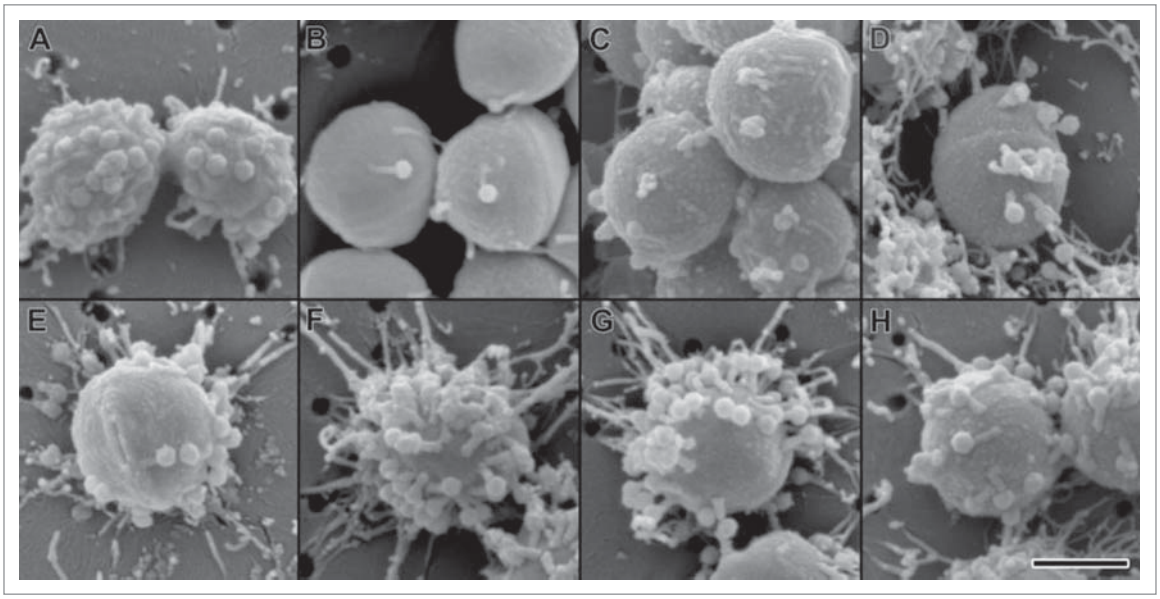

Figure 3. Scanning electron micrographs of phage bound to cells of *Staphylococcus aureus*. *S. aureus* strain ATCC11987 cells bound by the **novel** phage on membrane filters with 100 nm pores. (A) Bacteriophage K, (B) Bacteriophage SA0414$^{\Phi}$1 (C) Bacteriophage SA0414$^{\Phi}$2 (D) Bacteriophage SA0420$^{\Phi}$1, (E) Bacteriophage SA0420$^{\Phi}$1 (F) Bacteriophage SA0456$^{\Phi}$1, (G) Bacteriophage SA0470$^{\Phi}$1, (H) Bacteriophage SA0482$^{\Phi}$1, (I) Bacteriophage SA11987$^{\Phi}$1. Scale bar equals 500 nm.

The results indicate that multiple phage particles effectively recognize the *S. aureus* cell surface (Fig. 3A-H).

The S. aureus *novel phage group and phage K are distinct from each other*

Due to consistency in morphologies and lytic spectra between phage K and the novel phage, it was considered possible that these could be independent environmental isolates of phage K (Fig. 1A, 1B, and 2A). To assess this, *EcoR1* DNA restriction profiles of the novel phage were compared with phage K via pulse field gel electrophoresis (PFGE). The results show that phage K generates a pattern distinct from phage SA0420$^{\Phi}$1, SA0456$^{\Phi}$1, SA0470$^{\Phi}$1, SA0482$^{\Phi}$1, and SA11987$^{\Phi}$1 (Fig. 4). Additionally, comparison of the novel phage digestion patterns shows no difference in *EcoRI* digestion patterns, suggesting they are closely related (Fig. 4). Collectively, these results strongly suggest that the *S. aureus* phage are novel in nature and are not environmental isolates of phage K.

In vitro *inhibition of* S. aureus *growth by the novel phage group and phage K mixtures are synergistic*

Studies indicate *S. aureus* can develop resistance to the lytic effects of phage infection.[39] To determine if resistance to the novel phage group and phage K is observed among the clinical isolates, a growth curve analysis was performed utilizing the OmniLog® automated system. Our laboratory has modified the system to monitor and characterize phage infection at continuous intervals providing insight into phage resistance and effectiveness.[40] Phage K incubation with strain NSI0016 resulted in bacterial growth inhibition for 8 hours, indicating that resistance to Phage K arises in this strain (Fig. 5A). Phage SA0420$^{\Phi}$1, SA0456$^{\Phi}$1 and SA0482$^{\Phi}$1 inhibit bacterial growth for 10, 12, and 15 h, respectively. However, phage resistance is also evident after these incubation periods. Media-only or phage-only controls show baseline oxidation levels of reporter dye (Fig. 5A). These results suggest that all phage are virulent against strain NSI0016, but insufficient to control growth for time spans longer than 8–15 h.

To determine if emergence of phage-resistance could be reduced by using phage combinations, phage SA0420$^{\Phi}$1, SA0456$^{\Phi}$1 or SA0482$^{\Phi}$1, were tested with phage K (Fig. 5B). The combination of phage K with phage SA0420$^{\Phi}$1 inhibited growth for 20 h while SA0456$^{\Phi}$1 growth was inhibited for 26 h. In the case of phage SA0482$^{\Phi}$1, the combination with phage K inhibited growth of NSI0016 for 36 h. The effects of these phage combinations are more pronounced on strain 3195.CO1, where phage SA0420$^{\Phi}$1, SA0456$^{\Phi}$1 and SA0482$^{\Phi}$1 in combination with phage K completely inhibit bacterial growth (Fig. 5B). To assess

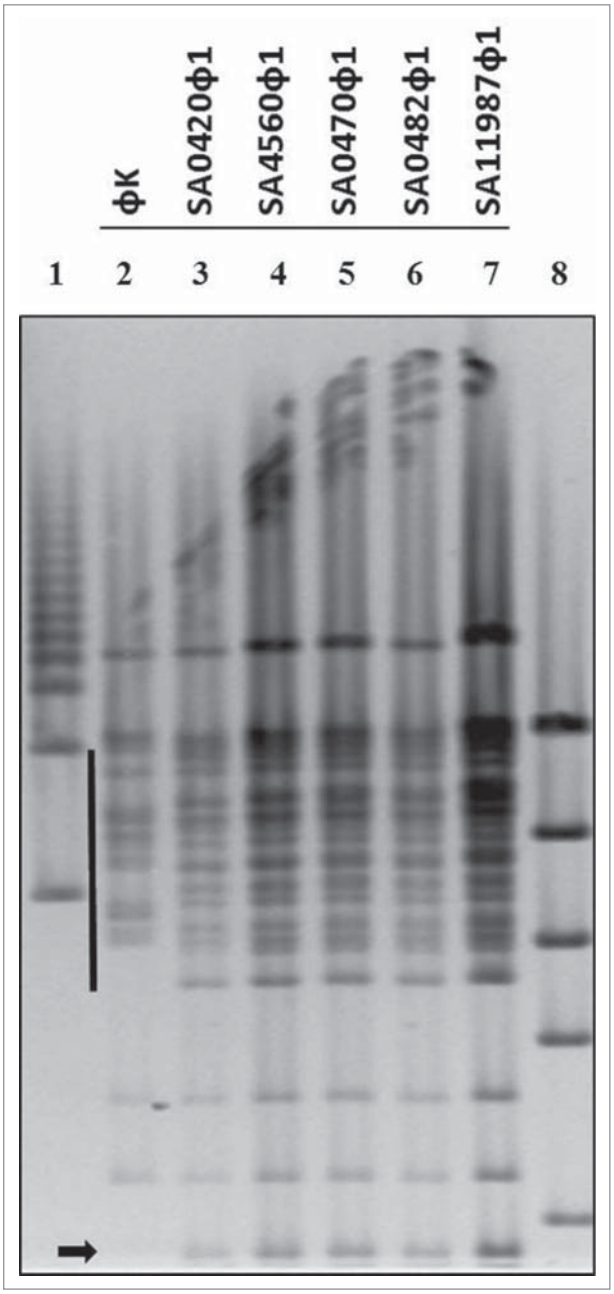

Figure 4. Analysis of *S. aureus* phages by Pulse field DNA analysis. Pulse field gel electrophoresis (PFGE) of purified S. aureus bacteriophage DNA digested with EcoR1. The solid line and arrow indicate regions where band patterns are distinct from phage K in comparison members in the novel phage group. Lanes 1 and 8 are molecular weight markers.

the possible additive effects of SA0420$^{\Phi}$1, SA0456$^{\Phi}$1, and SA0482$^{\Phi}$1 on *S. aureus*, a 3-phage mixture was prepared to a MOI identical to the single phage treatment. The results demonstrate that this phage combination can synergize to inhibit growth of NSI0016 and 3195.CO1 (Fig. 5A and 5B). These results show that combining phage K with SA0420$^{\Phi}$1, SA0482$^{\Phi}$1, SA0456$^{\Phi}$1 or a combination of the novel phage has a synergistic growth inhibition effect, suggesting the combination may reduce formation of phage resistance.

The S. aureus *phage require cell wall teichoic acid molecules for infection*

Studies have suggested that phage K utilizes teichoic acid molecules as cell surface receptors for infection.[26] The *tarO* gene encodes the N-acetyl glucosamine transferase, the enzyme responsible for initiating WTA synthesis in *S. aureus*. The catalytic activity of the *tarO* gene product can be inhibited by treatment with tunicamycin.[27,41] To elucidate the cell surface receptor utilized by the novel phage group, tunicamycin was used as an inhibitor of the N-Acetyl glucosamine transferase activity. Addition of 0.1 μg/ml of tunicamycin caused a 1000-fold inhibition in the ability of the novel phage group to infect bacteria (Fig. 6A). In comparison, phage K Infection was inhibited 100-fold by tunicamycin treatment (Fig. 6A). To assess the contribution of WTAs to phage infection of strains from our *S. aureus* collection, a sub-set of strains were screened for tunicamycin-mediated inhibition of phage infection. Exposure of strain 1028.N to tunicamycin caused a 100,000 fold inhibition of *S. aureus* infection by the novel phage group. This strongly suggests that teichoic acid molecules are involved in infection of the *S. aureus* clinical isolates in this study (Fig. 6B).

To further assess the WTA pathway involvement in the novel phage group infection of *S. aureus*, a series of null mutants were used. The strain RN4220 was selected as host due to the absence of a capsule, prophage, and restriction digestion modification systems known to produce immunity against phage infection.[29] EOP assay show that the novel phage group infect strain RN4220, albeit at a lower efficiency than phage K. Infection by phage SA0414$^{\Phi}$1 and SA0414$^{\Phi}$2 was only evident at the highest phage titer (Fig. 6C). These results indicate that this strain background is suitable for assessing the contribution of WTA biosynthetic enzymes to infection by the novel phage group. To assess further the involvement of the WTA pathway, isogenic null mutants of *tarO, tarM, tarS* and the Δ*tarM* Δ*tarS* double mutant were utilized.[27,29,35] The Δ*tarO* strain is impervious to infection by the novel phage group, however, the effects on phage K lysis inhibition are minimal on this assay (Fig. 6C). EOP

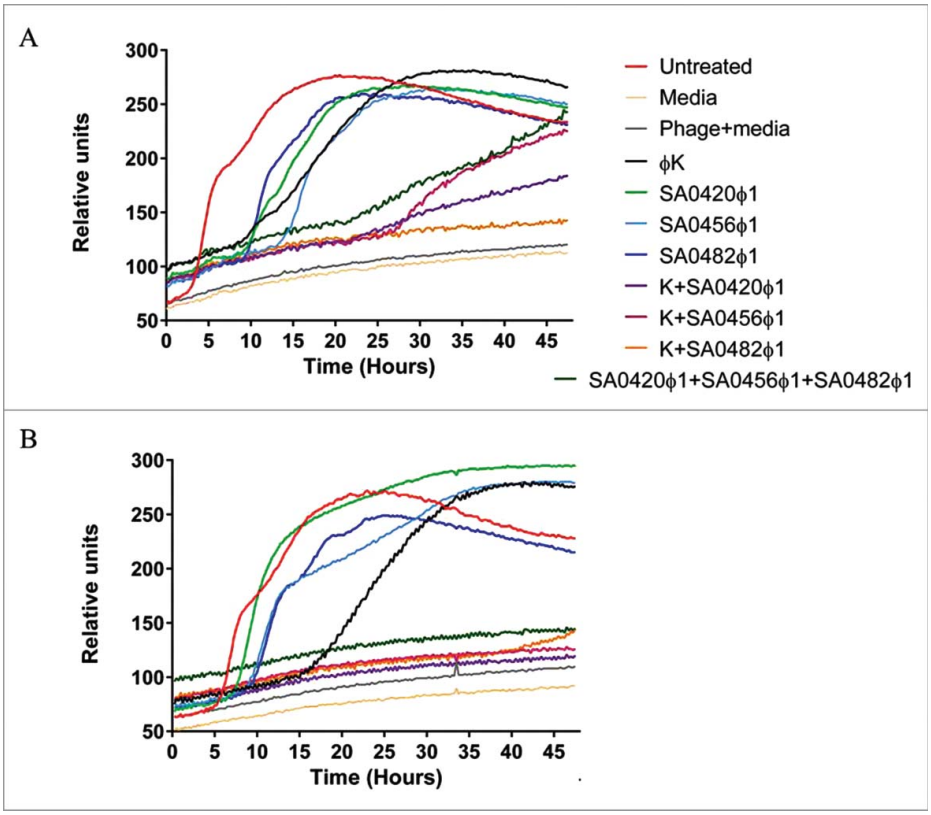

Figure 5. Analysis of bacteriophage cocktails on CA-MRSA and HA-MRSA Strains via the OmniLog® system. (A) Strain NSI0016 monitored for 48 h on the OmniLog® system. (B) Strain 3195.CO1 monitored for 48 h on the OmniLog® system. A total of 4×10^6 cells were added per well for each of the MRSA strains NSI0016 and 3195.CO1. The phage was added to a final Multiplicity of infection (MOI) of 2.5 and growth measured every 15 min for 48 h. The plots represent triplicate experiments, all combinations contain identical total phage quantities.

on Δ*tarS* mutant show complete inhibition of the lytic activity of the novel phage group, implicating *β*-GlcNAc moieties of the WTAs in phage infection. In contrast, the deletion of Δ*tarM* enhances the lytic activity of the phage. The Δ*tarM* Δ*tarS* double mutant shows a lytic phenotype that resembles the Δ*tarS* single mutant (Fig. 6C). These results indicate that *β*-GlcNAc moieties on WTA are essential for the novel phage infection of *S. aureus*.

Discussion

Reducing antimicrobial agent resistance is crucial for development of therapies against multiple-drug resistance in organisms such as *S. aureus*.[4] The results outlined in this article describe 7 novel polyvalent *S. aureus* phage virulent against 70% to 91% of the clinical isolates in our collection (Fig. 1A and 1B). Phage K has been reported as a broad spectrum phage against MRSA strains.[22] Here, we have expanded on those studies by finding phage K virulent against 82% of the of *S. aureus* strains (Fig. 1A and 1B). Importantly, the novel phage complements the virulence of phage K. For example, in strains NSC0096 and NSC0637, phage K is ineffective yet members of the novel phage group are virulent against these same strains (Fig. 1A). This makes phage K and all members of the novel phage group suitable candidates for a phage mixture for therapeutic treatment of *S. aureus* infections. Either phage K or the novel phage group fail to infect gram negative bacteria and are specific to *S. aureus*, showing minimal *staphylococcal* species cross virulence by infecting *S. hyicus* (Fig. 1B). One exception was phage SA0420$^{\Phi}$1 who infected several *Staphylococcus* species including *S. cohnii, S. epidermitis, S. haemoliticus, S. saprophiticus*, and *S. sciurii*. This suggests that phage SA0420$^{\Phi}$1 targets cell surface receptors common to all *Staphylococcus* species tested.

Electron micrograph studies demonstrate the novel phage group are members of the *Myoviridae* family, characterized by a polyhedral capsid, a long contractile tail and genomes larger than 140 Kb (Fig. 2A-H).[38]

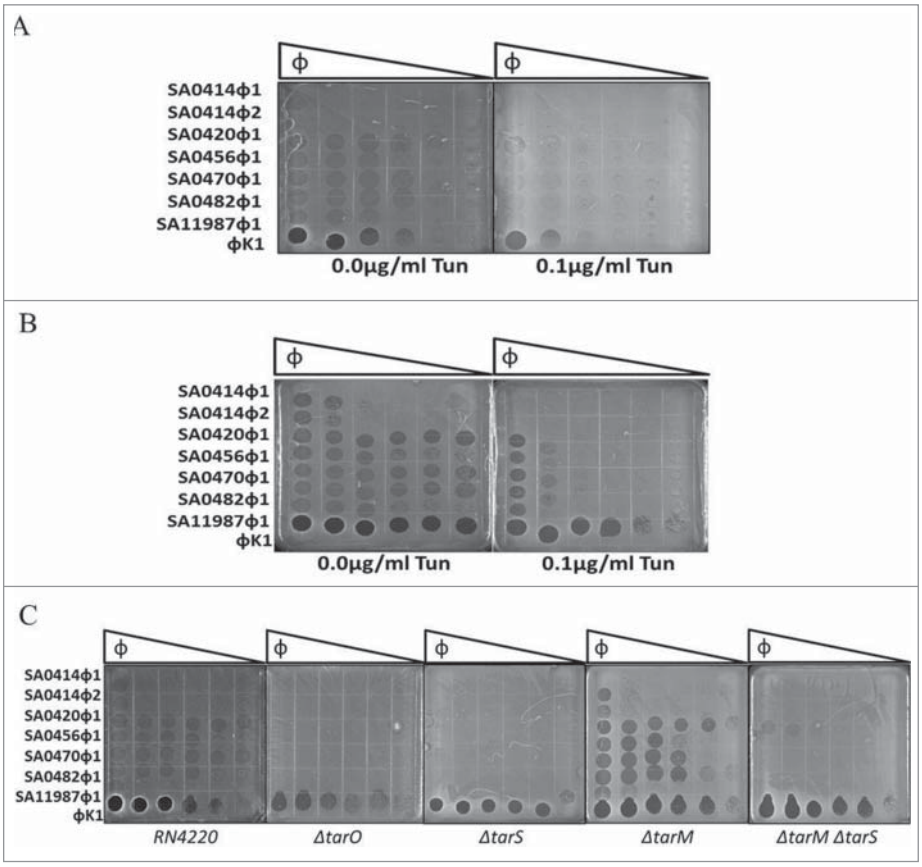

Figure 6. Cell wall teichoic acids are required for bacteriophage virulence. (A) Strain RN4220 infected with phage SA0414$^{\Phi}$1 SA0414$^{\Phi}$2, SA0420$^{\Phi}$1, SA0470$^{\Phi}$1, SA0482$^{\Phi}$1, SA0456$^{\Phi}$1, SA11987$^{\Phi}$1 and phage K in the absence or presence of 0.1 μg/ml tunicamycin. (B) Strain 1028.N CA-MRSA isolate treated with 0.1 μg/ml tunicamycin. (C) Strain RN4220 mutant derivatives tarO, tarS, tarM, tarM/tarS genes of the teichoic acid biosynthesis pathway treated with all phage in this study. Plates were incubated at 37°C for 12 h, a total 1×10^5 PFU/ml of each phage was used as the start point for a 10-fold serial dilution.

Indeed, our study determined that genome sizes of all the members of the novel phage group are 140 Kb or larger, consistent with members of the *Myoviridae* family (data not shown). The polyvalent nature of the novel phage showed a similarity with phage K, however, restriction analysis demonstrates otherwise (Fig. 4). Further distinction comes from lytic spectrum EOP analysis, where comparison of equal titers generates virulence profiles distinct for each strain-phage combination reflecting genetic differences among the novel phage (Fig. 1A and 1B). In the case of phage SA0414$^{\Phi}$1 has an average non-contracted tail size of 90.6nm and an average contracted tail size of 53.8 nm and an average capsid size 98.5 nm. In contrast, SA0414$^{\Phi}$2 has an average non-contracted tail size of 206.5 nm, an average contracted tail size of 112.5 nm and an average capsid size of 105.5 nm. Even though their lytic spectra are very similar, the difference in size establishes a morphological distinction between these phage (Fig. 1A, 1B and data not shown). Taken together, the results indicate that the novel phage are polyvalent in nature and effective against *S. aureus* clinical isolates.

This study shows that phage K resistance develops in strain NSI0016 after 8 h. Phage resistance is prevented or delayed by the addition of phage SA0420$^{\Phi}$1, SA0456$^{\Phi}$1 or SA0482$^{\Phi}$1. Different degrees of enhanced virulence are observed when combining phage K with SA0420$^{\Phi}$1, SA0456$^{\Phi}$1 or SA0482$^{\Phi}$1 (Fig. 5A and 5B). Additionally, the combination of phage SA0420$^{\Phi}$1, SA0456$^{\Phi}$1 and SA0482$^{\Phi}$1 effectively reduced phage-resistance on NSI0016 and 3195.CO1 (Fig. 5A and 5B). The synergistic effects observed in phage K combinations suggest that the novel phage group utilized an infection mechanism distinct from phage K. However, the additive effects observed in the SA0420$^{\Phi}$1, SA0456$^{\Phi}$1 and SA0482$^{\Phi}$1 triple combination indicate that there are differences in the mechanisms of infection among them. Perhaps, differences in novel phage affinity toward cell surface receptors

may alter phage replication kinetic in a particular host resulting in distinct latent periods and bust sizes.

Previous studies have implicated cell wall teichoic acids as important components for absorption of phage in the *Myoviridae* virus family.[26] Treatment with tunicamycin, an inhibitor of N-acetyl glucosaminidase activity, led to the inhibition of infection by the novel phage group suggesting a role of this modification in the infection mechanism (Fig. 6A). Importantly, the effect of tunicamycin is not confined the RN4220 strain for treatment of clinical isolate strain 1028.N1 shows a 10,000 fold reduction in phage infection (Fig. 6B). In other clinical isolates, a 10-fold reduction is observed (data not shown). The deletion of the N-acetyl glucosamine transferase, Δ*tarO*; impedes infection by members of the novel phage group, implicating cell wall teichoic acids as a key component for infection (Fig. 6C). Furthermore, the deletion of Δ*tarS*, the β N-acetyl glucosaminidase, rendered the cells insensitive to infection by the novel phage group. This suggests that β-N-acetyl glucosamine modification of cell wall teichoic acids act as cell surface receptors for the novel phage group (Fig. 6C). Interestingly, the deletion of *tarM*, the α-N-acetyl glucosaminidase, enhances the ability of the novel phage group to infect strain RN4220 (Fig. 6C). This may be due to increased β-N-acetyl glucosamine modification of WTAs enhancing binding of the novel phage group. Alternatively, the α-N-acetyl glucosaminidation may have an inhibitory effect on phage infection. Of note is the fact that the extent of WTA modification in *S. aureus* varies among strains. Some *S. aureus* strains have been reported to exclusively harbor α or β-GlcNAc modifications in WTAs while other strains consist of a mixture of both modifications.[27,33,34] This level of heterogeneity could explain the difference in phage susceptibility among *S. aureus* isolates. Saliently, phage K infection was not affected by Δ*tarS* or Δ*tarM* and only mildly by Δ*tarO*, suggesting that phage K may use other receptors besides WTAs. The enhancement of growth inhibition observed in phage K combinations with SA0420$^{\Phi}$1, SA0456$^{\Phi}$1 and SA0482$^{\Phi}$1 support the notion that the novel phage group use a common cell surface receptor distinct from that of phage K.

It is noteworthy that β-N-acetyl glucosamine modifications of WTAs have been linked to potentiation of β-lactam antibiotic resistance on MRSA strains.[27,29] β-N-acetyl glucosamine modifications serve as binding sites for the PBP2a, the enzyme responsible for β-lactam resistance in *S. aureus*, allowing for continuous cell wall synthesis. Interestingly, the WTA β-N-acetyl glucosamine modification needed for β-lactam resistance is also essential for novel phage group infection. An intriguing possibility lies in competition between the phage and PBP2a for a common site of action. Our preliminary studies suggest a phage-antibiotic synergy (PAS) effect of the novel phage group and β–lactam antibiotic (unpublished results).[42-44] This suggests that the novel phage group would be suitable candidates to augment β-lactam antibiotic treatment and strengthen the notion that the novel phage group are a useful tool to combat the emergence of MDR organisms (data not shown).

Several other studies have identified phage with virulence against methicillin resistant *S. aureus* strains.[22,23] However, few studies have identified phage with broad spectrum properties. In this study we have isolated 7 *S. aureus* polyvalent phage which possess the potential for use as treatment against antibiotic resistant *S. aureus* infections which may overcome the emergent phage resistance.

Abbreviations

CA-MRSA	Community acquired methicillin resistant *Staphylococcus aureus*
EOP	Efficiency of plating
HA-MRSA	Hospital acquired methicillin resistant *Staphylococcus aureus*
MDR	Multi-drug resistance
MOI	Multiplicity of infection
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin sensitive *Staphylococcus aureus*
PFGE	Pulse field gel electrophoresis.
PFU	Plaque forming units
SSTI	Skin and soft tissue infections

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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