EFFECT OF BACTERIOPHAGE 92 INFECTION IN COMBINATION WITH TEICOPLANIN ON MIXED-COMMUNITY METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* AND *STAPHYLOCOCCUS EPIDERMIDIS* BIOFILMS

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TABLE OF CONTENTS

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

ABSTRACT

EFFECT OF BACTERIOPHAGE 92 INFECTION IN COMBINATION WITH TEICOPLANIN ON MIXED-COMMUNITY METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* AND *STAPHYLOCOCCUS EPIDERMIDIS* BIOFILMS

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August 2013

SUPERVISING PROFESSOR: GARY M. ARON

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an aggressive pathogen, which is classified as a healthcare-associated infection (HAI). HAIs are a significant problem for the U.S. healthcare industry, with approximately 1.8 million patients affected each year. The emergence of antibiotic resistance among bacteria has resulted in the use of bacteriophage infection in combination with antibiotics to treat infection. *Staphylococcus epidermidis* is a commensal found in mixed-community biofilms with *S. aureus*. To date there have been no reports on the effect of phage and antibiotic on *S. aureus* and *S. epidermidis* mixed-community biofilms. This study reports the effect of the antibiotic teicoplanin in combination with *Staphylococcus* bacteriophage type 92 on the survival rates of mixed-community *S. aureus* and *S. epidermidis* 48 h biofilms. Teicoplanin is a glycopeptide antibiotic with a similar spectrum of activity to vancomycin

and used in the treatment of MRSA infections. The results demonstrate phage infection in combination with teicoplanin reduced *S. aureus* monoculture biofilm better (89% kill) than phage alone (40% kill) or teicoplanin alone (73% kill). Teicoplanin reduced *S. epidermidis* monoculture biofilm better (87% kill) than phage alone (24% kill) or teicoplanin in combination with phage (55% kill). In mixed-community biofilms, phage alone was more effective against *S. aureus* (96% kill) than teicoplanin (55% kill) or a combination of phage and teicoplanin (88%). The combination of teicoplanin and bacteriophage was more effective against *S. aureus* monocultures than either teicoplanin or phage alone, and increased susceptibility of *S. aureus* to phage infection was observed when present in mixed-community with *S. epidermidis*. However, *S. epidermidis* acquired teicoplanin resistance when present in mixed-community biofilms with *S. aureus*.

I
INTRODUCTION

Staphylococcus aureus and *Staphylococcus epidermidis* are common bacteria that can be found living harmlessly on skin and mucosal membranes. They are also opportunists and may cause a variety of infections at virtually any site in the human body (1). Infections caused by *S. aureus* include impetigo, folliculitis, and scalded skin syndrome (2). *S. aureus* also increases the mortality rates associated with bacteraemia, toxic shock syndrome, pneumonia, osteomyelitis, and endocarditis (3). Recently, *S. aureus* was identified in high frequency in chronic rhinosinusitis (4).

S. aureus possesses numerous virulence factors that either damage host tissue or evade host defense systems. Exotoxins that damage host tissue include hemolysins, catalases, proteases, and lipases (5). *S. aureus* also secretes exotoxins which direct cytolytic activity against host phagocytic cells, cause rapid induction of tissue-damaging tumor necrosis factor alpha, and have the ability to enter and survive within host phagocytic and non-phagocytic cells (6, 7, 8, 9). Cell walls of *S. aureus* contain numerous virulence factors that enable it to damage host tissue and evade host defense systems. Some of these include collagen binding protein, elastin binding protein, vitronectin binding protein, and fibronectin binding protein. Cell wall factors that enable *S. aureus* to evade host immunological defense mechanisms include multiple peptide resistance factor protein, which provide resistance to defensins, and capsular polysaccharides that resist phagocytosis (5). One virulence factor exclusive to the cell

wall of *S. aureus* is Staphylococcal surface protein A (SpA), which blocks the Fc portion of IgG, thereby inhibiting antibody-mediated phagocytosis (10). SpA may also cause apoptosis of B lymphocytes and impairs T lymphocyte responses (11).

S. epidermidis is a coagulase negative staphylococcal (CNS) commensal found on the skin and in the nasal cavities of entire populations (12). *S. epidermidis* is commonly found alongside methicillin-resistant *Staphylococcus aureus* in infection (13). For many years, *S. epidermidis* was found in blood samples of patients suffering from bacteremias and considered merely a contaminant. In the early 1990's, however, researchers determined that a significant portion of bacteremias were caused by *S. epidermidis* (14). Although *S. epidermidis* is less destructive than *S. aureus*, once *S. epidermidis* enters the bloodstream, it secretes a number of virulence factors including proteases, lipases, and enterotoxins (15, 16).

Of the numerous virulence factors possessed by both staphylococcal species, biofilm formation is the most problematic (17). Biofilms are communities of bacteria that can attach to a variety of different surfaces and conglomerate within an extracellular polymeric matrix. Once a staphylococcal biofilm has been established and covered by the signature glycocalyx barrier, multicellular clusters of bacterial cells are released and can metastasize and infect secondary locations (18). Cells encased in a biofilm extracellular polymeric matrix are more tolerant of antimicrobial influences than planktonic cells of the same species (19). Quorum sensing within a biofilm helps combat host immune responses by coordinating gene regulation in response to the immediate environment (20). Epidemiological evidence such as cell detachment from the aggregate

biofilm and relocation into the bloodstream indicates increased pathogen virulence as a result of biofilm formation, particularly in immunocompromised people (17).

Staphylococcal species have many different mechanisms that induce biofilm formation. There are numerous genetic contributors to *S. aureus* and *S. epidermidis* biofilm construction, but the general trend of biofilm formation follows a similar pattern. Specifically, cell wall adhesins and surface proteins are first expressed first during early colonization, whereas toxins responsible for tissue destruction are expressed following biofilm establishment (21).

The most documented mechanism of cell attachment in biofilms is the production of poly-*N*-acetylglucosamine and polysaccharide intercellular adhesin (PNAG/PIA) by the intercellular adhesion (*ica*) operon (22). It has been demonstrated however, that deletion of the *ica* operon does not result in decreased attachment kinetics of biofilms (23). This has been confirmed *in vivo* with catheter infections (24).

S. aureus has multiple avenues available to establish biofilms on abiotic surfaces (25). *S. aureus* is an extremely adaptable pathogen and numerous genetic determinants have been examined to elucidate the mechanism of biofilm formation. One study speculated that staphylococcal biofilm formation was affected by the accessory gene regulator (*agr*), a loci known to encode surface proteins (26). However, deletion of *agr* had no influence on biofilm formation (27). The staphylococcal accessory regulator (*sarA*), was also investigated since it regulates *agr*. While some strains of *S. aureus* exhibit decreased biofilm growth with deletion of *sarA*, *sarA* affects the expression of over 100 genes and signaling pathways, some of which have other consequences in

virulence factor production. Additional research will have to be performed to determine the viability of targeting *sarA* as a treatment (28).

Numerous other mechanisms of biofilm formation have been investigated with limited success. *S. aureus* has a complex array of surface proteins collectively referred to as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). These proteins likely serve as receptors for other proteins that develop on medical implants, thereby making common components of human fluid a substrate for growth (29). The major cell wall autolysin in *S. aureus* (Atl) is thought to be involved in cell adhesion to inert surfaces, as is the case with *Staphylococcus epidermidis* (30, 31). It was later determined, however, that while Atl plays a role in antibiotic-mediated lysis of *S. aureus*, deletion of the gene responsible for Atl did not significantly decrease virulence (30). Environmental factors were also found to enhance biofilm formation and include increased NaCl salinity, higher temperatures, and preferential anaerobic conditions (32, 33).

During the 1980's through the 1990's, uses of medical devices such as catheters, cerebrospinal shunts, and other prosthetics have dramatically increased. With the increased use of these devices, patients experienced an increased rate of device related infections, since these implants provide an ideal substrate for biofilm formation (34). While device related infections are a major cause of concern, a recent study involving prosthetic ankle implants showed that *S. aureus* and *S. epidermidis* proliferate and disseminate to locations from the site of initial infection (35). Another study revealed that *S. aureus* and *S. epidermidis* occur in different frequencies depending on the nature of the injury or damaged site, and not simply on the prosthetic implant (36).

Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as one of the most dangerous and widely publicized pathogens in the United States since its initial identification. MRSA is resistant to most beta-lactam class antibiotics, and accounts for up to 19,000 deaths per year in the United States (37, 38). In 2005, a study determined that persons entering correctional facilities have introduced different strains of MRSA into these institutions, increasing the possibility of MRSA outbreaks among inmates and their health care workers (39). Recent studies assert that almost 20% of patients who undergo surgery acquire nosocomial infection, which add $10 billion in costs to U.S. healthcare annually (38). Originally limited to nosocomial, or healthcare-acquired infections (HA-MRSA), MRSA has since been identified as a serious public health threat to persons with no affiliation with hospital settings, also known as community-acquired infections (CA-MRSA) (40). People living in densely populated, urban areas are at higher risk for infection by CA-MRSA (41). MRSA carriage rates among college students are also elevated, as compared to the general population (42). Recent evidence suggests that MRSA is replacing methicillin-susceptible *Staphylococcus aureus* (MSSA) as the common flora among people in the general public (43). From 2005-2008, MRSA infection rates increased from 13.5% to 25.7% in suburban skin and soft tissue infections in the United States (41).

The gene responsible for methicillin resistance in *S. aureus, mecA,* codes for a surface protein known as penicillin binding protein that is not commonly found on methicillin-susceptible strains of *S. aureus* (44). *mecA* is located on a mobile genetic element known as the staphylococcal cassette chromosome (SSC*mec*), which can be horizontally transferred to other staphylococcal species, thereby conferring methicillin-

resistance in the process (45). Although *S. epidermidis* usually attains methicillin resistance with acquisition of the *mecA* gene, it may exhibit methicillin resistance in the absence of *mecA* transcription (46).

One of the most common methods for treating beta-lactam resistant staphylococcal infections is the use of glycopeptide antibiotics, which inhibit peptidoglycan synthesis (47). Two such antibiotics are vancomycin and teicoplanin. While similar in bactericidal spectrum, teicoplanin is not currently FDA approved for use in the United States (48). In 2009, a comprehensive study determined that teicoplanin was no less effective than vancomycin, and had fewer adverse toxic events (49). Between 2000 and 2004, however, *S. epidermidis* strains showing teicoplanin resistance increased from 7.2% to 30.4% of cases in French hospitals, whereas *S. aureus* showed no increase in resistance (50). Since staphylococcal species easily attain antibiotic resistance, and some bacteriophages are known to degrade biofilm extracellular polymeric substance, attention has focused on the therapeutic use of bacteriophages (51).

Bacteriophages are viruses that have coevolved with bacteria and infect specific species or strains of bacteria (52). Bacteriophages are found naturally in the environment, where they can reach an abundance of up to 10^7 particles per milliliter in oceans (53). The first successful use of bacteriophage in disease occurred in 1919 when microbiologist Felix d'Herelle was able to treat infection due to *Shigella dysenteriae* in French soldiers during World War I (54). In 1921, Richard Bruynoghe and Joseph Maisin successfully used bacteriophages to treat staphylococcal skin infections (55). Despite its success as a treatment for infections, phage therapy is currently only available in Russia and Georgia. A major reason is the use of antibiotics in the United States has

overshadowed research in phage therapy (52). A recent study demonstrated that antibiotic resistant strains of *Pseudomonas aeruginosa* in patients with chronic otitis could be successfully treated with bacteriophage cocktails (56). A common concern of phage therapy is that it may not reach the site of infection. However, in 2011 a study demonstrated that phage could be effectively used to treat antibiotic resistant *P. aeruginosa* infections in lungs of mice by intranasal administration (57). Bacteriophages have been used to treat *S. aureus* skin infections with success, including those strains that display resistance to multiple antibiotics (58).

To date there have been few studies on the use of bacteriophages in combination with antibiotics to treat biofilm infections. *Klebsiella pneumoniae* biofilms have been successfully eradicated using a combination of amoxicillin and *K. pneumoniae* B5055 specific bacteriophage isolated from the environment (59). It has also been demonstrated that *K. pneumoniae* biofilms were eradicated more effectively by a combination of ciproflaxin and *K. pneumoniae* B5055 specific bacteriophage, than the use of ciproflaxin alone. The increase in biofilm eradication was attributed to a depolymerase produced by the bacteriophage (60). It was also recently demonstrated that *S. aureus* biofilms could be effectively treated using a combination of bacteriophage SAP-26 with rifampicin (61).

There have been few studies that explore the effect of antibiotic and bacteriophage in combination on the behavior of mixed-community biofilms. On prosthetic implants, *S. aureus* is commonly found in biofilms alongside *Staphylococcus epidermidis* (20). One study demonstrated that bacteriophage can effectively treat *Salmonella enterica* when it is in a mixed-community planktonic culture with

Escherichia coli B (62). Another study demonstrated that a viable reservoir of phages could be maintained within a mixed-community biofilm of *E. coli* and *P. aeruginosa* (63).

This study investigates the effect of teicoplanin and bacteriophage on the viability of monoculture and mixed-community biofilms of methicillin-resistant *S. aureus* and methicillin-susceptible *S. epidermidis.* The results show that teicoplanin in combination with phages were the most effective against monoculture *S. aureus* biofilms, compared to teicoplanin alone or phage alone. However, teicoplanin alone was more effective against monoculture *S. epidermidis* compared to use of phage alone or in combination with teicoplanin. In a mixed-community biofilm, phage alone was more effective than teicoplanin alone or in combination with phage. In addition, this study demonstrates (1) *S. aureus* can transfer teicoplanin resistance to *S. epidermidis* and (2) *S. aureus* susceptibility to phage infection increases in mixed-community biofilms.

II
MATERIALS AND METHODS

Bacteria and bacteriophage. Methicillin-resistant *Staphylococcus aureus* ATCC 37741) was from the American Type Culture Collection (Manassas, VA). *Staphylococcus epidermidis* (ATCC 12228) was generously provided by Robert McLean. (Texas State University-San Marcos, San Marcos, TX). Bacteriophage type 92 (ATCC 33741-B) was used to infect *S. aureus* and *S. epidermidis*.

Antibiotic preparation. Teicoplanin (CAS 61036-62-2, Sigma-Aldrich Co., St. Louis, MO) stocks were prepared in deionized water followed by filter sterilization (0.22 μm; Fisher 25-mm syringe filter; Fisher Scientific Inc., Dublin, Ireland). Teicoplanin at a concentration of 10 μg mL^{-1} was used for all experiments.

Growth of bacteria. *S. aureus* and *S. epidermidis* were grown in Tryptic Soy broth (TSB) (Accumedia Manufacturers, Inc., Lansing, Michigan) at 37°C in an orbital rotating shaker water bath (Lab-Line Instruments, Inc. model 3540 Orbital Shaker Bath, Melrose Park, IL).

Bacteriophage propagation. Bacteriophage 92 stocks were prepared by infecting early log phase *S. aureus* using the agar overlay technique as previously described (Adams, M. 1959). Briefly, 350 μL of early log phase *S. aureus* was added to 3.5 mL of 0.38% agar in TSB. 100 μL of 10^6 phage was added to yield just confluent lysis. Following 24 h at $37^{O}C$, phage was eluted from the agar overlay in 5 mL TSB at $4^{O}C$.

The eluant, which contained phage, was centrifuged at 4^{O}C for 20 min at 4000 rpm (Eppendorf Centrifuge model 5810 R, Hamburg, Germany). Supernatant was filtered (0.45 μm) and phage titers were determined by soft-agar overlay plaque assay (Adams, M. 1959).

Biofilm growth. Overnight cultures of *S. aureus* or *S. epidermidis* were diluted 1:500 and 200μL was aliquoted into clear, sterile, non-tissue treated 96-well plates (Fisher Scientific Co., Horizon Ridge, CT). For mixed-community biofilms, 50μL of each 18 h culture were diluted in 50mL TSB and 200μL of the mixed culture was aliquoted into plates. Plates were incubated for 48 h at 37^{O}C with rocking at 10^{O}, at 15 rpm (VWR Signature™ Incubating Rocking Platform Shaker, VWR, Houston, TX).

Biofilm growth curves. Following 48 h growth, supernatant was removed, biofilms were washed with 200 μL phosphate buffered saline (PBS) (Sigma-Aldrich, Co., St Louis, MO), and 200 μL of TSB was added to each biofilm. 20 μL of alamarBlue® (Life Technologies, Grand Island, NY) resazurin oxidation-reduction assay was then added to each biofilm, and absorbance was measured every 30 min for 12 h at 420 nm (OD_{420}). At the end of 12 h, supernatant was extracted and biofilms were washed with 200 μL PBS. Biofilms were sonicated for 1 min at 40 kHz (Branson Ultrasonics Corporation, Danbury, CT). Colony forming units (CFUs) were determined by dilution plating on TSA plates.

Determination of bacteriophage multiplicity of infection (MOI). Varying MOIs of bacteriophage were added to 48 h *S. aureus* biofilms followed by 20μL of alamarBlue®. Absorbance was determined (OD_{420}) every 60 min for 12 h. Addition of

bacteriophage at sufficiently high MOIs inhibited biofilm growth and reduced OD_{420} absorbance measurements. The lowest MOI of phage that significantly ($p<0.05$) inhibited growth of 48 h *S. aureus* biofilm was used for the remainder of experiments. Student's t-tests were used to determine significant differences.

Effect of bacteriophage and teicoplanin. Supernatant was removed from 48 h *S. aureus* biofilms and biofilms washed with 200 µL PBS. Biofilms were treated with either phage (MOI 10), teicoplanin (10 µg mL^{-1}), or a combination of both contained in 200 µL TSB at 37°C. At 12 h, biofilms were sonicated for 1 min at 40 kHz. Biofilms were sonicated for 1 min at 40 kHz, and colony forming units (CFU) determined by dilution plating on TSA plates.

Determination of increase in resistance to teicoplanin by *S. epidermidis*. Susceptibility of *S. epidermidis* to teicoplanin was determined using the Kirby-Bauer disk diffusion technique, as described by the Clinical and Laboratory Standards Institute (www.clsi.org) with TSA substituted for Mueller-Hinton agar. *S. epidermidis* susceptibility to teicoplanin was tested before and after exposure to *S. aureus* in mixed-community biofilms. *S. epidermidis* recovered from mixed-community biofilm was subcultured nine successive times and each generation was tested for susceptibility to teicoplanin.

Effect of *S. epidermidis* supernatant on *S. aureus* phage sensitivity. *S. aureus* sensitivity to phage was determined by growing *S. aureus* biofilms with 100 µL of *S. epidermidis* supernatant for 48 h at 37°C. Two samples of *S. epidermidis* supernatant were used for biofilm growth. The first sample was prepared from colonies of *S.*

epidermidis monoculture biofilms. The second sample was prepared from colonies of *S. epidermidis* mixed-community biofilms. Supernatants were prepared from stationary phase cultures of *S. epidermidis* centrifuged at 4000 rpm for 20 min and sterile filtered (0.45 μm). *S. aureus* biofilms were washed with 200 μL PBS and infected with phage at MOI 10. Biofilms were sonicated for 1 min at 40 kHz, and CFUs determined by dilution plating on TSA plates.

Transmission Electron Microscopy. 5 μL of 10^{10} PFU mL^{-1} phage were placed on 200 mesh count, Formvar carbon-coated copper grids (Electron Microscopy Sciences, Hatfield, PA) overnight at room temperature. Samples were stained with 2% uranyl acetate for one minute and viewed using a JEM-1200 EXII.

III
RESULTS

Effect of bacteriophage multiplicity of infection (MOI). The effect of phage multiplicities of infection on the growth of *Staphylococcus aureus* biofilms is shown in Figure 1. Infection of *S. aureus* with phage 92 at a multiplicity of infection (MOI) of 0.1 and 1.0 resulted in 0% kill and 14%, respectively. MOI of 10 resulted in 40% kill, which was the lowest MOI to significantly ($p < 0.001$) inhibit *S. aureus* biofilm growth. MOI 10 was used for future experiments.

Effect of bacteriophage, teicoplanin, and a combination on growth of *S. aureus* monoculture biofilms. The effect of phage, teicoplanin, and a combination on *S. aureus* monoculture biofilms is shown in Table 1 and Figure 2. Phage and teicoplanin alone resulted in 40% kill and 73% kill, respectively. A combination of phage and teicoplanin resulted in 89% kill, which indicated combination of phage and teicoplanin is more effective than either treatment alone.

Growth of *S. aureus* monoculture biofilms. The effect of phage and teicoplanin on *S. aureus* monoculture biofilms with alamarBlue® are shown in Figure 3. At 12 h, *S. aureus* biofilms treated with phage resulted in an optic density (OD_{420}) reading of 1.52. Biofilms treated with teicoplanin alone and a combination of phage and teicoplanin resulted in an OD_{420} of 1.12. All readings showed decreased biofilm growth when compared to readings of untreated biofilms (1.83). The data indicate

that there was no difference between teicoplanin alone or in combination with phage at inhibiting biofilm growth, but both were more effective than phage alone.

Effect of bacteriophage, teicoplanin, and a combination on growth of *Staphylococcus epidermidis* monoculture biofilms. The effect of phage and teicoplanin on *S. epidermidis* monoculture biofilms for 12 h is shown in Table 1 and Figure 4. *S. epidermidis* biofilms treated with phage and teicoplanin resulted in 24% kill and 87% kill, respectively. A combination of phage and teicoplanin resulted in 55% kill. The data indicate teicoplanin alone is more effective than phage or a combination of phage and teicoplanin.

Growth of *S. epidermidis* monoculture biofilms. The effect of teicoplanin and phage on *S. epidermidis* monoculture biofilms with alamarBlue® are shown in Figure 5. At 12 h, *S. epidermidis* biofilms treated with phage alone and a combination of phage and teicoplanin resulted in optic density (OD_{420}) readings of 1.45 and 1.49, respectively. Biofilms treated with teicoplanin alone resulted in an OD_{420} of 1.37. All readings showed decreased biofilm growth when compared to readings of untreated biofilms (1.54). The data indicate that there was no difference between phage alone or in combination with teicoplanin, but teicoplanin alone was somewhat more effective at inhibiting biofilm growth.

Effect of bacteriophage and teicoplanin on growth of mixed-community biofilms. The effect of phage and teicoplanin on the growth of mixed-community biofilms is shown in Table 2 and Figure 6. Mixed-community biofilms treated with phage 92 alone resulted in 96% kill and 25% kill of *S. aureus* and *S. epidermidis*,

respectively. Biofilms treated with teicoplanin alone resulted in 55% kill and 0% kill of *S. aureus* and *S. epidermidis,* respectively. Biofilms treated with a combination of phage and teicoplanin yielded 88% kill and 0% kill of *S. aureus* and *S. epidermidis,* respectively. The data indicates that phage alone was more effective than teicoplanin alone, or a combination of phage and teicoplanin, at *S. aureus* cell destruction. Additionally, the data indicate *S. epidermidis* increases with addition of teicoplanin.

Effect of *S. aureus* on *S. epidermidis* resistance to teicoplanin. The effect of *S. aureus* on *S. epidermidis* resistance to teicoplanin is shown in Table 3. *S. epidermidis* zones of inhibition averaged 26.8 ± 0.44 mm^2. There were an average of two resistant colonies within these zones of inhibition. After isolation from growth in a *S. aureus* mixed-community biofilm, *S. epidermidis* zones of inhibition decreased to an average of 20.6 ± 0.53 mm^2. There were an average of 89 resistant colonies within these zones of inhibition. The increased resistance to teicoplanin remained for subsequent subcultures, but by the 10^{th} subculture the average zone of inhibition increased to an average of 24.6 ± 0.53 mm^2. There were an average of 96 resistant colonies within these zones of inhibition. The data indicate that *S. epidermidis* obtains increased resistance to teicoplanin after growth with *S. aureus* in a mixed-community biofilm.

Effect of *S. epidermidis* supernatant on *S. aureus* bacteriophage sensitivity. The effect of phage on *S. aureus* biofilms grown in the presence of *S. epidermidis* supernatant is shown in Figure 7. Phage yielded 39% kill on biofilms grown with monoculture *S. epidermidis* supernatant. Phage yielded 50% kill on biofilms grown with mixed-community *S. epidermidis* supernatant. The data indicate that *S.*

epidermidis increases *S. aureus* susceptibility to lysis from phage infection in mixed-community biofilms.

TEM imaging of bacteriophage 92. Micrographs of phage 92 reveal icosahedral capsids approximately 62 nm in diameter and long, flexible tails approximately 175 nm in length. Using previously established taxonomy guidelines, phage 92 belongs to the family Siphoviridae (64).

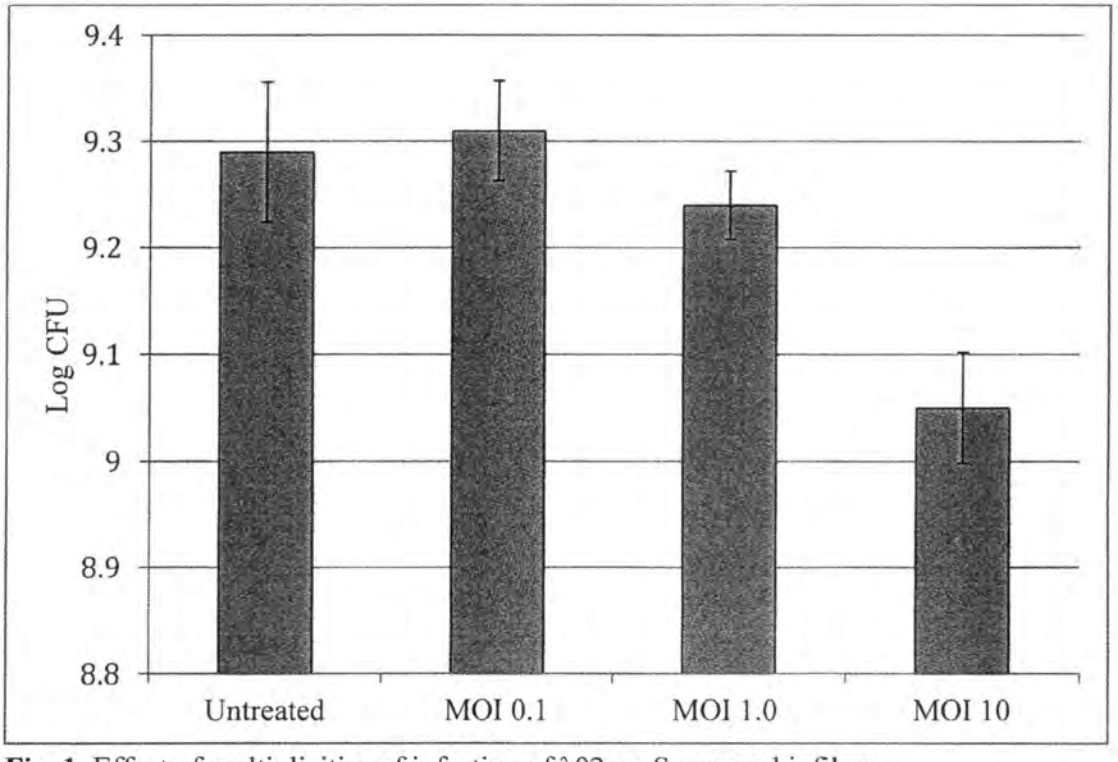

Fig. 1. Effect of multiplicities of infection of λ92 on *S. aureus* biofilms.

Table 1. Effect of λ92 and Teicoplanin on *Staphylococcus aureus* and *Staphylococcus epidermidis* monoculture biofilms.

	S. aureus CFU	% Kill	*S. epidermidis* CFU	% Kill
Untreated	$2.0x10^9$		$1.7x10^9$	
λ92	$1.2x10^9$	40	$1.3x10^9$	24
Teicoplanin	$5.4x10^8$	73	$2.2x10^8$	87
Combination	$2.2x10^8$	89	$7.6x10^8$	55

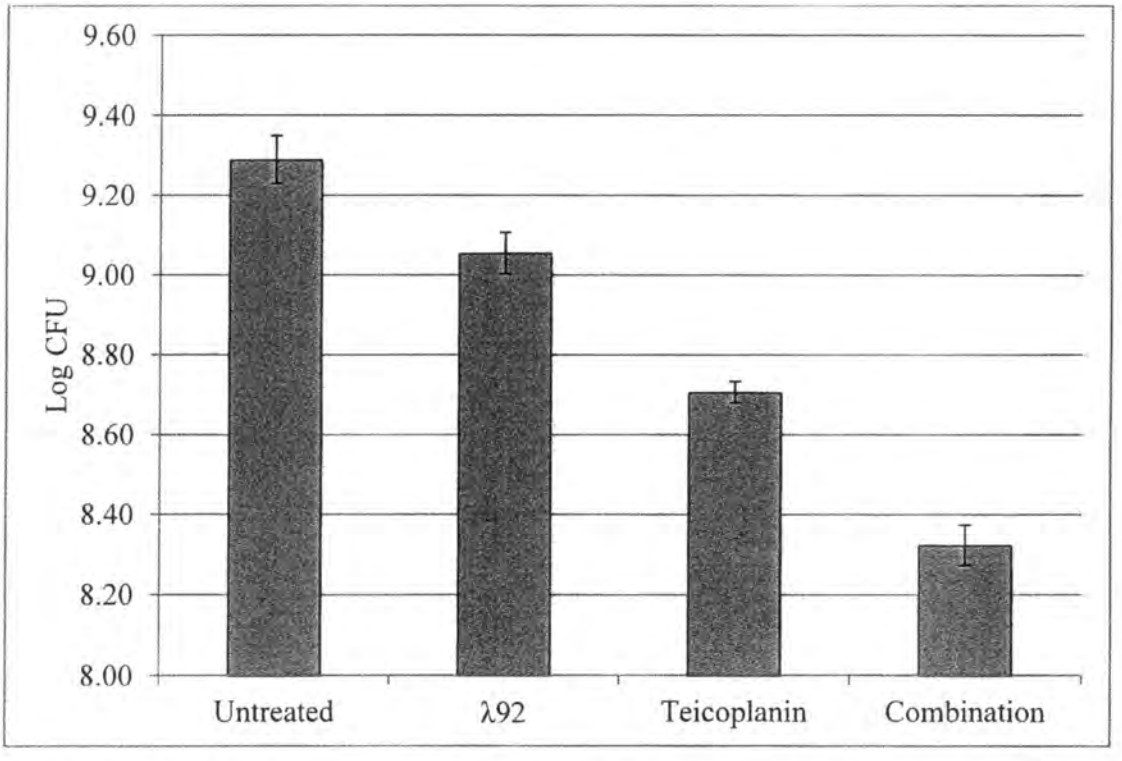

Fig. 2. Effect of λ92 MOI 10, teicoplanin 10 μg mL^{-1}, and a combination on *S. aureus* monoculture biofilms at 12 h.

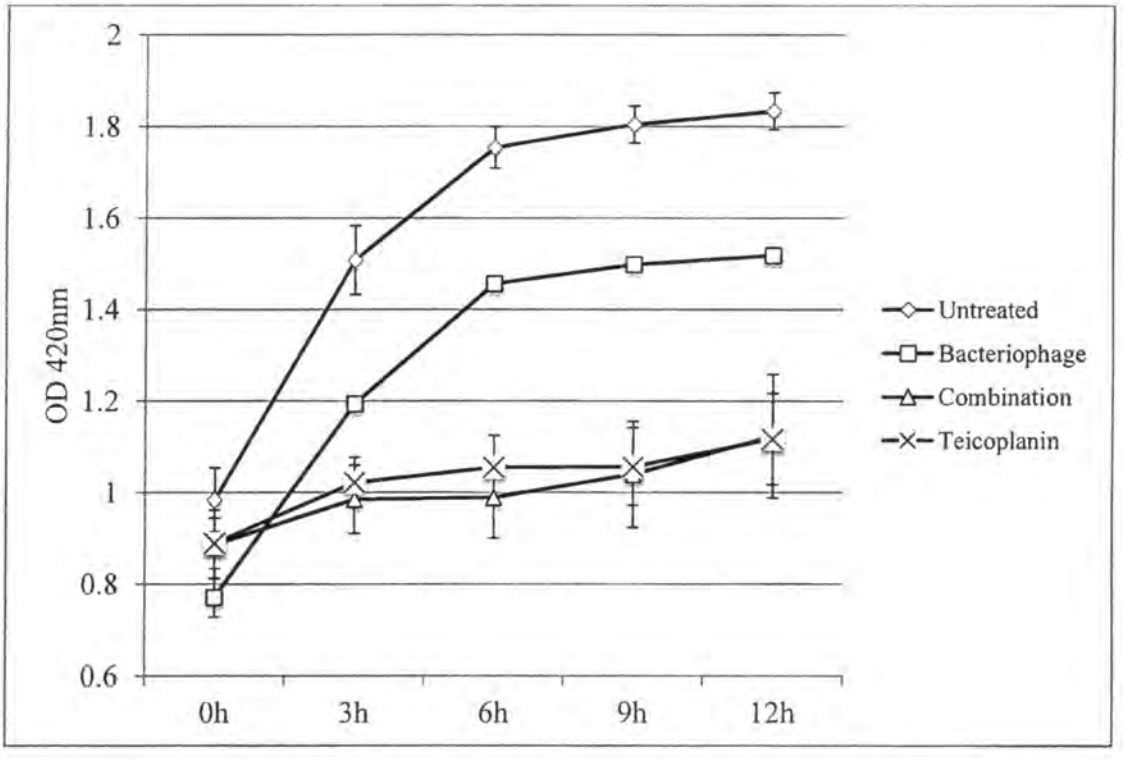

Fig. 3. Effect of λ92 MOI 10, teicoplanin 10 μg mL^{-1}, and a combination on *S. aureus* monoculture biofilms for 12 h.

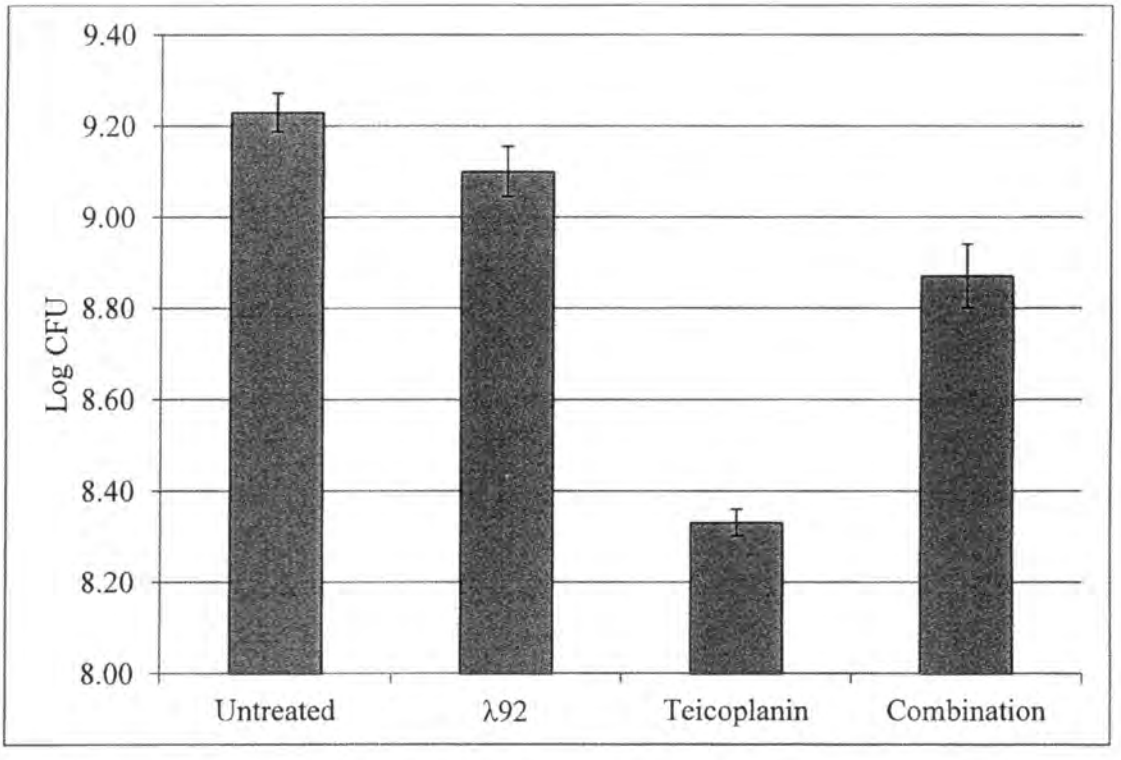

Fig. 4. Effect of λ92 MOI 10, teicoplanin 10 μg mL^{-1}, and a combination on *S. epidermidis* monoculture biofilms at 12 h.

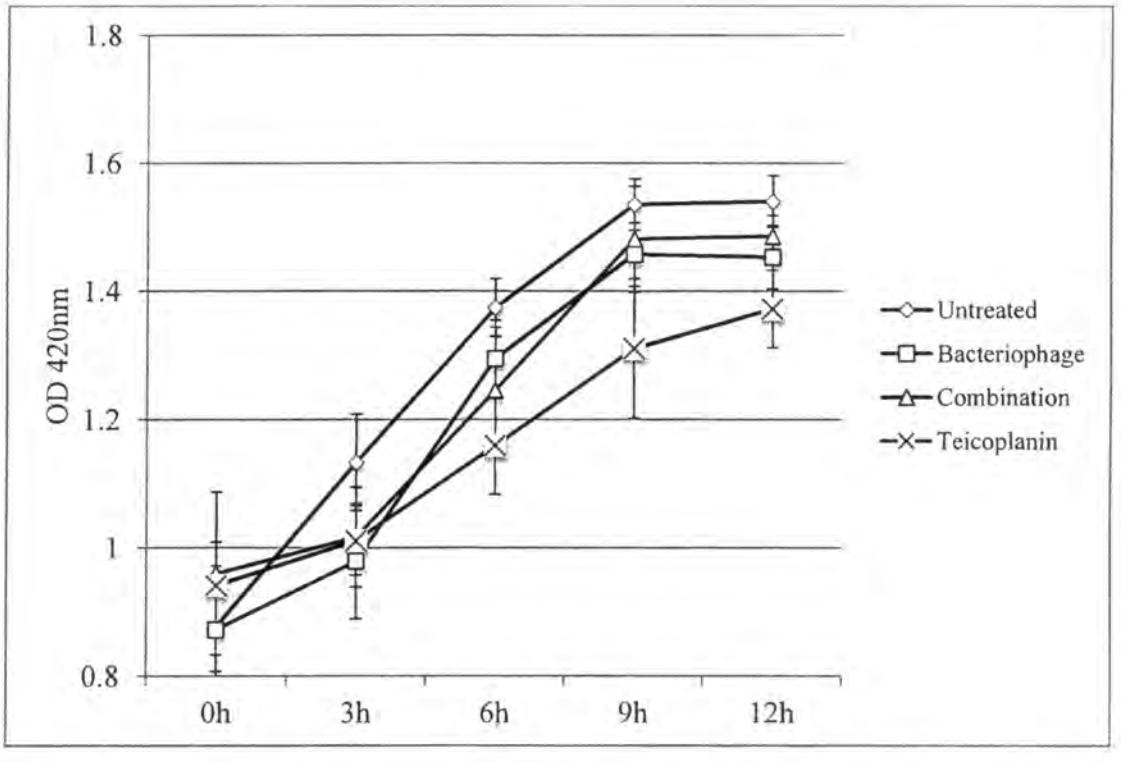

Fig. 5. Effect of λ92 MOI 10, teicoplanin 10 μg mL^{-1}, and a combination on *S. epidermidis* monoculture biofilms for 12 h.

Table 2. Effect of λ92 and Teicoplanin on *Staphylococcus aureus* and *Staphylococcus epidermidis* mixed-community biofilms.

	Total CFU	% Kill	*S. aureus* CFU	% Kill	*S. epidermidis* CFU	% Kill
Untreated	$6.8x10^9$		$5.8x10^9$		$1.0x10^9$	
λ92	$9.6x10^8$	86	$2.1x10^8$	96	$7.5x10^8$	25
Teicoplanin	$4.8x10^9$	29	$2.6x10^9$	55	$2.2x10^9$	0[a]
Combination	$3.0x10^9$	56	$6.9x10^8$	88	$2.3x10^9$	0[b]

[a] *S. epidermidis* CFUs increased by 174%.

[b] *S. epidermidis* CFUs increased by 188%.

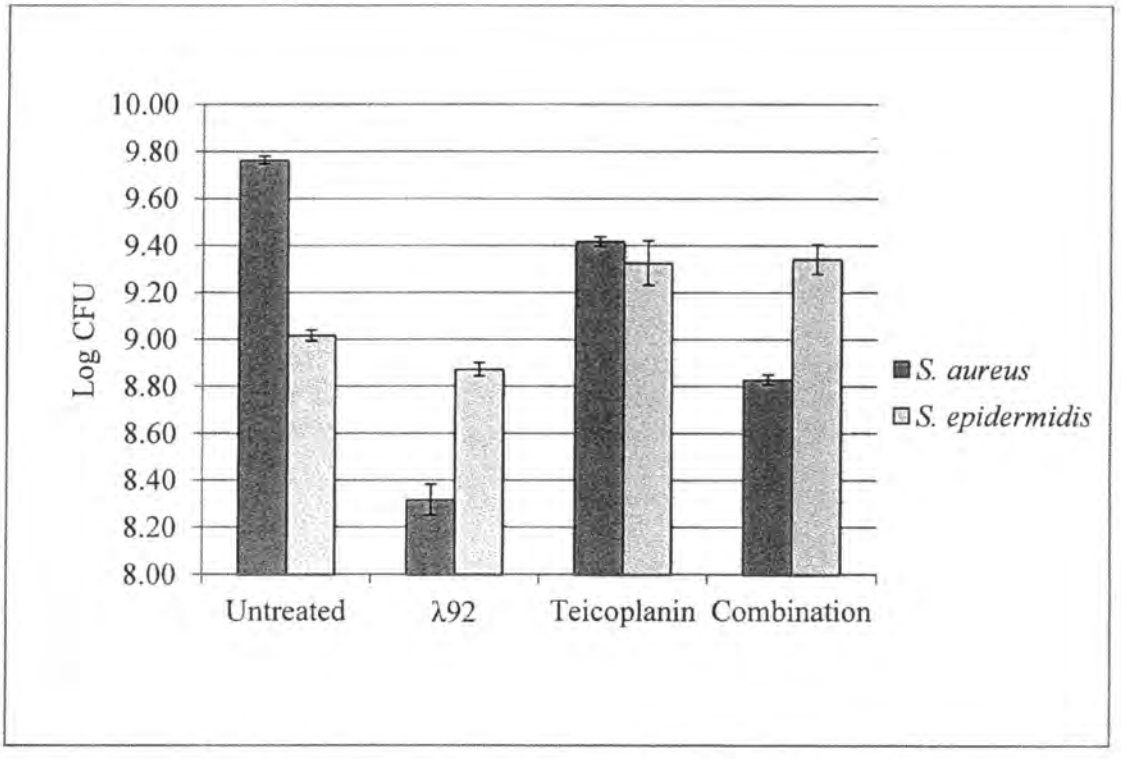

Fig. 6. Effect of λ92 MOI 10, teicoplanin 10 μg mL^{-1}, and a combination on *S. aureus* and *S. epidermidis* mixed-community biofilms at 12 h.

Table 3. *S. epidermidis* zones of inhibition and resistant colony counts from monoculture biofilms and mixed-community biofilms with *S. aureus*.

	0[a]	1[b]	2[b]	3[b]	4[b]	5[b]	6[b]	7[b]	8[b]	9[b]	10[b]
ZOI[c]	26.8	20.6	22.2	21.3	21.9	22.3	22	24.3	24.1	24.2	24.6
Colonies[d]	2	89	84	90	90	90	88	102	92	98	96

[a] *S. epidermidis* from monoculture biofilms

[b] *S. epidermidis* subcultured from mixed-community biofilms

[c] Zones of inhibition in mm^2

[d] Resistant colonies found inside of zones of inhibition

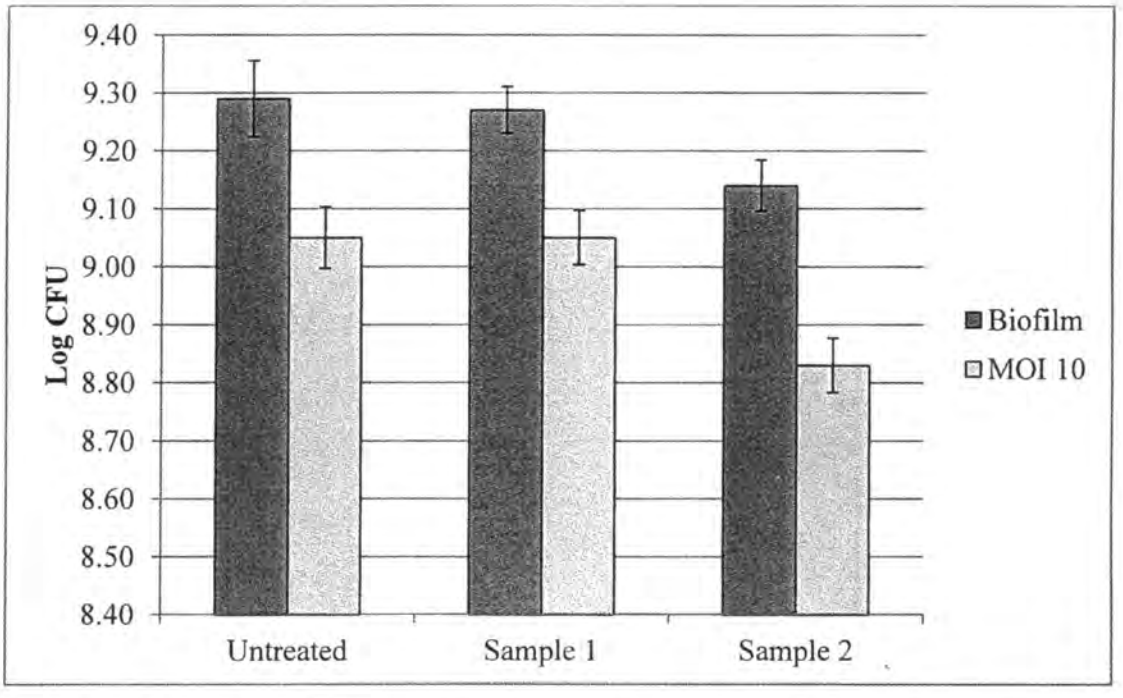

Fig. 7. Effect of λ92 MOI 10 on *S. aureus* biofilms grown with *S. epidermidis* supernatant samples. Sample 1 supernatant prepared from *S. epidermidis* monoculture biofilms. Sample 2 supernatant prepared from *S. epidermidis* from mixed-community biofilms.

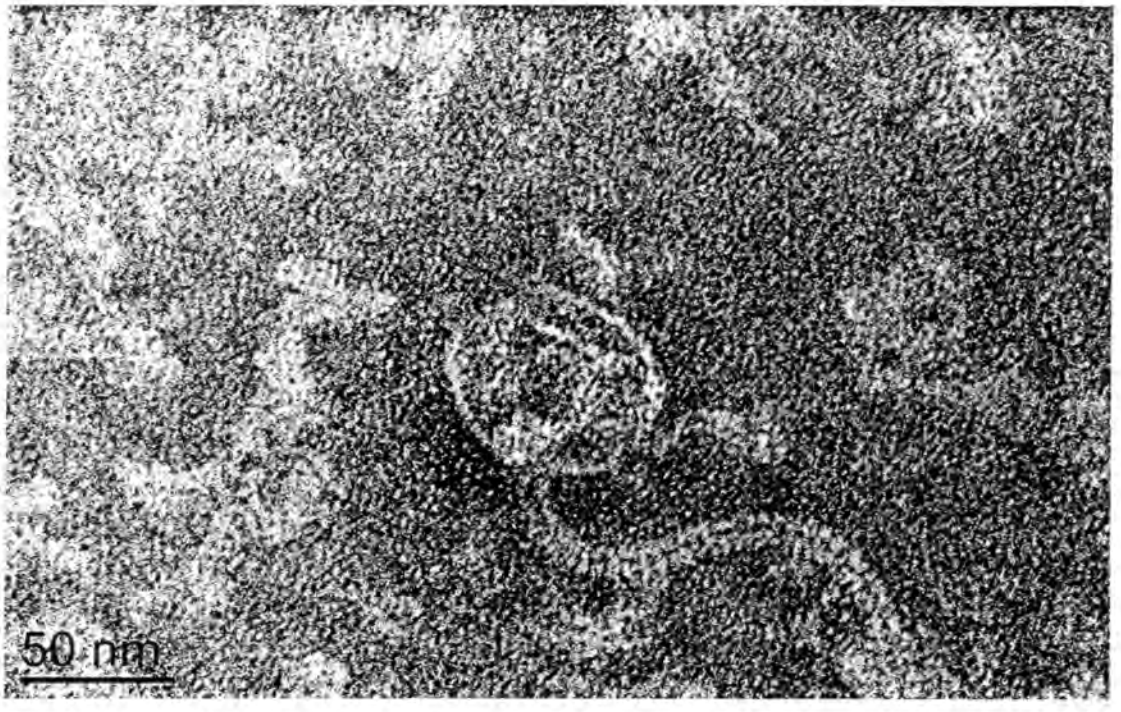

Fig. 8. Transmission electron micrograph of bacteriophage 92.

IV
DISCUSSION

Biofilms are difficult to treat in clinical settings due to increased resistance to antimicrobial agents (19). Considering the remarkable ability of *Staphylococcus aureus* to develop resistance to traditional therapies, comprehensive approaches to treat infections cause by *S. aureus* should be explored, with special attention to its natural environment, including mixed-community biofilms. The purpose of this study was to determine if a combination of bacteriophage with antibiotic is more effective at killing biofilms of methicillin resistant *S. aureus* and *Staphylococcus epidermidis* than either treatment alone. This study found that *S. aureus* and *S. epidermidis* biofilms respond to bacteriophage and antibiotic differently when cultured in mixed-community compared to monoculture biofilms.

The combination of teicoplanin and phage was most effective against *S. aureus* biofilms grown in monoculture (Table 1, Figure 2). Similar results have been observed in studies with *S. aureus* and *Klebsiella pneumonia* (59, 61). A combination of phage and antibiotic would be more effective than either phage or antibiotic alone since phage depolymerase can disrupt biofilm extracellular polymeric matrices (EPM) allowing antibiotics access (65). Also, disruption of EPM would cause an influx of nutrients, thereby increasing the metabolic activity of cells inside the biofilm. Since phage replication is enhanced in metabolically active cells and teicoplanin is effective only against actively growing cells, this would enhance biofilm destruction through increased

susceptibility to phage and teicoplanin (60). Finally, any antibiotic resistant cells present would be susceptible to bacteriophage infection and phage resistant cells would be susceptible to killing by antibiotics (59).

The combination of phage and teicoplanin did not result in increased destruction of *S. epidermidis* monoculture biofilms. Phage 92 is specific for *S. aureus* yet infection resulted in some lysis of *S. epidermidis* biofilms. Teicoplanin alone killed a significantly larger percentage of the *S. epidermidis* biofilm than a combination of phage with teicoplanin (Table 1, Figure 4). It is possible that the environmental stress caused by the action of phage 92 induced an adaptive response by *S. epidermidis,* which increased its tolerance to teicoplanin (66).

This study demonstrated altered susceptibility to phage and teicoplanin in mixed-community biofilms, when compared to the monoculture biofilms. *S. aureus* sensitivity to phage infection increased dramatically in mixed-community biofilms. Bacterial interaction in mixed-community biofilms is highly complex and effects vary considerably, from beneficial to mutually detrimental (67). One explanation for altered susceptibility is that *S. aureus* outcompetes *S. epidermidis* for available space. Approximately seven *S. aureus* colonies were isolated for each *S. epidermidis* biofilm colony recovered (Table 3). This phenomenon has been previously described as "Surface blanketing." "Surface blanketing" was first documented in a mixed-community biofilm where highly motile *Pseudomonas aeruginosa* quickly occupied available space, preventing *Agrobacterium tumefaciens* from initiating adhesion when cultured together (68).

S. epidermidis may also be responsible for the observed increased susceptibility of *S. aureus* to phage infection. *S. epidermidis* has been shown to secrete a serine protease (Esp), which disrupts *S. aureus* biofilm formation *in vivo* (69). Studies have found that the presence of a competing species can alter quorum signaling, which affects biofilm formation (70, 71). In order to elucidate the role of *S. epidermidis* in the increased susceptibility of *S. aureus* to phage infection, *S. aureus* biofilms were grown in the presence of *S. epidermidis* supernatants. The results demonstrated that *S. aureus* biofilms, grown with supernatant from *S. epidermidis* that had no prior exposure to *S. aureus*, grew equally well as *S. aureus* biofilms in normal biofilm growth conditions. Additionally, there was no difference in *S. aureus* phage susceptibility. *S. aureus* biofilms grown with supernatant from *S. epidermidis* cultured from a mixed-community biofilm, however, displayed a decrease in overall biofilm CFUs by 30%. Additionally, *S. aureus* susceptibility to phage increased from 43% to 50% (Figure 7). The decreased growth of *S. aureus* supports a study that determined an *S. epidermidis* serine protease produced in response to *S. aureus* decreased *S. aureus* biofilm elements (69, 72). Another study demonstrated that methicillin resistant *S. aureus* generally uses fibronectin binding protein and the major autolysin for early stage biofilm attachment, as compared to methicillin susceptible *S. aureus* (73). Fibronectin binding protein and the major autolysin may be the affected targets that caused decreased biofilm growth in this study. It is unclear whether *S. epidermidis* secretes a factor that also increases *S. aureus* sensitivity to phage directly, or if the increased susceptibility is a result of degradation of biofilm extracellular polymeric elements that provide a physical barrier for biofilm cells.

S. epidermidis biofilm CFUs in a mixed-community biofilm increased significantly in the presence of teicoplanin when compared to *S. epidermidis* in a mixed-community biofilm under normal growth conditions (Table 2, Figure 6). Glycopeptide resistant strains of bacteria have been shown to transfer resistance to other staphylococcal species (74). *S. aureus* used in this study is not teicoplanin resistant, therefore transfer of glycopeptide resistance would not be likely.

S. epidermidis was subcultured after growth in a *S. aureus* mixed-community biofilm to determine teicoplanin resistance according to Clinical and Laboratory Standards Institute (CLSI) guidelines (75). A decrease in the diameter of a zone of inhibition indicates increased *S. epidermidis* resistance to teicoplanin. Serial subcultures of ten generations of *S. epidermidis* retained increased resistance to teicoplanin. Additionally, the occurrence of teicoplanin-resistant colonies increased dramatically in all serial subcultures (Table 3). This may be similar to a phenomenon described in *S. aureus* clinical cases known as minimum inhibitory concentration (MIC) "Creep." MIC creep occurs in *S. aureus* infections as vancomycin MICs gradually increase over a period of years (76).

In addition to gene transfer and MIC creep, other factors may contribute to increased tolerance to teicoplanin by *S. epidermidis*. Resistance to glycopeptide antibiotics was first observed as a random, spontaneous phenomenon in coagulase-negative staphylococcal (CNS) species (77). Biofilms have been found to enhance tolerance to many different classes of antibiotics, including the glycopeptide antibiotic vancomycin, in *S. epidermidis* (78). Since *S. aureus* is able to outcompete *S. epidermidis* for available space, *S. aureus* may create a physical barrier to teicoplanin. It is also possible that *S.*

aureus cells killed by teicoplanin provide nutrition for *S. epidermidis* cells, as has previously been observed in *S. aureus* biofilms (19).

This study used a sub inhibitory concentration of teicoplanin when compared to concentrations commonly used to treat *S. aureus* infection in the United Kingdom (79). Low concentrations of antibiotic may provide an environment that enhances biofilm formation, as previously described in *S. epidermidis* (80). Use of sub inhibitory concentrations of antibiotics has been shown to act as chemical signals that stimulate biofilm growth in studies with *Pseudomonas aeruginosa* (81, 82). This increase of teicoplanin tolerance in *S. epidermidis* merits future consideration, since teicoplanin-resistant *S. epidermidis* has been recovered from numerous skin infections (83).

Mixed-community biofilms add a layer of complexity in the treatment of disease. This study agrees with previous studies that addition of bacteriophage to antibiotics can enhance pathogenic species biofilm destruction in monoculture biofilms (59, 61). However, this study also demonstrates when *S. aureus* is grown alongside a commensal species in a biofilm, phage is the most effective at biofilm destruction. Additionally, use of antibiotic encourages growth of the commensal species, which appears to attain virulence in the form of increased antibiotic resistance. This study emphasizes the clinical importance of studying different combinations of antibacterial treatments in mixed-community biofilms.

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