Development of Teicoplanin Tolerance by Staphylococcus epidermidis and Increased Susceptibility to Bacteriophage Type 92 by Methicillin-Resistant Staphylococcus aureus in Polymicrobial Biofilms

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Abstract

Staphylococcus epidermidis is a commensal commonly found in polymicrobial biofilms with Staphylococcus aureus. Given the increasing drug resistance in Staphylococci, we explored combination therapy with teicoplanin and bacteriophage type 92 (Siphoviridae) on the survival and antibiotic tolerance of methicillin-resistant S. aureus (MRSA) and S. epidermidis to teicoplanin and bacteriophage in 48-h pure and mixed culture biofilms. The combination of teicoplanin and bacteriophage was more effective against MRSA, but not S. epidermidis monocultures, than the use of either teicoplanin or phage alone. In polymicrobial biofilms, however, MRSA acquired increased susceptibility to phage infection and S. epidermidis acquired increased tolerance to teicoplanin, as well as increased fitness. The results demonstrate bacteriophage alone was more effective against MRSA and S. epidermidis in polymicrobial biofilms compared to the combination of bacteriophage and teicoplanin.

Keywords

Biofilms, Bacteriophage, Antibiotic, Tolerance, MRSA, Mixed culture

Introduction

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 [1,2]. Originally limited to nosocomial, or healthcare-associated infections (HA-MRSA), MRSA has since been identified as a serious public health threat to persons with no affiliation with hospital settings [3]. In the 2000s, MRSA emerged in community settings [4] including USA college campuses associated with clinical programs resulting in the distinction between HA-MRSA and community-acquired MRSA(CA-MRSA) [5]. The gene responsible for methicillin resistance in S. aureus, mecA, is not found on methicillin-susceptible strains of S. aureus [4]. A number of infections are associated with MRSA including those associated with medical devices, soft tissue abscess infections and occasionally more invasive conditions. MRSA infections are often polymicrobial and frequently include Staphylococcus epidermidis [6]. In medical device infections, MRSA often grows as surface-adherent biofilm communities. Biofilm growth results in increased antimicrobial resistance, particularly in a polymicrobial community [7].

Beta-lactam resistant staphylococcal infections are often treated with glycopeptide antibiotics, such as vancomycin and teicoplanin [8]. While similar to vancomycin in bactericidal spectrum, teicoplanin is not currently FDA-approved for use in the United States [8]. Outside the US, several studies have investigated the efficacy and potential side effects of teicoplanin in comparison to other glycopeptide antibiotics including vancomycin [9]. Since staphylococcal species easily attain antibiotic resistance, and some bacteriophages are known to degrade biofilm matrices, attention has refocused on the therapeutic use of bacteriophages [10].

Bacteriophages have been used since the earliest 20th century to treat a number of infections [11,12]. In Europe and North America phage therapy has been largely supplanted by antibiotic therapy [11] although it has still been pursued in the former Soviet Union and other eastern countries [13]. With the increasing resistance of bacterial infections to antibiotics, phage therapy is being reexamined. In this context, examples of experimental strategies include the use of phage cocktails targeting multiple bacterial strains [14] and combination phage-antibiotic therapy [15]. A number of phage depolymerase enzymes have been described, which enable phage to penetrate biofilm matrix polymers [10,16]. While these work well in monoculture biofilms, polymicrobial biofilms have variable matrix chemistries and our previous study [17] has shown matrix polymers from a non-target host organism to be capable of interfering with some phage. Here, we investigate the effectiveness of teicoplanin and bacteriophage combination therapy in monoculture and polymicrobial biofilms of methicillin-resistant S. aureus and methicillin-susceptible S. epidermidis. These two organisms are a frequent cause and commonly co-exist in transcutaneous medical device infections [6,18].

Materials and Methods

Bacterial strains, bacteriophage, media, and culture conditions

Methicillin-resistant Staphylococcus aureus (ATCC 37741),
Staphylococcus epidermidis (ATCC 12228), and Bacteriophage type 92 (ATCC 33741-B) [19] were obtained from the American Type Culture Collection (Manassas, VA). S. aureus and S. epidermidis were grown in Tryptic Soy broth (TSB) (Accuculture Inc., Lansing, Michigan) at 37°C in an orbital rotating shaker water bath. Bacteriophage type 92 was used to infect S. aureus and S. epidermidis. Bacteriophage 92 stocks were prepared by infecting early log phase S. aureus using the agar overlay technique as previously described [20]. Briefly, 350 µL of early log phase S. aureus was added to 3.5 mL of 0.38% (w/v) agar in TSB. 100 µL of 10^6 plaque-forming units (PFU)/ml phage was added to yield confluent lysis. Following 24 h at 37°C, phage was eluted from the agar overlay in 5 mL TSB at 4°C. The phage-containing liquid was centrifuged at 4°C for 20 min at 4000g (Eppendorf Centrifuge model 5810R, Hamburg, Germany). Supernatant was filtered (0.45 µm) and phage titers (PFU/ml) were determined by a soft-agar overlay assay [20]. Teicoplanin (Sigma-Aldrich Co. St Louis, MO) stocks were prepared in deionized water at 10 µg/mL was used for all experiments. During dilution plating of mixed cultures, S. aureus and S. epidermidis could be easily distinguished by growth on mannitol salt agar. A minimum of three biological replicates was performed for each study.

**Biofilm formation**

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

**Biofilm bacteriophage and antibiotic susceptibility assays**

Supernatant was removed from 48 h S. aureus biofilms and biofilms washed with 200 µL PBS. Biofilms were treated with either phage (multiplicity of infection (MOI) 10), teicoplanin (10 µg/mL), or a combination of both contained in 200 µL TSB at 37°C. After 12h treatment, 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 tolerance 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 [21] with TSA substituted for Mueller-Hinton agar. S. epidermidis susceptibility to teicoplanin was tested before and after exposure to S. aureus in polymicrobial biofilms. S. epidermidis recovered from polymicrobial biofilms was sub-cultured nine successive times and each sub-culture 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. Culture supernatant was obtained after 18 h planktonic culture from either S. epidermidis monoculture, or a mixed S. epidermidis – MRSA culture. Supernatants were prepared by centrifugation (3000 g for 20 min) followed by filtration through a 0.45 µm pore-size filter. S. aureus biofilms were washed with 200 µL PBS and infected with phage at MOI 10. Biofilms were enumerated by sonication and dilution plating as described above.

**Transmission electron microscopy (TEM)**

For TEM examination, 5 µL of 10^10 PFU/mL phage were placed on 200 mesh count, Formvar-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 at an accelerating voltage of 60 KV.

**Results**

**Phage type 92 characterization**

Phage type 92 was originally isolated in 1979 from MRSA isolates [19]. TEM examination of phage 92 (Figure 1), revealed icosahedral capsids approximately 62 nm in diameter and long, flexible tails approximately 175 nm in length. On the basis of viral morphology and established taxonomy guidelines [22], phage 92 belongs to the family Siphoviridae.

**Effect of bacteriophage and teicoplanin, on monoculture and mixed culture biofilms**

Growth of S. aureus and S. epidermidis following treatment with phage 92, teicoplanin and combination treatment is shown in Figure 2. We also calculated changes in fitness (expressed as a ratio of (log CFU treated culture/log CFU monoculture control)) which is shown in Figure 3. When compared to untreated controls, the most effective

**Figure 1:** TEM micrograph of Phage 92. Based on the flexible tail and icosahedral head [22], this phage should be classified in the family Siphoviridae.
phenomenon is that phage attack on the host \textit{S. aureus} facilitated competition from \textit{S. epidermidis}, which was minimally affected by the phage during monoculture growth. Of interest, growth in mixed culture resulted in less impact of teicoplanin against MRSA alone or in combination with phage 92.

In monoculture \textit{S. epidermidis} cultures, the most effective treatment was teicoplanin. This is not surprising as teicoplanin has been reported to be effective against this organism [9]. In contrast, mixed culture results were quite different. In untreated mixed-culture biofilms, MRSA outcompeted \textit{S. epidermidis} which is reflected both in culture numbers (Figure 2 control) and fitness calculations (Figure 3 control). Although MRSA is the host organism for phage 92, there was a slight decrease in \textit{S. epidermidis} numbers and fitness (P < 0.001) relative to the mixed culture control, suggesting that some phage influence or competition from MRSA was occurring in the presence of phage. In contrast to the reduced fitness seen in \textit{S. epidermidis} monocultures with teicoplanin alone or in combination with phage 92; the fitness of \textit{S. epidermidis} was significantly increased in mixed culture (P < 0.001 in comparison to monoculture results) when grown in mixed culture. We also observed that repeated subculture of \textit{S. epidermidis} with MRSA decreased teicoplanin susceptibility and the onset of resistance (Figure 4).

**Discussion**

The decreased antibiotic susceptibility of monoculture biofilms has been known for over 30 years [23] and a number of mechanisms for biofilm-mediated resistance have been described including altered growth rates within biofilms, biofilm-specific resistance genes and the presence of dormant persister subpopulations (reviewed in [24,25]). Biofilms including MRSA wound infections [6] typically contain polymicrobial communities and there is increasing interest in studying antimicrobial susceptibility in that context. Two studies showed that increased bacterial community diversity resulted in altered growth rates within biofilms, biofilm-specific resistance genes and the presence of dormant persister subpopulations (reviewed in [24,25]). Biofilms including MRSA wound infections [6] typically contain polymicrobial communities and there is increasing interest in studying antimicrobial susceptibility in that context. Two studies showed that increased bacterial community diversity resulted in altered growth rates within biofilms, biofilm-specific resistance genes and the presence of dormant persister subpopulations (reviewed in [24,25]).

In monoculture, MRSA biofilm cultures was combination phage and teicoplanin. Since phage require growing host cells in order to replicate, the enhanced success of the combination treatment suggests that teicoplanin did not completely block \textit{S. aureus} growth when added in combination with phage 92. Different results were seen during treatment of mixed culture \textit{S. aureus}. Here, the most effective treatment was phage alone. One likely explanation for this
with phase 92. The increased fitness of both MRSA and S. epidermidis during teicoplanin treatment in comparison to the monoculture results (P < 0.001) suggests that the two normally competitive organisms, exhibit synergy (facilitation) in the presence of teicoplanin. In previous work with environmental isolates, we found that the presence of one disinfectant (betadine) resistant organism could confer resistance to an entire mixed biofilm population [27] likely through facilitation, so this finding is not unprecedented.

In planktonic monoculture or mixed Pseudomonas aeruginosa and Escherichia coli cultures, we observed that the administration of sub-lethal concentrations of antibiotics or phage is followed rapidly by the onset of resistance through the planktonic populations likely through mutation and natural selection. In contrast, while biofilm populations of these organisms were protected by this mode of growth, a significant proportion of organisms within biofilms retained susceptibility to phage or antibiotics when released from biofilms by sonication, diluted and cultured on antibiotic- or phage-containing agar [15,17]. We tested the influence of previous co-culture growth on the teicoplanin susceptibility of S. epidermidis using a disk diffusion assay. We also measured the number of spontaneous teicoplanin-resistant colonies which grew within the zone of inhibition. During these studies, S. epidermidis was sub-cultured overnight up to ten times with MRSA and then purified and tested. As seen in Figure 4, there was a slight but significant (P < 0.01) decrease of susceptibility following the first sub-culture. In subsequent subcultures, while there was some variability in the zone of inhibition, it was still significantly less than the original culture (designated as subculture 0) before mixed culture growth. Similarly, there was an increase in the number of resistant colonies seen after the first sub-culture with the numbers increasing from 4 to > 90 in most cases. Although S. aureus (ATCC 37741) is resistant to methicillin, it is sensitive to teicoplanin, which would rule out horizontal gene transfer of a pre-existing resistance gene to S. epidermidis. Although we do not have a direct explanation for this phenomenon, it is possible that mixed culture growth of S. epidermidis with S. aureus activated mechanisms that enabled S. epidermidis to better tolerate exposure to teicoplanin. Further work will be needed to elucidate the mechanism(s) behind this phenomenon.

Conclusions

Many S. aureus infections involve polymicrobial interactions with S. epidermidis and biofilm growth [6]. In the current study, interactions between S. aureus and S. epidermidis strains used affected the efficacy of teicoplanin antibiotic and phage treatment. In some cases, when one organism was targeted (MRSA specific phase 92 in mixed culture), mixed culture interactions enhanced the treatment due to microbial competition, whereas in other cases in which both organisms were targeted by teicoplanin, the efficacy was reduced possibly due to facilitation interactions. Overall these data emphasize the importance of studying mechanisms of antimicrobial treatments in a polymicrobial biofilm growth system.

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Conflict of Interest

The authors declare no conflict of interest.

References