

## Original Article

# Adverse Effect of Staphylococci Slime on In Vitro Activity of Glycopeptides

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**SUMMARY:** Adhesion to biomaterial is assumed to be a crucial step in the development of staphylococcal foreign body infections. Production of extracellular slime has major implications for the development and implementation of therapeutic strategies. The effect of extracted slime was investigated on the activity of vancomycin, teicoplanin, linezolid, quinupristin/dalfopristin, rifampicin and ranbezolid against 10 clinical and 4 ATCC staphylococcal isolates. The slime extract caused a 2- to 16-fold increase in the MICs of vancomycin and teicoplanin, with a shift in the MIC<sub>90</sub> from 2 to 32 (vancomycin) and 2 to 16 (teicoplanin), whereas the MICs of linezolid and quinupristin/dalfopristin were only moderately affected. In time-kill studies, a significant decrease in bacterial killing (>3 log<sub>10</sub> cfu/ml) was observed with vancomycin and teicoplanin (4 × MIC) after addition of slime (5 and 20 mg/ml), whereas the effect of killing by linezolid and quinupristin/dalfopristin was very modest. The rifampicin and ranbezolid MICs and kill curves were not influenced by the addition of slime. The present study thus indicated that slime interferes with the antimicrobial effect of glycopeptide drugs (vancomycin, teicoplanin), and that for effective prevention and treatment of prosthetic device-related infections, appropriate and newer antibiotics such as ranbezolid should be considered.

## INTRODUCTION

Nosocomial infections that result in the formation of biofilms on the surface of prosthetic medical devices are a leading cause of sepsis and are often associated with colonization of the devices by staphylococci (1). Slime consolidates the bacterial adhesion and produces biofilm through a complex procedure (2). Subsequent transformation into invasive infection contributes significantly to morbidity and complications with the underlying illness for which these devices are used (3).

The mechanism of resistance of biofilm-forming staphylococci to chemotherapeutic agents is still unclear (4,5). Few reports have indicated that slime physically complexes and acts as a diffusion barrier and also contains polysaccharide that may interfere with the antimicrobial agents (6). Therefore, the standard susceptibility in vitro tests are often not predictive of the efficacy of antibiotics in treating prosthetic device-related infections (7). Ranbezolid (RBx 7644), an investigational oxazolidinone, has shown excellent activity against Gram-positive pathogens involved in prosthetic device-related infections (8,9). The present study was designed to investigate the in vitro effect of extracted slime on vancomycin, teicoplanin, linezolid, quinupristin/dalfopristin, rifampicin and ranbezolid.

## MATERIALS AND METHODS

**Bacterial isolates:** Slime producing (*Staphylococcus*

*epidermidis* ATCC 35984 and ATCC 35983), non-slime producing (*S. epidermidis* ATCC 12228 and *Staphylococcus aureus* ATCC 25923) and clinical isolates of *S. aureus* ( $n = 5$ ) and *S. epidermidis* ( $n = 5$ ) from patients with catheter-related blood stream infections were selected on the basis of their adherence properties. The isolates were tested for production of slime by a quantitative method as previously described (10).

**Preparation of slime extract:** The crude slime culture filtrate was prepared from a biofilm-positive strain of *S. epidermidis*, ATCC 35984, as per the procedure by Hussain and Hastings (11). Twenty-five microliters of bacterial suspension in normal saline ( $1 \times 10^9$  cfu/ml) was spread evenly on sterile membrane filters (diameter, 47 mm; porosity, 0.45  $\mu$ m; Millipore, Carrigtwohill, Ireland) placed on Trypticase Soya agar plates (Difco, Detroit, Mich., USA). After incubation at 37°C for 24 h, each filter was washed twice with phosphate buffer saline (PBS) of pH 7.2 to remove the non-adherent bacteria. Filters were placed in a flask containing 10 ml of normal saline and were blended properly. The solution was then centrifuged (Sorval, super T 21) at 4,000 g for 30 min at 4°C, and the supernatant was pooled and passed through a 0.25  $\mu$ m filter (Millipore). The supernatant was then concentrated in an ultrafiltration cell (Millipore) having a membrane with a molecular mass cutoff of 10 kDa, and freeze dried. The powder was reconstituted in distilled water to a concentration of 50 mg/ml.

**Antimicrobial agents:** Vancomycin, teicoplanin, linezolid, quinupristin/dalfopristin, rifampicin (commercial source) and ranbezolid (Ranbaxy Research Laboratories, Gurgaon, India) were dissolved in distilled water/buffer or the recommended solvent to yield a concentration of 1 mg/ml stock.

**Determination of MIC:** MICs were determined against 10 clinical staphylococcal isolates using MHB alone or MHB supplemented with extracted slime at a final concentration of

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0.5 or 2% (5 or 20 mg/ml) by the microbroth dilution method as per the NCCLS guidelines (12). Serial 2-fold dilutions of antibiotics were prepared and inoculum ( $10^6$  cfu/ml) was added. *S. aureus* ATCC 25923 was included in the study as a quality control. Each experiment was repeated twice to confirm the results. The MIC was defined as the lowest concentration of each antibiotic that inhibited visible growth of the organisms.

**Time-kill studies:** Time-kill kinetics experiments of vancomycin, teicoplanin, linezolid, quinupristin/dalfopristin, rifampicin and ranbezolid were performed in MHB alone or MHB supplemented with slime 0.5 or 2% (5 or 20 mg/ml) against slime-producing *S. epidermidis* ATCC 35984 and two clinical isolates, *S. epidermidis* RB 78 and *S. aureus* P 1500.

Growing cultures were added to medium and were exposed to  $4 \times$  MIC concentrations of drugs. Drug-free inoculated medium was also plated as a growth control. Samples were removed for colony counts at 0, 4, 8 and 24 h. Viable counts were determined by the serial dilution method. Plates were incubated for 24 h at  $35^\circ\text{C} \pm 2^\circ\text{C}$  and colonies were counted. Each experiment was repeated twice to confirm the results. Cfu/ml was plotted against time for each strain.

**Statistical analysis:** MIC values were analyzed by WHONET software (version 5.3), and the MIC<sub>50</sub>, MIC<sub>90</sub> and geometric mean were determined.

## RESULTS

**Slime production:** The selected bacteria showed different

levels of slime production, as observed by their different levels of adherence to microtiter plates, and the mean OD<sub>570nm</sub> values for *S. epidermidis* ATCC 35984 and ATCC 35983 were  $2.92 \pm 0.035$  and  $0.456 \pm 0.018$ , respectively. The clinical isolates *S. epidermidis* RB 78 and RB 76 and *S. aureus* O 128 and P 76 were low slime producers, whereas *S. epidermidis* RB 70, RB 81 and P 2076 and *S. aureus* P 1500, PC 85 and PC 81 were classified as high slime producers (Table 1).

**Effect of slime extract on MICs:** The MICs of the tested antibiotics against staphylococcal isolates are presented in

Table 1. Slime production by *S. aureus* and *S. epidermidis* isolates

Isolates	Optical density (OD <sub>570nm</sub> ) (Mean $\pm$ SD)
<i>S. epidermidis</i> RB 78	0.020 $\pm$ 0.006
<i>S. epidermidis</i> RB 70	1.769 $\pm$ 0.02
<i>S. epidermidis</i> RB 81	1.844 $\pm$ 0.026
<i>S. epidermidis</i> RB 76	0.344 $\pm$ 0.016
<i>S. epidermidis</i> P 2076	2.18 $\pm$ 0.019
<i>S. aureus</i> O128	0.065 $\pm$ 0.010
<i>S. aureus</i> P 1500	0.822 $\pm$ 0.012
<i>S. aureus</i> PC 85	2.46 $\pm$ 0.035
<i>S. aureus</i> PC 81	1.053 $\pm$ 0.022
<i>S. aureus</i> P 76	0.020 $\pm$ 0.016
<i>S. epidermidis</i> ATCC 35984	2.92 $\pm$ 0.035
<i>S. epidermidis</i> ATCC 35983	0.456 $\pm$ 0.018
<i>S. epidermidis</i> ATCC 12228	0.05 $\pm$ 0.016
<i>S. aureus</i> ATCC 25923	0.054 $\pm$ 0.010

Table 2. Effect of extracted slime (SE) on MICs of antimicrobial agents against staphylococcal isolates

Isolates	Vancomycin			Teicoplanin			Linezolid			Quinupristin/ dalfopristin			Rifampicin			Ranbezolid		
	SE (mg/ml)			SE (mg/ml)			SE (mg/ml)			SE (mg/ml)			SE (mg/ml)					
	0	5	20	0	5	20	0	5	20	0	5	20	0	5	20	0	5	20
<i>S. epidermidis</i> RB 78	2	4	8	1	4	8	2	2	4	0.06	0.125	0.5	0.03	0.03	0.03	0.06	0.06	0.06
<i>S. epidermidis</i> RB 70	1	8	16	1	4	8	1	1	2	0.125	0.125	0.25	>16	>16	>16	1	1	1
<i>S. epidermidis</i> RB 81	2	4	8	2	8	16	2	2	4	0.125	0.125	0.25	0.03	0.06	0.06	0.25	0.25	0.5
<i>S. epidermidis</i> RB 76	2	4	16	2	8	16	2	2	4	0.125	0.25	1	0.5	0.5	0.5	1	1	1
<i>S. epidermidis</i> P 2076	2	4	16	1	8	8	1	1	4	0.125	0.25	0.25	0.06	0.06	0.125	0.25	0.25	0.5
<i>S. aureus</i> O 128	2	8	32	1	4	8	2	2	2	0.25	0.25	0.5	0.125	0.125	0.25	0.25	0.25	0.5
<i>S. aureus</i> P 1500	1	4	8	0.25	2	4	1	2	4	0.25	0.5	1	0.03	0.03	0.03	1	1	1
<i>S. aureus</i> PC 85	1	4	8	2	4	8	2	2	4	0.25	0.5	2	>16	>16	>16	1	1	1
<i>S. aureus</i> PC 81	1	8	16	2	4	16	1	2	4	0.25	0.25	2	>16	>16	>16	1	1	2
<i>S. aureus</i> P 76	1	4	8	1	4	8	4	8	8	1	0.5	2	16	16	16	2	2	2
<i>S. epidermidis</i> ATCC 35984	2	8	32	2	4	16	2	4	8	0.25	0.5	1	0.03	0.03	0.06	0.25	0.5	0.5
<i>S. epidermidis</i> ATCC 35983	2	8	16	2	4	32	2	2	4	0.25	0.5	1	0.03	0.03	0.03	0.5	0.5	0.5
<i>S. epidermidis</i> ATCC 12228	1	8	16	1	8	16	2	4	8	0.25	1	1	0.03	0.03	0.03	0.5	0.5	0.5
<i>S. aureus</i> ATCC 25923	4	8	16	0.5	1	2	4	4	8	0.25	0.5	0.5	<0.03	0.03	0.03	2	2	2

Table 3. WHONET analysis to determine the effect of extracted slime (SE) at 5 and 20 mg/ml concentration on MICs of antimicrobial agents against staphylococcal isolates

Antibiotics	MIC <sub>50</sub>			MIC <sub>90</sub>			Geometrical mean		
	SE (mg/ml)			SE (mg/ml)			SE (mg/ml)		
	0	5	20	0	5	20	0	5	20
Vancomycin	2	4	16	2	8	32	1.56	5.65	13.79
Teicoplanin	1	4	8	2	8	16	1.16	4.20	9.75
Linezolid	2	4	2	4	8	4	1.81	2.32	4.41
Quinupristin/ dalfopristin	0.25	0.25	1	0.25	0.5	2	0.20	0.32	0.743
Rifampicin	0.03	0.06	0.06	32	32	32	0.283	0.313	0.364
Ranbezolid	0.5	0.5	0.5	2	2	2	0.55	0.578	0.705

Table 2. All isolates (high and low slime-producing staphylococci) showed an increase in MICs (2- to 8-fold) to vancomycin and teicoplanin with addition of the extracted slime even at 5 mg/ml. In the presence of slime (20 mg/ml) there was a 4- to 16-fold increase in MICs for vancomycin and teicoplanin, and the MIC<sub>90</sub> shifted from 2.0 to 32 (vancomycin) or 2.0 to

16 (teicoplanin) (Table 3, Fig. 1). There was a linear increase in MICs of vancomycin and teicoplanin detected with increased slime concentration against all tested isolates, whereas only a moderate increase in MICs (1- to 2-fold) was seen when slime was added to quinupristin/dalfopristin and linezolid.

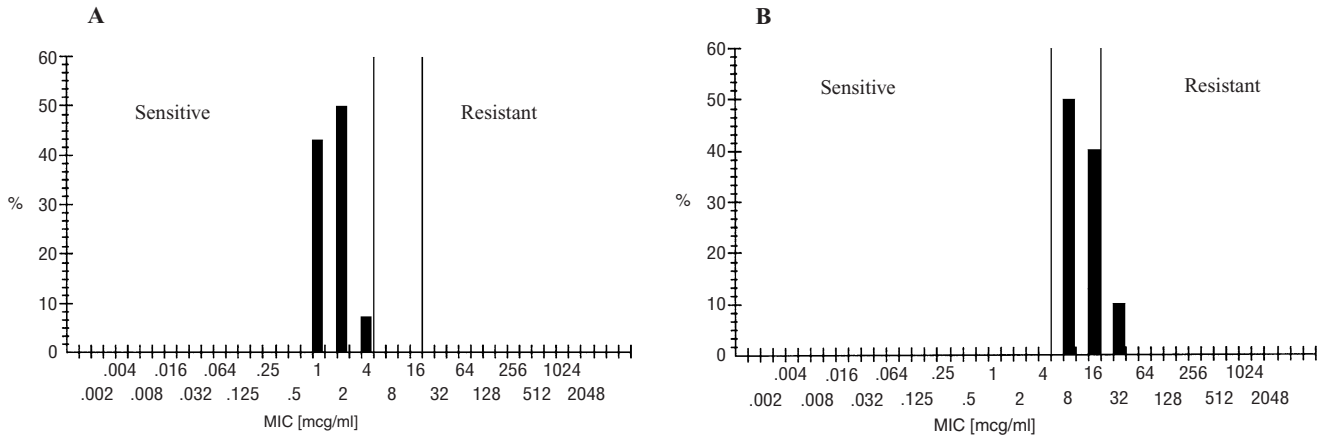


Fig. 1. Histogram of vancomycin in the absence and presence of slime extract (20 mg/ml). Horizontal thin lines depict vancomycin breakpoints against staphylococci ( $\leq 4$   $\mu\text{g/ml}$ , sensitive; 4-16  $\mu\text{g/ml}$ , intermediate; and  $\geq 32$   $\mu\text{g/ml}$ , resistant). (A) Vancomycin alone. MIC of vancomycin was 1-4  $\mu\text{g/ml}$ . (B) Vancomycin with slime. 4- to 16- fold increase in MIC (8-32  $\mu\text{g/ml}$ ), which was near to breakpoint for resistant staphylococci.

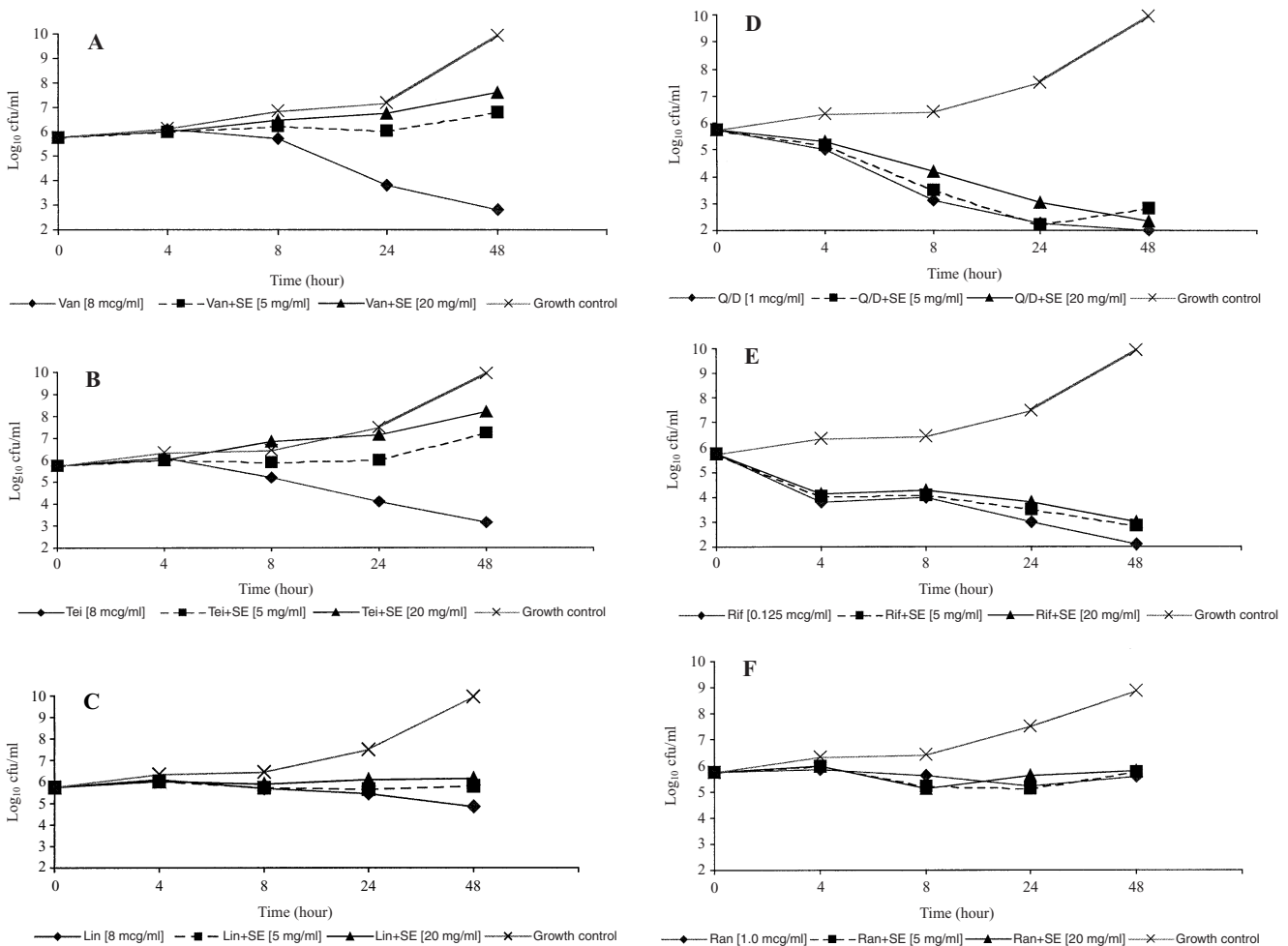


Fig. 2. Time-kill curve of *S. epidermidis* ATCC 35984 in presence of slime extract (5 and 20 mg/ml) and absence of slime. (A) Vancomycin (Van) 8  $\mu\text{g/ml}$ . (B) Teicoplanin (Tei) 8  $\mu\text{g/ml}$ . (C) Linezolid (Lin) 8  $\mu\text{g/ml}$ . (D) Quinupristin/dalfopristin (Q/D) 1  $\mu\text{g/ml}$ . (E) Rifampicin (Rif) 0.125  $\mu\text{g/ml}$ . (F) Ranbezolid (Ran) 1  $\mu\text{g/ml}$ .

The MICs of ranbezolid and rifampicin were not affected by the presence of slime, with the MIC<sub>90</sub> remaining at 2 and 32, respectively, with or without slime. The MICs for the QC strain *S. aureus* ATCC 25923 were in the range as per NCCLS guidelines.

**Effect of slime extract on kill kinetics:** In the kill kinetics experiment, vancomycin and teicoplanin showed a gradual decrease in bacterial killing against all the isolates in the presence of slime (5 and 20 mg/ml). This inhibition of the antibiotic effect was more pronounced and a reduction in killing ( $>3 \log_{10}$  cfu/ml) was observed after 12 h of incubation against *S. epidermidis* ATCC 35984 and the two clinical isolates *S. epidermidis* RB 78 and *S. aureus* P 1500, compared to drugs tested without slime extract. A linear relationship was observed between the concentration of extract and time in the presence of vancomycin. Bacterial killing by linezolid and quinupristin/dalfopristin were moderately affected ( $<1 \log_{10}$  cfu/ml) in the presence of slime against all three isolates, whereas rifampicin and ranbezolid activity remained unchanged (Fig. 2).

## DISCUSSION

Production of slime is characteristic of many strains of *S. epidermidis* and *S. aureus*. Transmission electron microscopic examination of antibody-stabilized biofilm preparation revealed that the exopolymeric matrix appears as fine fibers providing relatively thick, hydrated coatings around the cells (13). The ability to form a biofilm on the surface of a prosthetic device is probably a significant determinant of virulence for these staphylococci (14). All medical devices are made of synthetic material and are true foreign bodies; slime-producing staphylococci colonize them, forming sessile multicellular biofilm communities (4). Existing drugs are ineffective in treating isolates involved in prosthetic device-related infections (15,16). Vancomycin has emerged as a drug of choice in treating patients with methicillin-resistant staphylococci. However, resistance to vancomycin has been reported against slime-producing staphylococci (17,18). There have been at least two reports indicating that slime can interfere with drug activity; however, the mechanism of resistance is yet to be determined (19,20). We isolated the slime of a biofilm-producing strain by growing the strains on a membrane filter placed over solid media, to avoid addition of carbohydrate from the basal media. Further, slime was concentrated by ultrafiltration to obtain an extract with polysaccharide. Even at a concentration of 5 mg/ml, this slime extract exerted an antagonistic effect on vancomycin and teicoplanin, and a 2-8 fold increase in MICs was observed. Addition of 20 mg/ml slime extract resulted in a significant 4-16 fold increase in the MICs, which was near to the breakpoint for resistant staphylococci for vancomycin and teicoplanin. The activity of vancomycin and teicoplanin was deteriorated against both high and low slime-producing staphylococci. This could explain why these drugs are not effective against slime-producing bacteria involved in prosthetic device-related infections.

Reduced efficacy was also observed in the time-kill studies, where vancomycin and teicoplanin at  $4 \times$  MIC levels were unable to reduce the bacterial count in the presence of slime, and a significant decrease in bacterial killing ( $>3 \log_{10}$  cfu/ml) was observed, compared to drugs tested without slime extract. A linear relationship was observed between the slime concentration and bacterial killing. These results were in

accordance with the previous reports.

Slime moderately affected the activity of linezolid and quinupristin/dalfopristin and only a marginal decrease in the killing ( $<1 \log$  cfu/ml) of bacteria was observed, compared to drugs tested without slime. For rifampicin and ranbezolid, the MICs and kill kinetics were not influenced by the addition of slime.

The mechanism by which the extract interferes with the antimicrobial action of vancomycin remains to be elucidated. The mechanisms that have been proposed are that the glycoprotein/polysaccharide physically complexes with vancomycin and renders it biologically inactive, or that polysaccharides coat the cell wall and either serve as a barrier to vancomycin penetration because of their high molecular weight or interfere with its action on the cell wall itself (19,20). However, two reports have argued against the presence of an antibiotic diffusion barrier in biofilms (21,22). In our study, slime extract significantly reduced the antimicrobial effect of vancomycin and teicoplanin, whereas linezolid and quinupristin/dalfopristin activity were only modestly affected. The activity of rifampicin and ranbezolid were not affected by the presence of slime, and thus might be considered useful in treating prosthetic device-related infections. However, the use of rifampicin and quinupristin/dalfopristin cannot be recommended because of the rapid generation of resistance and occurrence of MLS<sub>B</sub> resistance among staphylococci (23,24).

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