Original article

Differential susceptibility of Staphylococcus epidermidis biofilms to vancomycin, daptomycin and linezolid between clinical isolates from neonates and adults

Maria Simitsopoulou, Paschalis Kadiltzoglou, Daniela Kyrpitzi and Emmanuel Roilides*

*Infectious Diseases Unit, 3rd Department of Pediatrics, Aristotle University School of Health Sciences, Hippokration Hospital, 54642 Thessaloniki, Greece

**A B S T R A C T**

This study aimed to compare the effects of vancomycin, linezolid and daptomycin against planktonic cells and biofilms of 32 bloodstream isolates derived from neonates and adults of three hospitals. Staphylococcus epidermidis biofilm formation was spectrophotometrically assessed following safranin staining. Susceptibility testing of planktonic and biofilm cells was performed by XTT reduction assay. MICs of vancomycin and daptomycin were 1 mg/L and that of linezolid 0.5 mg/L. At concentrations > 0.5 mg/L, complete eradication of planktonic cells was effected by all three antibiotics. The biofilm MICs for the three antibiotics were 3-7 twofold dilutions higher than the corresponding planktonic MICs (P<0.05). Vancomycin and linezolid exhibited similar median MICs against biofilms of neonatal isolates ranging from 16 to 32 mg/L with comparable damage (44%-84% for vancomycin vs 56%-77% for linezolid). Their MICs against biofilms of isolates from adults were 128 mg/L and 4 mg/L, respectively (P<0.001). Vancomycin showed lower MIC than daptomycin against biofilms of neonatal isolates (16 mg/L vs 64 mg/L, respectively; P<0.05). Biofilm MIC of linezolid was lower than the corresponding daptomycin MIC for both age groups (neonatal isolates: 16 mg/L vs 64 mg/L; adult isolates: 4 mg/L vs 64 mg/L, P<0.05). VAN and linezolid are equally effective against biofilms of neonatal blood isolates and both exhibit superior activity to daptomycin. In the adult group, linezolid is more efficacious than both vancomycin and daptomycin against biofilms, while vancomycin and linezolid exhibit similar anti-biofilm activities. Our results suggest that vancomycin and linezolid show better anti-biofilm activity than daptomycin against biofilms of neonates and linezolid have increased activity against biofilms of adults.

**Keywords:**

drug therapy, planktonic cells, biofilm formation, adhesins

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**Introduction**

Neonates in Neonatal Intensive Care Units (NICUs) are at high risk for developing central line-associated bloodstream infections (CLABSIs). Coagulase-negative staphylococci (CoNS), mostly Staphylococcus epidermidis, are the most frequent pathogens causing late-onset sepsis and device-associated infections in these settings. S. epidermidis, a common organism of the commensal microflora of human skin and mucous surfaces, has been recognized as a frequent nosocomial pathogen associated with infections of implanted medical devices particularly in critically ill and/or immunodeficient populations. The ability of the organism to adhere to prostheses and in trayvavenous devices and form biofilms, thereby diminishing the effect of host defense and drug therapy, plays significant role to its pathogenicity profile. Biofilm formation involves adherence and colonization of a surface, cell proliferation and accumulation in multilayers through production and excretion of polysaccharide intercellular adhesins. The produced extracellular matrix not only promotes biofilm development but also functions as a virulence factor, which hinders antibiotic penetration and effectiveness.

Vancomycin (VAN) has been historically the recommended glycopeptide antibiotic used as the initial choice of therapy of infections due to CoNs. More recently, it has been replaced at some degree by the newer antimicrobial agents of daptomycin (DAP) and linezolid (LIN), which are active against a range of Gram-positive organisms, including multi-drug resistant strains. Since VAN remains important for the treatment of catheter-related bloodstream infections caused by staphylococci, it is imperative to gain concrete knowledge about the activity profile of VAN compared to alternative antistaphylococcal antibiotics against staphylococcal biofilms in order to define significant shifts that will contribute to proper therapeutic guidance. Another consideration that points to the need for urgent alternative therapeutic regiments for successful in situ treatment of CoNs, is that most of the time removal of a central line, especially among the neonates who possess a relatively immature immune system, entails possible source of reinfection which may lead to an unfavorable outcome.
Accordingly, our aim in the present study was twofold: i) to compare the antibacterial activities of VAN, DAP and LIN against planktonic cells and established mature biofilms formed by S. epidermidis clinical strains isolated from blood of neonates and adults and ii) to investigate whether particular susceptibility trends to VAN, DAP or LIN exist between the isolates from the two age groups, neonates and adults. The results of this study may help optimizing the antibacterial therapy of these infections in neonates.

2. MATERIALS AND METHODS

2.1 Clinical isolates and growth conditions

We studied 32 clinical bloodstream isolates of S. epidermidis recovered from patients of three collaborating Institutions: The Hippokration and Papageorgiou General Hospitals of Thessaloniki, Greece and the University of Liverpool, UK between 2014 and 2015. The isolates collected from each hospital in Greece and in UK did not originate from cases of an epidemiological outbreak. CoNs bacteremia was defined as two positive blood cultures within 3 days of each other.

Twenty-one strains were isolated from similar number of late onset sepsis episodes in neonates and 11 strains were collected from sepsis episodes in adults. Identification of these isolates was performed using the Vitek II system or the API ID32C (both from bioMerieux, Marcy l’Étoile, France) according to the manufacturer’s instructions. Stocks were maintained at -30°C in 80% glycerol solution. Clinical isolates were revived after overnight incubation at 37°C on Mueller Hinton Agar plates (AppliChem, GmbH; Darmstadt, Germany). A loopful of each isolate was then transferred to 10 mL of Mueller Hinton Broth (AppliChem) supplemented with 0.25% glucose and incubated at 37°C for 2 h on a rocking table. The concentration of each grown culture was determined by measuring the optical density with the McFarland densitometer DEN-1 (1 McF=3x10^8organisms/mL).

2.2 Biofilm formation and quantification

Mature S. epidermidis biofilms (BF) were formed using 96-well microtiter polystyrene plates as previously described with minor modifications. Briefly, 200 μL of 106 bacteria/mL in Trypticase Soy Broth (TSB; AppliChem) were used to inoculate flat bottom 96-well sterile microtiter plates. After a 24h-incubation at 37°C, the plates were washed two times with phosphate-buffered saline solution (PBS: 0.02 M phosphate, 0.15 M NaCl; pH 7.2) and stained with 1% (w/v) safranin solution for 1 min. Excess stain was removed by two washes with distilled water and the plates were left upside down to air dry. BF formation was evaluated spectrophotometrically at OD 492 nm with a microplate reader (Chromate 4300; Palm City, FL). The following arbitrary classification was used to evaluate the extent of biofilm formation: if an isolate had an OD of <0.100, it was classified as non-BF producer; if the OD was > 0.100 and ≤ 0.280, the isolate was classified as an intermediate BF producer, whereas if the OD was >0.280, the isolate was classified as strong BF producer. The value of 0.100 was chosen as the lower cut-off value defined as 3 standard deviations above the mean OD value of control wells that contained 1% safranin without the organism (negative control). The value of 0.280 was determined as the upper cut-off value defined as 2x the lower cutoff OD value.

2.3 Antimicrobial agents

Vancomycin (VAN) was obtained from Sigma-Aldrich Co. (Athens, Greece); daptomycin (DAP) from Merck and Co. Inc. (Whitehouse Station, NJ); and linezolid (LIN) from Pfizer Inc. (New York, NY). The stock solutions for VAN, DAP and LIN (2 mg/mL, each) were maintained at -35°C for up to one month. A portion from each antibiotic stock was further diluted to 1.024 mg/L in TSB and used to prepare a series of twofold dilutions ranging from 0.007-256 mg/L.

2.4 Antibiotic treatment and assessment of biofilm damage

One hundred microliters of the above indicated serially double-strength twofold diluted concentrations of each drug were added to corresponding microtiter wells containing 100 μL of 24-hour mature biofilms (106 bacteria/mL) or planktonic cells (5x10^5 bacteria/mL) and the plates were incubated at 37°C for an additional 24 h. For controls, biofilms or planktonic cells were incubated in the presence of 200 μL of TSB without antibiotics under otherwise identical conditions. The in vitro activity of each drug against biofilms or planktonic cells was assessed by using an XTT [2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide]-reduction assay as described previously. Antibiotic activity was calculated according to the following formula: percent bacterial damage = (1-X/C) x 100, where X is the absorbance of experimental wells and C is the absorbance of control wells. The minimum inhibitory concentrations (MICs) for biofilms and planktonic cells were determined as the minimum antibacterial concentration that caused ≥50% bacterial damage compared to the untreated controls. MICs for planktonic cells were also determined by broth microdilution according to EUCAST guidelines. Planktonic MIC values obtained by EUCAST and XTT methods were in good agreement with either test employed. To prove soundness of statistical analysis, differences in bacterial damage between planktonic and biofilm cells were analyzed with quantitative results obtained by the XTT assay.

2.5 Statistical analysis

Each concentration of VAN, DAP and LIN for every clinical isolate was tested in pentaplicate and each untreated control was tested in sixteen replicates per experiment. The average values of these replicates were used in the data analysis to determine mean ± SE for each condition. All isolates were studied three times on independent experiments. The differences in biofilm formation between the control strain ATCC 35984 and S. epidermidis isolates of neonates and adults and the differences in the bacterial damage between planktonic cells and biofilms of the isolates were assessed by one way ANOVA with Bonferroni post-test. The differences in drug MICs for each antibiotic between biofilms of neonates and adults and the differences in MICs for VAN versus the MICs for DAP or LIN against S.
epidermidis biofilms within each age group were evaluated by the parametric unpaired t test, Welsh corrected. Data were analyzed using Instat v3 biostatistics software (GraphPad Inc., San Diego CA). A two-tailed P value of <0.05 was considered significant.

3. RESULTS

3.1 Biofilm formation of S. epidermidis isolates

Among the 21 isolates recovered from neonates, 13 were biofilm formers and 8 were non-biofilm formers (38.1%). In contrast, among the 11 isolates from adult patients, only one strain was non-biofilm former (9.1%, P=0.049). A total of nine non-biofilm formers were excluded from the study and the remaining 23 were used for the subsequent antibacterial susceptibility studies. S. epidermidis isolates from adults produced biofilms of significantly higher intensity than neonates and control (0.176 ± 0.03 vs 0.125 ± 0.01, P=0.03; Fig. 1).

3.2 Susceptibility of S. epidermidis planktonic cells and biofilms to antibiotics

Planktonic cells: The three antibiotics exhibited similar antibacterial activities against planktonic cells of the 23 S. epidermidis isolates, as median MICs of VAN and DAP were 1 mg/L and the median MIC of LIN was 0.5 mg/L (Table 1). Of note, at concentrations above corresponding MIC’s all three antibiotics rapidly achieved 100% bacterial damage of the planktonic cells of isolates derived from both neonates and adults (Fig. 2-4).

Biofilms: The MICs of the three antibiotics were 3-7 twofold dilutions higher for biofilms than for planktonic cells of isolates derived from both neonates and adults (P<0.05; Table 1). Comparative analysis of the efficacies of the 3 antibiotics against biofilms of isolates from neonates vs. adults showed that VAN had significantly better activity against biofilms of neonatal isolates than that exhibited by DAP and LIN (VAN: 16 vs. 128 mg/L; DAP: 64 vs. 64 mg/L; and LIN: 16 vs. 4 mg/L, respectively; P<0.05, Table 1). Comparative analysis within each age group revealed that, while VAN and LIN MICs were equal and significantly lower than that of DAP against biofilms of neonatal isolates, LIN showed superior activity against biofilms of adult isolates (for adult isolates: LIN, 4 mg/L; VAN, 128 mg/L; DAP, 64 mg/L; P<0.05, Table 1).

The highest biofilm damage achieved by VAN, DAP and LIN in neonatal and adult isolates was at 256 mg/L and ranged from 72% to 98% (Fig. 2-4). At concentrations 128 and 256 mg/L DAP achieved the highest damage of biofilms of both neonatal and adult isolates as compared to VAN and LIN (P<0.001, Fig. 2-4). Furthermore, at low concentrations, VAN ranging from 0.015 to 0.5 mg/L and LIN ranging from 0.015 to 0.125 mg/L caused significantly higher damage against biofilms of both neonates and adults than planktonic cells (VAN: 30%-40% vs. 9%-17% for planktonic cells; LIN: 18%-38% vs. 4%-18%, P<0.05; Fig. 2 and Fig. 4). However, at concentrations >0.25 mg/L the expected recalcitrance of biofilms to the antibiotics as compared to planktonic cells was in effect. The above phenomenon of bacterial damage was not observed with DAP (Fig. 3).

Table 1: MICs of VAN, DAP and LIN for planktonic cells and biofilms of S. epidermidis clinical isolates from neonates and adults determined by the XTT assay

<table>
<thead>
<tr>
<th>Source of isolates (n=23)</th>
<th>VAN</th>
<th>DAP</th>
<th>LIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Planktonic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biofilms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neonates (n=13)</td>
<td>1 (1-2)</td>
<td>16 (4-64)</td>
<td>1 (1-2)</td>
</tr>
<tr>
<td>Adults (n=10)</td>
<td>1 (1-2)</td>
<td>128 (128-256)</td>
<td>1 (1-2)</td>
</tr>
</tbody>
</table>

Figure 1. Biofilm densities of S. epidermidis bloodstream isolates. Clinical isolates from neonates (n=13) and adults (n=10) producing biofilm are compared to the biofilm-documented S. epidermidis control ATCC 35984 strain. The asterisk shows significant differences between S. epidermidis biofilm-formers isolated from neonates and adults (P<0.05). All isolates were studied at least three times on independent experiments.

Figure 2. Bacterial damage of planktonic cells and biofilms of S. epidermidis bloodstream isolates from neonates and adults caused by different concentrations of VAN. Bacterial damage was assessed by XTT assay. Results are means ± SE of drug-induced % bacterial damage of planktonic cells (PL, dark bars) and biofilms (BF, open bars) of all isolates. Asterisks show significant differences between planktonic cells and corresponding biofilms for the concentrations indicated by horizontal lines (P<0.01). The discontinuous line denotes the 50% cutoff of MICs.
4. DISCUSSION

CoNS infections are an important cause of morbidity and potential mortality, including prolonged hospital stay and increased health care costs in infants and adults. In the hospital environment, staphylococcal biofilms constitute a major problem that needs to be recognized and managed especially among the immunocompromised patients. Among CoNS, *S. epidermidis* is the most frequent organism identified in patients with indwelling medical devices, due to a number of evading mechanisms the organism possesses against host defenses that promote persistence, colonization and commencement of diseases. Glycopeptides targeting cell wall integrity, such as vancomycin constitute the first choice of treatment for CoNS infections. However, due to inherent tolerance of biofilms and the emerging vancomycin resistance there is an urgent need to gain additional information on the efficacy of novel antistaphylococcal antibiotics with Gram-positive coverage.

Daptomycin, a cyclic lipopeptide, causes membrane disturbance by intercalating its lipophilic tail in the membrane, whereas linezolid, an oxazolidinone, inhibits protein synthesis. Little and controversial are the data about the anti-staphylococcal activities of VAN, DAP or LIN against biofilms.

The present study compares the in vitro activities of VAN with LIN and DAP within and among the two study groups of neonates and adults. The comparative analysis showed that susceptibilities of *S. epidermidis* biofilm-grown cells isolated from neonates and adults were antibiotic-dependent. VAN and LIN were equally effective against biofilms of neonates and both showed superior activity to DAP. In the adult age group, LIN was more efficacious than both VAN and DAP against biofilms, while VAN and DAP exhibited similar anti-biofilm activities. The inherent biofilm resistance phenotype to antibiotics became clear when we compared the susceptibility profile of planktonic cells with that of biofilms; the free-living forms were significantly more susceptible to all three antibiotics than the biofilms across both age groups. It is noteworthy that while the three antibiotics seemed to have similar efficacies against planktonic cells, a significant antibiotic-dependent diversion in their susceptibility profile became evident against biofilm-grown cells. Such observation suggests that optimal antibiotic therapy might differ with different stages of infection, however this is just a hypothesis.

While in vitro reports published up to date have not studied differences in the susceptibility profiles of biofilms between neonates and adults to VAN and LIN, the antibiofilm activities of the above antibiotics in this study were similar with those demonstrated by others. Specifically, two previous studies investigating the efficacy of VAN and LIN against biofilms formed either on microtiter plates or using a catheter-embedded model showed that *S. epidermidis* isolates were susceptible to both VAN and LIN; although the latter antibiotic was considered to be a more appropriate treatment option against biofilms, neither agent sterilized biofilm-embedded bacteria. Another study on antibiotic susceptibility of coagulase-negative staphylococci isolated from very low birth weight infants showed that all 27 staphylococcal clinical strains remained susceptible to VAN reducing the density of preformed biofilms but...
without being able to eradicate the CoNS biofilms. Experiments designed to correlate biofilm formation with antibiotic resistance in 107 S. epidermidis blood isolates showed that the reduced susceptibility to VAN on preformed biofilms was due to the presence of antibiotic resistant subpopulations of S. epidermidis; the authors reasoned that the reduced VAN activity could be due to a change in the thickening of the bacterial cell wall leading to failure of antibiotic uptake. Such VAN heteroresistance in hospital settings may have shown increased mortality rates, but alternative therapeutic regimens for staphylococcal infections exist including those with LIN and DAP.

Contradicting results are reported on DAP activity against S. epidermidis biofilms in comparison to VAN or LIN. While one in vitro study reported that reductions in biofilm were observed for VAN but not for DAP, another study concluded that DAP was most effective antibiotic compared to LIN even though at peak serum concentrations LIN caused the highest reduction of biofilm biomass. An in vivo time-kill study performed using an experimental model of a foreign-body and systemic infection of mice demonstrated that DAP produces a concentration-dependent bactericidal effect. Based on our results, DAP at ≥128 mg/L may achieve higher biofilm damage in both study populations compared to VAN and LIN, but the effect is seen at clinically unachievable concentrations and does not reach biofilm eradication. A recent study on DAP bioavailability and localization in S. aureus biofilms visualized by fluorescence imaging reported that, most antibiotic molecules freely move through the biomass reaching their target, but DAP even at concentrations >100 mg/L is poorly effective against a subset of cells with low metabolic activity. The authors concluded that staphylococcal biofilms are resistant to DAP due to structural modifications of the cell membrane, a strain dependent effect.

While at low concentrations VAN and LIN caused significantly higher damage against biofilms of both neonates and adults than planktonic cells (Fig. 2 and Fig. 4), at higher concentrations the expected recalcitrance of biofilms to the antibiotics as compared to planktonic cells was in effect. Although this was a repeated phenomenon through all the experiments performed, because of the low level of damage caused, it is unlikely to have a major impact on the clinical course of patients.

Although in vitro data have indirect applicability to clinical settings, the novelty produced by our results is the significantly different susceptibility profile of VAN against biofilms of strains isolated from neonates and adults. Our results on VAN efficacy raise the question of whether or not staphylococcal biofilms in neonates, possessing an immature immune system and thus providing an easier environment for bacterial growth, have different structural and metabolic requirements that make VAN more efficacious against preformed biofilms in neonates than in adults. Understanding the factors that drive the establishment of probable beneficial effects between an immature immune system, antibiotic efficacy and microbial biofilms requires sustained efforts and probably more work on the animal models used currently. In this study, the relatively small sample size and the absence of age subgroups within each population constitute limitations, which could potentially lead to over-interpretation in favor of a particular antibiotic. Therefore, additional studies need to be performed with higher patient enrollment and additional cohorts of patients.

5. CONCLUSIONS

In conclusion, our results suggest that VAN and LIN show better anti-biofilm activity than DAP against biofilms of neonates and LIN have increased activity against biofilms of adults. Although biofilm eradication may not be possible, reduction in colonization could keep bacterial load under control until removal of central catheters or other indwelling devices in cases where this is feasible. Regarding the most optimal therapy in biofilm control, additional animal models of foreign-body infections need to be performed in order to compare the efficacy of VAN with LIN or DAP and gain valuable information on the status of immune responses, bacterial burden and survival rates.

In neonates, the failure to establish immune tolerance leads to important mortality and morbidity and is particularly crucial for the survival of premature infants that are even more susceptible to infections and sepsis.

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