Inhibition of biofilm maturation by linezolid in meticillin-resistant Staphylococcus epidermidis clinical isolates: comparison with other drugs

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Biofilm resistance mechanisms are multifactorial and vary from one organism to another. The purpose of this study was to investigate the efficacy of linezolid against indwelling device-related meticillin-resistant Staphylococcus epidermidis (MRSE) biofilm, and compare this with other antimicrobials. MICs, minimum biofilm inhibitory concentrations (MBICs) and minimum biofilm eradication concentrations (MBECs) were determined by the microtitre plate method. Fourteen and thirteen isolates from patients with indwelling device-related bacteraemia (IDB) and indwelling device colonization not associated with bacteraemia, respectively, were assessed.

High MBIC was associated with a high intensity of biofilm formation (gentamicin r = 0.796; linezolid r = 0.477; rifampicin r = 0.634; tigecycline r = 0.410; and vancomycin r = 0.771), but this correlation was not observed with MBEC. Linezolid demonstrated better in vitro antimicrobial activity than other antimicrobials (MBIC – gentamicin P < 0.001, rifampicin P = 0.019, vancomycin P = 0.008; MBEC – gentamicin P < 0.001, rifampicin P = 0.002, vancomycin P < 0.001). Biofilm growth inhibition was strongly associated with biofilm formation intensity; however, biofilm eradication was not cell number dependent. MRSE biofilm eradication would represent a huge advance for IDB, although high concentrations of gentamicin, linezolid, rifampicin, tigecycline and vancomycin were required for that. In general, linezolid reached better in vitro concentrations and was demonstrated to be highly active against MRSE biofilms by inhibiting their growth during biofilm formation.

INTRODUCTION

Staphylococcus epidermidis biofilm creates many barriers against successful antimicrobial therapy, adversely affecting the treatment of indwelling device-related infections by adhering to foreign surfaces and forming a matrix-like coating, preventing immunological factors and antibiotic penetration (Costerton et al., 1999; Mah & O’Toole, 2001; Donlan & Costerton, 2002; Arciola et al. 2012). Hence, these infections usually require device removal as well as systemic antimicrobial therapy. However, access vein loss, device replacement and the high cost of this procedure indicate saving the infected device when the clinical situation allows it (Mermel et al., 2001; Arciola et al. 2012).

Bacterial biofilm is highly refractory to antimicrobial treatment, which has serious consequences for the therapy of infections that involve biofilm (Suci et al., 1998). Molecular mechanisms of antimicrobial resistance in biofilm are not the same as for planktonic bacteria, since biofilm formation is accompanied by global genetic regulatory changes. The biofilm lifestyle affords bacteria a 10- to 1000-fold increase in antimicrobial resistance compared to their planktonic counterparts, and many of these genetic changes render the constituent bacteria resistant to antimicrobials (Mah & O’Toole, 2001; Stewart & Costerton, 2001; Patel, 2005; Antunes et al., 2011). The resistance of biofilm to antimicrobials may be associated with limited antimicrobial diffusion through the biofilm matrix (Suci et al., 1994), physiological changes (Dagostino et al., 1991) and a reduced growth rate of bacteria in biofilms (Duguid et al., 1992).

Currently, despite all the biofilm virulence and resistance mechanisms, there is a strong and constant need to find an
antimicrobial that effectively kills biofilm-forming microorganisms and those already encased in biofilms. The measurement of minimum biofilm eradication concentration (MBEC), and more recently, minimum biofilm inhibitory concentration (MBIC), has been suggested as a laboratory assay to evaluate antimicrobial activity against mature biofilm (Anwar et al., 1990; Sandoe et al., 2002). In the present study, we used an in vitro polystyrene microtitre plate biofilm model to determine the MIC, MBIC and MBEC for linezolid against indwelling device-related meticillin-resistant *S. epidermidis* (MRSE) biofilm, and compared these with the results with other antimicrobials.

**METHODS**

**Study design and bacterial strains.** *S. epidermidis* strains were recovered from patients attending the Complexo Hospitalar Santa Casa de Misericórdia de Porto Alegre (CHSCMPA), Porto Alegre, Brazil. These strains were recovered from patients with indwelling device-related bacteraemia (IDB) and from patients with indwelling device colonization not associated with bacteraemia (IDC), between August 2010 and January 2011. For IDB, routine blood cultures were performed and the strains were recovered after isolation of the microorganism in 5% sheep blood agar. For IDC, routine cultures were performed as described by Maki et al. (1977), where colony counts above 15 were considered a positive result.

All isolates were stored at ~20°C and all microbiological analyses were performed at the Gram-positive Cocci Laboratory, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, Brazil. All isolates were previously confirmed as *S. epidermidis* using screening and confirmatory methods (Antunes et al., 2008; Bannerman, 2011), and screened for meticillin resistance using 30 μg cefoxitin discs (CLSI, 2011); this was confirmed further by the presence of meca (Zhang et al., 2005).

**Group definitions.** Bacteraemia was defined as ≥2 consecutive 3 day-interval paired positive blood cultures with MRSE. IDC was defined as a bacteraemia where the primary source of the infection was the indwelling device, in patients with temperature >38°C, chills and septic appearance. IDC was defined as a positive culture and a negative peripheral blood culture for the same microorganism.

**Antimicrobials.** Gentamicin, linezolid, rifampicin and vancomycin analytical powders were provided by Sigma-Aldrich. Tigecycline powder was a gift from Wyeth Pharmaceuticals.

**Biofilm determination and quantification.** Biofilm formation ability was determined by microtitre plate assay, and optical density results were scored and interpreted as described elsewhere (Stepanović et al., 2007). Briefly, 180 μl 1% glucose-trypticase soya broth (TSB) (Becton Dickinson) was added to a sterile 96-well polystyrene flat-bottom microtitre plate (TPP Techno Plastic Products), followed by 20 μl 1x10⁸ c.f.u. ml⁻¹ bacterial suspension (a total of 1x10⁹ c.f.u. ml⁻¹). The plates were incubated for 24 h at 35±2°C under static conditions. After incubation and broth removal, the plate was washed three times with sterile saline, and attached bacteria were fixed with methanol and left to air dry overnight in an inverted position. Finally, adherent bacteria were stained with 0.5% crystal violet.

To quantify biofilm, optical density was measured at 492 nm using the Expert Plus microtitre plate reader (Asys Hitech). The optical density cut-off value (ODc; negative control optical density at 492 nm) was defined as threefold the SD above the negative control (in practical terms, a reading around 0.090 at 492 nm) and the intensity of biofilm formation was categorized as strong (2ODc ≤ OD₄₉₂ ≤ 4ODc) or weak (ODc<OD₄₉₂<2ODc).

**MIC determination.** Conventional MICs of gentamicin, linezolid, rifampicin, tigecycline and vancomycin were determined in all isolates in duplicate, by twofold serial broth microdilution (CLSI, 2012). *Staphylococcus aureus* ATCC 29213 was tested as a quality control.

**MBIC and MBEC determinations.** MBIC and MBEC experiments were performed using a modified version of the Calgary biofilm device method (Ceri et al., 1999; Cafiño et al., 2010). In brief, 20 μl 10⁸ c.f.u. ml⁻¹ bacterial suspensions were added to 180 μl 1% TSB, placed into a sterile 96-well polystyrene flat-bottom microtitre plate (TPP Techno Plastic Products) and incubated overnight at 35°C without shaking, to allow bacterial attachment. Non-adherent cells were removed by gentle washing three times with sterile saline solution (150 μl 0.9% NaCl). The plates were left to air dry for 15 min. Serial twofold dilutions of each antimicrobial agent in cation-adjusted Mueller–Hinton broth (CAMHB) were added to the microplates followed by incubation at 35°C for 24 h. MBIC was defined as the minimal antimicrobial concentration at which there was no observable bacterial growth in wells containing adherent microcolonies, i.e. the minimal concentration that inhibited the release of planktonic bacteria from biofilm.

After MBIC measurement, the broth was removed and wells were washed three times with sterile saline solution (150 μl 0.9% NaCl) and antimicrobial-free CAMHB added, followed by incubation for 24 h at 35°C. MBEC was defined as the minimal antimicrobial concentration at which bacteria fail to regrow after antimicrobial exposure, i.e, the minimal concentration required for eradicating the biofilm. All determinations were performed in duplicate.

**Statistical analysis.** Mann–Whitney U and Spearman rank correlation tests were used for continuous non-normally distributed data and multiple comparisons were performed using the Kruskal–Wallis test followed by Dunn's post hoc test for simultaneous pairwise inference. Differences were considered statistically significant at *P*≤0.05. All statistical tests were performed using spss software version 16.0 (SPSS).

**RESULTS AND DISCUSSION**

*S. epidermidis* infections are considered extremely recalcitrant to therapy mainly due to treatment failure associated with its ability to form biofilm in medical devices, from where these multilayered bacteria aggregates are very hard to remove (Mack et al., 2006). When it comes to biofilm, several parameters must be evaluated regarding antimicrobial resistance. With the advent of multidrug-resistant micro-organisms and recurring treatment failures caused by biofilm-forming bacteria, new treatment approaches must be assessed to improve outcomes. This study was mainly designed to evaluate linezolid activity against MRSE biofilm.

A total of 38 consecutive *S. epidermidis* isolates were recovered; 20 were obtained from patients with IDB and 18 from patients with IDC. Among IDB isolates, four were meticillin susceptible and two did not form biofilm. Among IDC isolates, two were meticillin susceptible and three did not form biofilm. These 11 isolates were excluded from the study and the remaining 14 IDB-MRSE and 13
IDC-MRSE isolates were assessed. An explanation for these non-biofilm-forming isolates may be phase variation, that is one of the strategies employed by pathogenic bacteria to switch on the expression of proteins according to the environment. So, even if these non-biofilm-forming isolates harboured the genetic ability to produce biofilm, it does not necessarily mean that biofilm will be produced (Conlon et al., 2004; Tormo et al., 2007).

There was no difference between the groups (IDB and IDC) regarding MIC, MBIC and MBEC. All MRSE were susceptible to linezolid, tigecycline and vancomycin, 18 were susceptible to rifampicin (66.7 %) and 12 to gentamicin (44.4 %) by MIC determination (Table 1). These susceptible isolates were then submitted to antimicrobial exposure after in vitro adherence on polystyrene microtitre plates and the results were extremely high when compared to the MIC. MBEC varied from one to seven dilutions higher than MBIC. The MBIC and MBEC for other antimicrobials were statistically higher when compared with linezolid, except for tigecycline in which no difference was observed (Table 1). Linezolid resistance has remained relatively uncommon among staphylococci. However, recent reports of linezolid resistance among coagulase-negative Staphylococcus (CNS) at medical centres raises concerns (Potoski et al., 2006; Dandache et al., 2009). Because of the high prevalence of CNS biofilm in our setting (Antunes et al., 2010, 2011; Reiter et al., 2011) and around the world (Dandache et al., 2009; Fredheim et al., 2009; Jain & Agarwal, 2009), and the increasing prevalence of higher antimicrobial resistance rates among these biofilm-forming isolates (Cha et al., 2011), future potential treatments for staphylococcal infections mediated by the formation of biofilms are compromised.

MBEC/MIC and MBIC/MIC ratios were calculated to verify how much higher the antimicrobial concentrations were when tested against adherent cells in comparison with planktonic cells. These ratios describe the importance of antimicrobial concentration detection in biofilm, since the results demonstrated an estimation of significant differences between planktonic and sessile cells in terms of antimicrobial performance. Rifampicin presented the highest ratios, followed by gentamicin, as shown in Fig. 1. Both graphs correlate the cumulative number of isolates with each ratio value, and it was observed that linezolid, tigecycline and vancomycin demonstrated statistically lower ratios than rifampicin and gentamicin, evidenced by each antimicrobial curve tendency (Fig. 1). A lower slope of the curve indicated a worse performance of the correspondent antimicrobial against biofilm, i.e. it is necessary to use higher concentrations (P<0.001). The impact of these ratios is evident when treatment failure occurs in cases where physicians have prescribed appropriate antimicrobial doses.

Linezolid presented better concentrations against adherent cells than other antimicrobials tested, even when compared to vancomycin. These results raise many questions and worries about the questionable activity of vancomycin against biofilm, which was already demonstrated to be better than daptomycin, tigecycline, ceftriaxone and azithromycin (Presterl et al., 2009) and worse than rifampicin and ciprofloxacin (Qu et al., 2009, 2010). We found that vancomycin activity was similar to linezolid and tigecycline, and higher than gentamicin and rifampicin activities against MRSE biofilm. However, linezolid seemed to be a more indicated treatment option against biofilm, as seen in other studies (Rodríguez-Martínez et al., 2007; Bayston et al., 2012).

It is important to know the biofilm-producing ability and micro-organism at the species level, since there may exist critical differences between these features. Antunes et al. (2010) demonstrated that MIC results for staphylococci did not change when the isolate was not capable of biofilm formation.

Table 1. MIC, MBIC and MBEC results of planktonic and adherent MRSE isolates

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Planktonic bacteria</th>
<th>Adherent bacteria*</th>
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<td></td>
<td>MIC range</td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
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<tr>
<td>Gentamicin</td>
<td>&lt;0.125–16&gt;16</td>
<td>&lt;0.125</td>
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<tr>
<td>Linezolid</td>
<td>0.125–1</td>
<td>0.5</td>
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<td>Rifampicin</td>
<td>&lt;0.03–32</td>
<td>0.06</td>
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<tr>
<td>Tigecycline</td>
<td>0.06–2</td>
<td>1</td>
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<tr>
<td>Vancomycin</td>
<td>0.5–4</td>
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*MBIC and MBEC determination was performed only for isolates under each antimicrobial breakpoint (susceptible by MIC): gentamicin n=12; linezolid n=27; rifampicin n=18; tigecycline n=27; vancomycin n=27.
†Statistically significant differences were found between linezolid MBIC values and gentamicin (P<0.001), rifampicin (P=0.019) and vancomycin (P=0.008) MBIC values.
‡Statistically significant differences were found between linezolid MBEC values and gentamicin (P<0.001), rifampicin (P=0.002) and vancomycin (P<0.001) MBEC values. There was no significant difference between linezolid and tigecycline MBIC (P=0.148) and/or MBEC (P=0.278) values.
production. Likewise, Raad et al. (2007) showed that linezolid and vancomycin were less effective against meticillin-resistant *S. aureus* biofilm than other antimicrobials, as also demonstrated by Rose & Poppens (2009), but not as demonstrated by our study with MRSE.

Spearman’s coefficients from correlation between the MBICs and the intensity of biofilm formation (the OD$_{492}$ reading) demonstrated regular and strong correlation for all antimicrobials (Fig. 2). Weak biofilm intensity was associated with lower MBICs for all antimicrobials.

**Fig. 1.** Comparison between each antimicrobials tested and their respective MBEC/MIC (a) and MBIC/MIC (b) ratios. The cumulative number of isolates shows the antimicrobial efficiency pattern, a greater efficiency is demonstrated by the earlier dropping of the curve for linezolid, tigecycline and vancomycin. Statistically significant differences were found between the following pairs: linezolid compared with gentamicin, linezolid compared with rifampicin; tigecycline/gentamicin, tigecycline/rifampicin; vancomycin/gentamicin and vancomycin/rifampicin ($P<0.001$). ◆, Gentamicin; ■, linezolid; ▲, rifampicin; X, tigecycline; ●, vancomycin.

**Fig. 2.** Correlation between each antimicrobial MBIC (µg ml$^{-1}$) and OD$_{492}$. Spearman’s coefficients were calculated: gentamicin, $r=0.796$ $P=0.002$; linezolid, $r=0.477$ $P=0.012$; rifampicin, $r=0.634$ $P=0.005$; tigecycline, $r=0.410$ $P=0.034$; and vancomycin, $r=0.771$ $P<0.001$. Each point refers to the mean OD$_{492}$ at each MBIC.
However, even linezolid demonstrated lower MBICs compared with other antimicrobials, the correlation between these MBICs and intensity was poor. Rodriguez-Martinez et al. (2007) demonstrated that linezolid penetration in S. epidermidis biofilms was significantly greater than vancomycin, as well as other studies that have demonstrated a reduced vancomycin penetration through S. aureus and S. epidermidis biofilms (Jefferson et al., 2005; Singh et al., 2010).

However, when the MBECs and the intensity of biofilm formation were compared, there was no statistically significant correlation among the antimicrobials (gentamicin $r=0.298$, $P=0.06$; linezolid $r=-0.009$, $P=0.966$; rifampicin $r=0.237$, $P=0.344$; tigecycline $r=0.345$, $P=0.078$; and vancomycin $r=0.082$, $P=0.684$). Complete biofilm eradication was not associated with biofilm intensity and this difference cannot be explained by the present study. Maybe biofilm growth inhibition is more likely to be related to cell number because the majority of these cells could be at their basal metabolism status, surviving in the environment thanks to the biofilm community. Then, after therapy discontinuation, these cells could repopulate the biofilm, independently of the antimicrobial concentration that has been applied to it.

There are a lot of contradictory facts about the behaviour of staphylococcal biofilms in response to antimicrobials, and our study helps to show how difficult it is to treat bacteria living in biofilms considering our results. Due to inherent resistance to antimicrobial agents, it is extremely important to reach an effective antimicrobial concentration at the treatment site. Although no antimicrobial provides complete biofilm eradication, linezolid seemed to be highly active against MRSE biofilm by inhibiting its growth at reachable concentrations.

ACKNOWLEDGEMENTS

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul.

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