In vitro interference of tigecycline at subinhibitory concentrations on biofilm development by Enterococcus faecalis

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Objectives: Since biofilm formation is the hallmark of Enterococcus faecalis isolates, the aim of this study was to quantify biofilm formation in the presence of subinhibitory concentrations of tigecycline.

Methods: Interference of tigecycline on biofilm formation was spectrophotometrically quantified using 20 biofilm-producing E. faecalis isolates with tigecycline MICs of 0.12 (8 strains) or 0.25 mg/L (12 strains). Biofilm production was measured in antibiotic-free tryptic soy broth supplemented with 1% glucose and compared with biofilm production in the same medium with tigecycline at subinhibitory concentrations (0.25× or 0.5× MIC, similar to trough concentrations in serum or concentrations in the colon after a standard dose) by reading the optical density at 450 nm (OD450) after staining with Crystal Violet.

Results: In the presence of subinhibitory tigecycline concentrations, pooled OD450 values for the 20 strains [median (IQR)] were significantly lower than those for controls: 0.468 (0.379–0.516) for antibiotic-free controls versus 0.295 (0.200–0.395) for 0.25× MIC tigecycline (P<0.001) and 0.287 (0.245–0.479) for 0.5× MIC tigecycline (P<0.001), with significant differences between pooled OD450 values obtained with each concentration of tigecycline (P=0.022). In 17 out of 20 (85%) strains the OD450 obtained with 0.25× MIC tigecycline was significantly (P<0.05) lower than the basal OD450, while this occurred in 12 out of 20 (60%) strains with 0.5× MIC.

Conclusions: In vitro tigecycline subinhibitory concentrations were able to interfere with biofilm formation by E. faecalis.

Keywords: slime, Enterococcus, glycylcyclines

Introduction

Biofilm formation is an important factor in the pathogenesis of enterococcal infections, with Enterococcus faecalis producing biofilm more often than Enterococcus faecium. Enterococci in biofilms are more resistant to antibiotics than planktonically growing enterococci, thus the potential impact of biofilm formation could be clinically significant. A previous in vitro study determining minimum biofilm inhibitory concentrations showed that very high ampicillin, vancomycin and linezolid concentrations were required to inhibit enterococcal biofilms in vitro.1 In an in vitro catheter-related bacteremia model, the lack of linezolid and vancomycin activity against biofilm embedded organisms appeared as the primary reason for microbiological failure.2 Resistance to antibiotics and to phagocytosis of cells embedded in biofilms frequently requires removal of the medical device, which may not always be possible, as the only appropriate cure of enterococcal biofilm-associated infections.

One of the major goals in modern clinical microbiology is the development of strategies capable of reducing biofilm infections, with compounds not only active against planktonic cells but also able to interfere with or decrease biofilm development. Since tigecycline exhibits the highest intrinsic activity among common antibiotics used against E. faecalis (MIC90=0.25 mg/L),3 although with differences between MICs and MBCs (tolerance), the aim of this study was to quantify biofilm formation by E. faecalis in the presence of subinhibitory concentrations of tigecycline.

Materials and methods

Strains

A total of 56 recent clinical isolates (50 E. faecalis and 6 E. faecium), 30 from Hospital Central de la Defensa Gomez Ulla (Madrid, Spain) and 26...
(including the 6 E. faecium isolates) kindly supplied by Hospital Universitario Gregorio Marañón (Madrid, Spain), were screened to assess their capability of biofilm production, following the methods described below. The 6 E. faecium strains had been isolated from peritoneal fluid (5 strains) and blood (1 strain), and the 50 E. faecalis strains had been isolated from urine (25 strains), blood (22 strains), intra-abdominal abscess (1 strain), catheter (1 strain) and sputum (1 strain). At screening, isolates were classified for biofilm production by spectrophotometrical measurement [optical density at 450 nm (OD\textsubscript{450})], using the absorbance of non-bacterial-inoculated wells (OD\textsubscript{450} = 0.055) as the negative control. Isolates were classified as biofilm non-producers (OD\textsubscript{450} ≤ 0.055), weak (OD\textsubscript{450} = 0.056–0.200), moderate (OD\textsubscript{450} = 0.201–0.350) or strong (OD\textsubscript{450} > 0.350) biofilm producers. The 20 isolates showing the highest OD\textsubscript{450} values in the screening phase were chosen.

**Susceptibility testing**

The MICs of tigecycline were determined three times by microdilution following CLSI recommendations.\textsuperscript{4} Modal values were considered.

**Biofilm production**

Biofilm production was spectrophotometricaly assessed\textsuperscript{5} using 96-well polystyrene microtitre plates (Masterlab S.L., Madrid, Spain). Bacterial suspensions in tryptic soy broth (TSB; Pronadisa, Torrejón de Ardoz, Madrid, Spain) supplemented with 1% glucose (turbidity equivalent to that of a 0.5 McFarland standard) were used. Wells (0.2 mL) were inoculated with 0.15 mL of bacterial suspensions and 0.05 mL of supplemented TSB containing the corresponding tigecycline concentrations or supplemented TSB without antibiotic (antibiotic-free controls). Final tigecycline concentrations in the 0.2 mL samples were 0.25× MIC or 0.5× MIC for the strains tested. Plates were covered and incubated (37°C, 5% CO\textsubscript{2}) for 24 h. Afterwards, the contents of each well were removed by decanting, and the wells were washed with sterile distilled water. The empty wells were allowed to dry for 30 min, then 0.2 mL of Crystal Violet (25%) stain was added to each well. After 5 min, excess stain was rinsed off by decanting, and the plate was washed. After the well had been allowed to dry for 30 min, 0.2 mL of 25% HCl was added to each well, and, after 1 min, the ODs of stained adherent bacterial films were read using a Micro-ELISA auto-reading spectrophotometer (Labsystems Multiskan, Helsinki, Finland) at 450 nm. E. faecalis ATCC 29212 was used as positive control (strong producer, OD\textsubscript{450} = 0.611), and non-bacterial-inoculated wells were used as negative controls (OD\textsubscript{450} = 0.055). Experiments with each strain were performed 12 times.

**Statistical analysis**

For each strain, median (IQR) values were calculated from the OD\textsubscript{450} values obtained in the absence and presence of tigecycline. OD\textsubscript{450} values from antibiotic-free medium and tigecycline-containing medium were compared individually and globally (all 20 strains) using the Wilcoxon test. The comparison between OD\textsubscript{450} values obtained with urinary strains and those with strains from blood was performed by the Mann–Whitney test. In addition, per-strain percentages of reduction in median OD\textsubscript{450} in the presence of tigecycline versus controls (antibiotic-free wells) were calculated.

**Results**

All E. faecium isolates tested in the screening phase were biofilm non-producers (4 isolates) or weak producers (2 isolates). Among the screened 50 E. faecalis isolates, 1 (2%) was a non-producer, 23 (46%) were weak producers, 15 (30%) were moderate producers and 11 (22%) were strong producers. The 11 strong producers and the 9 moderate producers with the highest OD\textsubscript{450} values obtained in the screening phase were chosen for the study. Table 1 shows the origin of the strains, MICs of tigecycline, OD\textsubscript{450} values [median (IQR)] obtained in basal experiments (antibiotic-free controls) and in the presence of 0.5× and 0.25× MIC tigecycline, and percentage reduction in median basal OD\textsubscript{450} caused by tigecycline. In antibiotic-free experiments, OD\textsubscript{450} values obtained with isolates from urine (median values 0.349–0.750) were significantly (P < 0.027) higher than those obtained with isolates from blood (median values 0.254–0.498).

In the presence of subinhibitory tigecycline concentrations, global OD\textsubscript{450} values [median (IQR)] were significantly (P < 0.001) lower than in controls: 0.468 (0.379–0.516) for antibiotic-free controls versus 0.295 (0.200–0.395) for 0.25× MIC tigecycline and 0.287 (0.245–0.479) for 0.5× MIC tigecycline. Significant global OD\textsubscript{450} differences were obtained between the two concentrations of tigecycline (P = 0.022). In 17 out of 20 (85%) strains the OD\textsubscript{450} obtained with 0.25× MIC tigecycline was significantly (P < 0.05) lower than basal OD\textsubscript{450}, while this occurred in 12 out of 20 (60%) strains at 0.5× MIC. The reduction from basal OD\textsubscript{450} was higher with 0.25× than with 0.5× MIC tigecycline in 16 out of 20 (80%) strains, although the difference was only significant (P < 0.05) in 5 (25%) strains.

**Discussion**

E. faecalis causes 85%–90% of enterococcal infections and the remaining are caused by E. faecium where acquisition of genes encoding resistance to penicillin, aminoglycosides (high level) and glycopeptides is a cause of concern. In contrast, resistance in E. faecalis is mainly functional (phenotypic) because virtually all isolates produce biofilm, and cells embedded in biofilms are highly resistant to antibiotics due to restricted penetration of antimicrobials, heterogeneous metabolic activity, different gene expression patterns compared with planktonic cells and cell-to-cell communication systems.

In the screening phase of the present study, 98% of E. faecalis isolates produced biofilm to varying degrees (54.2% moderate or strong producers), whereas only 33.4% of E. faecium isolates were biofilm producers (weak). Biofilm production is the hallmark of E. faecalis, with isolates from intravascular-cather-related infections producing more biofilm.\textsuperscript{6} A previous study that tested 352 isolates from urinary tract infections showed that all isolates produced biofilm.\textsuperscript{7} In the present study, significantly higher biofilm formation (higher OD\textsubscript{450} in antibiotic-free controls) was measured when testing isolates from urine than those from blood. Different studies have identified several environmental, phenotypic and genetic factors associated with E. faecalis biofilm production; however, much more research is needed for a better understanding of the regulation of biofilm production.

Once the biofilm is completely developed, conventional antibiotic treatments fail to eradicate the biofilm layers,\textsuperscript{8} and very high enterococcal-biofilm-eradicating concentrations (not achievable in vivo) are required.\textsuperscript{9} Due to this, strategies aimed at preventing biofilm formation acquire importance. In the present study, we investigated the ability of tigecycline to interfere with biofilm formation using physiological subinhibitory tigecycline concentrations. MICs of tigecycline for the 20 studied E.
faecalis strains were in the upper range of susceptibility (0.12 or 0.25 mg/L) according to EUCAST and BSAC breakpoints (≤0.25 mg/L). The tigecycline concentrations used were similar to trough concentrations in serum (0.06 mg/L) after the standard dose, or to concentrations in the colon (0.10 mg/L) using a penetration rate (AUCcolon/AUCserum) of 1.73. Using these concentrations significantly lower biofilm formation was observed when tigecycline was present in the medium: reductions in 11 of 20 (55%) strains with 0.5×MIC tigecycline concentrations, and in 17 of 20 (85%) with 0.25×MIC. However, the reason for the significantly greater reduction obtained with 0.25×MIC than with 0.5×MIC for 5 studied strains remains to be explored since, in the present study, OD450 reductions were not related to MICs or to the magnitude of biofilm formation in the absence of the antibiotic.

Enterococcal biofilms are important not only in intravascular catheters, biliary stent implants and gastrostomy devices, but also in the catheterized urinary tract; these provide ideal conditions for biofilm development, inducing complications in patient care. A previous animal model highlighted the potential benefit of tigecycline in preventing enterococcal ureteral stent infections. The present study shows that tigecycline was able to interfere with biofilm formation in vitro by E. faecalis. Further studies exploring the activity of achievable supra-inhibitory concentrations in vivo against formed E. faecalis biofilms are warranted.

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Transparency declarations
None to declare.

### Table 1. MICs of tigecycline for the 20 studied strains, OD450 (median [IQR]) values for controls and with tigecycline in the medium at concentrations equal to 0.25×MIC or 0.5×MIC, and the percentage reduction of the median OD450 control values (in antibiotic-free medium) caused by the presence of tigecycline

<table>
<thead>
<tr>
<th>Origin</th>
<th>Strain</th>
<th>MIC (mg/L)</th>
<th>Basal OD450 (control)</th>
<th>0.25×MIC OD450</th>
<th>reduction (%)</th>
<th>0.5×MIC OD450</th>
<th>reduction (%)</th>
</tr>
</thead>
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<tr>
<td>urine</td>
<td>18</td>
<td>0.12</td>
<td>0.750 (0.643–0.828)</td>
<td>0.513 (0.431–0.674)</td>
<td>31.6</td>
<td>0.575 (0.451–0.741)</td>
<td>23.3</td>
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<td></td>
<td>12</td>
<td>0.12</td>
<td>0.580 (0.477–0.716)</td>
<td>0.543 (0.389–1.025)</td>
<td>6.4</td>
<td>0.516 (0.366–0.767)</td>
<td>11.1</td>
</tr>
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<td></td>
<td>22</td>
<td>0.25</td>
<td>0.517 (0.465–0.703)</td>
<td>0.497 (0.449–0.596)</td>
<td>3.9</td>
<td>0.391 (0.349–0.452)</td>
<td>24.5</td>
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<tr>
<td></td>
<td>14</td>
<td>0.12</td>
<td>0.514 (0.427–0.599)</td>
<td>0.423 (0.380–0.462)</td>
<td>17.7</td>
<td>0.396 (0.331–0.452)</td>
<td>22.9</td>
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<tr>
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<td>21</td>
<td>0.25</td>
<td>0.497 (0.434–0.565)</td>
<td>0.219 (0.192–0.335)</td>
<td>55.9</td>
<td>0.191 (0.158–0.232)</td>
<td>61.6</td>
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<td>20</td>
<td>0.25</td>
<td>0.490 (0.444–0.651)</td>
<td>0.343 (0.320–0.499)</td>
<td>10.1</td>
<td>0.340 (0.307–0.391)</td>
<td>30.6</td>
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<td>19</td>
<td>0.12</td>
<td>0.447 (0.409–0.546)</td>
<td>0.266 (0.185–0.332)</td>
<td>40.4</td>
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<td>1</td>
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<td>0.381 (0.339–0.462)</td>
<td>0.284 (0.260–0.312)</td>
<td>25.6</td>
<td>0.240 (0.215–0.257)</td>
<td>37.0</td>
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<tr>
<td></td>
<td>10</td>
<td>0.12</td>
<td>0.349 (0.321–0.468)</td>
<td>0.245 (0.195–0.287)</td>
<td>29.8</td>
<td>0.236 (0.212–0.273)</td>
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<tr>
<td>blood</td>
<td>50</td>
<td>0.25</td>
<td>0.498 (0.422–0.549)</td>
<td>0.252 (0.180–0.370)</td>
<td>49.4</td>
<td>0.301 (0.190–0.371)</td>
<td>39.6</td>
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<tr>
<td></td>
<td>41</td>
<td>0.25</td>
<td>0.489 (0.444–0.544)</td>
<td>0.395 (0.340–0.483)</td>
<td>19.2</td>
<td>0.321 (0.286–0.390)</td>
<td>34.4</td>
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<td>47</td>
<td>0.12</td>
<td>0.386 (0.327–0.416)</td>
<td>0.290 (0.220–0.354)</td>
<td>24.8</td>
<td>0.300 (0.219–0.369)</td>
<td>21.8</td>
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<td>17</td>
<td>0.25</td>
<td>0.382 (0.361–0.438)</td>
<td>0.373 (0.241–0.443)</td>
<td>2.1</td>
<td>0.291 (0.204–0.351)</td>
<td>23.8</td>
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<td>36</td>
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<td>0.379 (0.332–0.403)</td>
<td>0.185 (0.151–0.218)</td>
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<td>0.156 (0.146–0.188)</td>
<td>58.8</td>
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<td>0.25</td>
<td>0.327 (0.269–0.354)</td>
<td>0.107 (0.058–0.145)</td>
<td>67.2</td>
<td>0.104 (0.080–0.113)</td>
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<td></td>
<td>38</td>
<td>0.12</td>
<td>0.280 (0.257–0.292)</td>
<td>0.245 (0.231–0.279)</td>
<td>12.5</td>
<td>0.197 (0.186–0.259)</td>
<td>29.5</td>
</tr>
<tr>
<td></td>
<td>49</td>
<td>0.12</td>
<td>0.245 (0.226–0.291)</td>
<td>0.250 (0.206–0.276)</td>
<td>−2.0</td>
<td>0.210 (0.170–0.262)</td>
<td>14.5</td>
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<td>catheter</td>
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<td>0.561 (0.485–0.632)</td>
<td>28.3</td>
<td>0.556 (0.485–0.632)</td>
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<td>0.574 (0.561–0.607)</td>
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<td>0.161 (0.138–0.200)</td>
<td>61.6</td>
<td>0.189 (0.159–0.239)</td>
<td>55.1</td>
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</table>

*a* P<0.05 versus basal OD450.

*b* P<0.05 versus OD450 obtained with tigecycline at 0.5×MIC.

*c* Increase in OD450 relative to basal OD450.
References


