Measurement of ampicillin, vancomycin, linezolid and gentamicin activity against enterococcal biofilms

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Background: Enterococci frequently cause biofilm infections but susceptibility of clinical isolates growing in biofilms has not been investigated. The minimum biofilm eradicating concentration (MBEC) has been suggested as a guide to treatment of biofilm infections. We measured an alternative endpoint, the minimum biofilm inhibitory concentration (MBIC) and compared the results with MIC and MBC.

Objectives: To compare the MIC, MBC and MBIC of ampicillin, vancomycin and linezolid against enterococcal biofilms, to assess the impact of additional gentamicin and correlate findings with clinical outcome.

Methods: MIC and MBC were measured using standard techniques. MBICs were measured using a modification of the Calgary biofilm device method. Fifty-eight enterococcal isolates from episodes of intravascular catheter-related bloodstream infection were tested.

Results: Tolerance to ampicillin, vancomycin and linezolid was seen in 93%, 100% and 93% of isolates, respectively. MIC90s of ampicillin, vancomycin and linezolid were all 4 mg/L for Enterococcus faecalis isolates. MBC90s of ampicillin, vancomycin and linezolid for E. faecalis isolates were 1024, >128 and 2048 mg/L, respectively. MBIC90s of ampicillin, vancomycin and linezolid for E. faecalis isolates were 8192, 4096 and 4096 mg/L, respectively. Results for Enterococcus faecium were similar for vancomycin and linezolid but this species was generally more resistant to ampicillin. Adding 10 mg/L gentamicin had a variable effect on MIC, MBC or MBIC, which was not predictable by gentamicin susceptibility on disc testing.

Conclusions: Very high concentrations of ampicillin, vancomycin and linezolid are required to inhibit enterococcal biofilms in vitro. Combining these agents with gentamicin significantly reduced MIC, MBC and MBIC against only a proportion of enterococcal isolates. No correlation between MBIC and outcome was found.

Keywords: Enterococcus, enterococci, susceptibility, intravascular catheter-related bloodstream infections

Introduction

Biofilm infections represent a major challenge in current medical practice. Although these infections often occur on medical devices, removal of the device is sometimes unsafe or impractical. Antibiotics can occasionally eradicate biofilm infections, e.g. intravascular catheter-related bloodstream infection (CRBSI), but it is unclear which patients can be treated successfully in this manner. It has been shown that monotherapy with either vancomycin or ampicillin frequently fails to eradicate enterococcal biofilms from patients with CRBSI, when the colonized catheter is left in situ. Failure occurs in spite of apparent susceptibility of the causative organism to such antibiotics in vitro. This suggests that selection of antimicrobial agents for the eradication of microbial biofilms is flawed when based on traditional susceptibility testing methods, such as the broth MIC or breakpoint determination and may result in treatment failure. The aim of this work was to undertake preliminary investigations of an alternative susceptibility testing method that may be more useful in the setting of biofilm infections. We measured a novel variable ‘minimum biofilm inhibitory concentration’ (MBIC) and compared these values with the MIC and MBC of various antibiotics against enterococcal biofilms. We assessed the impact of additional...
gentamicin and, where possible, correlated values with clinical outcomes.

**Methods**

**Bacterial strains**

Enterococci from 58 episodes of CRBSI, including 49 *Enterococcus faecalis* and nine *Enterococcus faecium*, were studied. The 61 episodes of CRBSI from which the test isolates were derived, were caused by 25 different Strain-types of *E. faecalis* and 10 different Strain-types of *E. faecium*. *E. faecalis* Strain-type A caused 22 of these episodes. *E. faecalis* NCTC 12697 was used as the control strain.

**Antibiotics**

Ampicillin, gentamicin and vancomycin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Linezolid powder was a gift from Pharmacia and Upjohn Inc. (Kalamazoo, MI, USA). High-level gentamicin resistance was determined using 200 μg gentamicin discs.

**MICs and MBCs**

MICs were determined by broth microdilution based on a method published previously. To determine MBC, 5 μL was aspirated from wells where there was no visible growth in the MIC experiment and plated onto horse blood agar (HBA; Oxoid, Basingstoke, UK) plates and incubated overnight at 37°C in air. The MBC was read as the lowest antibiotic concentration to kill 99.9% of the initial inoculum. When antibiotics were tested in combination with gentamicin, it was added at a fixed concentration of 10 mg/L to each antibiotic dilution.

**MBIC**

Each MBIC experiment was conducted in parallel with MIC and MBC determination. Test isolate, grown on HBA, was inoculated into 10 mL of BHI broth, then incubated at 37°C, stationary, in air, overnight. Control isolate broth culture (200 μL) was added to each well of one row of a 96 well microtitre plate, leaving column 12 empty. The empty well provided a biofilm-free control peg for each isolate. Test isolate broth culture (200 μL) was added to each well of a row of the microplate. This was repeated for other test organisms using the remaining rows. A polystyrene lid with 96 pins (Transferable solid phase screening system, Nalge Nunc International, Rochester, NY, USA 14602), designed to fit into a 96 well microplate with the pegs touching either the bottom or the sides of the wells, was placed onto the microplate containing the broth cultures and incubated on an orbital shaker (200 rpm) at 37°C in air for 18–20 h.

Volumes of antibiotic (200 μL) were added to each well of a second microtitre plate at concentrations in the range 0–8192 mg/L in columns 1–12, respectively. When gentamicin was used, a fixed concentration of 10 mg/L was added to each antibiotic dilution. After overnight incubation, the 96 pin lid was removed from the microtitre plate, shaken gently over a discard pot to remove excess broth and then washed once by immersion of the 96 pin lid in a microtitre plate containing 200 μL of sterile 0.9% (w/v) sodium chloride in each well. The 96 pin lid was then placed onto the microtitre plate containing antibiotics and incubated, stationary, in air for 20 h at 37°C. After incubation, the 96 pin lid was removed from the microtitre plate, shaken gently over a discard pot and washed once by immersion in 0.9% (w/v) sodium chloride. Wells of column 1 of the microtitre plate, which had not contained antibiotic, were examined visually for turbidity as an indication that biofilm had formed on the pegs.

The 96 pin lid was then placed onto a microplate containing 200 μL of fresh BHI broth in each well and was incubated overnight, stationary, at 37°C in air. After incubation, the lid was removed and the wells were examined for evidence of turbidity. It was ensured that all wells of column 1, which had contained biofilm pegs without exposure to antibiotics, were turbid and that all wells of column 12, which had contained pegs without biofilm, had no sign of growth. The lowest antibiotic concentration showing no growth after exposure to biofilm was recorded as the MBC. Assays were repeated at least once and, provided results were within two doubling dilutions of each other, the highest reading was recorded for analysis.

**Quantification of biofilm on pegs**

To quantify biofilm, the pegs were broken off using a small screwdriver to fracture the polystyrene at the base of the peg. Pegs were transferred into sterile bijou bottles containing 1 mL of sterile saline and sonicated for 15 min. A Whitley automatic spiral plater (Don Whitley Scientific Ltd., Shipley, UK) was used to plate-out and Protocel software (Synbiosis UK, Cambridge, UK) was used to count colony forming units (cfu).

**Interpretation of data**

Results were expressed in terms of MIC90, MBC90, MBIC90 and ranges. Tolerance was defined as a ratio of MBC to MIC ≥32. The effects of antibiotic combinations on MIC, MBC and MBIC of individual isolates were assessed by calculating the ratio of single antibiotic exposure to combination exposure for each isolate. Ratios greater than two, representing more than two doubling dilutions reduction with the combination treatment, and less than 0.25, representing at least two doubling dilutions increase with the combination treatment, were considered ‘significant’ in this descriptive analysis.

In addition, the sets of MIC data for each species using single antibiotic and combined with gentamicin were collectively compared using the Wilcoxon signed-rank test, using a cut-off *P* value of 0.05 to reject the null hypothesis, i.e. that there was no significant difference in MICs for the population of isolates tested with and without the addition of gentamicin. Wilcoxon signed-rank test was also used to compare MBCs with and without gentamicin for each antibiotic and MBIC with and without gentamicin for each antibiotic.

**Results**

The quantities of biofilm that formed on pegs of seven randomly selected isolates are shown in Table 1. Table 2 summarizes the findings for each antibiotic.

**Ampicillin**

Tolerance was seen in 48/49 (98%) *E. faecalis* and 6/9 (66%) *E. faecium* isolates. Additional gentamicin produced a ‘significant’ reduction in the MICs obtained for 11 (22%) *E. faecalis* and 2 (22%) *E. faecium* isolates. Ampicillin MBCs were reduced by the addition of gentamicin in 27 (55%) *E. faecalis* isolates and one (11%) *E. faecium* isolates. Gentamicin caused a ‘significant’ increase in MBC against five (10%) *E. faecalis* and two (22%) *E. faecium* isolates. MBICs of ampicillin were reduced by the addition of gentamicin in 12 (24%) *E. faecalis* and 3 (33%) *E. faecium* isolates. High-level susceptibility to gentamicin on disc diffusion testing had a sensitivity of 100% for predicting a significant reduction in MBC with the addition of gentamicin, but
Enterococcal biofilm eradication

Specificity (33%) and positive predictive value (58%) were poor. The ability of high-level gentamicin susceptibility to predict synergistic effects on MBIC and MIC was inferior to that for MBC (data not shown).

In the statistical analysis of all E. faecalis isolates, the addition of gentamicin produced a significant reduction in MBC of ampicillin ($P = 0.031$, Wilcoxon signed-rank test) but did not have a significant impact on MBIC or MIC. In the analysis of all E. faecium isolates there was no statistically significant difference in MIC, MBC or MBIC between exposure to ampicillin alone and in combination with gentamicin ($P > 0.05$, Wilcoxon signed-rank test).

### Linezolid

Tolerance of linezolid was seen in 54/58 (93%) isolates. The addition of gentamicin ‘significantly’ reduced the MIC of just three isolates, two E. faecalis and one E. faecium. In contrast, linezolid MBC and MBIC values were ‘significantly’ reduced by the addition of gentamicin in 57 and 36% of isolates, respectively. When all E. faecalis isolates were analysed collectively, there was a statistically significant reduction in MIC, MBC and MBIC when linezolid was combined with gentamicin ($P \leq 0.0005$, Wilcoxon signed-rank test). The combination only produced a significant reduction in MBIC for E. faecalis isolates ($P = 0.043$, Wilcoxon signed-rank test).

### Vancomycin

Vancomycin was tested against 21 E. faecalis and three E. faecium isolates, chosen because this agent was used during therapy for the infections caused by these isolates. All isolates were vancomycin-tolerant with MBCs > 128 mg/L. The addition of gentamicin ‘significantly’ reduced the MIC for only one isolate but reduced the MBC and MBIC for 50 and 38% of isolates, respectively.

In the analysis of all E. faecalis isolates, the addition of gentamicin produced a significant reduction in both vancomycin MIC and MBIC for E. faecalis ($P \leq 0.012$, Wilcoxon signed-rank test). MBCs were not included in this analysis because they were not quantified above 128 mg/L. Gentamicin had no significant impact on vancomycin MIC or MBIC for E. faecium in this analysis.

### Discussion

In *in vitro* susceptibility to ampicillin or vancomycin by routine disc diffusion testing of enterococcal isolates causing intravascular CRBSI was not predictive of treatment success when the catheter was left in situ. 2 We therefore measured the MBIC, a novel endpoint for *in vitro* susceptibility testing and compared results with standard MIC, MBC and clinical outcome. Equipment equivalent to the Calgary biofilm device was used but we simplified this method, using lack of visible growth in broth culture exposed to pegs as an indicator of biofilm inhibition, rather than quantifying biofilm formed on each peg to shorten the processing time. 2 Peg biofilm load ($10^2$-$10^5$ cfu) was similar to dental biofilm and the organism load used for MIC testing. MBIC$_{90}$ of ampicillin, linezolid and vancomycin were $10^3$ times greater than MIC$_{90}$, at least twice the MBC$_{90}$, and well above serum levels that are achievable or sustainable in patients.

Ampicillin and vancomycin are often combined with gentamicin for treatment of serious enterococcal infections on the basis of presumed synergy. The addition of gentamicin to linezolid or vancomycin produced a significant reduction in MBC and MBIC when

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**Table 1.** Quantification of peg biofilm before exposure to ampicillin and after exposure, at the observed minimum biofilm inhibitory concentration (MBIC)

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Biofilm CFU/mL</th>
<th>Pre-antibiotic exposure</th>
<th>At measured MBIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. faecalis 26</td>
<td>1.7 x 10$^5$</td>
<td>1330</td>
<td></td>
</tr>
<tr>
<td>E. faecalis 29</td>
<td>1.3 x 10$^5$</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>E. faecalis 35</td>
<td>1.6 x 10$^5$</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>E. faecalis 59</td>
<td>6.2 x 10$^4$</td>
<td>470</td>
<td></td>
</tr>
<tr>
<td>E. faecalis 75</td>
<td>1.5 x 10$^3$</td>
<td>405</td>
<td></td>
</tr>
<tr>
<td>E. faecalis 104</td>
<td>1.4 x 10$^2$</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>E. faecalis 110</td>
<td>1.4 x 10$^2$</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*The MBIC was above the upper limit of detection (i.e. >8192 mg/L) so no colony count could be performed.*

**Table 2.** MIC$_{90}$s, MBC$_{90}$s, MBIC$_{90}$s and MIC ranges of ampicillin, linezolid and vancomycin against enterococcal isolates from episodes of intravascular catheter-related bloodstream infection

<table>
<thead>
<tr>
<th></th>
<th>MIC ($mg/L$)</th>
<th>MBC ($mg/L$)</th>
<th>MBIC ($mg/L$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC$_{90}$</td>
<td>Range</td>
<td>MBC$_{90}$</td>
</tr>
<tr>
<td><strong>E. faecalis (n)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin (49)</td>
<td>4</td>
<td>0.5–8</td>
<td>1024</td>
</tr>
<tr>
<td>Linezolid (49)</td>
<td>4</td>
<td>2–8</td>
<td>2048</td>
</tr>
<tr>
<td>Vancomycin (21)</td>
<td>4</td>
<td>2–8</td>
<td>&gt;128</td>
</tr>
<tr>
<td><strong>E. faecium (n)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin (9)</td>
<td>128</td>
<td>8–128</td>
<td>8192</td>
</tr>
<tr>
<td>Linezolid (9)</td>
<td>4</td>
<td>2–4</td>
<td>1024</td>
</tr>
<tr>
<td>Vancomycin (3)</td>
<td>4</td>
<td>2–4</td>
<td>&gt;128</td>
</tr>
</tbody>
</table>

$n$, number of isolates tested.
in at least a third of the isolates tested. No antagonism was seen with these antibiotic combinations. Although gentamicin enhanced the effects of vancomycin and linezolid against some *E. faecalis* biofilms it did not have a marked effect on the activity of ampicillin in this respect. In these experiments, high-level susceptibility to gentamicin by disc diffusion did not necessarily predict a reduction in MIC, MBC or MBIC. Examination for aminoglycoside resistance determinants to explain this discrepancy was not undertaken. The mechanism of antagonism seen for some isolates is not known but has been noted previously with a different aminoglycoside.\(^9\) In contrast to previous studies\(^{10,11}\) we showed some reduction in MBC and MBIC when gentamicin was combined with linezolid, but methodological differences preclude direct comparisons.

An attempt was made to correlate MBICs with the treatment and outcomes of the episodes of CRBSI from which the clinical isolates were derived but the number of cases where the catheter remained *in situ* \(n = 13\) were too small to justify statistical analysis of susceptibility data in relation to outcome. The infections in three patients that were cured with ampicillin (or amoxicillin) and gentamicin, with the intravascular catheter *in situ*, were caused by isolates that had ampicillin MBIC 1024–2048 mg/L and showed a one doubling-dilution reduction in MBIC with the addition of gentamicin in only two cases (‘non-significant’ by the descriptive analysis used herein). The MBCs of ampicillin for these isolates (range 64–1024 mg/L) were significantly reduced to 32 mg/L by the addition of gentamicin. However, with the small number of cases, there was no clear cut-off value of either MBC or MBIC that correlated with a successful outcome. For the patients in whom the intravascular catheter was removed, an isolate with a MIC indicating resistance had a poor sensitivity (50\%) and predictive value (50\%) of failure of therapy.

This work demonstrates the very high concentrations of ampicillin, vancomycin and linezolid required to inhibit enterococcal biofilms *in vitro* and may explain why monotherapy with these agents frequently fails to eradicate biofilm infections. The poor predictive value of MICs in enterococcal CRBSI treatment has been demonstrated and confirms the previous results from disc diffusion testing. It also shows how combining vancomycin or linezolid or ampicillin with gentamicin can reduce the MBIC against some, but not all, enterococcal isolates. Data from large numbers of clinical episodes would be required to define the relationship between MBIC and clinical outcome before any advantages over MIC could be assessed. We hope that this work will stimulate the investigation of susceptibility tests that have more relevance to biofilm infections than current methods.

### Acknowledgements

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### Transparency declarations

J. S. received funds towards this research from Pharmacia (now Pfizer). M. H. W. has acted as a consultant on advisory boards for Pfizer.

### References


