In vitro antibacterial activity of tigecycline against clinical isolates of linezolid-intermediate and linezolid-resistant *Enterococci* by time-kill assay

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ABSTRACT

Introduction: Enterococci have become the third leading cause of nosocomial bacteraemia, an infection which is significantly associated with the risk of developing infective endocarditis. Linezolid provides high rates of clinical cure and microbiologic success in complicated infections due to *Enterococcus spp.* However, several instances of emergence of resistance during linezolid treatment have been reported. The aim of this study was evaluate the activity of tigecycline against linezolid-intermediate (LIE) and linezolid-resistant *enterococcus faecalis* (LRE) by the time-kill assay.

Methods: Five isolates of LRE and two isolates of LIE were used in this study. Minimum inhibitory concentration (MIC) was determined by broth dilution following the guidelines from the Clinical and Laboratory Standards Institute (CLSI). Time-kill assay was employed to access the in vitro response profile of tigecycline.

Results: All seven isolates presented MIC of 0.125 µg/mL. Tigecycline activity was individually evaluated according to CLSI criteria. This antibiotic showed bactericidal activity against three of the five isolates of LRE and bacteriostatic activity against the other isolates.

Conclusions: Tigecycline presented both bacteriostatic and bactericidal activity against tested isolates, which is an important data that must be considered for new studies.

Keywords: *Enterococcus; anti-infective agents; bacterial growth*

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Enterococci have become the third leading cause of nosocomial bacteraemia, an infection which is significantly associated with the risk of developing infective endocarditis. The enterococci pose a considerable therapeutic problem since they display resistance to a wide range of antimicrobial agents, including aminoglycosides, penincilins and glycopeptides. The problematic treatment of enterococcal infections emphasizes the need for evaluation of new effective therapeutic options[1].

A product of these efforts has been the development of linezolid, a synthetic antibiotic that was the first commercially available drug from the group of oxazolidinones. Linezolid provides high rates of clinical cure and microbiologic success in complicated infections due to *Enterococcus spp*. However, several instances of emergence of resistance during linezolid treatment have been reported in clinical isolates of enterococci and staphylococci[2].

In Brazil, bacteria from the genus *Enterococcus* are responsible for about

10% of all nosocomial infections[3]. The presence of linezolid-intermediate enterococci (LIE) and linezolid-resistant enterococci (LRE) has been described in nosocomial infections over the last years[4]. However, despite these findings, there are no available studies on the relationship of LIE and LRE with regard to tigecycline.

The antimicrobial agent tigecycline, the first glycylcycline available for clinical use, was licensed by the US Food and Drug Administration (FDA) in 2005. This drug inhibits bacterial protein translation by binding to the 30S ribosomal subunit and blocking the entry of amino-acyl tRNA molecules into the A site of the ribosome, which prevents the incorporation of amino acid residues into elongating peptide chains. Tigecycline, a derivative of minocycline, is not affected by any of the known tetracycline resistance determinants. In general, tigecycline is considered bacteriostatic; however, bactericidal activity has been demonstrated against isolates of *S. pneumoniae* and *L. pneumophila*[5].

The reaction of these microorganisms in the face of new drugs such as tigecycline has been tested through classic assays, taking into consideration only the bacterial growth response to antimicrobial concentrations, as seen in minimum inhibitory concentration (MIC) tests. Conversely, the time-kill assay has been employed to further assess in vitro response profile, accounting not only for different antimicrobial concentrations but also for different exposure times.

In order to evaluate the time dependence of tigecycline activity, we have undertaken time-kill experiments against clinical isolates of LIE and LRE.

METHODS

The present study includes 2 clinical isolates of *Enterococcus faecalis* recovered from different patients and characterized as LIE, as well as 5 isolates characterized as LRE. Isolates were collected from different hospitals in the city of Porto Alegre, State of Rio Grande do Sul, Brazil, between 2008 and 2010.

All isolates were characterized as *E. faecalis* according to a previously described biochemical key[6].

An additional identification and a susceptibility profile were obtained using the Walk-Away MicroScan™ system (SIEMENS). Also, the susceptibility profile was confirmed with the disc diffusion method (Kirby-Bauer), according to Clinical and Laboratory Standards Institute (CLSI) guidelines, for the following antibiotics: linezolid and tigecycline[7].

The MIC assay as well as the previous growth (until the inoculum reaches the log phase, in order to perform in time-kill assay) were conducted using freshly prepared (< 12 h old) cation supplemented Mueller-Hinton Broth (MHB) (Becton Dickinson & Co, Maryland, USA).

Trypticase Soy Agar (HiMedia Laboratories Ltd, Mumbai, India) was used for colony counts during the time-kill assay; quality control of media was performed using *E. faecalis* ATCC 29212.

The antimicrobial agent tigecycline was provided by Wyeth Pharmaceuticals (Collegeville, PA).

MICs were determined by broth macro dilution following CLSI guidelines[7]. Tubes containing serial dilutions of tigecycline, within a range of 0.0312 to 1 µg.mL-1, were used. The FDA-approved MIC (µg/mL) susceptibility breakpoints of tigecycline against *E. faecalis* (vancomycin-susceptible isolates only) was ≤ 0.25 for susceptible microorganisms (source: Tigecycline package insert; Wyeth Pharmaceuticals, Philadelphia, PA).

Time-kill assays were performed twice independently according to guideline M26-A of the CLSI using polypropylene 96-well deep well plates. Suspensions were prepared in flasks containing 50 ml of fresh MHB and inoculated with 10 µl of the seven tested organisms in logarithmic growth phase, determined through a growth curve from a previous experiment (not shown), and then adjusted to obtain a final inoculum of 5 x 105 colony-forming units (CFU).mL-1.

The time-kill kinetics of tigecycline was tested at the MIC (0.125 µg.mL-1) – obtained by broth dilution susceptibility testing –, three dilutions above the MIC (0.25 µg.mL-1, 0.5 µg.mL-1and 1 µg.mL-1), and two dilutions below the MIC (0.0625 µg.mL-1 and 0.0312 µg.mL-1). Viability counts were performed after 0, 2, 4, 8, 12, and 24 hours of incubation at 35°C.

Plates were incubated at 35°C for 24 h, and colony counts were determined. A growth control was included for each isolate tested. We also included a quality control strain, as recommended by the CLSI[7].

Bactericidal activity was defined as a reduction of 99.9% (≥ 3 log10) in the total count of CFU per milliliter of the original inoculum. Bacteriostatic activity was defined as the maintenance of the original inoculum concentration or a reduction of less than 99.9% (< 3 log10) in the total count of the original inoculum[8].

RESULTS

All seven isolates showed a MIC of 0.125 µg.mL-1 for tigecycline.

Tigecycline exhibited bacteriostatic activity against all the LIE isolates and two of the LRE (table 1). A bactericidal activity was found against the other 3 isolates of LRE for at least two concentrations above the MIC at 12 or at 24 hours (figures 1, 2 and 3).

Table 1: Reduction in log CFU.mL-1 (observed – initial).

Concentrations (µg.mL^{-1})	1	0.5	0.25	0.125	0.0625	0.0312	C+
Isolate number							
1 - LIE							
2h	-0,07	-0,36	-0,30	-0,57	-0,58	-0,34	0,33
4h	-1,19	-1,12	-0,66	-0,59	-0,59	-0,13	0,44
8h	-1,28	-1,01	-1,21	-0,94	-0,68	0,09	0,44
12h	-2,08	-0,28	-0,95	-1,23	-1,35	0,42	0,44
24h	-2,26	-2,56	-2,08	-1,28	-1,51	0,44	0,44
2 - LIE							
2h	-0,98	-1,27	-0,87	-1,06	-1,04	-0,15	-0,09
4h	-1,44	-1,48	-1,76	-1,17	-0,66	0,24	0,24
8h	-2,16	-2,16	-1,51	-1,40	-0,82	0,15	0,24
12h	-2,46	-1,81	-2,46	-1,44	-0,53	0,24	0,24
24h	-2,76	-2,06	-2,16	-2,29	0,24	0,24	0,24
3 - LRE							
2h	-0,86	-1,45	-1,05	-1,08	-1,07	-0,55	-0,09
4h	-1,15	-1,29	-1,30	-1,29	-1,05	-0,29	0,32
8h	-1,98	-1,57	-1,90	-1,43	-0,56	0,06	0,32
12h	*	-1,43	-1,45	-1,73	-0,46	0,32	0,32
24h	*	*	-2,08	-1,73	0,32	0,32	0,32
4- LRE							
2h	-0,77	-0,79	-0,79	-0,59	-0,51	-0,24	0,09
4h	-1,34	-1,52	-1,29	-0,96	-0,29	0,00	0,30
8h	-1,24	-1,92	-1,92	-1,21	-0,21	0,30	0,30
12h	-2,70	-2,00	-1,40	-1,59	-0,35	0,30	0,30
24h	*	*	-2,00	-1,32	0,30	0,30	0,30
5 - LRE							
2h	-0,80	-0,72	-0,60	-0,60	-0,65	-0,61	0,06
4h	-0,84	-0,75	-0,80	-0,69	-0,72	-0,31	0,46
8h	-1,03	-0,90	-0,86	-0,94	-0,17	0,46	0,46
12h	-1,85	-1,59	-1,43	-1,20	0,19	0,46	0,46
24h	-2,07	-2,24	-1,70	-1,37	0,46	0,46	0,46
6 - LRE							
2h	-0,82	-1,11	-1,12	-0,79	-0,97	-0,66	-0,03
4h	-1,08	-1,30	-1,14	-1,01	-0,85	-0,37	0,40
8h	-1,75	-1,70	-1,45	-1,28	-0,08	0,40	0,40
12h	-2,12	-1,90	-1,52	-2,30	0,27	0,40	0,40
24h	-2,30	-2,60	-1,42	-1,82	0,40	0,40	0,40
7 - LRE							
2h	-1,93	-1,59	-1,93	-1,36	-1,08	-0,77	-0,05
4h	-1,86	-1,50	-1,86	-1,36	-1,04	-0,53	0,23
8h	-2,23	-2,10	-1,93	-1,34	-0,57	0,11	0,29
12h	*	*	-2,71	-2,10	-0,01	0,26	0,29
24h	*	*	*	-2,71	0,29	0,29	0,29

* represents bactericidal activity (≥ 3 log10 CFU/mL); LIE: linezolid-intermediate *Enterococcus*; LRE: linezolid-resistant Enterococcus. CFU: colony-forming unity.

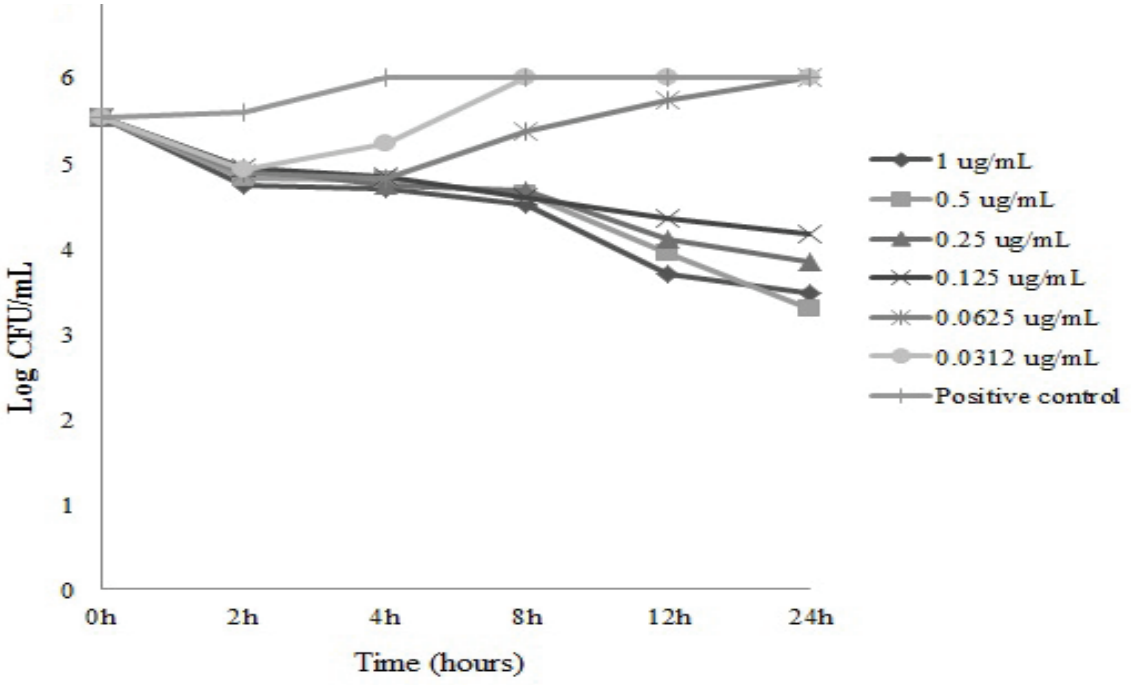

Figure 1: Time-kill curves for Linezolid-Resistant isolate number 3 after incubation with tigecycline for a 24 hours period of time.

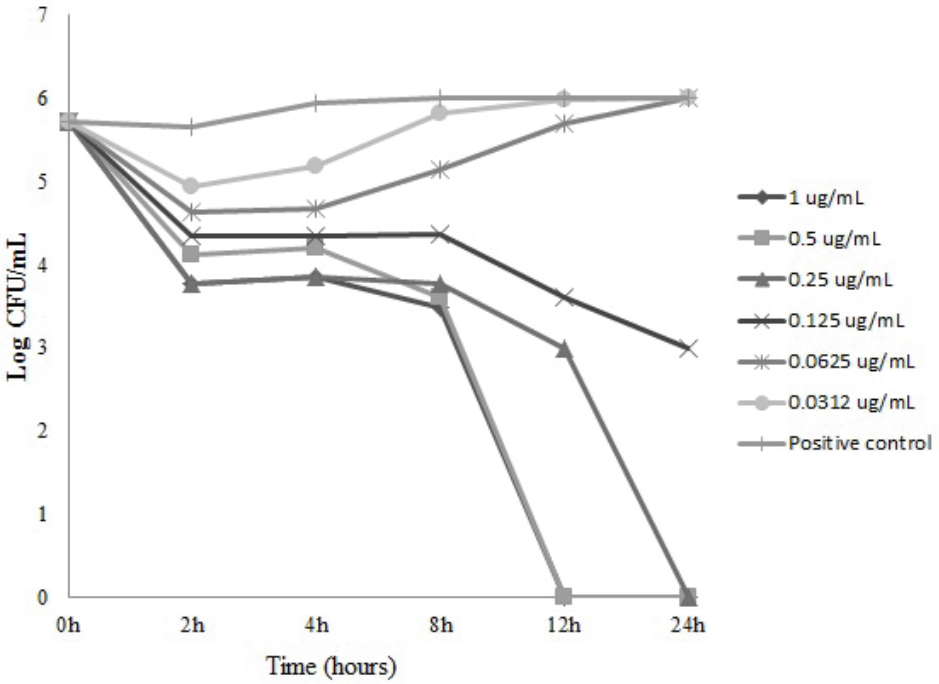

Figure 2: Time-kill curves for Linezolid-Resistant isolate number 4 after incubation with tigecycline for a 24 hours period of time.

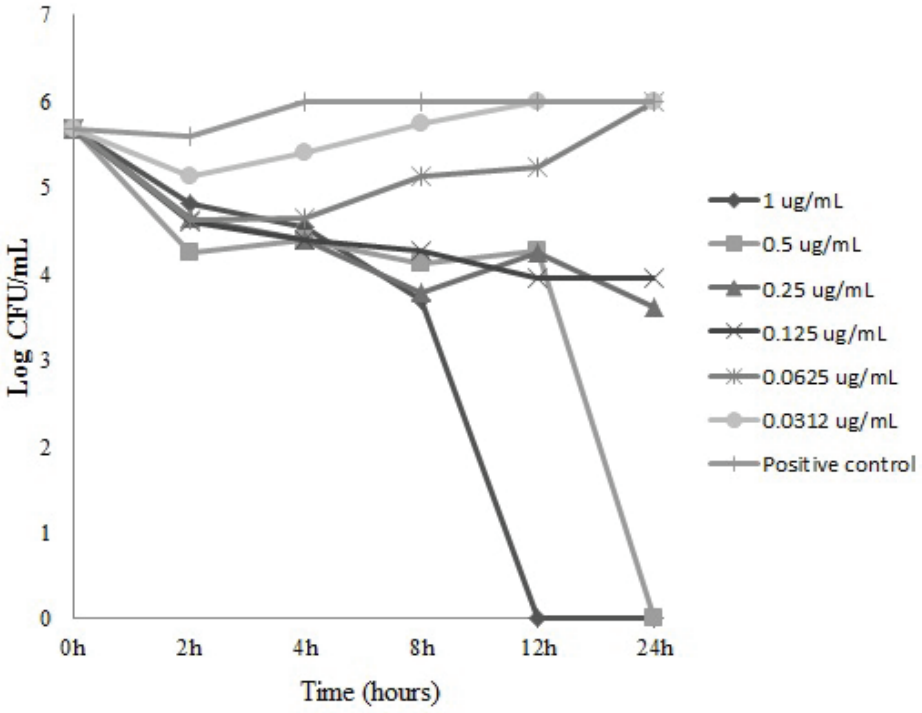

Figure 3: Time-kill curves for Linezolid-Resistant isolate number 7 after incubation with tigecycline for a 24 hours period of time.

When the mean of the two LIE isolates was evaluated, the antimicrobial presented a progressive reduction of bacterial growth after 2 hours at the concentrations of MIC, 2 x MIC, 4 x MIC, and 8 x MIC. At the concentration of 0.5 x MIC, tigecycline reduced the bacterial presence at 2, 4, 8 and 12 hours, although a bacterial regrowth occurred at 24 hours. At 0.25 x MIC, there was reduction only at 2 hours and 12 hours the bacterial growth was the same as at growth control.

As for LRE isolates, similarly to LIE isolates, tigecycline showed as in a progressive reduction in bacterial growth at concentrations MIC, 2 x MIC, 4 x MIC and 8 x MIC at the times of 2, 4, 8, 12 and 24 hours. At the concentrations below MIC, there was a reduction only at 2 hours. After this time a bacterial regrowth was found.

DISCUSSION

Currently, insufficient data have been published on tigecycline against linezolid-intermediate and linezolid-resistant *E. faecalis*. Considering the previous data, the tigecycline MIC approved by the FDA for *E. faecalis* is 0.25 µg.mL-1. Our study found a MIC lower than FDA breakpoint, showing accordance with a study that tested MICs for the same drug in different situations. In this study, tigecycline MIC performed with freshly prepared Mueller-Hinton broth for *E. faecalis* ranged from 0.03 to 0.12 µg.mL-1[9]

The bacteriostatic effect of tigecycline found in this study confirmed previous data. However, it is necessary to consider that the authors of the above mentioned study employed linezolid-resistant *Enterococcus faecium*, a species that presents high rates of resistance to antimicrobials in comparison with *E. faecalis*[8].

The bactericidal effect found in our study has not yet been reported in literature for LIE and LRE isolates. This activity is defined as a reduction of 99.9% (≥ 3 log10) of the total count of CFU per milliliter of the original inoculum. Our results showed a complete reduction of the bacterial growth, a fact that makes it impossible to calculate the log of CFU.ml-1 (figures 1, 2. and 3). That is the reason that a number was not shown in Table 1.

Furthermore, more studies involving LIE and LRE against new pharmaceutical drugs are necessary, as well as in vivo studies to evaluate more precisely the activity of these antimicrobials.

This study has two limitations that must be

considered. The first is the small number of isolates and the second is the absence of a genetic profile analysis.

Therefore, the antimicrobial analysis of this study can be considered an important step on the overall analysis of tigecycline. Both bactericidal and bacteriostatic activity of tigecycline demonstrates the importance of this antimicrobial against LIE and LRE isolates and must be considered for new studies.

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