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# Linezolid Compared with Eperezolid, Vancomycin, and Gentamicin in an In Vitro Model of Antimicrobial Lock Therapy for *Staphylococcus epidermidis* Central Venous Catheter-Related Biofilm Infections

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**Central venous catheter (CVC)-related infection (CVC-RI) is a common complication of CVC use. The most common etiological agents of CVC-RI are gram-positive organisms, in particular, staphylococci. An in vitro model for the formation of biofilms by *Staphylococcus epidermidis* ATCC 35984 on polyurethane coupons in a modified Robbins device was established. Biofilm formation was confirmed by electron microscopy and was quantified by determination of viable counts. Mueller-Hinton broth was replaced with sterile physiological saline (control) or a solution of vancomycin (10 mg/ml), gentamicin (10 mg/ml), linezolid (2 mg/ml), or eperezolid (4 mg/ml). Viable counts were performed with the coupons after exposure to antimicrobials for periods of 24, 72, 168, and 240 h. The mean viable count per coupon following establishment of the biofilm was $4.6 \times 10^8$ CFU/coupon, and that after 14 days of exposure to physiological saline was $2.5 \times 10^7$ CFU/coupon. On exposure to vancomycin (10 mg/ml), the mean counts were $2.5 \times 10^7$ CFU/coupon at 24 h, $4.3 \times 10^6$ CFU/coupon at 72 h, $1.4 \times 10^5$ CFU/coupon at 168 h, and undetectable at 240 h. With gentamicin (10 mg/ml) the mean counts were $2.7 \times 10^7$ CFU/coupon at 24 h, $3.7 \times 10^6$ CFU/coupon at 72 h, $8.4 \times 10^6$ CFU/coupon at 168 h, and $6.5 \times 10^6$ CFU/coupon at 240 h. With linezolid at 2 mg/ml the mean counts were $7.1 \times 10^5$ CFU/coupon at 24 h and not detectable at 72, 168, and 240 h. With eperezolid (4 mg/ml) no viable cells were recovered after 168 h. These data suggest that linezolid (2 mg/ml) and eperezolid (4 mg/ml) achieve eradication of *S. epidermidis* biofilms more rapidly than vancomycin (10 mg/ml) and gentamicin (10 mg/ml).**

Central venous catheters (CVCs) are inserted in more than 20 million hospital patients in the United States alone each year (15). A CVC-related bloodstream infection (CR-BSI) rate of 2.3 per 1,000 catheter days has been estimated for nonmedicated short-term CVCs (6). The attributable mortality rate is estimated to be 12 to 25%, with additional health care costs of the order of $33,000 to $35,000 per incident (6). The predominant microorganisms associated with CVC-related infections (CVC-RIs) are *Staphylococcus epidermidis* and *Staphylococcus aureus*. Other important pathogens include *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Candida albicans* (10, 22, 24).

CVC-RI is associated with establishment of a microbial biofilm on the surface of the CVC (16, 17, 22, 23, 26). Treatment of a CVC-RI with conventional antimicrobial agents based on standardized antimicrobial susceptibility test results is frequently unsuccessful, and removal of the CVC is required in many cases. Standardized antimicrobial susceptibility tests are performed with rapidly growing planktonic cells. The bacteria in biofilm communities attached to surfaces are much less susceptible to killing by antimicrobial agents (8, 21). Failure of conventional antimicrobial therapy alone to eradicate a CVC-RI is the clinical corollary of this difference in the antimicrobial susceptibilities of planktonic and biofilm bacteria. The relative antimicrobial resistance of a biofilm may be associated with restricted diffusion of antimicrobials through the biofilm matrix (14, 28), physiological changes and gene expression or repression due to the biofilm mode of growth (1, 7), and a decreased growth rate of the bacteria in biofilms (2, 8, 9, 11).

Removal of the CVC, and therefore of the associated biofilm, has been considered an integral part of the approach to therapy of CR-BSIs. However, removal of a CVC, in particular, of a long-term tunneled CVC in a critically ill patient, is associated with significant practical problems and costs. It has recently been proposed that a decision to attempt therapy with the CVC in situ may be considered when the episode of CR-BSI is considered to represent a low to moderate risk of a poor outcome (22). Under such circumstances, antimicrobial lock therapy may be used in an attempt to salvage long-term tunneled catheters. Antimicrobial lock therapy is the instillation of high concentrations of antimicrobial agent into the lumens of infected CVCs for extended periods. Antimicrobial lock therapy, recently reviewed by Berrington and Gould (4), is based on the expectation that exposure of biofilms to very high concentrations of antimicrobials for extended periods may overcome the relative antimicrobial resistance of biofilm bacteria (3–5, 18). A variety of agents have been used empirically, including glycopeptides and aminoglycosides. However, the evidence base for selection of the agents and antimicrobial concentrations is very limited.

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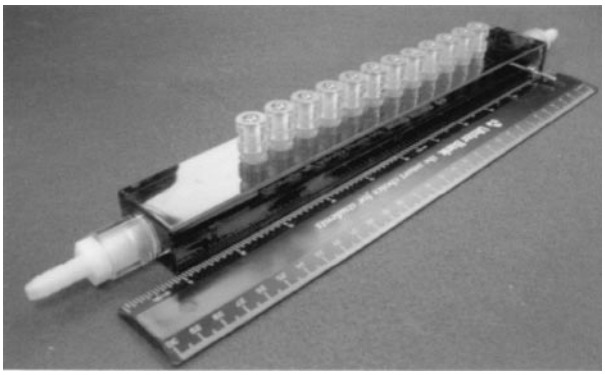

FIG. 1. MRD used in this study.

Linezolid is the first oxazolidinone antimicrobial agent introduced into clinical use. The oxazolidinone family of antimicrobial agents acts by inhibition of protein synthesis (27). The spectrum of activity includes most gram-positive organisms, including isolates resistant to beta-lactams and glycopeptides (12). The project described here compared the effectiveness of high concentrations of linezolid and eperezolid with higher concentrations of vancomycin and gentamicin against *S. epidermidis* biofilms in an in vitro model of CVC-RI.

## MATERIALS AND METHODS

**Organisms and culture conditions.** *S. epidermidis* ATTC 35984 (oxacillin resistant) was selected for the present study. The organism was maintained at −70°C in brain heart infusion broth with 15% glycerol, and on each occasion the biofilm was established from the original stock.

**Antimicrobial agents.** Antimicrobial agents were obtained as powders of defined potency, as follows: vancomycin and gentamicin were obtained from Sigma-Aldrich Corp. (St. Louis, Mo.), and linezolid and eperezolid were donated by Pharmacia & Upjohn (Pharmacia Ireland Ltd., Dublin, Ireland). Linezolid solution intended for intravenous administration was used in a number of instances, as detailed below, because of the relatively large quantities of linezolid required and limits on the quantity of powder of defined potency that was readily available. The MICs of gentamicin, linezolid, eperezolid, and vancomycin for *S. epidermidis* ATCC 35984 were determined by the agar dilution method of the National Committee for Clinical Laboratory Standards (NCCLS) (20). *S. aureus* ATCC 29213 was used as the control.

**In vitro model of biofilm development and antimicrobial lock technique.** A biofilm was grown by using a modified Robbins device (MRD; Tyler Research Corporation, Edmonton, Alberta, Canada) (21) coupled with a continuous-culture system. The MRD, constructed from acrylic (28 cm), contained 12 sampling ports (Fig. 1). Polyurethane coupons (surface area, 50 $mm^2$) manufactured from medical-grade polyurethane (Bayer Polymers, Leverkusen, Germany) were placed in each sampling port. Before each experiment the device was cleaned and sterilized with ethylene oxide gas. A continuous culture of *S. epidermidis* ATCC 35984 was established in a continuous-culture system (100 ml) fed with Mueller-Hinton broth (MHB) at a flow rate of 1 ml $min^{-1}$. Steady state was achieved over a period of 24 h. The MRD was coupled with the continuous-culture system and irrigated (1 ml $min^{-1}$) for 6 h with *S. epidermidis* from the continuous-culture system to seed the biofilm, followed by irrigation for 18 h (2 ml $min^{-1}$) with sterile MHB to allow establishment of a biofilm. In control experiments, MHB was then replaced with sterile physiological saline (0.9%) or saline containing heparin (heparin sodium injection B.P., 1,000 U/ml; Leo Laboratories Ltd., Princes Risborough, Bucks, United Kingdom) at 10 U/ml for 336 h.

For the antimicrobial lock experiments the MRD was disconnected from the flow of medium and a solution containing vancomycin (10 mg/ml; pH 4), gentamicin (10 mg/ml; pH 5), linezolid (2 mg/ml; pH 5), or eperezolid (4 mg/ml; pH 7) was instilled into the cavity of the MRD for periods of 24, 72, 168, and 240 h. The antimicrobial solution was static in the device throughout the period of exposure to simulate the conditions of antimicrobial lock therapy. For vancomycin, gentamicin, and eperezolid, heparin was not included in the antimicrobial solution. Linezolid (2 mg/ml) was initially evaluated alone and was subsequently evaluated in combination with heparin at a concentration of 10 U/ml for a period of 72 h. The MRD was maintained at 37°C throughout the period of exposure to saline or antimicrobial solution. At the end of each period of antimicrobial lock, the antimicrobial lock solution was cultured on Mueller-Hinton agar to determine if any viable planktonic organisms were present. The stabilities of the antimicrobials over the period of exposure were assessed by immunoassay (vancomycin and gentamicin) on a TDX/FLX instrument (Abbott Laboratories, Abbott Park, Ill.) or bioassay (linezolid and eperezolid) (25).

The organisms in the biofilms were quantified by performing viable counts. Coupons were removed from the MRD and washed with 20 ml of sterile physiological saline (0.9%) to remove planktonic and loosely adherent cells and to remove the antimicrobial residue. Individual coupons were subjected to high-speed vortexing in 5 ml of sterile saline for 15 s, followed by sonication for 30 min at 45 to 49 kHz (model 28x ultrasonic cleaner; NEY Dental Inc., Bloomfield, Conn.). Preliminary experiments indicated that this process removed essentially all biofilm from the coupons and that sonication was not associated with a loss of viability of the cells in suspension. In each experiment, the viable counts for six coupons were established and the mean viable count was determined. Mean viable counts were determined on three separate occasions after establishment of the biofilm and after 14 days of exposure to saline. Similarly, experiments involving antimicrobial exposure were performed in duplicate or triplicate. In addition to determination of viable counts, a coupon from each experiment was also placed in sterile MHB for 48 h at 37°C with agitation to maximize the possibility of detection of any viable cells in the biofilm.

In experiments in which viable organisms were recovered from the biofilm, the organisms were subcultured on Mueller-Hinton agar and preserved at −70°C as described above. The organisms were confirmed to be coagulase-negative staphylococci by use of the Vitex GPI card (Biomerieux, Marcy l'Etoile, France). The MIC of the antimicrobial to which recovered viable cells had been exposed was determined by the NCCLS agar dilution method (20).

**SEM.** The biofilm was visualized by scanning electron microscopy (SEM). Following the washing step, the biofilm was fixed with 3% glutaraldehyde for 30 min and dehydrated through a graded series of aqueous ethanol (50 to 100%) for durations of 15 to 30 min. Specimens were treated with 1 drop of hexamethyl disilazane (Sigma-Aldrich Corp.), mounted on aluminum stubs with quick-drying silver paint (Agar Scientific Ltd., Stansted, United Kingdom), coated in gold with an EMSCOPE SC500 gold sputter coater, and viewed and photographed with a scanning electron microscope (Hitachi Corp., Tokyo, Japan).

## RESULTS

**MICs.** The MICs for *S. epidermidis* ATCC 35984 were as follows: gentamicin, 2 μg/ml; vancomycin, 2 μg/ml; linezolid, 2 μg/ml; and eperezolid, 0.5 μg/ml. The strain was confirmed to be *mecA* positive by PCR (data not shown).

**In vitro model of biofilm development and antimicrobial lock technique.** After the organisms were seeded from the continuous-culture system (6 h) and irrigated with MHB for 18 h, the mean viable count was determined to be $4.6 \times 10^8 \pm 2.7 \times 10^8$ CFU/coupon and the presence of a biofilm was confirmed by SEM. The mean viable counts following exposure to saline, saline containing heparin at 10 U/ml, or the antimicrobial solutions are presented in Table 1. After 72 h of exposure to linezolid (2 mg/ml), 240 h of exposure to vancomycin (10 mg/ml), or 168 h of exposure to eperezolid (4 mg/ml), the coupons were sterile on the basis of an absence of growth from coupons following 48 h of incubation in MHB and the lack of detectable organisms by determination of viable counts. Inclusion of heparin at a concentration of 10 U/ml did not impair the activity of linezolid against the biofilm. SEM confirmed that the biofilm matrix was still present on the coupon surface. Significant biofilm killing was not achieved with gentamicin (10 mg/ml) at any of the time intervals examined. Recovery of viable organisms from the MRD following prolonged antimicrobial exposure was not associated with acquired resistance in the planktonic phase of growth, as determined by the NCCLS

TABLE 1. Effects of saline and antimicrobial solutions on viability of *S. epidermidis* ATCC 35984 biofilm[a] in an MRD

| Lock period (h) | Mean ± SD no. of CFU/coupon[b] | | | | | |
|---|---|---|---|---|---|---|
| | Sterile saline (0.9%) | Sterile saline (0.9%) and heparin (10 U/ml) | Vancomycin (10 mg/ml) | Gentamicin (10 mg/ml) | Linezolid (2 mg/ml) | Eperezolid (4 mg/ml) |
| 24 | ND[c] | ND | $(2.5 \pm 2.1) \times 10^7$ (1)[d] | $(2.7 \pm 1.3) \times 10^7$ (1) | $(7.1 \pm 6.2) \times 10^5$ (3) | $(6.7 \pm 4.0) \times 10^6$ (1) |
| 72 | ND | ND | $(4.3 \pm 3.5) \times 10^6$ (1) | $(3.7 \pm 2.1) \times 10^6$ (1) | NVCR[e] (3) | $(1.3 \pm 0.6) \times 10^6$ (2) |
| 168 | ND | ND | $(1.4 \pm 1.2) \times 10^5$ (2) | $(8.4 \pm 3.6) \times 10^6$ (1) | NVCR (1) | NVCR (2) |
| 240 | ND | ND | NVCR (2) | $(6.5 \pm 2.4) \times 10^6$ (2) | NVCR (1) | ND |
| 336 | $(2.5 \pm 1.5) \times 10^7$ (3) | $(9.2 \pm 2.3) \times 10^6$ (2) | ND | ND | ND | ND |

[a] Average biofilm size before antimicrobial lock experiments, $(4.6 \pm 2.7) \times 10^8$ CFU/coupon ($n = 3$).
[b] The surface area of the coupon was 50 $mm^2$.
[c] ND, not determined.
[d] The values in parentheses indicate the numbers of replicates.
[e] NVCR, no viable cells recovered.

agar dilution method. The antimicrobial concentrations in the MRD at 37°C declined by 9% for linezolid after 72 h; 36 and 3% for vancomycin and gentamicin, respectively, after 240 h; and 16% for eperezolid after 168 h.

## DISCUSSION

Therapy for a CR-BSI with systemic antimicrobial therapy alone while the CVC remains in situ is frequently associated with a recurrence of infection following the discontinuation of antimicrobial therapy. However, removal of the CVC may not be necessary in all cases, and antimicrobial lock therapy represents an opportunity to salvage infected CVCs. The application of antimicrobial lock therapy may be most appropriate for those with CR-BSIs associated with long-term tunneled catheters in which the infecting organism is of low virulence (22). A recent review of antimicrobial lock therapy has suggested a period of antimicrobial lock of up to 10 days. However, this may pose practical problems in patients in whom ongoing central venous access is essential. Evidence on which to base selection of the optimal antimicrobial agent, the optimal concentration, and the optimal duration of antimicrobial lock therapy is limited in particular for newer agents. Our results suggest that for *S. epidermidis*-associated CR-BSIs, a 10-day period of antimicrobial lock may be appropriate for vancomycin but that a much shorter duration of antimicrobial lock may be sufficient for linezolid. The results obtained with eperezolid suggest that the enhanced effectiveness of eperezolid relative to that of vancomycin is a property of the oxazolidinone class of antimicrobial agents. These findings raise uncertainty concerning the appropriateness of the use of gentamicin for antimicrobial lock therapy for *S. epidermidis* CR-BSIs. Our finding that heparin does not inhibit the activity of linezolid against *S. epidermidis* biofilms may be of practical importance and may allow clinicians to include heparin in antimicrobial lock solutions to prevent thrombosis in the CVC during the period of lock (4).

The relatively rapid killing of the *S. epidermidis* biofilm with linezolid (2 mg/ml) relative to that achieved with vancomycin (10 mg/ml) was an unexpected finding that has not previously been reported (Table 1). This effect was not attributable to carryover of residual linezolid from the biofilm matrix, as the coupons were washed to remove residual linezolid and no linezolid activity was detectable on bioassay of the saline solution following resuspension of cells from the coupon (results not shown). The observed effect is unlikely to be attributable to a component of the intravenous solution used other than linezolid, as the other components do not have potent antibacterial activities. On the basis of conventional studies with planktonic-phase bacteria, vancomycin is generally considered bactericidal, while linezolid is considered bacteriostatic. Furthermore, Wilcox et al. (29) have reported that the concentrations of vancomycin are higher than those of linezolid in intravascular catheter-associated biofilms. The relevance of the distinction between bacteriostatic and bactericidal activities for therapeutics is uncertain, and recent clinical studies suggest that the activity of linezolid is superior to that of vancomycin for therapy of lower respiratory tract infections associated with methicillin-resistant *S. aureus* (R. G. Wunderink, S. K. Cammarata, R. V. Croos-Dabrera, and M. H. Kollef, Abstr. 40th Annu. Meet. Infect. Dis. Soc. Am., abstr. 179, 2002). The study of Gander et al. (13), which evaluated the activity of linezolid relative to those of other antimicrobial agents, including glycopeptides and quinuprisitin-dalfopristin, did not report effects similar to those that we have observed. However, the model used and the concentrations and the duration of exposure are not comparable to those used in our study and do not simulate antimicrobial lock therapy in the same way as our model does. Gander et al. (13) used a model consisting of a cylindrical paper sleeve encasing a compacted mesh of cellulose fibers. The biofilm is formed on the cellulose fiber. Antimicrobial exposure occurs by continuous perfusion through the model, and the impacts of antimicrobial agents on the biofilm are evaluated through enumeration of the bacteria eluted from the biofilm rather than through direct examination of the biofilm itself.

We speculate that the bacteria in a biofilm may have a relatively rapid turnover of enzymes and may be susceptible to inhibition of protein synthesis. However, the cells could be dividing relatively slowly and could possibly be insensitive to the effects of agents that inhibit cell wall synthesis. This hypothesis is not consistent with the lack of an effect of gentamicin, since the principle mechanism of action of aminoglycosides is also inhibition of protein synthesis. Other factors that may contribute to differences in the rates of killing of *S. epidermidis* biofilms by antimicrobial agents include differences in the levels of penetration of the antimicrobials into the biofilm and the possible existence of foci of anaerobiosis and low pH in the biofilm matrix. Aminoglycosides are relatively inactive

under conditions of anearobiosis or low pH. Recently, Monzon et al. (19), using a very different model of an *S. epidermidis* biofilm and a different measure of biofilm killing, also reported that erythromycin, rifampin, and tetracycline (bacteriostatic agents that act by inhibition of protein synthesis) exhibited activities superior to that of vancomycin against an *S. epidermidis* biofilm. However, clindamycin yielded results comparable to those obtained with vancomycin.

The model of *S. epidermidis* used in the present study is intended to replicate as closely as possible the biofilm formed in CVCs in a highly standardized way. At present there is no universally accepted in vitro model of CVC biofilm infection. While our model simulates a catheter lumen with medical-grade polyurethane coupons under flow conditions, the model is complex; and this poses limitations on the number of strains, antimicrobial agents, and antimicrobial concentrations that can be tested. Due to these limitations, each antimicrobial agent was studied at a single concentration. In the case of vancomycin and gentamicin, the concentrations were selected on the basis of a suggested concentration for antimicrobial lock therapy (4). For linezolid, the concentration selected was the concentration commercially available for intravenous administration and is close to the maximum solubility of linezolid (2.9 mg/ml). The concentration used for eperezolid was also close to the maximum solubility (4.2 mg/ml). There was not a constant ratio between the conventional MIC of each agent and the concentration chosen for antimicrobial lock therapy, and dose-response curves were not prepared. We cannot exclude the possibility that some of the differences between antimicrobial agents observed relate to the use of suboptimal concentrations of some agents, and we also cannot directly correlate the data with clinical use.

Notwithstanding the limitations of the present study, it may be appropriate to consider the use of linezolid for antimicrobial lock therapy in selected patients. Well-structured clinical trails are the optimal approach to defining appropriate antimicrobial agents and appropriate concentrations for antimicrobial lock therapy. However, such studies are likely to be difficult to establish, and further in vitro work may help to define the parameters that should be addressed in clinical trials or animal studies. Future work to develop a model that produces results that correlate with those observed in this study but in a format that is better adapted to the screening of multiple bacterial isolates at multiple concentrations of antimicrobial agents is planned.

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