Evaluation of linezolid for the treatment of *Clostridium difficile* infection caused by epidemic strains using an *in vitro* human gut model

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Objectives: Therapeutic options in *Clostridium difficile* infection (CDI) are limited. We examined linezolid activity *in vitro* and potential therapeutic efficacy using a gut model of CDI.

Methods: MICs were determined by agar incorporation for 118 diverse *C. difficile* faecal isolates, including epidemic strains and strains with reduced susceptibility to metronidazole. CDI was established in two gut model experiments using *C. difficile* epidemic strains (ribotypes 027 and 106) and linezolid was dosed to achieve human gut concentrations.

Results: Linezolid demonstrated good *in vitro* activity against 98% of the isolates. Two isolates (PCR ribotypes 023 and 067) demonstrated resistance to linezolid, although supplementary susceptibility testing of ribotype 023 isolates did not detect further resistance. In a gut model that simulates CDI, linezolid reduced the duration of cytotoxin production by *C. difficile* PCR ribotype 027 without influencing viable counts of vegetative forms of the organism. *C. difficile* PCR ribotype 106 viable counts declined at a faster rate than those of PCR ribotype 027 following dosing with linezolid, but cytotoxin titres declined at a similar rate to an untreated control. Gut flora perturbation occurring on linezolid exposure reversed after drug cessation. Recrudescence of spore germination with subsequent cytotoxin was seen with the *C. difficile* ribotype 106 strain. Resistance to linezolid was not detected either during linezolid instillation or post-dosing.

Conclusions: Linezolid may reduce toxin levels, as reported in staphylococci and streptococci. Further evaluation is warranted of the effect of linezolid on expression of *C. difficile* toxin, and to investigate potential recurrence of CDI following cessation of linezolid.

Keywords: spores, toxin, recurrence

Introduction

The incidence of *Clostridium difficile* infection (CDI) has increased in recent years, particularly disease attributed to an apparently hyper-virulent *C. difficile* strain that has been identified as 027 by PCR ribotyping, NAP1 by PFGE and BI by restriction endonuclease analysis. CDI is a major financial burden upon healthcare systems worldwide; a recent report estimated that in the USA alone the annual costs associated with CDI were $3.4 billion. CDI is almost exclusively associated with prior antimicrobial therapy and classes of antimicrobial agents with a noted predisposition for induction of CDI include lincosamides, aminopenicillins and cephalosporins (particularly third generation). Antimicrobial agents that induce CDI are hypothesized to perturb the stable colonic microflora, reduce host colonization resistance and thus facilitate *C. difficile* spore germination, proliferation and toxin production.

Treatment strategies for CDI have changed little over the past two decades. Oral metronidazole (400–500 mg three times daily) or vancomycin (125 mg four times daily) are most commonly used to treat CDI. Early studies demonstrated little difference between metronidazole and vancomycin in terms of response or recurrence rates, although response time was faster with the latter. More recent reports have questioned the efficacy of metronidazole therapy for CDI, particularly for disease caused by epidemic *C. difficile* ribotype 027. These
studies have reinforced the need to evaluate the efficacy of new antimicrobial agents for the treatment of CDI. We have used a triple-stage chemostat human gut model to investigate both antimicrobial induction\textsuperscript{15–19} and treatment of CDI.\textsuperscript{20–23}

Linezolid is an oxazolidinone antimicrobial agent active principally against Gram-positive bacteria by inhibiting protein synthesis via targeting of bacterial 23S rRNA.\textsuperscript{24} Linezolid is not currently used to treat CDI, but can inhibit exotoxin production\textsuperscript{25,26} and has been reported to have good activity against \textit{C. difficile}.\textsuperscript{27,28} Susceptibilities of 118 \textit{C. difficile} isolates (including epidemic strains and isolates with reduced susceptibility to metronidazole\textsuperscript{29}) to linezolid were determined in a preliminary study using an agar incorporation method. We then examined the efficacy of linezolid in treating simulated CDI caused by epidemic \textit{C. difficile} PCR ribotypes 027 and 106 in separate experiments using a triple-stage chemostat human gut model.

\section*{Materials and methods}

\textbf{C. difficile strains}

The \textit{C. difficile} ribotype 027 strain evaluated in the \textit{in vitro} human gut model was isolated during an outbreak of CDI at the Maine Medical Center (Portland, ME, USA) in 2005 and was supplied via Dr Robert Owens (Maine Medical Center). The \textit{C. difficile} PCR ribotype 106 strain evaluated in the \textit{in vitro} human gut model was isolated from a symptomatic female patient with CDI at the Leeds General Infirmary in 2007 (Leeds, UK). A total of 32 genotypically distinct \textit{C. difficile} isolates (by PCR ribotyping), 11 each of the three most commonly encountered \textit{C. difficile} PCR ribotypes in the UK (ribotypes 001, 106 and 027) and 22 \textit{C. difficile} isolates with reduced susceptibility to metronidazole\textsuperscript{25} were used in linezolid susceptibility studies. Linezolid susceptibilities of the epidemic \textit{C. difficile} strains studied in prior gut model experiments were also determined, i.e. PCR ribotypes 001 (n = 1), 106 (n = 1) and 027 (n = 2). PCR ribotyping was performed by Dr Warren N. Fowlie [\textit{C. difficile} Ribotyping Network for England and N. Ireland (CDRN), Leeds General Infirmary, Leeds, UK] using the method of Stubbs et al.\textsuperscript{30} Additionally, linezolid MICs for 27 \textit{C. difficile} PCR ribotype 023 faecal isolates recovered via the CDRN were determined.

\textbf{Triple-stage chemostat human gut model}

We have described previously the use of a triple-stage chemostat human gut model to study the interplay between antimicrobial agents, the indigenous gut microflora and \textit{C. difficile}.\textsuperscript{15–23} The gut model was validated against physicochemical and microbiological measurements from the intestinal contents of sudden-death victims.\textsuperscript{31} The gut model is, however, limited by an inability to simulate immunological and secretory events that occur within the human colon. The gut model comprised three pH-maintained (pH 5.5 ± 0.1, vessel 1; pH 6.2 ± 0.1, vessel 2; and pH 6.8 ± 0.1, vessel 3) fermentation vessels, top-fed by growth medium at a controlled rate [dilution rate (D) ≈ 0.015 h\textsuperscript{-1}]. Constituents and preparation of growth medium for the gut model were as described previously.\textsuperscript{15} The gut model was inoculated with a faecal emulsion (~10% w/v in pre-reduced PBS) prepared from \textit{C. difficile}-negative faeces of five elderly (≥65 years) volunteers. Faecal donors were in good health and received no antimicrobial therapy for at least 3 months prior to commencement of this study.

\textbf{Enumeration of gut microflora and \textit{C. difficile} cytotoxin titres}

Gut bacterial populations and \textit{C. difficile} numbers were enumerated as described previously.\textsuperscript{15} Gut microflora populations cultured were total facultative anaerobes, total anaerobes (facultative+obligate), lactose-fermenting Enterobacteriaceae, enterococci, lactobacilli, bifidobacteria, total \textit{Clostridium} spp., \textit{Bacteroides fragilis} group, \textit{C. difficile} total viable counts (vegetative \textit{C. difficile}+spores) and \textit{C. difficile} spore viable counts. \textit{C. difficile} cytotoxin production was monitored using a Vero cell cytotoxicity assay as described previously.\textsuperscript{15} Indigenous gut microflora populations from vessel 1 of the gut models were not determined; only \textit{C. difficile} total viable counts, spore counts and cytotoxin titres were quantified. Additionally, \textit{C. difficile} selective agar containing 4 mg/L linezolid was used to monitor selection for \textit{C. difficile} PCR ribotype 106 or 027 resistant to linezolid once dosing of the drug commenced.

\textbf{Experimental design}

Time periods for this experiment are displayed in Figure 1. Following inoculation of the gut model with faecal emulsion (day 0) the medium pump was started and no further interventions were made for 13 days. Gut microflora were enumerated every 2 days. \textit{C. difficile} spores (~10\textsuperscript{7} cfu) were prepared as described previously\textsuperscript{15} and inoculated into vessel 1 on day 14. Viable counts of \textit{C. difficile} and the indigenous gut microflora and \textit{C. difficile} cytotoxin titres were monitored daily thereafter. After 7 days another single inoculum of \textit{C. difficile} spores was instilled into vessel 1 followed by 150 mg/L ceftriaxone once daily for 7 days. Ceftriaxone was instilled to reflect the concentration observed in faeces of patients and volunteers.\textsuperscript{15} Instillation of linezolid commenced once high-level cytotoxin titres [≥4 relative units (RU)] were observed for at

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Schematic representation of time periods in gut model experiments with linezolid. Time periods correspond to: A, steady-state equilibration period; B, \textit{C. difficile} internal control (antimicrobial-free) period; C, ceftriaxone instillation period; D, \textit{C. difficile} induction period; E, linezolid instillation period; and F, post-dosing recovery period. CD, \textit{C. difficile} spores; CRO, ceftriaxone; LZD, linezolid. *Variable duration depending on \textit{C. difficile} strain under study.}
\end{figure}
least two consecutive days. Linezolid (PNU-100766, Lot # 1000113796, 7.1 mg/L; Pfizer, Inc., CT, USA33) was instilled into vessel 1 of the gut model twice daily for 7 days. Following cessation of linezolid instillation, gut bacterial populations and C. difficile cytotoxin titres were monitored for a further 14 days.

Antimicrobial assay and MIC determination

Samples (1 mL) from each vessel of the gut model were centrifuged (15 min, 16,000 g) and the supernatants sterilized by filtration through 0.22 μm syringe filters. Sterilized culture supernatants were stored at −70°C prior to antimicrobial assay. Linezolid concentrations were determined by large plate bioassay (Leeds) and by a previously validated HPLC method (BCARE Bristol34). Chromatography was performed on a Hypersil S50 column (HPLC Technology Ltd, Macclesfield, UK) using a mobile phase of methanol/water/phosphoric acid (30:69:1) with the addition of 2 g/L heptane sulphonic acid (Sigma Chemical Co.) and the pH adjusted to 4.5. The assay response was linear over the concentration range 0.05–100 mg/L, with a lower limit of quantification of 0.1 mg/L for linezolid. Recovery was in the range 95%–110%, and intra-day accuracy and precision were assessed by the use of quality control standards with limits of accuracy of 10% (actual = 9.5%) and coefficient of variability for precision of <5% (actual = 2.3%).

Microbiological bioassay

Spore suspensions (4×10⁸ cfu/mL) of the indicator organism Bacillus subtilis ATCC 6633 were prepared to seed bioassay agar. Briefly, 30 mL volumes of LB broth were inoculated with a fresh (24 h) culture of B. subtilis ATCC 6633 and incubated at 35°C for 48 h with continuous agitation. Cultures were centrifuged at 3000 g for 15 min, the supernatants discarded and the pellets alcohol shocked in 6 mL of 50% (v/v in saline) ethanol for at least 1 h. Spore preparations were stored at ambient temperature until required. B. subtilis spore suspension (500 μL) was added to 100 mL of molten Mueller–Hinton agar (50°C), mixed by inversion and agar poured into 245 mm² bioassay dishes. Agars were allowed to set at room temperature, following which 25 wells (9 mm in diameter) were removed from the agar using a cork borer. Linezolid calibrators (30 μL, 4–64 mg/L) and filter-sterilized samples from the gut model were randomly assigned to bioassay wells in triplicate. Bioassay plates remained at ambient temperature for 4 h before overnight aerobic incubation at 37°C. Zone diameters were measured using calipers accurate to 0.1 mm. Calibration lines were plotted from squared zone diameters and unknown concentrations from culture supernatants determined. All assays were performed in duplicate. Coefficient of variation values were typically 15% for linezolid bioassays and R² values for calibration lines all >0.96. The limit of detection for the linezolid bioassay was 3 mg/L.

Linezolid MIC determination

Agar incorporation MICs were determined using previously published methods.35 Antimicrobial agents evaluated in MIC studies were linezolid, metronidazole and vancomycin. All antimicrobial stock solutions were prepared in deionized water and sterilized by filtration through 0.22 μm syringe filters. Briefly, C. difficile strains were cultured in pre-reduced Schaedler’s anaerobic broth overnight to an inoculum density of ~10⁶ cfu/mL and diluted 1:10 in sterile pre-reduced saline. One microlitre of diluted culture (~10⁴ cfu) was then applied to antimicrobial-containing or control agar (Wilkins Chalgren agar) using a multipoint inoculator. Antimicrobial-containing plates and growth controls were incubated at 37°C in an anaerobic atmosphere for 48 h. MICs were determined in duplicate for all antimicrobials. The MIC was determined as the antimicrobial concentration where an absence or marked reduction in growth (multiple tiny colonies, hazy or fine film of growth or one or two colonies) compared with the growth control was observed. Linezolid MICs for an additional panel of C. difficile PCR ribotype 023 isolates (n = 27) were also determined.

Results

Susceptibility of C. difficile to linezolid

Linezolid demonstrated overall equivalent activity to metronidazole and vancomycin against a large collection of clinical C. difficile isolates (Table 1). Furthermore, linezolid demonstrated equivalent activity to vancomycin against a panel of C. difficile recently shown to have reduced susceptibility to metronidazole; geometric mean MICs were 1.17 and 1.13 mg/L for linezolid and vancomycin, respectively. If the panel of C. difficile with reduced susceptibility to metronidazole was excluded from the analysis, MIC₉₀ values of all antimicrobial agents were identical (1 mg/L), while MIC₉₀ of linezolid and vancomycin were identical (1 mg/L) with metronidazole one doubling dilution higher (2 mg/L) (data not shown). Single isolates of two C. difficile strains (PCR ribotypes 023 and 067) were resistant to linezolid (BSAC/CLSI breakpoint MIC >4 mg/L) with MICs of 8 mg/L. Analysis of a further panel (n = 27) of C. difficile ribotype 023 demonstrated no resistant isolates, with a geometric mean linezolid MIC of 1.33 mg/L (MIC₉₀/₉₀ = 1/2 mg/L) (data not shown). No further C. difficile PCR ribotype 067 could be obtained for supplementary linezolid MIC determination despite a pan-European search, indicating the relative rarity of this ribotype.

Equilibration period and internal C. difficile control period

In general, observations in vessels 2 and 3 of the gut model were very similar, and therefore we have concentrated on data from vessel 2 of each experiment in this report. The composition of the indigenous gut microflora in C. difficile PCR ribotype 027 and 106 gut models was similar, and both achieved steady-state by the end of period A. Indigenous gut microflora were dominated by obligate anaerobes, e.g. B. fragilis group and bifidobacteria.

### Table 1. MICs (mg/L) of linezolid for C. difficile

<table>
<thead>
<tr>
<th>Geometric mean MIC</th>
<th>n</th>
<th>Linezolid</th>
<th>Metronidazole</th>
<th>Vancomycin</th>
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<tbody>
<tr>
<td>Total</td>
<td>91</td>
<td>1.15</td>
<td>1.31</td>
<td>0.88</td>
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<td>MIC₉₀</td>
<td>91</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>range</td>
<td>91</td>
<td>1–8</td>
<td>0.125–8</td>
<td>0.5–4</td>
</tr>
<tr>
<td>Genotypically distinct</td>
<td>32</td>
<td>1.33</td>
<td>0.36</td>
<td>0.79</td>
</tr>
<tr>
<td>11×001, 11×106 and 11×027</td>
<td>33</td>
<td>1.00</td>
<td>1.66</td>
<td>0.86</td>
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<td>Fully susceptible to metronidazole</td>
<td>69</td>
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<td>0.82</td>
<td>0.81</td>
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<td>Reduced susceptibility to metronidazole</td>
<td>22</td>
<td>1.17</td>
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<td>1.13</td>
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001, PCR ribotype 001; 106, PCR ribotype 106; 027, PCR ribotype 027.

#Two individual C. difficile isolates demonstrated linezolid MICs of 8 mg/L.

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**Source:** Linezolid and C. difficile infection model

**Author:** JAC

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001, PCR ribotype 001; 106, PCR ribotype 106; 027, PCR ribotype 027.

#Two individual C. difficile isolates demonstrated linezolid MICs of 8 mg/L.
and also *Lactobacillus* spp. (data not shown). *C. difficile* was not isolated during the equilibration period. Following its instillation, *C. difficile* remained as spores and cytotoxin was undetectable for the remainder of the period (period B, Figure 2a and b).

**Effects of ceftriaxone instillation**

Instillation of ceftriaxone facilitated similar alterations in indigenous gut microflora composition in both gut models. A deleterious effect of ceftriaxone instillation against bifidobacteria (4 log_{10} cfu/mL), *B. fragilis* group (2 log_{10} cfu/mL), lactobacilli (2 log_{10} cfu/mL) and lactose-fermenting Enterobacteriaceae (2 log_{10} cfu/mL) was observed, although *B. fragilis* group viable counts increased again by the end of ceftriaxone instillation. *Enterococcus* spp. were unaffected by ceftriaxone instillation and viable counts increased by 2 log_{10} cfu/mL by the end of ceftriaxone instillation. Following a second instillation of *C. difficile* spores concurrent to ceftriaxone instillation, *C. difficile* remained quiescent for 2 and 3 days, respectively, in the PCR ribotype 027 and 106 gut models (period C, Figure 2a and b). A marked increase in *C. difficile* total viable count over spore count was observed after 3 and 4 days of ceftriaxone instillation, respectively, in the PCR ribotype 027 and 106 gut

![Figure 2.](image-url)
models. *C. difficile* total viable counts increased to 6.5 log_{10} cfu/mL and stabilized at this concentration in both gut models for the remainder of the ceftriaxone instillation period. *C. difficile* cytotoxin was detected after 4 and 5 days of ceftriaxone instillation, respectively, in the PCR ribotype 106 and 027 gut models. By the end of ceftriaxone instillation *C. difficile* cytotoxin titres were 3 RU (PCR ribotype 106) and 4 RU (PCR ribotype 027). No *C. difficile* spore germination, proliferation or cytotoxin production was observed in vessel 1 of either gut model (data not shown).

**Linezolid instillation**

Instillation of linezolid commenced 2 days after cessation of ceftriaxone dosing in the *C. difficile* PCR ribotype 027 gut model (day 29) and 1 day later in the *C. difficile* PCR ribotype 106 gut model (day 30).

**Concentrations of linezolid in the gut model**

Linezolid was instilled into vessel 1 of the gut model at 7 mg/L twice daily, which according to theoretical mass balance calculations should have reached steady-state concentrations of 16.22 mg/L in vessel 1 of the gut model (calculations not shown). Linezolid was detectable in all vessels of the gut models using both assay methods (Figure 3a and b). Linezolid concentrations demonstrated by microbiological bioassay were lower than those by HPLC. Peak linezolid concentrations in vessel 1 of the *C. difficile* PCR ribotype 027 gut model were substantially higher than those in vessels 2 and 3 and also the corresponding PCR ribotype 106 gut model. A malfunction of the growth medium pump in the *C. difficile* PCR ribotype 027 gut model (day 30/31) occurred, which may explain the markedly higher concentration of linezolid in vessel 1 of the gut model at that time. Consequently, linezolid was undetectable by HPLC in vessel 3 of the *C. difficile* PCR ribotype 027 gut model until 4 days after commencement of linezolid instillation, i.e. 2 days after linezolid was detected by HPLC in vessel 3 of the corresponding PCR ribotype 106 gut model. Peak linezolid concentrations were similar in vessels 2 and 3 of both gut models (Figure 3a and b).

**Effects of linezolid on gut microflora and *C. difficile* in the PCR ribotype 106 gut model**

Linezolid instillation facilitated minor reductions in viable counts of *Bifidobacterium* spp., *Lactobacillus* spp. and *Enterococcus* spp., 

![Figure 3](chart.png)

**Figure 3.** (a) Concentrations of linezolid (mg/L) in *C. difficile* PCR ribotype 106 gut model. Horizontal dashed line is limit of detection for bioassay. (b) Concentrations of linezolid (mg/L) in *C. difficile* PCR ribotype 027 gut model. Horizontal dashed line is limit of detection for bioassay. Asterisk denotes timing of pump malfunction; V, vessel.
and more substantial declines of 2.5 and 3 log viable counts of Clostridium spp. and B. fragilis group, respectively (pre-dosing counts of 8 and 7.5 log cfu/mL, respectively). Lactose-fermenting Enterobacteriaceae viable counts showed a substantial 5.5 log_{10} cfu/mL increase from 4.0–5.1 log cfu/mL to 9.5–9.9 log cfu/mL by the end of linezolid instillation. C. difficile total viable counts declined rapidly in vessels 2 and 3 of the gut model, with only C. difficile spore forms demonstrable 4 days after commencement of linezolid dosing (period E, Figure 2a and b). C. difficile cytotoxin titres declined after 3 days of linezolid instillation. Declines in viable counts of C. difficile PCR ribotype 106 vegetative forms (Figure 4a) following commencement of linezolid dosing were slightly faster than observations from the C. difficile PCR ribotype 027 gut model (Figure 4a). C. difficile PCR ribotype 106 cytotoxin titres declined at a similar rate to untreated (control) cytotoxin titres (PCR ribotype 027) (Figure 4b).

**Effects of linezolid on gut microflora and C. difficile in the PCR ribotype 027 gut model**

Alterations in viable counts of indigenous gut microfloras following commencement of linezolid instillation were similar to those observed in the PCR ribotype 106 gut model. The rate of decline in C. difficile total viable counts following instillation of linezolid was similar to that observed in an untreated ceftriaxone gut model study (Figure 4a). C. difficile PCR ribotype 027 cytotoxin titres following linezolid instillation were undetectable 6 days earlier than those measured in an untreated ceftriaxone gut model study (Figure 4b).

**Observations following cessation of linezolid instillation**

Viable counts of all the indigenous gut microfloras adversely affected by linezolid instillation recovered during the post-dosing recovery period in both gut models. Linezolid was undetectable (<3 mg/L) in vessels 1, 2 and 3 of the PCR ribotype 106 gut model 3, 4 and 6 days, respectively, after cessation of antibiotic instillation, as measured by bioassay (Figure 3). Linezolid was undetectable in the PCR ribotype 027 gut model after 2, 1 and 3 days in vessels 1, 2 and 3, respectively, as measured by bioassay. Increased C. difficile PCR ribotype 106 total viable counts over spore counts were observed in vessels 2 and 3 of the gut model 9 days after cessation of linezolid instillation, and subsequently C. difficile cytotoxin was detectable (Figure 2a). No additional cycle of growth and cytotoxin production was observed in the PCR ribotype 027 gut model following cessation of linezolid instillation (Figure 2b). No C. difficile colonies were recovered from selective agars containing 4 mg/L linezolid during either linezolid instillation or the post-dosing recovery period.

**Discussion**

Linezolid had good activity against 98% of the C. difficile isolates in this study, reflecting prior susceptibility studies. Two C. difficile isolates (ribotypes 023 and 067) were resistant to linezolid (MIC=8 mg/L). Of these two PCR ribotypes, 023 accounted for 3% of CDI reports in a recent European study, while 067 is a very uncommon cause of CDI. Evaluation of a further panel of C. difficile PCR ribotype 023 demonstrated no additional resistant isolates, and therefore linezolid resistance in C. difficile appears to be rare and not related to ribotype clones. Resistance to linezolid in C. difficile was first documented by Ackermann et al. who showed low-level resistance (6 mg/L) in 6 of 192 clinical C. difficile isolates. This group reported elevated linezolid MICs associated with erythromycin and clindamycin resistance, and we also observed this in ribotypes 023 and 067 upon further investigation (MIC 128–>256 mg/L of both antimicrobials). However, in contrast to Ackermann et al., both were susceptible to moxifloxacin (MIC 2 mg/L). Ackermann et al. did not perform DNA typing on the C. difficile isolates, and therefore it is difficult to determine the epidemiological significance of the cross-resistance they described. Mechanisms of resistance to linezolid have been documented in some Gram-positive bacteria, but not in C. difficile, although linezolid MICs up to 24 mg/L have been reported. Failure to isolate C. difficile from gut model samples, collected during and after prolonged exposure, that were plated onto linezolid breakpoint agar, suggests there is a low propensity for resistance development via clonal selection. Indeed, Schmidt et al. failed to select resistant C. difficile using an agar-based method over three bacterial generations. The acquisition/development of linezolid resistance in C. difficile using other experimental techniques warrants further study especially if linezolid may be considered a potential treatment for CDI in the future.

In the present gut model experiments we used ceftriaxone to induce C. difficile spore germination, proliferation and high-level cytotoxin production, as previously demonstrated with cefotaxime. Cephalosporin (particularly second and third generation) administration is a risk factor for CDI in vivo. Ceftriaxone dosing aimed to simulate expected in vivo antibiotic concentrations. Pletz et al. demonstrated 152 mg/kg and 258 mg/kg ceftriaxone in the faeces of patients 4 days and 8 days after commencing therapy, respectively, which is reflective of the ceftriaxone concentrations we observed in vessel 1 (if mg/kg are assumed approximately equal to mg/L). Ceftriaxone was detectable at only low concentrations in vessel 2 of the gut models (<5 mg/L) and was undetectable in vessel 3 of the gut models (data not shown). The lack of detectable bioactive ceftriaxone in the vessels of the gut model that simulate the distal colon suggests that either drug degradation/inactivation or non-specific binding occurred. Notably, the impact of ceftriaxone on the indigenous gut microfloras was reflective of prior studies in our laboratory (S. D. Baines, G. S. Huscroft, S. L. Todhunter, J. Freeman and M. H. Wilcox, unpublished data) and also prior in vivo studies. Germination of C. difficile PCR ribotype 106 and 027 spores was observed only in vessels 2 and 3 of the gut model. Ceftriaxone MICs for both strains used in the gut model experiments were 32 mg/L, and thus the supra-MIC concentrations of ceftriaxone in vessel 1 of the gut model were probably inhibitory to C. difficile proliferation and cytotoxin production. Spore germination studies using phase-contrast microscopy in our laboratory suggested that supra-MIC concentrations of ceftriaxone in vessel 1 of the gut model were probably inhibitory to C. difficile proliferation and cytotoxin production. An alternative explanation for the lack of C. difficile spore germination, proliferation and cytotoxin production in vessel 1 of the gut model could be that the more...
acidic (pH 5.5) conditions within this vessel were not conducive to spore germination. A recent study by Wheeldon et al.45 investigated chemical and physical factors that may influence C. difficile spore germination; pH 6.5–7.5 was optimum for C. difficile spore germination, and the rate and extent of germination was reduced at pH 5.5 and 8.5. Lode et al.33 demonstrated mean faecal concentrations of linezolid of 7.1 mg/kg (+2.6 mg/kg) in volunteers after 4 days of linezolid dosing. We therefore instilled linezolid into the gut model to achieve 7 mg/L in vessel 1. Linezolid levels differed between the two models over the first 3 days, with a substantial peak in vessel 1 concentrations on day 3 of dosing in the 027 model. This is almost certainly due to the pump malfunction described earlier. However, there was concurrence of linezolid concentrations in vessels 2 and 3. Peak linezolid concentrations and profiles in vessels 2 and 3 of both models were similar (Figure 3a and b). There was a discrepancy between bioassay and HPLC-derived data, particularly in the 106 model, with peak levels determined by bioassay almost half those measured by HPLC (vessel 1: 7.2 versus 16.9 mg/L; vessel 2: 9.6 versus 16.7 mg/L; vessel 3: 9.2 versus 15.9 mg/L). This is within one doubling dilution and may simply be due to the bioassay operating near to its limit of detection (3 mg/L), and consequently zone sizes being difficult to measure accurately. Additional measurement by HPLC was undertaken to give better sensitivity, given the expected low concentrations of linezolid. The antimicrobial effects of linezolid against the indigenous gut microfloras within the vessels of the gut model largely reflected prior in vivo studies in healthy volunteers.33 Obligate anaerobe populations and lactobacilli were most adversely affected by linezolid instillation, while viable counts of other facultative anaerobes (lactose-fermenting Enterobacteriaceae) increased. Declines in adversely affected bacterial groups were slow in comparison with other therapeutic agents that have been examined in the gut model (glycopeptides and metronidazole), and probably reflect the pharmacodynamic action of linezolid. Linezolid is bacteriostatic against most susceptible organisms and as such activity against the indigenous gut microfloras in the gut model would result in a slow decline in numbers. Crucially, indigenous gut microfloras within the gut model may not be actively proliferating upon commencement of antimicrobial instillation. Hence, metabolically active antimicrobials such as linezolid may potentially demonstrate reduced inhibitory activity. Upon instillation of linezolid into the model, C. difficile

Figure 4. (a) Comparative C. difficile PCR ribotype 027 and 106 total viable counts (log_{10} cfu/mL) in vessel 2 of the gut model in linezolid-treated and control (PCR ribotype 027) ceftriaxone gut model experiments. Day 0 corresponds to the day of experiment preceding C. difficile spore germination. (b) Comparative C. difficile PCR ribotype 027 and 106 cytotoxin production profiles in linezolid-treated and control (PCR ribotype 027) ceftriaxone gut model experiments.
populations were in stationary/early decline phase of growth, i.e. proliferating slowly or not at all, and were releasing cytotoxin in both experiments. Linezolid instillation was accompanied by declines in viable counts of vegetative *C. difficile* PCR ribotypes 027 and 106, but the rate and magnitude of decline were not substantially different from that observed in control 027 (untreated) experiments. Thus, we cannot conclude that the decline in *C. difficile* viable counts was a consequence of linezolid instillation. However, duration of cytotoxin detection was shorter in the linezolid-treated *C. difficile* PCR ribotype 027 gut model compared with an untreated control experiment. We have not evaluated *C. difficile* PCR ribotype 106 in a prior control (untreated) ceftriaxone gut model experiment; therefore whether duration of cytotoxin production was shorter following linezolid treatment remains to be definitively determined. This observation of reduced duration of toxin detection may reflect inhibition by linezolid of toxin gene translation, as has been demonstrated in staphylococci.25,26 Gerber et al46 used a simple batch culture method to demonstrate earlier toxin production and increased toxin gene transcription rates by *C. difficile* strains exposed to sub-MIC concentrations of metronidazole, vancomycin and linezolid, but not clindamycin. This may also indicate antimicrobial-mediated effects in *C. difficile* at the transcription level. We did not formally evaluate the ability of linezolid to induce *C. difficile* germination and toxin production in the gut model here. However, recrudescence of germination and toxin production by ribotype 106 showed post-germination toxin production by day 3, compared with day 2 following ceftriaxone induction (Figure 2a), which is in contrast to the findings of Gerber et al.46 This highlights the potential for conflicting results obtained in batch culture and continuous culture systems, which produce different bacterial growth dynamics; we believe that the latter are more reflective of CDI in vivo.

Linezolid concentrations differed between the gut models over the course of the initial 3 days of dosing. High peaks in vessel 1, with correspondingly low values in vessels 2 and 3 of the 027 model can be accounted for by the pump malfunction, which occurred on day 30/31. Linezolid concentrations quickly stabilized the following day and were similar to those in the 106 model for the duration of the dosing period. There was little discernible effect apart from a slight delay in detectable linezolid in vessel 3; there was no apparent effect upon *C. difficile* viable counts.

Following cessation of linezolid instillation viable counts of gut microflora generally increased, suggesting that linezolid does not promote sustained bacterial disruption. We observed a further cycle of *C. difficile* PCR ribotype 106 spore germination, proliferation and cytotoxin production following cessation of linezolid instillation. The significance and reproducibility of these observations remain to be determined. We have demonstrated a similar apparent recurrence in simulated CDI in the gut model following cessation of metronidazole instillation in *C. difficile* PCR ribotype 001 (UK strain) and 027 (USA strain).23 Interestingly, we demonstrated a similar recurrence of simulated CDI following cessation of vancomycin instillation in *C. difficile* PCR ribotype 027 (USA strain) experiment, but not in a comparator experiment with a UK PCR ribotype 027 strain or PCR ribotype 106 strain.27 Whether certain *C. difficile* PCR ribotypes are more likely to demonstrate spore recrudescence following the removal of (therapeutic) antimicrobial pressure remains to be determined.

Results from the CDI gut model have been shown to be predictive of clinical response, i.e. exposure of *C. difficile* to antimicrobials with a known propensity to induce CDI in vivo (e.g. cefotaxime, clindamycin and fluoroquinolones) facilitated germination, proliferation and high-level cytotoxin production within the gut model.18–23 Conversely, antimicrobial agents not readily associated with development of CDI in vivo (e.g. piperacillin/tazobactam, tigecycline and mecillinam) failed to facilitate sustained *C. difficile* germination, proliferation and high-level cytotoxin production.15–17 Prior gut model experiments examining treatment agents for clindamycin-induced CDI within the gut model have included evaluations of metronidazole (*C. difficile* PCR ribotypes 001 and 027), vancomycin (*C. difficile* PCR ribotypes 001, 106 and 027), ramoplanin (*C. difficile* PCR ribotype 001) and telovamer (*C. difficile* PCR ribotype 001).20–23

Given the data in the present study, further evaluation is warranted of the effect of linezolid on expression of *C. difficile* toxin, linezolid resistance in *C. difficile* and to investigate potential recurrence of CDI following removal of linezolid from the gut model.

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**References**


