Evaluation of antimicrobial activity of ceftaroline against *Clostridium difficile* and propensity to induce *C. difficile* infection in an *in vitro* human gut model

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**Objectives:** To examine the effects of exposure to ceftaroline or ceftriaxone on the epidemic *Clostridium difficile* strain PCR ribotype 027 and the indigenous gut microflora in an *in vitro* human gut model. Additionally, the MICs of ceftriaxone and ceftaroline for 60 *C. difficile* isolates were determined.

**Methods:** Two triple-stage chemostat gut models were primed with human faeces and exposed to ceftaroline (10 mg/L, twice daily, 7 days) or ceftriaxone (150 mg/L, once daily, 7 days). Populations of indigenous gut microorganisms, *C. difficile* total viable counts, spore counts, cytotoxin titres and antimicrobial concentrations were monitored throughout. MICs were determined by a standard agar incorporation method.

**Results:** In the gut model, both ceftaroline and ceftriaxone induced *C. difficile* spore germination, proliferation and toxin production, although germination occurred 5 days later in the ceftaroline-exposed model. Toxin detection was sustained until the end of the experimental period in both models. No active antimicrobial was detected in vessel 3 of either model, although inhibitory effects on microflora populations were observed. Cefaroline was ~8-fold more active against *C. difficile* than ceftriaxone (geometric mean MICs, 3.38 versus 28.18 mg/L; MIC90s, 4 versus 64 mg/L; and MIC ranges, 0.125–16 versus 8–128 mg/L).

**Conclusions:** Cefaroline, like ceftriaxone, can induce simulated *C. difficile* infection in a human gut model. However, low *in vivo* gut concentrations of ceftaroline and increased activity against *C. difficile* in comparison with ceftriaxone mean that the true propensity of this novel cephalosporin to induce *C. difficile* infection remains unclear.

**Keywords:** cephalosporins, chemostat, MICs

**Introduction**

*Clostridium difficile* infection (CDI) is particularly common amongst the hospitalized elderly. The emergence of an apparently hypervirulent *C. difficile* strain, which has been identified as PCR ribotype 027, NAP1 by PFGE and BI by restriction endonuclease analysis, led to an increased incidence of CDI.1–4 Although CDI attributed to ribotype 027 strains is decreasing in the UK,5 it remains a key potential healthcare-associated infection and a major financial burden upon healthcare systems worldwide. Recent systematic reviews have estimated that the incremental costs associated with CDI range from £4577 to £8843 across Europe6 and from US$4846 to US$8570 in the USA.7 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).8–10 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. The antimicrobial spectrum of activity does not necessarily correlate with the risk of induction of CDI in vivo. Antimicrobial agents with broad-spectrum activity may...
differ markedly in their propensity to induce CDI both in vitro and in vivo. For example, piperacillin/tazobactam is active against Gram-positive, Gram-negative and anaerobic bacteria, but is considered low risk for CDI induction. Conversely, third-generation cephalosporins are relatively high-risk antibiotics for CDI and yet generally have a spectrum of activity that is narrower than that of piperacillin/tazobactam.

We have used a triple-stage chemostat human gut model to investigate both antimicrobial predisposition to induction of CDI and efficacy of therapeutic interventions for clindamycin-induced CDI. These results from the gut model have correlated well with clinical observations. Antimicrobial agents with a propensity to induce CDI facilitated the germination of C. difficile spores and sustained high-level cytotoxin production (e.g. cefotaxime, clindamycin and fluoroquinolones), whereas those not closely associated with CDI did not (piperacillin/tazobactam and tigecycline). We have recently demonstrated the propensity of ceftriaxone to induce simulated CDI in the gut model.

Ceftaroline, the active metabolite of the prodrug ceftaroline fosamil, is a new cephalosporin approved in the USA for community-acquired bacterial pneumonia and acute bacterial skin and skin structure infection. It was also recently given European Medicines Agency approval for community-acquired pneumonia (CAP) and complicated skin and soft tissue infection (cSSTI). Here, we compare the effect of exposure to ceftriaxone or ceftaroline on the epidemic C. difficile strain PCR ribotype 027 and the indigenous gut microflora in the human gut model. We have also determined the susceptibilities of 60 C. difficile isolates to these cephalosporins.

Materials and methods

C. difficile strains

The C. difficile ribotype 027 strain evaluated in the in vitro human gut model was isolated during an outbreak of CDI at Maine Medical Centre (Portland, ME, USA) in 2005 and was supplied via Dr Robert Owens. A total of 30 genotypically distinct C. difficile isolates (by PCR ribotyping) and 10 each of three commonly encountered C. difficile PCR ribotypes in the UK (ribotypes 001, 106 and 027) were used in cephalosporin susceptibility studies. PCR ribotyping was performed by Dr Warren N. Fawley (Leeds Teaching Hospitals NHS Trust) using the method of Stubbs et al.

Triple-stage chemostat human gut model

The gut model was validated against physicochemical and microbiological measurements from the intestinal contents of sudden-death victims.

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 of 280 mL volume (vessel 1) and 300 mL volume (vessels 2 and 3), top-fed by growth medium at a controlled rate (D = 0.015 h⁻¹). Constituents and preparation of growth medium for the gut model were as described previously. The gut models were inoculated with a faecal emulsion (~10% w/v in pre-reduced PBS) prepared from C. difficile-negative faeces of five healthy elderly (≥65 years) volunteers. Faecal donors were in good health and received no antimicrobial therapy for >3 months prior to the commencement of this study.

Enumeration of gut microflora and C. difficile cytotoxin titres

Gut bacterial populations and C. difficile numbers were enumerated as described previously. The gut microflora populations cultured were as follows: total facultative anaerobes, total anaerobes (facultative-obligate), lactose-fermenting Enterobacteriaceae, enterococci, lactobacilli, bifidobacteria, total Clostridium spp., Bacteroides fragilis group, C. difficile total viable counts (vegetative C. difficile + spores) and C. difficile spore viable counts. C. difficile cytotoxin production was monitored using a Vero cell cytotoxicity assay as described previously. Indigenous gut microflora populations from vessel 1 of the gut models were not determined; only C. difficile total viable counts, spore counts and cytotoxin titres were quantified.

Experimental design

The time periods for this experiment are displayed in Figure 1. Following inoculation of the gut model with faecal emulsion (day 0), the media pump was started and no further interventions were made for 12 days. Gut microflora were enumerated every 2 days. C. difficile spores (~10⁷ cfu) were prepared as described previously and inoculated into vessel 1 on day 13. Viable counts of C. difficile and the indigenous gut microflora and C. difficile cytotoxin titres were monitored daily thereafter. After 7 days, another single inoculum of C. difficile spores was instilled into vessel 1 followed by either 10 mg/L ceftaroline twice daily for 7 days or 150 mg/L ceftriaxone once daily for 7 days. Each dose was a single aliquot of drug to achieve the desired concentration in vessel 1. The dosing regimen aimed to reflect reported faecal levels of antibiotic in vivo. Following cessation of cephalosporin instillation, gut bacterial populations and C. difficile cytotoxin titres were monitored for a further 9 days.

Antimicrobial assay and MIC determination

Samples from the gut model were stored at −20°C prior to antimicrobial bioassay. Cephalosporin concentrations were determined using an in-house large-plate microbiological bioassay. Briefly, the indicator organism, Escherichia coli ATCC 25922, was inoculated onto Mueller–Hinton agar measurements from the intestinal contents of sudden-death victims. 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 of 280 mL volume (vessel 1) and 300 mL volume (vessels 2 and 3), top-fed by growth medium at a controlled rate (D = 0.015 h⁻¹). Constituents and preparation of growth medium for the gut model were as described previously. The gut models were inoculated with a faecal emulsion (~10% w/v in pre-reduced PBS) prepared from C. difficile-negative faeces of five healthy elderly (≥65 years) volunteers. Faecal donors were in good health and received no antimicrobial therapy for >3 months prior to the commencement of this study.

**Figure 1.** Schematic representation of gut model experimental time periods. CD, C. difficile spores; CPT, ceftaroline; CRO, ceftriaxone. Antimicrobials were instilled for 7 days. *Period D was curtailed by 5 days due to experimental difficulties.*
agar (Oxoid, UK) and incubated overnight at 37°C. A standard inoculum (equivalent turbidity to that of a 0.5 McFarland standard, ≈10⁸ cfu/mL) was prepared in sterile PBS (Sigma, UK). Mueller–Hinton (100 mL) agar was sterilized by autoclaving and subsequently cooled to 50°C before addition of the E. coli suspension (1 mL). Inoculated molten agar was mixed by inversion and poured into 245 mm² square bioassay dishes. Twenty microlitres of filter-sterilized (0.22 μm) culture, ceftaroline calibrator (1–128 mg/L) or ceftriaxone calibrator (1–1024 mg/L) were assigned randomly to bioassay plate wells (9 mm diameter). The bioassay plates were incubated overnight at 37°C and zone diameters measured using calipers accurate to 0.1 mm. Calibration lines were plotted from squared zone diameters of known concentrations, and used to determine unknown concentrations in culture supernatants. All assays were performed in duplicate. The coefficient of variation values were typically 10% for ceftaroline bioassays and 15% for ceftriaxone bioassays; R² values for calibration lines for ceftriaxone and ceftaroline were all >0.97 and >0.98, respectively. The lower limits of detection for both bioassays were 1 mg/L. The MICs of ceftaroline and ceftriaxone for 60 C. difficile isolates were measured according to a reference agar dilution method.29

Results

Evaluation of ceftaroline and ceftriaxone in the gut model

Only data from vessel 3 of the gut model are presented graphically in this report. Populations of indigenous gut microflora were stable by the end of period A in both ceftaroline and ceftriaxone experiments (Figures 2 and 3). The gut microflora composition was similar when comparing the ceftaroline and ceftriaxone experiments. The instillation of a single inoculum of C. difficile PCR ribotype 027 spores (period B) did not adversely affect any enumerated bacterial group in the ceftaroline experiment (Figure 2). Viable counts of lactose-fermenting Enterobacteriaceae and Bifidobacterium spp. declined by 1 log₁₀ cfu/mL during period B in the ceftriaxone experiment (Figure 3). C. difficile populations remained comprised principally of spores during period B and cytotoxin was not detected in either experiment (Figure 4).

![Figure 2](https://academic.oup.com/jac/article-abstract/68/8/1842/867769)

Figure 2. Viable counts (log₁₀ cfu/mL) of (a) obligate anaerobes and (b) facultative anaerobes in vessel 3 of the ceftaroline-exposed model.
Effect of cephalosporin instillation

Ceftaroline

Instillation of ceftaroline elicited little adverse effect overall on the enumerated indigenous gut microflora in either vessel 2 (data not shown) or vessel 3 of the gut model (period C, Figure 2). However, by the end of 7 days of ceftaroline instillation, *Bifidobacterium* spp. viable counts declined by 2 log₁₀ cfu/mL and viable counts of *Clostridium* spp. declined by 1 log₁₀ cfu/mL. Bifidobacterial viable counts continued to decline after cessation of ceftaroline instillation and declined in total by 4.5 log₁₀ cfu/mL before eventually starting to increase towards the end of period D.

*C. difficile* remained as spores and cytotoxin was undetected in vessel 1 of the gut model for the duration of the ceftaroline experiment (data not shown). Germination of *C. difficile* spores and subsequent proliferation, i.e. increased total viable counts over spore counts, were observed in vessels 2 (data not shown) and 3 following cessation of ceftaroline instillation (Figure 4). *C. difficile* cytotoxin was detected 3 and 4 days after the initial increase in *C. difficile* numbers in vessels 2 and 3, respectively (day 31 in vessel 3). *C. difficile* remained as vegetative cells until the end of the experiment and cytotoxin titres were 4 relative units (RU).

Ceftriaxone

During the initial period of ceftriaxone instillation, small declines (~1 log₁₀ cfu/mL) in viable counts of lactose-fermenting Enterobacteriaceae, *Lactobacillus* spp. and total facultative anaerobes occurred, but then increased by the end of period C (Figure 3). *Bifidobacterium* spp. viable counts declined by ~1.5 log₁₀ cfu/mL by the end of the ceftriaxone instillation period. Enterococcal viable counts increased by 3 log₁₀ cfu/mL towards the end of the ceftriaxone instillation in both vessels 2 and 3. A greater adverse effect of ceftriaxone instillation was observed in vessel 2 of the gut model than in vessel 3; declines in the viable counts of
B. fragilis group (2 log_{10} cfu/mL) and lactose-fermenting Enterobacteriaceae (2.5 log_{10} cfu/mL) were observed (data not shown). The viable counts were more variable during period D, which coincided with a loss of the visible biofilm coating the inside of vessel 1 of the gut model.

C. difficile remained as spores and cytotoxin was undetected in vessel 1 for the duration of the ceftriaxone experiment (data not shown). The germination of C. difficile spores and subsequent proliferation were observed 3 days after the commencement of ceftriaxone instillation in vessel 3 (period C, Figure 4) and 1 day subsequently in vessel 2 (data not shown). C. difficile cytotoxin was detected 2 days after the initial increase in C. difficile viable counts and reached a peak titre of 4 RU. Vegetative populations of C. difficile declined following the cessation of ceftriaxone instillation, until total viable counts were identical to C. difficile spore counts. Cytotoxin titres were 3 RU by the end of the experiment.

**Cephalosporin concentrations in the gut model**

Ceftaroline was detectable only in vessel 1 of the gut model and the concentration peaked at 34 mg/L on the last day of instillation (data not shown). In vessel 1, ceftaroline was undetectable 3 days after antimicrobial instillation ceased. Ceftriaxone was detectable in vessels 1 and 2 of the gut model, but not in vessel 3 (data not shown). Concentrations peaked at 293 and 1.5 mg/L in vessels 1 and 2, respectively. Ceftriaxone was undetectable 3 and 1 day after antimicrobial instillation ceased in vessels 1 and 2, respectively.

**Susceptibility of C. difficile to ceftaroline and ceftriaxone**

Ceftaroline was ≈8-fold more active than ceftriaxone against the panel of C. difficile strains evaluated in this study; the geometric mean MICs were 3.38 versus 28.18 mg/L and the MIC ranges were 0.125–16 versus 8–128 mg/L, respectively (Table 1). The MICs of ceftaroline and ceftriaxone for the C. difficile PCR ribotype 027 strain utilized in the experiments with the gut model were 8 and 32 mg/L, respectively (individual strain data not shown). Cefaroline and ceftriaxone MICs for E. coli ATCC 25922 were both 0.06 mg/L, which is within the accepted tentative MIC range for ceftaroline (Cerexa Inc.) and the accepted MIC range for ceftriaxone (BSAC, 2008).

**Discussion**

Ceftaroline, the active metabolite of the prodrug ceftaroline fosamil, is a new cephalosporin with *in vitro* bactericidal activity against Gram-positive and common Gram-negative pathogens. Clinical data show that ceftaroline fosamil is efficacious in the treatment of cSSTI and CAP.27,28,30 – 32 Cephalosporin (particularly third generation) administration is associated with an increased incidence of CDI, although it remains unclear what is the relative risk for specific agents.6 – 8 The gut model has been shown to produce results that correlate well with clinical observations.11,14,17 – 23 In the present study, we instilled the cephalosporins ceftaroline and ceftriaxone, such that each dose elicited 10 and 150 mg/L, respectively, in vessel 1 of the gut models. Given that many cephalosporins are excreted at higher levels in the upper regions of the GI tract compared with those detected in faeces, we hypothesized that the concentrations in vessels 2 and 3 of the gut model would be lower than those in vessel 1. For ceftriaxone, the dosing strategy aimed to reflect the magnitude of ceftriaxone concentrations observed in the faeces of patients. Pletz et al.26 demonstrated 152–258 mg/kg ceftriaxone in the faeces of patients 4–8 days after commencing therapy, which is reflective of the ceftriaxone concentrations observed in

![Figure 4.](https://academic.oup.com/jac/article-abstract/68/8/1842/867769) Comparative C. difficile total viable counts (TVC) and spore counts (SP) (log_{10} cfu/mL) and cytotoxin titres (CYT) (RU) in ceftaroline (CPT) and ceftriaxone (CRO) gut model (vessel 3) experiments.
Ceftaroline in a human gut model

vessel 1 of the gut model in the present study (assuming mg/kg is approximately equal to mg/L).

The selection of a dosing strategy for ceftaroline was more difficult, given the paucity of published data on the faecal excretion of the drug. Unlike ceftriaxone, which is excreted primarily in the bile, ceftaroline is eliminated primarily by renal excretion, which may lead to lower faecal levels of the drug. Only 6% of radiolabelled drug/metabolite was detected in faeces 48 h after a single labelled 600 mg/L intravenous dose, which corresponds to ~36 mg. However, this is likely to be an overestimate of the active concentration in faeces, as inactive open-ring metabolites would still contain the label. Panagiotidis et al. detected no active ceftaroline in faeces after thirteen 600 mg intravenous doses given at 12 hourly intervals, suggesting that the majority of the radiolabel detected in faeces may be due to the presence of inactive metabolites. Bioactive ceftaroline was detectable in vessel 1 of the gut model, but not in the distal vessels, correlating with a previously published lack of detection in faeces. Ceftriaxone inactivation in faecal supernatants from volunteers treated with ceftriaxone. Edlund et al. evaluated the impact of cephalosporin administration on the intestinal microflora of volunteers and also assayed for β-lactamase activity. Interestingly, statistically significant elevations in β-lactamase activity were observed in samples from the final day of treatment versus pre-treatment samples. Cephalosporinases may be produced by both aerobic and anaerobic Gram-negative bacilli; 3%–70% of B. fragilis are resistant to β-lactam antimicrobials via the production of enzymes.

Table 1. MICs of ceftaroline and ceftriaxone (mg/L) for C. difficile

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<th>Ceftaroline</th>
<th>Ceftriaxone</th>
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<tr>
<td></td>
<td>n</td>
<td>geometric mean MIC</td>
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<tr>
<td>All C. difficile isolates</td>
<td>60</td>
<td>3.38</td>
</tr>
<tr>
<td>Genotypically distinct ribotypes</td>
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<td>4.81</td>
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<tr>
<td>Three most common ribotypes</td>
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<tr>
<td>ribotype 027</td>
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<td>2.74</td>
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<tr>
<td>ribotype 106</td>
<td>10</td>
<td>2.91</td>
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correlated ceftriaxone inactivation in faecal supernatants with the presence of Bacteroides spp. As the activity of β-lactam antibiotics involves an irreversible interaction with bacterial transpeptidases, no β-lactam will be detected by bioassay once this has occurred; this may help to explain the lack of detectable cephalosporin in downstream vessels of the gut model. The low levels of radiolabel detected in patient faeces indicate that in vivo the concentration of inactive metabolized ceftaroline in faecal samples is also low.

The impact of ceftaroline on the indigenous gut microflora was minimal, with the exception of Bifidobacterium spp., correlating with the absence of detectable (>1 mg/L) active ceftaroline from vessels 2 and 3. The initial declines in the viable counts of B. fragilis group, total facultative anaerobes and total clostridia suggest that either active ceftaroline (below the limit of bioassay) was present in vessels 2 and 3 or that populations (excluding B. fragilis group) were inhibited by ceftaroline in vessel 1 of the gut model, resulting in fewer bacteria moving distally in the gut model. The impact of ceftaroline on the indigenous gut microflora was similar to that elicited by ceftriaxone and was reflective of previously published in vivo data; however, in contrast to ceftaroline, the instillation of ceftriaxone elicited a substantial increase in the viable counts of Enterococcus spp., which remained elevated until the end of the experiment. Previously reported effects of ceftaroline on the gut microflora of healthy volunteers (using faecal samples) noted declines in bifidobacteria (~2 log₁₀ cfu/g), Lactobacillus spp. (~1 log₁₀ cfu/g) and E. coli (~2 log₁₀ cfu/g) populations, and an increase in clostridia (~2 log₁₀ cfu/g).

Ceftaroline was ~8-fold more active than ceftriaxone against tested C. difficile strains and 4-fold more active against the C. difficile 027 strain (MICs 8 and 32 mg/L) used in the gut model experiments. Hence, supra-MIC levels of cephalosporins were achieved in vessel 1 of both gut model experiments, but not in vessels 2 and 3. Although both ceftaroline and ceftriaxone facilitated C. difficile spore germination, proliferation and high-level cytotoxin production at the concentrations tested, the timings differed between antibiotics. C. difficile spore germination and proliferation occurred 5 days earlier with ceftriaxone than with ceftaroline. The reasons for this difference are unclear. Interestingly, C. difficile cytotoxin production occurred 1 day after spore germination in the ceftriaxone experiment, but 3 and 4 days after spore germination in vessels 2 and 3, respectively, for ceftaroline. In prior studies with C. difficile 027, cytotoxin
Detection usually occurred 1 day after spore germination. The delay in cytotoxin production/release associated with ceftaroline likely reflects the balance between antibiotic-mediated effects on the gut flora and on C. difficile. Bifidobacterium spp. were the most predominant component of the gut microflora that were adversely affected by both ceftaroline (2.5 log₁₀ cfu/mL) and ceftriaxone (1 log₁₀ cfu/mL) when C. difficile spore germination and proliferation occurred. Reduced viable counts of Bifidobacterium spp. (+B. fragilis group) correlated with C. difficile spore germination and proliferation in all prior experiments with clindamycin and recently with cefotaxime and fluoroquinolones. We have previously demonstrated a shift to an indigenous gut microflora that is dominated by facultatively anaerobic bacterial groups, rather than obligate anaerobes before C. difficile germination, but this was not observed to the same extent in the present study. These observations suggest that other factors are involved in induction of CDI. Antimicrobial agents may stimulate C. difficile directly to germinate and proliferate, rather than these events being solely attributable to disruption of the indigenous gut microflora (i.e. colonization resistance), although this remains to be demonstrated. Notably, Denève et al. demonstrated that exposure to subinhibitory concentrations of both clindamycin and ampicillin increased the expression of genes encoding several C. difficile adhesins (slpA, Cwp66 and Fbp68) and a protease (Cwp84). In addition, DNA microarray studies evaluating the in vitro response of a single C. difficile strain to antibiotic stresses demonstrated that agents may differentially affect gene expression. There is little clinical evidence suggesting a propensity of ceftaroline to induce CDI; testing for C. difficile during clinical trials was not mandated, but during four Phase 3 studies only two patients were identified with CDI. We have shown that ceftaroline induces CDI in the gut model, but further clinical studies are required to determine the relative risk of CDI associated with this antibiotic, especially given the increased activity against C. difficile in comparison with ceftriaxone and the low, but potentially variable, concentrations of this antibiotic in the large bowel.

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Forest own ceftaroline, but the original owner was Cerexa; it was the latter that funded the study and reviewed the study protocol, which was written by the Leeds team. Cerexa made no alteration to the experimental study design. A draft manuscript was commented on by Forest prior to a final version being submitted for publication. The editorial assistance, provided by Scientific Therapeutics Information Inc., was funded by Forest Research Institute, Inc.

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