Mixed infection by *Clostridium difficile* in an *in vitro* model of the human gut

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**Objectives:** *Clostridium difficile* infection (CDI) is still a major clinical challenge. Previous studies have demonstrated multiple distinct *C. difficile* strains in the faeces of patients with CDI; yet whether true mixed CDI occurs *in vivo* is unclear. In this study we evaluated whether two distinct *C. difficile* strains could co-germinate and co-proliferate in an *in vitro* human gut model.

**Methods:** An *in vitro* triple-stage chemostat was used to study the responses of two PCR ribotype 001 *C. difficile* strains following exposure to ceftriaxone at concentrations observed *in vivo* (7 days). *C. difficile* viable counts (vegetative and spore forms), cytotoxin titres and indigenous microflora viable counts were monitored throughout the experiment.

**Results:** Both *C. difficile* strains germinated and proliferated following exposure to ceftriaxone. Cytotoxin production was detected in the gut model following *C. difficile* spore germination and proliferation. Ceftriaxone elicited reduced viable counts of *Bifidobacterium* spp. and elevated viable counts of *Enterococcus* spp.

**Conclusions:** These data suggest that multiple *C. difficile* strains are able to proliferate concurrently in an *in vitro* model reflective of the human colon. Previous studies in the gut model have reflected clinical observations so clinicians should be mindful of the possibility that multiple *C. difficile* strains may infect patients. These observations augment recent human epidemiological studies in this area.

**Keywords:** spores, resistance, epidemiology, ceftriaxone

**Introduction**

*Clostridium difficile* infection (CDI) is the leading cause of infectious diarrhoea in healthcare institutions worldwide. US healthcare costs attributable to CDI were recently estimated as ~$500 million. Understanding how infection occurs and is spread is crucial to optimizing interventions. Recurrent CDI may be due to either relapses attributable to the original infecting strain or re-infections with a different strain. Notably, Eyre et al. recently demonstrated, using multilocus sequence typing, the presence of mixed *C. difficile* strains in ~7% of CDIs. Despite such observations, it is unclear whether multiple *C. difficile* strains are capable of truly coinfesting a patient, i.e. both strains proliferate and produce toxins, or if the perturbed colonic environment is only able to support growth and toxin production by a single strain.

We have previously used a triple-stage chemostat human gut model to investigate the induction of CDI and also the efficacies of therapies. Results from the gut model correlate well with clinical observations. We have now examined the effects of antibiotic exposure on two distinct *C. difficile* strains and the gut microflora in a mixed-infection gut model.

**Materials and methods**

**C. difficile strains**

Two *C. difficile* ribotype 001 strains were studied; both isolated from cytotoxin-positive faeces of patients with CDI at Leeds General Infirmary (Leeds, UK). The clindamycin-resistant (MIC ≥256 mg/L) metronidazole-susceptible (MIC ≤0.25 mg/L) *C. difficile* (CD-CR, strain P62) isolate was *erm(B)* positive. The mechanism of reduced susceptibility to metronidazole in the other *C. difficile* isolate (CD-RM, strain 11/11, MIC 8 mg/L) is undetermined; this strain was clindamycin susceptible (MIC 2 mg/L). PCR ribotyping was performed as described previously.

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**Gut model**

The gut model was previously validated against physico-chemical/ microbiological measurements from the intestinal contents of victims of sudden death and provides a reproducible and gut-reflective environment for studying *C. difficile*. The gut model comprises three pH-maintained vessels (pH 5.5 ± 0.1 – 6.8 ± 0.1) inoculated with pooled *C. difficile*-negative faeces.

**Enumeration of gut microflora and *C. difficile* cytotoxin**

Gut bacterial populations and *C. difficile* were enumerated as described previously. The *C. difficile* isolates were distinguished by culture on agar that contained antibiotics: CD-CR was isolated on Brazier’s CCEYL agar containing 8 mg/L clindamycin and CD-RM was isolated on Brazier’s CCEYL agar containing 1 mg/L metronidazole. *C. difficile* spore counts were determined using alcohol-shock. Total *C. difficile* viable counts and spore counts were also determined on CCEYL agar. Total *C. difficile* cytotoxin titres were monitored by Vero cell cytotoxicity assay and expressed as log₁₀ relative units (RU).

**Experimental design**

Following inoculation of the gut model with faecal emulsion (day 0) no further interventions were made for 13 days (period A). *C. difficile* spores (10⁶ cfu per strain) were prepared as described previously and inoculated into vessel 1 on day 13 (period B). After 7 days (day 20) a further single inoculum of *C. difficile* spores was instilled into vessel 1 followed by 150 mg/L ceftriaxone once-daily for 7 days (period C). *C. difficile* was instilled to reflect the concentration observed in faeces of patients and volunteers. Following cessation of cephaparin instillation, gut bacterial populations and *C. difficile* cytotoxin titres were monitored for a further 14 days (period D).

**Antimicrobial bioassay**

Gut model samples were stored at –20°C before ceftriaxone bioassay. Mueller-Hinton agar (100 mL volumes) was autoclaved, cooled (50°C), seeded with 10⁶ cfu *E. coli* ATCC 25922 and poured into bioassay dishes. Twenty microlitre aliquots of filter-sterilized (0.22 μm) culture or calibrator (1–1024 mg/L) were assigned randomly in duplicate to wells (9 mm). Plates were incubated overnight (37°C) and zone diameters were measured. Coefficient of variation values were 15% and R² values for calibration lines were >0.98. The limit of detection was 1 mg/L.

**Results**

**Specificity of CCEYL breakpoint agar**

Preliminary experiments demonstrated that CD-CR and CD-RM were unrecoverable on metronidazole- and clindamycin-containing agar, respectively. Multilocus variable tandem-repeat analysis (MLVA) was performed on *C. difficile* recovered on each antibiotic selective agar; this confirmed the correct MLVA profile for each strain.

**Response of *C. difficile* and gut microflora to ceftriaxone**

Vessel 3 gut model data are shown graphically in this report. Total viable bacterial counts were predominantly stable before ceftriaxone instillation. Both *C. difficile* populations remained quiescent before ceftriaxone instillation and exited gut model vessels at similar rates. Cultivable gut microfloras at this time were dominated by obligate anaerobes; particularly *Bifidobacterium* spp. and *Bacteroides fragilis* group, whereas *Clostridium* spp. and facultative anaerobes were less numerous (Figure 1). Ceftriaxone instillation elicited declines in lactose-fermenting Enterobacteriaceae (2 log₁₀ cfu/mL) and *Lactobacillus* spp. (1 log₁₀ cfu/mL), yet *Enterococcus* spp. increased (≥3 log₁₀ cfu/mL). The *B. fragilis* group were largely unaffected by ceftriaxone, but *Bifidobacterium* spp. declined markedly (Figure 1). The spore populations of both *C. difficile* strains germinated during ceftriaxone dosing (total viable counts increased over spore counts) 3 and 4 days after commencement for CD-CR and CD-RM strains, respectively (Figure 2). *C. difficile* germination and proliferation were similar in vessel 2, but were absent in vessel 1. Maximal CD-RM and CD-CR total viable counts were similar in vessels 2 and 3. *C. difficile* cytotoxin was detected on the last day of ceftriaxone dosing in vessels 2 and 3, and was absent in vessel 1. Ceftriaxone was detectable in vessel 1 (peak concentration 150 mg/L) and concentrations declined rapidly and fell below the limits of detection on day 29 (2 days after ceftriaxone cessation).

**Bacterial ecology after ceftriaxone dosing**

Lactose-fermenting Enterobacteriaceae and *Bifidobacterium* spp. normalized midway through period D; yet *Enterococcus* spp. remained elevated. CD-RM and CD-CR total viable counts declined in vessel 3 during the first 4 days of period D; CD-CR strain counts declined at a greater rate than those of CD-RM, and re-converged with CD-CR spore counts. CD-RM viable counts did not decline in vessel 2 after ceftriaxone cessation, in contrast to CD-CR total viable counts, which declined sharply by 2 log₁₀ cfu/mL. *C. difficile* germination was delayed in vessel 1 until days 31 (CD-CR) and 33 (CD-RM), following which increased total viable counts were observed in vessel 3. By the end of the experiment, indigenous microfloras normalized to steady-state levels, except *Enterococcus* spp., which remained elevated. *C. difficile* total viable counts were 1 log₁₀ cfu/mL higher than spore counts and cytotoxin was undetectable.

**Discussion**

Cephaparin administration is a documented risk factor for CDI. Ceftriaxone dosing in the present study aimed to simulate the expected in vivo concentrations. Pietz et al. demonstrated 152 mg/kg and 258 mg/kg ceftriaxone in faeces from patients 4 and 8 days after commencing therapy, respectively; these levels reflect the ceftriaxone concentrations observed in gut model vessel 1 (assuming mg/kg = mg/L). We were unable to detect active drug in gut model vessels 2 or 3. Similarly low levels of detectable ceftriaxone in vessels 2 and 3 of the gut model have been noted previously. Inactivation of β-lactams in faeces may occur through enzymatic hydrolysis or adsorption to macromolecules. Absence of detectable ceftriaxone in the distal gut model vessels was probably a result of one or both of these mechanisms. Ceftriaxone elicited a minimally deleterious effect against the gut microflora; *Bifidobacterium* spp. and to a lesser extent lactose-fermenting Enterobacteriaceae were most inhibited. Proliferation of CD-CR and CD-RM strains occurred similarly, within 1 day during ceftriaxone dosing.
This suggested that both strains responded to similar germinant(s)/stimuli in the model and that there was no obvious fitness cost associated with each resistance phenotype during germination/proliferation phases. However, differences in growth were observed as *C. difficile* strains entered stationary/decline phases; total vegetative CD-CR declined more rapidly than CD-RM and re-converged with spore counts. Total viable counts of both strains then increased, while the indigenous gut microflora remained perturbed. We observed similar *C. difficile* PCR ribotype 027 biphasic growth previously. The second cycle of proliferation may have been a result of delayed spore germination (after washout of bioactive ceftriaxone) in gut model vessel 1 and transfer of germinated spores/vegetative cells downstream. Maximal cytotoxin expression observed in this study was 2–3 log\textsubscript{10} RU lower than for other epidemic *C. difficile* we have studied. The concentrations of *C. difficile* toxins released *in vitro* and *in vivo* are known to vary\textsuperscript{7,19} and in the present study it is unlikely that the lower cytotoxin production was a consequence of the resistance phenotype of either strain. Although we detected cytotoxin production in this experiment, we could of course not demonstrate if one or both strains were responsible. However, given that spores of both *C. difficile* isolates germinated and proliferated in a similar way, it is likely that both strains contributed to the measured titre. The significance of the smaller decline phase of CD-RM is unclear; this may be an intrinsic trait of this *C. difficile* strain or somehow connected to the resistance phenotype. Further gut model studies are required to assess the profile of growth/decline phases.

This study clearly demonstrated that two distinct *C. difficile* strains proliferated concurrently in a complex *in vitro* model following antimicrobial stimulation. These data supplement epidemiological studies performed in patients with CDI, which identified multiple *C. difficile* PCR ribotypes in the faeces of these patients\textsuperscript{2–4} and the hypothesis that *in vivo* coinfection by more than one *C. difficile* strain is possible. This body of evidence indicates that *C. difficile* epidemiological studies should ideally examine multiple colony picks and use highly discriminatory fingerprinting techniques, such as MLVA or whole genome sequencing\textsuperscript{10,20} to ensure that potentially misleading data are not reported. Whether some *C. difficile* strains, for example epidemic ribotypes such as 027 and 078, actually outcompete others in the gut is unknown, but warrants further study. The phenomenon of mixed infection is important, especially as hospitalized patients in particular may be exposed to multiple *C. difficile* strains.

**Figure 1.** Viable counts (log\textsubscript{10} cfu/mL, mean ± SEM) of gut microflora in vessel 3 of the gut model.
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Transparency declarations
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References
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