Activity of vancomycin against epidemic *Clostridium difficile* strains in a human gut model

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Objectives: Vancomycin and metronidazole remain the only primary options for the treatment of *Clostridium difficile* infection (CDI). Recent reports have suggested a superior clinical response to vancomycin therapy compared with metronidazole, but this has been difficult to prove or explain. There are few robust *in vitro* data of the effects of antibiotic treatment of CDI in a gut reflective setting.

Methods: We used clindamycin to induce high-level toxin production by two epidemic *C. difficile* PCR ribotypes in a human gut model of CDI. Vancomycin was instilled into the models to achieve *in vivo* faecal concentrations. *C. difficile* populations and toxin titres, and gut bacterial populations and vancomycin levels were monitored before, during and after vancomycin instillation.

Results: Clindamycin treatment elicited *C. difficile* germination and high-level cytotoxin production. Vancomycin reduced total viable counts and cytotoxin titres of both *C. difficile* PCR ribotypes, with no evidence of recurrence before the model runs were ended. *C. difficile* PCR ribotype 027 populations exhibited greater germination capacity than did PCR ribotype 106. Vancomycin was more rapidly effective against the greater numbers of PCR ribotype 027 vegetative forms. Vancomycin showed no activity against *C. difficile* spores.

Conclusions: Bacteriological response to vancomycin varies between strains causing CDI, possibly correlating with the extent of germination capacity. Vancomycin effectively reduced vegetative forms and cytotoxin titres of both of the epidemic *C. difficile* PCR ribotypes evaluated, but showed no anti-spore activity. Comparison with the results of a previous gut model study showed that vancomycin was more effective than metronidazole in reducing *C. difficile* PCR ribotype 027 numbers and cytotoxin titres.

Keywords: PCR ribotype 027, toxin, colitis

Introduction

*Clostridium difficile* is the aetiological agent of pseudomembranous colitis and is implicated in ~30% of cases of antibiotic-associated colitis.¹,² Most antimicrobials have been implicated, but clindamycin, third-generation cephalosporins and aminopenicillins are particularly noted for their propensity to induce *C. difficile* infection (CDI).³–⁵ CDI is a significant cause of morbidity, particularly in elderly hospital patients. In 1996, the additional costs due to CDI were over £4000 ($6000) per case in the UK,⁶ while a more recent study estimated the annual healthcare costs associated with management of CDI in the USA at over $3.2billion.⁷ Increases in CDI incidence and poor outcome have been associated with the emergence of a *C. difficile* PCR ribotype 027 (NAP 1).⁸,⁹

 Therapeutic choices in CDI are limited, and conventional antimicrobial treatment remains either metronidazole or vancomycin. However, recent reports have shown that metronidazole is inferior to vancomycin, notably in severe cases of CDI.⁸–¹１ It remains unclear whether there are strain-dependent differences in response to antibiotic therapy of CDI. We have previously described an *in vitro* human gut model of CDI that yields results consistent with clinical and animal model data.¹²–¹⁶ The aim of this study was to evaluate the effects of vancomycin on antibiotic-induced growth and toxin production in this gut model, using two epidemic *C. difficile* strains.
Vancomycin activity against *C. difficile* in a gut model

**Materials and methods**

*C. difficile* strains

We investigated two strains of *C. difficile*. *C. difficile* PCR ribotype 027 was isolated during an outbreak of severe CDI at Stoke Mandeville Hospital, Buckinghamshire, UK. *C. difficile* PCR ribotype 106 was isolated from a symptomatic patient in Leeds, UK. Both isolates were confirmed by PCR ribotyping by the *C. difficile* Ribotyping Network for England.\(^{11}\)

**CDI gut model**

The gut model was developed and validated against the caecal contents of sudden death victims by MacFarlane et al.\(^{16}\) We have previously described its modification and use as a model of CDI.\(^{12–16}\) Briefly, the gut model comprises three fermentation vessels top-fed by growth medium at a controlled rate (\(D = 0.015\) h\(^{-1}\)) and operating in a weir cascade system. Each vessel operates at a controlled pH to reflect the increasing alkalinity of the gut. Thus vessel 1 (280 mL) operates at a low pH (5.5) and high substrate availability while vessels 2 and 3 (300 mL) maintain a more neutral pH (6.2 and 6.8, respectively) and lower substrate availability. The gut model was primed with an emulsion of pooled faecal samples and allowed to equilibrate in respect of bacterial populations at a total retention time of 66.7 h (V1 = 16.7 h, V2 = 25 h, V3 = 25 h).

**Preparation of the gut model**

Human faeces were collected from five healthy elderly volunteers (>_65 years) with no history in the previous 2 months of antibiotic treatment. Faecal samples were transported and screened for the presence of *C. difficile* by anaerobic culture as previously described.\(^{12–16}\) A 10% (w/v) coarse-filtered faecal slurry was prepared and used to inoculate the gut model as previously described.\(^{12–16}\) The media pump was started and the system allowed to achieve steady-state bacterial populations for ~9 days. The model was sampled daily thereafter to enumerate the total *C. difficile* and spore counts, to determine toxin titres and to quantify gut bacterial populations as previously described.\(^{12–15}\)

Gut model growth medium was prepared as previously described.\(^{12–16}\)

**Experimental procedure**

High-level *C. difficile* toxin production was induced using clindamycin as previously described,\(^{14,16}\) and the experimental time-course is presented in Figure 1. Briefly, at steady-state (period A) *C. difficile* spores (~10⁷ cfu)\(^{12–16}\) were added to vessel 1 and bacterial populations enumerated daily from thereon. There were no further interventions for 7 days (period B). A further inoculum of *C. difficile* spores (~10⁷ cfu) was instilled into vessel 1 of the gut model (day 17), in addition to 33.9 mg/L cldiamycin (Pfizer) every 6 h for 7 days (period C) to reflect the mean concentration observed in faeces after standard therapy.\(^{19}\) Following cessation of clindamycin instillation, bacterial populations and *C. difficile* cytotoxin titres were monitored until high-level cytotoxin production [≥4 relative units (RU)] was observed for at least two consecutive days (period D).

**Antimicrobial assay**

Following commencement of vancomycin instillation (period E), aliquots from all vessels of the gut model were centrifuged at 16 000 g, and the supernatants removed and then stored at −20°C. Vancomycin concentrations in culture supernatants were determined using an in-house large-plate multi-well diffusion microbiological bioassay with the indicator organism *Staphylococcus aureus* NCTC 6571. Briefly, 100 mL volumes of pH-adjusted (pH 7.8–8) antibiotic medium number 1 (Oxoid, UK) supplemented with 1 M para- amino benzoic acid were prepared following the manufacturer’s instructions and cooled to 50°C following autoclaving. One millilitre of an overnight culture of the indicator organism (at a turbidity equivalent to that of a 0.5 McFarland standard) was inoculated into the cooled agar and mixed by inversion, following which the agar was poured into a 245 mm\(^2\) bioassay dish (Nunc, Denmark) and allowed to set and dry. Twenty-five wells were cut into the seed agar using a number 5 cork borer and were filled with 20 μL of either a randomly assigned vancomycin stock (1–128 mg/L) or filter-sterilized (0.22 μm) culture sample from the gut model. Inoculated agars were refrigerated (4°C) for 5 h to allow antimicrobial diffusion whilst minimizing bacterial growth, following which they were incubated aerobically at 37°C for 24 h. Zone diameters (mm) were measured with callipers (according to the manufacturer’s instructions) accurate to 0.1 mm and a calibration line produced by plotting squared zone diameters of calibrated Vancomycin standards against their log₂ concentration. Log₂ vancomycin concentrations in samples from the gut model were determined from calibration lines and converted into actual concentrations using a 2\(^x\) conversion. All assays were performed in duplicate. The limit of detection for this microbiological bioassay was 8 mg/L.

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**Figure 1.** Schematic representation of experiment.
Results

Populations of indigenous bacteria were largely stable throughout period A, and following the addition of *C. difficile* spores in both *C. difficile* PCR ribotype 106 and 027 experiments (period A, Figure 2). Following clindamycin instillation, total anaerobe counts remained fairly steady, although fluctuations in numbers of individual anaerobic components were noted. *Bifidobacterium* spp. were the most adversely affected, with viable counts declining below the limits of detection (~2 log₁₀ cfu/mL) by the end of period C, while lactose fermenters increased in number. *Bacteroides fragilis* group populations initially declined following instillation of clindamycin, but stabilized or increased by the end of period C in both experiments. Facultatively anaerobic bacterial groups were largely unaffected by clindamycin instillation. Lactobacilli decreased by ~2 log₁₀ cfu/mL during clindamycin instillation, but regained their original numbers thereafter.

Instillation of vancomycin commenced 11 days and 12 days after cessation of clindamycin addition in *C. difficile* PCR ribotype 027 and 106 models, respectively. Vancomycin concentrations peaked in vessel 3 at 554 mg/L (Figure 3, *C. difficile* PCR ribotype 027 experiment) and 599 mg/L (Figure 4, *C. difficile* PCR ribotype 106 experiment). Populations of the *B. fragilis* group and lactose fermenters declined during the period of clindamycin instillation, but stabilized or increased by the end of period C in both experiments. Facultatively anaerobic bacterial groups were largely unaffected by clindamycin instillation.

**Behaviour of *C. difficile***

*C. difficile* spores remained quiescent in the absence of antimicrobial disruption in both experiments, decreasing in number as vessel contents were diluted (period B, Figures 3 and 4). During clindamycin instillation, *C. difficile* populations principally comprised spores, and cytotoxin was undetectable (period C, Figures 3 and 4). Germination and proliferation of *C. difficile* spores were observed 6 and 7 days after the cessation of clindamycin instillation in *C. difficile* PCR ribotype 027 and 106 experiments, respectively. Increased recovery of *C. difficile* PCR ribotype 106 spores was observed following germination, proliferation and cytotoxin production (days 29–34, Figure 4), but not in the corresponding *C. difficile* PCR ribotype 027 experiment. *C. difficile* cytotoxin was detected 1 and 2 days after germination of *C. difficile* PCR ribotype 027 and 106 spores, respectively. Following the start of vancomycin instillation, *C. difficile* total counts decreased to equivalence with *C. difficile* spore counts within 1 day in both experiments (period E, Figures 3 and 4). *C. difficile* remained as spores for the remainder of the experiments, even after vancomycin concentrations declined below the limits of detection.

**Discussion**

We have previously reported the use of the gut model to study both the propensity of antibiotics to induce CDI and the efficacy of therapeutic agents. Gut model results correlate well with clinical data on CDI risk and treatment efficacy. In the present study, we used clindamycin to induce germination of *C. difficile* spores and high-level toxin production by two clinically relevant *C. difficile* strains (PCR ribotypes 027 and 106), prior to treatment with vancomycin in accordance with a clinically relevant dosing regimen. In agreement with previous gut model studies, *C. difficile* spore germination, proliferation and high-level toxin production by both strains were observed ~7 days after the end of clindamycin instillation. We have previously demonstrated that this timing correlates with the decline in the concentration of the inducing antibiotic to below the MIC for the *C. difficile* strain under study. Clindamycin instillation...
resulted in similar declines in gut bacterial populations to those previously documented.\(^\text{14,16}\) Populations had recovered to predosing levels (except bifidobacteria) prior to the administration of vancomycin, whereupon they were again deleteriously affected. Despite the marked effect of vancomycin upon the gut flora, which was similar in magnitude to that following clindamycin administration, germination and toxin production by \(C.\) difficile were not observed. This reinforces our previous gut model data that indicate that the relationship between antibiotic-mediated depletion of colonization resistance and \(C.\) difficile proliferation and toxin production is neither simple nor the only mechanism underlying CDI.\(^\text{12–16}\) Investigations with the gut model using extended time frames and other \(C.\) difficile ribotypes could help to further understand this relationship.

Vancomycin was effective in reducing vegetative cells of both \(C.\) difficile strains, but demonstrated no activity against spores. This concurs with \textit{in vitro} batch culture experiments where vancomycin showed no demonstrable activity against spores of three \(C.\) difficile ribotypes using three separate methods (S. D. Baines, unpublished data). Both \(C.\) difficile PCR ribotype 027 and 106 populations had a 2 log\(_{10}\) cfu/mL decrease in total viable counts within the vancomycin dosing period, but numbers of the former strain decreased earlier (4 versus 7 days). As reported previously,\(^\text{16}\) the \(C.\) difficile PCR ribotype 027 population contained a greater proportion of vegetative cells in the post-germination period than did the comparator strain. Since vancomycin is effective only against vegetative \(C.\) difficile cells, the pronounced decline in total \(C.\) difficile PCR ribotype 027 counts may be attributable to this. In contrast, \(C.\) difficile PCR ribotype 106 populations contained a relatively greater proportion of spores and therefore the decrease in total viable counts was less rapid, but ultimately of similar magnitude. In a similar experiment, metronidazole (dosed to achieve \textit{in vivo} concentrations) also exerted a more pronounced effect upon...
C. difficile PCR ribotype 027 populations when compared with PCR ribotype 001. Again, this correlated with the higher proportion of vegetative cells seen for C. difficile PCR ribotype 027 compared with PCR ribotype 001.

Interestingly, there was a sustained increase in C. difficile PCR ribotype 106 spore numbers as germination and proliferation proceeded. We have previously demonstrated differences in sporulation capacity between C. difficile strains, particularly PCR ribotype 001, which was until recently the most prevalent C. difficile ribotype in the UK. C. difficile PCR ribotype 106 is now the second most prevalent strain in England. Investigation of virulence determinants, including sporulation capacity, may provide an insight into the reasons behind the emergence of this C. difficile ribotype.

Toxin production occurs only when C. difficile is in its vegetative state, following spore germination and the decrease of antimicrobial concentrations to sub-MIC levels. Vancomycin concentrations in the gut model (>550 mg/L) far exceeded the MIC for C. difficile. By contrast, we found that the levels of metronidazole were not consistently inhibitory, particularly in the distal part (vessel 3) of the system where antimicrobial concentrations were markedly lower than in the proximal gut model vessels. We postulated that bacterial inactivation of metronidazole within vessels 1 and 2 of the gut model may account for these observations. We observed no such reduction in vancomycin concentrations in the present experiments. Bacterial inactivation of vancomycin by intrinsically resistant microorganisms is not thought to be a significant factor in vancomycin therapy for CDI. Cytotoxin titres decreased to undetectable levels for PCR ribotype 027 and PCR ribotype 106 at 9 and 14 days, respectively, after the commencement of vancomycin. This may be a reflection of the inevitable differences in experiments of such durations. Prolonged toxin production, which is associated with metronidazole, toxin production by C. difficile PCR ribotype 027, 16 may be less likely to occur in the presence of high levels of vancomycin. At subinhibitory antibiotic concentrations, as may be more likely during treatment with metronidazole, toxin production by C. difficile PCR ribotype 027 in particular may continue beyond that by comparator strains. These in vitro findings are consistent with the slower and less consistent responses in CDI treatment with metronidazole that have been recognized more commonly since the emergence of C. difficile PCR ribotype 027.

The potential for recurrent C. difficile spore germination and toxin production after metronidazole or vancomycin instillation in the gut model is also likely to be influenced by the very different respective concentrations of these antibiotics. We have previously evaluated the efficacies of two existing and one novel antimicrobial to treat clindamycin-induced CDI in the gut model. Recurrence of C. difficile spore germination, proliferation and high-level cytotoxin production were observed after cessation of metronidazole but not vancomycin instillation. We did detect a transient increase in C. difficile numbers in one vessel of the gut model for one strain (vessel 2, PCR ribotype 027) after vancomycin addition ceased, but this was not associated with sustained growth or high-level toxin production. Symptomatic recurrence of CDI following the cessation of antimicrobial therapy occurs in ~20–30% of individuals and is a major drawback of current treatment options. Some recent reports have noted increased CDI recurrence rates associated with metronidazole treatment. Our results suggest that recurrent C. difficile germination and toxin production are less likely following therapy with vancomycin compared with metronidazole, and may help to explain the clinically observed higher rate of recurrence sometimes associated with the latter.

Up until the emergence of C. difficile PCR ribotype 027, the efficacies of metronidazole and vancomycin in CDI were considered similar, although a slower response had been noted with the former agent. More recently, Zar et al. reported a superior clinical response for vancomycin in severe CDI, and Al-Nassir et al. described a slower and less consistent microbiological response to metronidazole. Notably, two large randomized Phase III studies of tolevamer found greater response rates in severe cases for vancomycin-treated CDI cases. It remains unclear, however, whether the likelihood of poor treatment response is explained primarily by epidemic strains such as C. difficile PCR ribotype 027. It is possible that subtypes of such strains are associated with poor outcome, but this remains unproven. Furthermore, we recently reported reduced susceptibility to metronidazole among C. difficile PCR ribotype 001 strains collected in 2005 (geometric mean MIC = 5.94 mg/L) versus those collected in 1995 (geometric mean MIC = 1.03 mg/L; P << 0.001). This phenomenon may further complicate an emerging trend for reduced metronidazole efficacy. Continued surveillance and further well-controlled prospective studies of the relative efficacies of the two agents are warranted.

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