Evaluation of the effect of oritavancin on *Clostridium difficile* spore germination, outgrowth and recovery

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**Objectives:** Previous work suggests oritavancin may be inhibitory to *Clostridium difficile* spores. We have evaluated the effects of oritavancin exposure on *C. difficile* spore germination, outgrowth and recovery.

**Methods:** Germination and outgrowth of *C. difficile* spores exposed to different concentrations of oritavancin, vancomycin, or metronidazole (0.1–10 mg/L) were monitored at 0, 2, 4, 6, 24 and 48 h using phase-contrast microscopy. Recovery of antimicrobial-exposed spores was determined by viable counting on Brazier's modified CCEYL agar. Persistence of oritavancin activity on spores after washing was determined by measuring activity against a *Staphylococcus aureus* lawn.

**Results:** Oritavancin, vancomycin and metronidazole exposure did not prevent germination of phase-bright spores to phase-dark spores, but did inhibit further outgrowth into vegetative cells. The inhibitory effect of oritavancin persisted after washing, whereas the inhibitory effects of vancomycin and metronidazole did not. Oritavancin exposure affected spore recovery; fewer spores were recovered after washing following oritavancin exposure than vancomycin exposure. The extent of this effect was dependent on PCR ribotype, with recovery of ribotype 078 spores completely prevented, but recovery of ribotype 001 spores only slightly affected. Spores exposed to oritavancin, but not vancomycin, retained antimicrobial activity after washing, indicating adherence of oritavancin, but not vancomycin, to the spore surface.

**Conclusions:** Oritavancin may adhere to spores, potentially causing early inhibition of germinated cells, preventing subsequent vegetative outgrowth and spore recovery. This may prevent some recurrences of symptomatic *C. difficile* infection that are due to germination of residual spores following antibiotic therapy.

**Keywords:** adherence, outgrowth inhibition, recurrence

**Introduction**

*Clostridium difficile* infection (CDI) is a major cause of morbidity, with recurrence rates of ~25%.1,2 Fidaxomicin is associated with an ~50% reduction in the recurrence of non-ribotype 027 CDI, but not of recurrence due to ribotype 027.3,4 highlighting the requirement for additional treatment options. *C. difficile* forms spores resistant to heat, alcohol, disinfectants and antimicrobials,5,6 which can persist in the hospital environment and act as a source of disease transmission or recurrence (reinfection).7,8 Few studies have evaluated antimicrobial activity against *C. difficile* spores. Oritavancin is a novel, semi-synthetic lipoglycopeptide with good activity against vegetative *C. difficile*.9 We have previously reported that oritavancin is highly effective in treating simulated CDI in an in vitro human gut model10,11 and has no propensity to induce CDI.12 We noted reduced detection of *C. difficile* spores following oritavancin exposure compared with vancomycin exposure. In this study, we have evaluated the activity of metronidazole, vancomycin and oritavancin against *C. difficile* spores and the effects on spore germination and outgrowth.

**Methods**

**Preparation of *C. difficile* spores**

*C. difficile* strains were inoculated onto modified Brazier’s CCEYL (containing 5 mg/L lysozyme) agar, incubated anaerobically at 37 °C for 48 h and subcultured onto 10 Columbia blood agar (CBA) plates (incubated anaerobically at 37 °C for 10 days). Growth was removed from CBA plates and emulsified in 2 mL of sterile saline/ethanol (50% v/v). The suspension was periodically mixed at ambient temperature for 1 h prior to centrifugation at 16,000 g for 10 min. The supernatant was discarded and the pellet resuspended in 1 mL of sterile saline.
The purity of spore preparations was determined by phase-contrast microscopy. If necessary, phase-bright (PB) spores were separated from phase-dark (PD) spores and cellular debris using density gradient centrifugation (2000 g) following layering onto 50% (w/v) Urografin 370.13

**Enumeration of C. difficile**

*C. difficile* spore preparations were diluted in pre-reduced saline (10-fold serially to 10⁻⁴). Twenty microlitres of appropriate dilutions were inoculated on modified Brazier’s CCEYL agar in triplicate (incubated anaerobically at 37°C for 48 h). Growth of 20–200 colonies were counted and the cfu per millilitre were calculated. *C. difficile* total viable counts and dormant spore counts in germinating cultures were enumerated the same way, distinguished by alcohol shock (50% ethanol, 30 min) prior to serial dilution for spores.

**Phase-contrast microscopy**

Populations of visible *C. difficile* PB spores (dormant), PD spores (germinated) and vegetative cells (VCs; outgrowth) were determined by transferring 50 μl of culture or suspension to glass microscope slides (Superfrost plus, Thermo Scientific). Slides were air dried and heat fixed (50°C). Molten Wilkins–Chalgren agar was overlaid on the sample and a coverslip was applied. Slides were read within 24 h under a Leica DM2000 phase microscope (magnification ×1000, PH3). The numbers of PB and PD spores and VCs were counted from at least three fields of view to a total of ~100 entities and relative percentages were calculated. Triplicate counts were determined from each slide.

**Spore germination and outgrowth experiments**

**C. difficile strains evaluated**

Four epidemic *C. difficile* PCR ribotypes14 were evaluated in spore germination and outgrowth experiments. *C. difficile* PCR ribotype 027 (210) was isolated in an outbreak of CDI in North America (Maine Medical Center, Portland, ME, USA). *C. difficile* PCR ribotypes 001 (P24) and 106 (106-36/64) were both isolated on Leeds General Infirmary from symptomatic CDI patients. *C. difficile* PCR ribotype 078 (078-Beaumont) was isolated from a symptomatic patient at Beaumont Hospital, Dublin, Ireland.

**Phase-contrast microscopy of antibiotic-exposed spores**

Brazier’s broth was prepared by heating 48 g/L Brazier’s CCEYL agar (50°C, 1 h), then removing solid agar by filtration. Sodium thioglycolate (2.28 mg/L) and lysozyme (5 mg/L) were added after sterilization, and the broth adjusted to pH 7. *C. difficile* spores (10² cfu/mL, 1 mL) were suspended in 30 mL Brazier’s broth incorporating 0.1, 1 or 10 mg/L metronidazole, vancomycin or oritavancin, or a non-antimicrobial-containing control. Samples were removed at 0, 1, 2, 4, 6, 24, 32 and 48 h and slides prepared for phase-contrast microscopy as described above. Percentages of PB spores, PD spores and VCs were recorded in triplicate. The effects of antimicrobial concentration on spore germination (conversion of PB spores to PD spores) and outgrowth (conversion of PD spores to VCs) were determined by comparison with controls.

**Post-antibiotic effect spore germination assays**

*C. difficile* ribotype 027 spores (10² cfu/mL) were exposed to 10 mg/L metronidazole, vancomycin, or oritavancin for 1 h, washed three times in sterile PBS, resuspended in PBS and inoculated into Brazier’s broth. Samples were removed and prepared for phase-contrast microscopy as described above.

**Viable counting of antibiotic-exposed spores**

At each timepoint used for phase-contrast microscopy (above) alcohol-shocked and non-alcohol-shocked samples were centrifuged, washed five times in pre-reduced PBS, diluted and plated onto modified Brazier’s CCEYL plates to enumerate cell viability. Plates were read after 48 h and viable counts determined as described above. Due to spore availability, this was not done for ribotype 106.

**C. difficile spore binding assays with oritavancin**

*C. difficile* PCR ribotype 001 spores (10² cfu/mL) were suspended in P80, P80+oritavancin (350 mg/L), sterile water±vancomycin (350 mg/L) or metronidazole (9.3 mg/L). *C. difficile* spores were centrifuged (16000 g, 5 min), the supernatants removed and pellets resuspended in the appropriate diluent. This process was repeated 10 times. Ten microlitres of the resuspended spore suspension was inoculated onto the surface of a lawn of S. aureus NCTC 6571 on CBA. Agar plates were incubated aerobically (37°C, overnight). The diameters of zones of inhibition were recorded.

**Statistical analyses: phase-contrast microscopy**

Percentage data were transformed using an arcsine transformation and analysed using a Wilcoxon signed-rank test. P < 0.05 was considered statistically significant.

**Results**

**Phase-contrast microscopy of antibiotic-exposed spores**

A low concentration of oritavancin (0.1 mg/L) was not inhibitory to germination or outgrowth of *C. difficile* PCR ribotype 027 spores compared with the control (Figure 1). When spores were exposed to 1 or 10 mg/L of oritavancin, outgrowth of PD spores to VCs was inhibited (Figure 1).

When exposed to 0.1 or 1 mg/L of vancomycin or metronidazole there was no effect on PCR ribotype 027 spore germination or outgrowth compared with controls (Figure 1). Exposure to 10 mg/L of vancomycin or metronidazole inhibited PCR ribotype 027 spore outgrowth.

There was no effect of any antibiotic at any concentration on spore germination (Figure 1).

Rates of spore germination and outgrowth of control samples of PCR ribotypes 106, 001 and 078 varied from those of PCR ribotype 027, as did the effects of antimicrobials (data presented elsewhere).15,16 However, for all strains, oritavancin, vancomycin and metronidazole inhibited spore outgrowth to varying degrees, but had no effect on spore germination.

**Post-antibiotic effect spore germination assays**

Spores exposed to all antimicrobial agents converted from PB to PD at a similar rate to control samples. Vancomycin- or metronida- zole-exposed PD spores showed comparable outgrowth to control samples; however, oritavancin-exposed spores showed a reduction in outgrowth of PD spores to VCs. After 24 h, oritavancin-exposed samples contained ∼20% VCs, whereas control samples contained ∼80% VCs (data not shown).
Figure 1. Percentage ($\pm$ range) of *C. difficile* ribotype 027 phase-bright (PB) spores, phase-dark (PD) spores and vegetative cells (VCs) exposed to (a) 0.1 mg/L, (b) 1 mg/L or (c) 10 mg/L metronidazole (MTZ), vancomycin (VAN), or oritavancin (ORI). C, control.
Viable counting of antibiotic-exposed spores

For all ribotypes, recovery of viable spores and VCs after washing following vancomycin exposure was similar to control samples; however, there was considerable strain-to-strain variation observed for the oritavancin-exposed samples (Figure 2). For ribotype 001, the number of spores and VCs recovered after washing was comparable for oritavancin- and vancomycin-exposed samples, and was similar to that of non-antimicrobial-exposed controls (Figure 2b). For ribotype 027, after exposure to both vancomycin and oritavancin, comparable numbers of VCs and spores compared with controls were recovered at 0 h, but for oritavancin-exposed samples, only sporadic recovery at around the level of detection was observed thereafter, whereas recovery of vancomycin-exposed VCs and spores was comparable to that in control samples (Figure 2a). This was even more pronounced for ribotype 078, where no spores or VCs were recovered from 2 h post-oritavancin exposure (Figure 2c).

C. difficile spore binding assays with oritavancin

C. difficile spores (controls and vancomycin-exposed) were not inhibitory to growth of the S. aureus lawn, whereas oritavancin-exposed C. difficile spores retained inhibitory activity against S. aureus after 0, 1, 2, 4, 5, 6, 9 and 10 washes (data not shown).

Discussion

We have previously noted an apparent enhanced activity of oritavancin against spores when compared with vancomycin. Spore germination/outgrowth experiments using phase-contrast microscopy demonstrated that all antimicrobials evaluated at supra-MIC levels inhibited C. difficile spore outgrowth but not germination. This is similar to recently reported results for fidaxomicin and vancomycin. Interestingly, the outgrowth of C. difficile PCR ribotype 027 spores exposed to 10 mg/L of oritavancin (but not vancomycin and metronidazole) was significantly inhibited after thorough washing, indicating persistence of the antimicrobial effect. These data suggest that oritavancin is not directly affecting the germination of the spore, but persists to a greater extent than other antibiotics, affecting subsequent VC outgrowth. Oritavancin is functionally related to ramoplanin and the lantibiotic nisin, which have also been reported to inhibit both Bacillus subtilis and Clostridium sporogenes spore outgrowth. We have seen similar reductions in spore detection within the human gut model after ramoplanin and oritavancin instillation. The mechanism of inhibitory activity against outgrowing C. difficile spores is not fully elucidated, but may potentially be a consequence of oritavancin binding directly to spores. Oritavancin adheres to plastic surfaces and filters, probably mediated by electrostatic charge, and this can be reduced by the surfactant polysorbate 80. Teicoplanin is known to bind to inert surfaces to a greater extent than vancomycin, and this may also be due to an electrostatic charge effect. Oritavancin may bind directly to spores, as washed oritavancin-exposed spores inhibited S. aureus growth. Here, we show that spores exposed to oritavancin before washing had substantially reduced viability compared with those exposed to other antimicrobials before washing, although this effect varied between C. difficile strains. Oritavancin exhibited little effect on the recovery of PCR ribotype 001 spores, but completely inhibited the recovery of ribotype 078 spores. This may indicate that the adherence of oritavancin to spores is dependent on a spore coat characteristic that varies between strains. However, as only one strain of each ribotype was analysed here, differences may also be present between different strains of the same ribotype.
Adherence of oritavancin to dormant spores would locate it to the cell surface, potentially allowing it to exert rapid anti-*Clostridium difficile* activity as the spores break dormancy. This effect may be particularly critical following treatment in vivo. If little or no ‘free’ or ‘bioavailable’ antimicrobial remained within the gut lumen, adherence of oritavancin to spores may retain it at the required site of action for a longer period. Previous data indicate that this phenomenon may occur in an *in vitro* human gut model.10,11 Oritavancin has been shown to have activity against *S. aureus* cultures in stationary phase and within biofilms, where other antimicrobials such as vancomycin, teicoplanin, linezolid, or daptomycin did not to the same extent.23 This was postulated to be due to the mechanisms of action, including disrupting membrane potentials and permeability.23 Oritavancin surface adherence may cause persistence within biofilms and on cell surfaces, and hence persistence at the site of action, thereby enhancing activity.

Our data support further exploration of the mechanism of interaction between oritavancin and *C. difficile* spores to further understand this phenomenon and its potential effects in vivo.

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