Analysis of Clostridium difficile biofilms: Imaging and antimicrobial treatment

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Background: Clostridium difficile, a spore-forming Gram-positive anaerobic bacillus, is the most common causative agent of healthcare-associated diarrhoea. Formation of biofilms may protect C. difficile against antibiotics, potentially leading to treatment failure. Furthermore, bacterial spores or vegetative cells may linger in biofilms in the gut causing C. difficile infection recurrence.

Objectives: In this study, we evaluated and compared the efficacy of four antibiotics (fidaxomicin, surotomycin, vancomycin and metronidazole) in penetrating C. difficile biofilms and killing vegetative cells.

Methods: C. difficile biofilms grown initially for 48 or 72 h using the colony biofilm model were then treated with antibiotics at a concentration of 25 % MIC for 24 h. Vegetative cells and spores were enumerated. The effect of treatment on biofilm structure was studied by scanning electron microscopy (SEM). The ability of fidaxomicin and surotomycin to penetrate biofilms was studied using fluorescently tagged antibiotics.

Results: Both surotomycin and fidaxomicin were significantly more effective than vancomycin or metronidazole (P < 0.001) at killing vegetative cells in established biofilms. Fidaxomicin was more effective than metronidazole at reducing viable spore counts in biofilms (P < 0.05). Fluorescently labelled surotomycin and fidaxomicin penetrated C. difficile biofilms in ,1 h. After 24 h of treatment, SEM demonstrated that both fidaxomicin and surotomycin disrupted the biofilm structure, while metronidazole had no observable effect.

Conclusions: Fidaxomicin is effective in disrupting C. difficile biofilms, killing vegetative cells and decreasing spore counts.

Clostridium difficile is a spore-forming, Gram-positive, anaerobic bacillus that is responsible for one of the most common types of hospital-associated infections.1,2 A 2011 study reported that C. difficile was responsible for nearly half a million infections and was associated with 29000 deaths in the USA alone.3 C. difficile infection (CDI) risk factors include a history of hospitalizations, advanced age and chronic health issues.4 In addition, dysbiosis of intestinal flora following prolonged use of antibiotics is associated with an increase in the risk of CDI.1,4,5 In the past, CDI was more common in the hospital setting; however, it is becoming increas-ingly common within the community setting, where infection can occur in people devoid of the obvious risk factors.6 Many clinically important C. difficile strains form biofilms,7 wherein a matrix of extracellular polymeric substances functions as a physical and chemical diffusion barrier to antibiotic penetration and provides a potential reservoir for C. difficile spore persist-ence.8–10 The ability of this pathogen to form endospores allows the bacteria to evade the antimicrobial effects of treatment and cause a high level of recurrence, as spores linger in the gut long after completion of the treatment regimen. Furthermore, nutrient limitation within biofilms slows the growth rate of bacteria and activates the general stress response.8 The combined effect of these features means that antimicrobial tolerance of bacteria within biofilms can be as high as 100- to 1000-fold greater than the MIC for the planktonic counterpart.11 This has serious implications for patient health and appears to play a crucial role in the development of chronic and persistent infections.9,12 However, when biofilms are disrupted, the cells are once again vulnerable.13,14 In this study, the activities of four antibiotics were tested using the colony biofilm model (CBM).15 Vancomycin and metronidazole were evaluated for in vitro efficacy against established C. difficile biofilms and compared with fidaxomicin, a narrow-spectrum macrocyclic antibiotic, and surotomycin, a cyclic lipopeptide antibiotic that was in development until recently. We further evaluated the ability of these antibiotics to penetrate and disrupt biofilm structure.
Materials and methods

C. difficile strain and growth conditions

C. difficile strain ATCC BAA-1382 (historical strain 630, PCR ribotype 078; ATCC, Manassas, VA, USA) was used throughout the study. Overnight cultures from frozen stocks were grown at 37 °C, anaerobically, in modified brain heart infusion supplemented (mBHIS) broth (BHI medium plus 5 g/L yeast extract, 0.1 M glucose and 0.03% L-cysteine, with an adjusted final concentration of 50 mg/L calcium chloride). All media used in the study were adjusted to 50 mg/mL CaCl2. All chemicals were obtained from Fisher Scientific (Pittsburgh, PA, USA).

Determination of MICs

The MICs of the test antibiotics were determined by agar dilution following CLSI guidelines. Metronidazole and vancomycin were obtained from Sigma-Aldrich (St Louis, MO, USA); surrotomycin and fidaxomicin were supplied by Merck & Co., Inc. (Kenilworth, NJ, USA). All antibiotics were serially diluted in cooled mBHIS or Mueller-Hinton II agar and poured into Petri dishes. The plates were inoculated with 10 μL drops of an overnight culture of C. difficile (10⁶ cfu/mL). Plates were incubated anaerobically at 37 °C for 24 h. The lowest concentration of the antibiotic to inhibit visible growth was considered as the MIC of the particular antibiotic. MIC determination was performed in triplicate.

Colony model biofilms

C. difficile biofilms were grown using the CBM. Prior to inoculation, black polycarbonate membrane filters (0.22 μm pore size, 25 mm in diameter; GE Osmonics, Inc., Minnetonka, MN, USA) were sterilized in a laminar flow hood under germicidal light for 10–15 min per side. The UV-sterilized membranes were then placed on mBHIS agar. The membranes were inoculated with 10 μL aliquots of the overnight culture (approximately 10⁸–10⁹ cfu by viable plate count on mBHIS agar) and allowed to dry for 15–30 min before being incubated at 37 °C under anaerobic conditions. Inoculum cell densities were confirmed by serial dilution and drop plate counts on mBHIS agar. The membranes were aseptically transferred to fresh mBHIS agar plates under anaerobic conditions every 24 h. Biofilms were grown for a total of 72–96 h (48–72 h growth and 24 h antibiotic treatment). For treatment, the membranes were transferred to mBHIS agar plates containing antibiotics for 24 h. Control biofilms were transferred to mBHIS agar plates that did not contain antibiotics. Initial successive CBM experiments were conducted using C. difficile strain 630 biofilms treated with 100 × MIC or 50 × MIC for 24 h. All these treatments, except vancomycin, resulted in a complete kill, whereas 25 × MIC treatments resulted in partial kill of the biofilms, as desired for comparing the antibiotics. However, the results were highly variable, no spores were recovered and two colony morphologies were observed. For these reasons, a new culture of C. difficile (ATCC BAA-1382) was used for subsequent experiments and antibiotics were used at 25 × MIC. For vegetative cell counts, a total of five repeat experiments were conducted. Spore counts were obtained for four of these five experiments and an additional three experiments were conducted with spore counts only, for a total of seven experiments with spore counts.

Viable plate count analysis

Viable plate counts of both spores and vegetative cells were performed by placing the membrane with the biofilm in Dey/Engley neutralizing broth (Difco, BD Diagnostics, Sparks, MD, USA) and vortexing for 30 s. Vegetative bacteria were enumerated on mBHIS agar. Similarly, spores were enumerated on mBHIS agar supplemented with 0.1% sodium taurocholate, after heat killing vegetative cells at 80 °C for 20 min. Viable plate count data were log₁₀ transformed to determine log densities (LDs). Based on this, mean LDs (MLDs) were calculated. For antibiotic treatment, log reductions were calculated relative to untreated control biofilms for each experiment. Mean log reductions (MLRs) were calculated as an average of all the repeat experiments. The analysis of variance (ANOVA) general linear model (Minitab version 17, State College, PA, USA) was used to compare the MLRs. Vegetative cell data and spore data were analysed separately. A general linear ANOVA model was fitted to each data set with a 95% CI. Experiment was included as a random effect, while treatment and time (biofilm growth time prior to treatment) were included as fixed effects. Pair-wise comparisons between treatments (antibiotics) were made using the Tukey method at 95% confidence.

Treatment with fluorescently labelled fidaxomicin and surrotomycin

Evaluation of antibiotic penetration by surrotomycin and fidaxomicin was performed by treating C. difficile biofilms with fluorescently labelled surrotomycin and fidaxomicin, supplied by Merck & Co., Inc., Kenilworth, NJ, USA. Surrotomycin and fidaxomicin were fluorescently labelled with boron-dipyrromethene [BODIPY FL NHS ester (succinimidyl ester)]. The label attachment was assigned to the ornithine nitrogen position of surrotomycin and to one of the phenolic OH groups at positions 2 or 4 on fidaxomicin. The fluorescent antibiotics were purified by reverse-phase chromatography (H₂O/acetoniitride and 0.1% formic acid) and characterized by H1-NMR. Stock solutions of fluorescent antibiotics were prepared by the addition of 10 mg of fluorescent stain to 1.0 mL of DMSO or the addition of 11.6 mg of the stain to 1.16 mL of DMSO for surrotomycin and fidaxomicin, respectively, and stored at −20 °C. Absorbent pads (API002500, EMD Millipore Corporation, Billerica, MA, USA) were saturated with 0.5 mL of mBHIS broth and then 10 μL of the fluorescent antibiotic stock solution was added to each pad to achieve the treatment concentration of 200 mg/L. Biofilms grown for 72 h were transferred to an absorbent pad soaked with fluorescent antibiotic for 1 or 24 h. The membranes were immersed in optimum cutting temperature compound (Sakura Finetek USA, Inc., Torrance, CA, USA), cryo-sectioned and examined using confocal microscopy. Thin sections (5 μm) were cut at −20 °C using a Leica CM1850 cryostat and placed on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA). The sections were examined using a Nikon Eclipse E-800 microscope equipped for epifluorescence or differential interference contrast (DIC). Epifluorescence and DIC images were captured using a CoolSNAP EZ cooled CCD camera (Photometrics, Tucson, AZ, USA) and processed using MetaMorph image analysis software version 7.8.1 (Molecular Devices, LLC, Sunnyvale, CA, USA).

Scanning electron microscopy (SEM)

Biofilms were fixed with paraformaldehyde overnight, cryo-embedded and cryo-sectioned for examination by SEM. The sections were dehydrated in a graded ethanol series (50%, 70%, 90%, 95%, 100% and 100%), air dried, sputter coated with iridium (10 nM) and examined using a Zeiss Supra 55VP FE-SE microscope.

Results

Determination of treatment concentration

The C. difficile MICs on MHII agar were 1.0 mg/L for surrotomycin, 0.13 mg/L for fidaxomicin, 8.0 mg/L for vancomycin and 0.5 mg/L for metronidazole. These MICs were then used to determine the appropriate concentration for treatment of C. difficile biofilms in the remaining experiments. The activity of the fluorescently labelled compounds was decreased 8-fold for BODIPY-labelled surrotomycin and >32-fold for BODIPY-labelled fidaxomicin (data not shown), as previously reported for other antibiotics.

When C. difficile biofilms grown for 24 or 48 h were treated with fidaxomicin, surrotomycin and metronidazole at a concentration of
100 \times \text{MIC} \text{ for a further 24 h, post-treatment counts were below the detection limit of quantification or } > 7.5 \text{ log reduction for both spores and vegetative cells, thus indicating complete killing. In contrast, following 24 or 48 h of growth, a 24 h treatment of the biofilms with vancomycin at 100 \times \text{MIC resulted in 2.7 and 1.1 log reductions, respectively. A similar complete kill of vegetative cells with spore levels below the levels of detection was observed when antibiotics were used at a concentration of 50 \times \text{MIC}, whereas 25 \times \text{MIC resulted in only partial killing. As partial killing is desirable for comparison of the effect of antibiotics on biofilms, this concentration was deemed appropriate for further investigation in this study.}

**Viable plate counts**

There were no significant differences in the LDs of vegetative cells within biofilms grown for 72 h (range 7.2–8.9 log cfu/mL, MLD 8.3±0.4 log cfu/mL) or 96 h (range 7.7–8.7 log cfu/mL, MLD 8.2±0.4 log cfu/mL) (Figure 1). The LD of spores within biofilms was more variable, with a range 2.4–5.9 log cfu/mL, MLD 3.7±1.0 log cfu/mL at 72 h and with a range 2.8–5.9 log cfu/mL, MLD 4.0±0.9 log cfu/mL at 96 h (Figure 1). In order to compare the efficacy of the antibiotics in killing *C. difficile* within biofilms, CBMs grown for 48 or 72 h were treated with antibiotics at 25 \times \text{MIC for 24 h (total growth time of 72 and 96 h, respectively). This is a clinically relevant concentration for treatment, as gastrointestinal concentrations of surotomycin in patients with CDI have been shown to greatly exceed MIC concentrations.}^{18} \text{ This is similar to observations in patients treated with metronidazole, vancomycin and fidaxomicin.}^{19-21} \text{ There were no significant differences in the vegetative cell total MLR between biofilms grown for 48 and 72 h prior to antibiotic treatment (} P = 0.161). \text{ Therefore, the log reduction data for both growth times were included in the same ANOVA model. The MLR ± standard deviation for biofilms treated with fidaxomicin (MLR 2.2±1.2) was significantly greater than for vancomycin (} P < 0.001, \text{ MLR 0.7±0.4}) \text{ and metronidazole (} P < 0.001, \text{ MLR 0.4±0.6}) (Figure 2). Similarly, MLR for biofilms treated with surotomycin (MLR 2.9±0.6) was significantly greater than vancomycin (} P < 0.001, \text{ MLR 0.7±0.4}) \text{ and metronidazole (} P < 0.001, \text{ MLR 0.4±0.6}) (Figure 2).

For the spore count, biofilm growth time had a significant effect on the total MLR (} P = 0.026). \text{ Thus, the data were analysed in separate ANOVA models. For surotomycin and fidaxomicin, the log reductions were numerically lower for the 96 h biofilm compared with 72 h biofilm. This was further confirmed via real-time imaging. Biofilms grown in treatment imaging flow 196 cells were imaged before and after treatment with fluorescently labelled surotomycin (see Supplementary Materials and methods). In contrast, for vancomycin and metronidazole, the log reductions were higher for the biofilms cultured for 96 h compared with 72 h. Pairwise comparisons were analysed similarly as reported above. No significant differences were observed for spore MLR for biofilms grown for 48 h prior to treatment (72 h total growth). In the case of biofilms grown for 72 h prior to antibiotic treatment (96 h total growth), MLR for biofilms treated with fidaxomicin (MLR 2.3±0.9) was significantly greater than that for metronidazole (} P = 0.005, \text{ MLR 1.0±0.3}) (Figure 3). The MLRs in spore counts for 96 h cultured biofilms were numerically and statistically similar for vancomycin (MLR 1.5±1.1) \text{ and surotomycin (} P = 0.981, \text{ MLR 1.6±1.3}).

**Treatment with fluorescently labelled fidaxomicin and surotomycin**

In order for antibiotics to have an effect on bacteria within biofilms, it is essential that they are able to penetrate through the structure. Therefore, to study the ability of fidaxomicin and surotomycin to penetrate through *C. difficile* biofilms, the antibiotics were tagged with fluorescent probes. The final concentration of BODIPY-labelled compounds (200 mg/L) is likely higher than the MIC of the labelled compounds and within the solubility margin of
fidaxomicin. Therefore, the concentrations of the BODIPY-labelled compounds were considered physiologically relevant.

These fluorescently labelled antibiotics were used to treat CBM biofilms grown for 72 h. Within 1 h, BODIPY-labelled surotomycin was detected throughout the biofilm (Figure 4a). However, in the untreated control biofilms, no detectable green fluorescence was observed (data not shown). After 24 h, cells within the biofilm exhibited intense fluorescence, indicating that the antibiotic accumulated both in and on individual cells within the biofilm (Figure 4b). This was further confirmed via real-time imaging. Biofilms grown in treatment imaging flow cells were imaged before and after treatment with fluorescently labelled surotomycin. Fluorescence was found to increase steadily for the first 90 s as the antibiotic entered the flow cell and was followed by a more gradual increase in intensity as it accumulated within biofilms (Figure S1, available as Supplementary data at JAC Online).

BODIPY-labelled fidaxomicin appeared to penetrate more rapidly than surotomycin, within 2 min, at which point significant fluorescence was detected throughout the biofilm (data not shown). Similar results were seen with exposure times of 5 min to 1 h (Figure 5). As the exposure time increased, fluorescence seemed to be more uniformly distributed among the biofilm sections. Overall, fluorescent fidaxomicin penetrated the biofilms faster than fluorescent surotomycin (Figures 4 and 5). Also, microscopy indicated that biofilms were thinner after 24 h of treatment with fluorescent fidaxomicin, indicating a disruptive activity of this antibiotic on biofilm structure (data not shown).

**SEM**

Cryo-sections of the biofilms were examined using SEM to identify any changes in the biofilm structure following treatment with antibiotics. Biofilms were grown for 48 and 72 h and

Figure 3. Effect of antibiotic treatment on spores within biofilms. Mean $\log_{10}$ reductions in spores were calculated for *C. difficile* colony biofilm models grown for 48 and 72 h and treated for 24 h with $25 \times$ MIC of test antibiotics (72 and 96 h of total growth, respectively). The antibiotics tested were surotomycin (SUR; 25 mg/L), fidaxomicin (FDX; 3.25 mg/L), vancomycin (VAN; 200 mg/L) and metronidazole (MTZ; 12.5 mg/L). Error bars indicate 1 SD from the mean.

Figure 4. Penetration of biofilm by fluorescently labelled surotomycin. Confocal laser scanning microscopy images of cross-sections of *C. difficile* colony biofilm models after treatment with fluorescently labelled surotomycin for 1 h (a) and 24 h (b).
subsequently treated with antibiotics for 24 h. Surotomycin (Figure 6c and Figure S2c) and fidaxomicin (Figure 6d and Figure S2d) treatment, but not metronidazole treatment (Figure 6b and Figure S2b), caused disruption of the biofilm structure. Compared with control biofilms, those treated with metronidazole did not exhibit any change in biofilm structure (Figure 6a versus 6b and Figure S2a versus S2b). Biofilms treated with surotomycin and fidaxomicin had fewer cells and more voids as compared with metronidazole (Figure 6b–d and Figure S2b–d).

**Figure 5.** Penetration of biofilm by fluorescently labelled fidaxomicin at 1 h. Combined epifluorescence and DIC images of *C. difficile* biofilm sections after treatment with fluorescent fidaxomicin shows that the antibiotic penetrates through the biofilms and kills the cells within, thereby leading to thinning of the biofilm.

**Figure 6.** Cross-sectional SEM images of 72 h control biofilms (a) and biofilms treated for 24 h with metronidazole (b), surotomycin (c) and fidaxomicin (d). An asterisk indicates the bottom of the biofilm.
Discussion

In this study, we tested the ability of four different antibiotics (vancomycin, metronidazole, fidaxomicin and surotomycin) to penetrate and kill bacteria within C. difficile biofilms. Our study demonstrated that fidaxomicin and surotomycin are significantly more effective at killing vegetative C. difficile within biofilms in vitro. Furthermore, fidaxomicin led to significant decreases in bacterial spores within biofilms.

The biofilm mode of growth is considered to be the natural state for most bacteria and is ubiquitous in nature. While it is difficult to replicate all of the complex characteristics of a biofilm in vitro, techniques such as CBM permit preliminary testing of antibiotics with biofilms. In this study, C. difficile biofilms were grown for 48 or 72 h and subsequently treated with antibiotics for 24 h at a concentration of 25 × MIC. MLRs of vegetative cells in antibiotic-treated biofilms relative to the untreated control showed that both surotomycin and fidaxomicin were significantly more effective than vancomycin or metronidazole in killing C. difficile. In addition, MLR of spores upon fidaxomicin exposure was more than double that of metronidazole (2.3 versus 1.0 log cfu/mL; P = 0.005), indicating that fidaxomicin is significantly more effective than metronidazole in reducing C. difficile spore counts within biofilms. Previous studies have shown that formation of biofilms induces sporulation and suppresses germination in C. difficile. Suppression of germination not only enables efficient dissemination via spores but could also play an important role in recurring infection. Spores shed into the environment are difficult to eradicate because of their resistance to heat, chemicals and radiation. Moreover, spores linger in the gut until completion of a treatment regimen and can cause recurrent infection. Any antibacterial treatment that could reduce spore levels may be effective in preventing recurrence of infection. A previous study showed that fidaxomicin is a potent inhibitor of C. difficile sporulation and was effective even after the sporulation process was initiated. Moreover, fidaxomicin was found to persist on C. difficile spores and fidaxomicin-exposed spores did not show vegetative growth after 24 or 48 h. In comparison, vancomycin did not persist on C. difficile spores, with outgrowth of vancomycin-exposed spores occurring within 24 h of washing. This may also explain the effectiveness of fidaxomicin against C. difficile spores within biofilms. Additionally, direct access to spores was also shown to inhibit outgrowth of vegetative cells from germinated spores.

This study substantiates results from previous Phase 3 studies comparing vancomycin and fidaxomicin, which showed greater clinical cure without recurrence within 4 weeks’ follow-up after treatment (sustained clinical response) in patients treated with fidaxomicin as compared with those treated with vancomycin (P = 0.001; P = 0.006), although longer-term surveillance is necessary in clinical practice. Fidaxomicin was also more effective in preventing recurrence in different subgroups exhibiting various risk factors for CDI. Thus, it is possible that the efficacy of fidaxomicin in killing vegetative cells and reducing spore counts within biofilms contributes to its effectiveness in preventing CDI recurrence.

Fluorescently labelled antibiotics were used to further evaluate the ability of surotomycin and fidaxomicin to penetrate C. difficile biofilms and their rates of penetration. Fluorescent surotomycin rapidly penetrated C. difficile biofilms in <1 h and continued to accumulate within the structure, while fidaxomicin penetrated established C. difficile biofilms within 2 min. In order for any antibacterial to be effective against C. difficile, it has to negate the protective biofilm structure and kill both vegetative cells and spores. Therefore, we analysed the structures of untreated control biofilms and those treated with each of the antibiotics by SEM. Biofilms treated with surotomycin and fidaxomicin displayed substantial changes to biofilm structure. In both cases, microscopy revealed that biofilms had fewer cells after treatment with surotomycin or fidaxomicin and had larger areas without cells. Thus, both of these antibiotics kill bacteria and clear large sections of the biofilm, thereby penetrating inside the structure to exert their bactericidal effects. As the fluorescently labelled antibiotics were not tested for stability, we are unable to exclude the possibility of the labels coming off as they penetrate through the biofilms. However, the fluorescent studies combined with the SEM studies that show changes in the biofilm structure would argue against the fluorescent label falling off.

A limitation of this study is that only single-species C. difficile biofilms were examined, whereas in vivo it is highly likely that C. difficile biofilms would be part of a community of indigenous gut microbiota, as has been studied using a pooled human faecal emulsion in biofilm model studies. Little is known about changes in physical properties in multi-species biofilms. Depending on the species present, multi-species biofilms may be more or less permeable to treatment chemistries. The small number of replicates also means that the standard deviations for log reductions in vegetative cells and spores are relatively large. The concentrations of antibiotics tested were selected to be higher than the MIC50 of surotomycin, fidaxomicin and vancomycin, and to allow comparison as stated in the Materials and methods section. However, it is important to note that in human patients the concentrations observed in faecal samples or in the gut lumen are much higher, closer to 1000 × MIC.

This study indicates that fidaxomicin and surotomycin are significantly more efficacious at killing C. difficile within biofilms in the in vitro models used. As the human gut harbours a complex microbial community, which exists both in planktonic forms and in sessile biofilms, the interactions between the various microorganisms within the human gut and the effect of antibiotics on bacteria within biofilms in vivo are understandably difficult to duplicate. Our study indicates that fidaxomicin and surotomycin are effective in penetrating and killing bacterial cells within biofilms and that fidaxomicin was highly efficacious in reducing bacterial spores inside biofilms. However, the clinical relevance of the data for surotomycin is limited. The development of surotomycin has been discontinued given the lack of benefit over vancomycin in preventing recurrences demonstrated in a Phase 3 trial. The manner in which our findings reflect the in vivo relationship between biofilm and antibiotics remains to be seen.

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