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Clostridium difficile

A novel method for imaging the pharmacological effects of antibiotic treatment on *Clostridium difficile*

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ABSTRACT

Clostridium difficile is a significant cause of nosocomial-acquired infection that results in severe diarrhea and can lead to mortality. Treatment options for *C. difficile* infection (CDI) are limited, however, new antibiotics are being developed. Current methods for determining efficacy of experimental antibiotics on *C. difficile* involve antibiotic killing rates and do not give insight into the drug's pharmacologic effects. Considering this, we hypothesized that by using scanning electron microscopy (SEM) in tandem to drug killing curves, we would be able to determine efficacy and visualize the phenotypic response to drug treatment. To test this hypothesis, supraMIC kill curves were conducted using vancomycin, metronidazole, fidaxomicin, and ridinilazole. Following collection, cells were either plated or imaged using a scanning electron microscope (SEM). Consistent with previous reports, we found that the tested antibiotics had significant bactericidal activity at supraMIC concentrations. By SEM imaging and using a semi-automatic pipeline for image analysis, we were able to determine that vancomycin and to a lesser extent fidaxomicin and ridinilazole significantly affected the cell wall, whereas metronidazole, fidaxomicin, and ridinilazole had significant effects on cell length suggesting a metabolic effect. While the phenotypic response to drug treatment has not been documented previously in this manner, the results observed are consistent with the drug's mechanism of action. These techniques demonstrate the versatility and reliability of imaging and measurements that could be applied to other experimental compounds. We believe the strategies laid out here are vital for characterizing new antibiotics in development for treating CDI.

1. Introduction

Clostridium difficile is a gram-positive, rod-shaped anaerobic bacteria that is the most common cause of hospital-associated infection [1,2]. Roughly half a million Americans are affected by this bacteria each year and the prevalence and severity of *Clostridium difficile* infection (CDI) has increased rapidly [3,4]. Currently, there are very few medications available to treat CDI spurring the extensive focus on developing new antibiotics for treatment. With many new antimicrobials in developments, a thorough characterization of antibiotic effects will be necessary.

Current methods available for evaluating antibiotic efficacy are by *in vitro* standard killing curve measurements [5,6]. To perform these measurements, bacteria are allowed to incubate in broth with or without drug at specified time points and then are subsequently plated to determine how many colonies form, which indicates cell growth and viability. Although these measurements are reliable and reveal the overall outcomes of antibiotic treatment, they are rudimentary and do not capture the cells' direct phenotypic response to the medication. By evaluating the cell's morphological response to treatment, it is possible to grasp a better understanding of the antibiotic's pharmacological effects.

Due to its extremely small size, *Clostridium difficile* is difficult to image without high magnification [7]. Thus, the task of evaluating

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the direct antibiotic effects on *C. difficile* is technically demanding. However, by utilizing scanning electron microscopy, we hypothesized that it would be possible and relatively quick to clearly observe small changes that occur to *C. difficile* in response to stressors such as antibiotics. Moreover, we thought that it would also be possible to determine the drugs' pharmacological effects by evaluating the phenotypic properties (cell wall-acting antibiotic versus cell metabolism-acting antibiotics). To test these hypotheses, we assessed the phenotypic differences in *C. difficile* cells upon exposure to vancomycin, metronidazole, fidaxomicin, and ridinilazole [8–10]. By implementing SEM microscopy in tandem with time kill curves, we show that these techniques can be complementary and allow for the rapid dissection of drug effects on the bacterial morphology and/or metabolism.

2. Experimental methods

2.1. Bacterial strain, culture and killing kinetics

C. difficile strain R20291 (BI/NAP1/027) was grown on blood agar plates for 48 h at 37 °C in anaerobic chamber and isolated colonies were used to make pre-cultures for further experiments. MICs for vancomycin, metronidazole, fidaxomicin, and ridinilazole were determined by broth microdilution in 0.1% sodium taurocholate brain heart infusion (BHI) medium. Cultures of *C. difficile* were prepared by inoculating one colony to BHI medium. The ridinilazole killing curve was adapted from Basseres et al. [11] After 24 h incubation at 37 °C in anaerobic chamber, pre-cultures were diluted 1:100 to approximately 10^6 CFU/mL in fresh BHI supplemented with 0.1% sodium taurocholate and the appropriate concentration of antibiotic (T0). Total viable counts were determined at T0; T24 and T48 (hours) of antibiotic treatment. 1 mL of culture was supplemented with 100 μL of killed yeast solution and centrifuged for 1 min at 10000 rpm. The supernatant was removed and the pellet resuspended in fresh BHI. 100 μL of sample serial dilutions were spread on blood agar plates in duplicate and incubated at 37 °C in anaerobic chamber for 48 h prior to colony counting. The limit of detection (LOD) for these assays was 500 CFU/mL. Bactericidal was defined as a ≥ 3 $\log_{10}$ reduction in viability relative to the starting inoculum.

2.2. Scanning electron microscopy (SEM) preparation

Cells were collected following drug treatment at T0, T6, and T24 and concentrated using centrifugation. The cells were then fixed using standard protocols [7,12] and re-diluted in water for imaging. Aliquots of the bacteria were allowed to incubate for 30 min on coverslips. The coverslips were then dried at room temperature and prepared for coating procedures. Cells were coated with 20 nm of gold coating using a Denton Desk II Sputtering System. Following sputtering, the gold coated coverslips were prepared for imaging. Collectively, this procedure roughly takes 1 h after harvesting the cells.

Following cell coating, coverslips were transferred to a FEI XL-30FEG scanning electron microscope. Following equilibration and calibration, working distance was set to 5 mm and high resolution mode was used to image the cells. Images were taken between 15.0 and 5.0 kV at designated magnifications.

2.3. Image analysis and statistics

After images were acquired, they were opened in FIJI (http://fiji.sc/Fiji). Scale bars were calibrated and cell length was determined using the multi-measure function as described previously [13]. To quantify cell wall activity, we applied a semi-automatic approach to quantify the deformation of a cell. A few landmarks were manually selected to represent the cell wall. A least square method was used to fit an ellipse to the selected landmarks. An ellipse with the best fit creates a region of interest (ROI) around the cell. This ellipse acts as a benchmark to define a deformation score. Image intensity thresholding was combined with morphological operations to segment out the cell. The deformation score S_i of a cell i is defined as the following,

$$d_i = \frac{S_i - S_e}{S_e}, \quad (1)$$

where S_i is the surface of the cell I and S_e is the surface of its best-fit ellipse e. Fig. 3A and B illustrates of the quantification method. Experiments were performed at least in duplicate and >17 cells were measured per group.

Statistical analysis was performed using SPSS statistics software (IBM). Data are expressed as the mean ± SEM. Data were assessed for significance using a *t*-test or one-way ANOVA as appropriate. A probability value of $P < 0.05$ was considered significant.

3. Results

3.1. Antibiotic killing curves

MIC values were found to be 1 μg/mL for vancomycin, 0.5 μg/mL for metronidazole, and 0.06 μg/mL for fidaxomicin and ridinilazole. Kill curves of vancomycin, metronidazole, fidaxomicin, and ridinilazole against *C. difficile* at supra-MIC concentrations (4xMIC and 40xMIC) demonstrated a decrease of total CFU/mL of treated samples at 24 and 48 h. In all cases the reduction observed was bactericidal, more than 3 $\log_{10}$ using supra-MIC concentrations (Fig. 1). These measurements are consistent with the effects demonstrated previously [14,15].

3.2. Evaluating morphologic antibiotic effects on C. difficile

To determine the treatment effect of vancomycin, metronidazole, fidaxomicin, and ridinilazole on *C. difficile*, the cells were prepared and imaged using a high-resolution SEM. Control cells were intact and numerous throughout the studies (Fig. 2). In contrast, the cell wall of vancomycin-treated cells was completely damaged, which was worsened as the cells were exposed for longer periods of time (Fig. 2). Metronidazole-treated cells were significantly smaller in size than control cells demonstrating a potential effect on cell growth and metabolism (Fig. 2). Fidaxomicin and ridinilazole treated cells were smaller in size and the cell wall seemed to be affected, although not to the same extent as the vancomycin-treated cells. Collectively, these results demonstrate the different phenotypic response by *C. difficile* to antibiotics with different mechanisms of action.

3.3. Quantifying the antibiotic effects

To identify whether the antibiotic treatments had specific effects on the cell wall or cell size, we used a high-throughput image analysis pipeline to measure cell deformation and cell length. To measure cell deformation, landmarks on the cell wall were manually selected for least square ellipse fitting. After setting the region of interest (ROI), images were segmented to define the cell wall boundaries. Within the ROI after thresholding, the images with the smoothest, more intact features were given a lower score (Fig. 3A) and the images with the roughest features were given a higher score depicting more deformations (scores range from 0 to 1; Fig. 3B). By doing this, we were able to determine that