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Bile acid sensitivity and *in vivo* virulence of clinical *Clostridium difficile* isolates

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Abstract

Clostridium difficile is an anaerobic bacterium that causes diarrheal illnesses. Disease onset is linked with exposure to oral antibiotics and consequent depletion of secondary bile acids. Here we investigate the relationship between *in vitro* secondary bile acid tolerance and *in vivo* disease scores of diverse *C. difficile* strains in mice.

Keywords

Clostridium difficile; secondary bile acid

Clostridium difficile is an anaerobic, spore-forming bacterium that causes an increasing number of antibiotic-associated intestinal infections in the hospital and community setting^{1–3}. Exposure to *C. difficile* spores leads to a range of outcomes, from asymptomatic carriage to pseudomembranous colitis with life-threatening toxic megacolon⁴. Individuals most at risk for severe infection are those over 65 years of age with recent exposure to oral antibiotics and weakened immune systems^{5–6}. In addition, virulent *C. difficile* strains exhibit significant genetic variability, and new strains are continuing to be discovered^{7–9}. *C. difficile* toxin production^{10–11}, sporulation^{12–13}, flagella structure¹⁴, and germination efficiency^{15–16} have all been proposed to contribute to virulence, yet the bacterial phenotypes that influence disease outcome in the host remain incompletely defined. However, it is known that bile salts play an important role in the germination and growth of *C. difficile* in the mammalian host^{17–22}.

Bile acids are amphipathic molecules produced by the liver and released by the gallbladder in response to food entering the intestine. They serve a critical role in the absorption of dietary fats and are increasingly appreciated as modulators of microbial populations in the gut. Humans synthesize their own primary bile acids^{23–24}, which can then be dehydroxylated by a subset of commensal bacteria to form secondary bile acids (primarily deoxycholic acid and lithocholic acid). The relative concentrations of bile acids are especially important in *C. difficile* infections. The primary bile acid taurocholic acid induces

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germination of metabolically latent spores^{18,25}, while secondary bile acids inhibit the growth of vegetative, toxin-producing cells^{18,26–28}. Only a select group of bacteria produce the enzymes necessary to dehydroxylate primary bile acids, and they are sensitive to killing by many commonly prescribed antibiotics^{23,29}. Recent work in our lab and others has shown that antibiotic treatment in mice shifts the bile acid pool towards a predominance of primary bile acids, and that the restoration of secondary bile acids to pre-antibiotic levels is associated with resistance to *C. difficile* infections^{27–28,30}. However, this work has been conducted with a limited number of *C. difficile* strains. It remains to be determined if clinically relevant isolates exhibit heterogeneity in response to secondary bile acids, and if that heterogeneity associates with observed differences in virulence with a murine model of infection.

To address this question, we tested fecal samples obtained from patients undergoing allogeneic hematopoietic stem cell transplantation for the presence of *C. difficile* organisms by a PCR-based assay for *C. difficile* toxin B. While some patients had been diagnosed with *C. difficile*, other patients had not been diagnosed with *C. difficile* and thus were asymptomatic carriers³¹. We isolated individual *C. difficile* colonies from the infected stool and typed them using multi-locus sequence typing (MLST)³². Of the 21 isolates collected, 16 were members of clade 1 (including sequence types 2,6,12,42,46,58,107), two were members of clade 2 (sequence type 1, also classified as ribotype 027), and 1 each was a member of clade 4 and 5 (STs 39 and 11, respectively)^{32–33}. We prepared purified spore suspensions for each clinical isolate according to published protocols³⁴.

For the *in vivo* experiments, wild-type C57BL/6 mice (Jackson labs) were rendered susceptible to *C. difficile* by replacing their drinking water with antibiotic-containing water for three days. We used a cocktail of three antibiotics: metronidazole, (0.25 g·L⁻¹, Sigma-Aldrich), neomycin (0.25 g·L⁻¹, Sigma-Aldrich), and vancomycin (0.25 g·L⁻¹, NOVA-PLUS). Two days after stopping antibiotic water, mice were additionally treated with 200 µg clindamycin (Sigma-Aldrich) via intraperitoneal injection. We inoculated mice 24 hours later with 100 spores of individual clinical isolates via oral gavage, infecting five mice with each isolate. After infection, mice were monitored daily for indicators of morbidity and given a disease score³⁵. Additionally, fecal pellets were collected 1, 2, 7, and 14 days after infection and quantified for *C. difficile* burden³⁶.

We found that infection with *C. difficile* clinical isolates caused a range of disease severity in mice (Fig. 1A–B). The most virulent strains were members of clade 2 (MLST1/ribotype 027) and clade 5 (MLST11, also classified as ribotype 078) (Fig. 1A). These strains approached and/or surpassed the disease score of our reference strain, *VPI10463*, a notoriously virulent strain^{13,37}. Members of clade 4 (1 isolate) and clade 1 (16 isolates) had heterogeneous disease scores but consistently produced lower morbidity than *VPI10463* (Fig. 1A and B, respectively). The differences in observed morbidity could not be attributed to differences in colonization ability of the clinical isolates, as fecal samples from all infected mice harbored in excess of 10⁶ colony forming units per gram two days after infection (Fig. 1C), and mice remained heavily infected throughout the two-week experiment (data not shown).

We next investigated the impact of secondary bile acids on the growth kinetics of the isolates in an *in vitro* setting. Anaerobic culturing at 37°C was performed and growth was monitored by measurement of optical density of each clinical isolate over a 22-hour period. Each isolate was grown under three treatment conditions: (1) vehicle (brain-heart infusion media supplemented with yeast extract and cysteine), (2) vehicle with 0.01% lithocholic acid, and (3) vehicle with 0.01% deoxycholic acid (BHI and yeast extract from BD Biosciences, others from Sigma-Aldrich). We repeated each experiment twice, for a total of three trials.

Figure 2 reports the growth curves of the laboratory reference strain *VP110463* in response to treatment with lithocholic acid (LCA, Fig. 2A) and deoxycholic acid (DCA, Fig. 2D). In addition, each of the examined clinical isolates was inhibited by LCA and DCA, although to varying degrees (see Fig. 2B and 2E for growth comparison during the exponential phase, and 2C and 2F for growth comparison after cultures reached the stationary phase). In some cases, variation in bile acid tolerance was seen even in strains classified as the same MLST group (see Fig. 2B, MLST 2's, and Fig. 2E, MLST 1's as examples).

Because each of the clinical isolates was unique in terms of secondary bile acid tolerance and murine *in vivo* disease score, we next explored the relationship between these two variables (Figure 3). We found that at two days after infection, the clinical isolates rated most virulent exhibited a trend towards increased lithocholic acid resistance ($p = 0.108$, Fig. 3A, orange points). This trend achieved statistical significance with the clinical isolates that caused morbidity beyond the window of acute infection. *C. difficile* isolates with the highest disease score one and two weeks after infection were also the isolates most tolerant to LCA exposure *in vitro* ($p = 0.017$ at week 1 (blue points), $p = 0.037$ at week 2 (gray points)). In contrast, we did not find a significant correlation between host disease scores and tolerance to deoxycholic acid (Fig. 3B).

Antibiotics of various classes disrupt the normal balance of bile acids in the mouse intestine, decreasing the relative concentrations of secondary bile acids as they increase susceptibility to *C. difficile* infections^{27–28,38}. Administration of a cocktail of bacteria that contained a species capable of converting primary bile acids to their secondary counterparts (*Clostridium scindens*) is sufficient to increase resistance to *C. difficile* challenge. The link between antibiotic administration, secondary bile acid depletion, and increased susceptibility to *C. difficile* infections has been well documented in the literature^{27–30}; however, the impact of strain type on bile acid tolerance and *in vivo* virulence has remained largely unexplored.

Our results demonstrate that for a small cohort of *C. difficile* clinical isolates, *in vitro* tolerance to lithocholic acid administration is associated with higher disease scores in the mouse model. The association is stronger in later stages of the infection (one and two weeks after spore administration), during which time the microbiota is recovering from antibiotic treatment and secondary bile acids are returning to their pre-treatment levels^{27,29}. In contrast, no association was found for *in vivo* disease scores and deoxycholic acid tolerance. Little is known about the response of *Clostridial* species to bile acid stress; studies with other gram positive organisms suggest strain variation in bile acid sensitivity is common^{39,40}. Further investigation into the mechanism of LCA and DCA tolerance may help explain the observations presented in this study.

Growth in the presence of toxic metabolites is just one way an individual *C. difficile* strain can cause disease in the host; other contributing factors include toxin production, germination efficiency, sporulation efficiency, and flagellar structure^{10–16}. Future studies will test the hypothesis that bile acid tolerance acts in combination with other virulence mechanisms. For example, a relative increase in lithocholic acid tolerance may extend a given *C. difficile* strain's ability to produce toxin and allow it to cause persistent disease in the host. Our study is limited both by the small sample size of clinical isolates used and by their restricted phylogenetic diversity (most are members of clade 1). Additional relationships between secondary bile acid tolerance and *in vivo* virulence could be elucidated with a larger, more diverse cohort of clinical isolates.

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Highlights

- *C. difficile* isolates collected from patients cause a range of disease in mice
- These isolates exhibit variable tolerance to secondary bile acids *in vitro*
- Tolerance to lithocholic acid is associated with higher disease scores in mice

Figure 1. *In vivo* disease scores and bacterial burden of *C. difficile* clinical isolates
(A) Disease scores for wild type C57BL/6 mice infected with *C. difficile* isolates identified as MLST1/Clade 2 (red, two isolates), MLST39/Clade 4 (purple), and MLST11/Clade 5 (orange) as compared with the reference strain, VPI10463 (black). **(B)** Disease scores for wild type mice infected with *C. difficile* isolates identified as members of Clade 1 (blue, various MLST types) as compared with VPI10463 (black). **(C)**. *C. difficile* burden as measured from mouse fecal content two days after infection. Numbers at the base of the bars indicate MLST classification. $n = 5$ mice for each clinical isolate tested, data shown are mean $\pm$ SEM. d = day; CFU/g = colony forming units per gram of feces.

Figure 2. *In vitro* growth suppression of *C. difficile* clinical isolates by the secondary bile acids lithocholic acid and deoxycholic acid

(A,D) Growth kinetics of *C. difficile* strain VPI10463 in the absence (solid line, bounded by light gray region) or presence (dotted line, bounded by dark gray region) of lithocholic acid (A) and deoxycholic acid (D), as measured in the change in optical density. (B–C) Tolerance of each *C. difficile* clinical isolate to administration of lithocholic acid and (E–F) deoxycholic acid at indicated time points. Numbers at the base of the bars indicate MLST classification. All experiments were conducted three times, data shown are mean $\pm$ SEM.

Figure 3. Comparison of secondary bile acid tolerance and *in vivo* disease score of clinical *C. difficile* isolates

Tolerance to (A) lithocholic acid or (B) deoxycholic acid during exponential growth was compared to the mean *in vivo* disease scores recorded two days (orange), one week (blue), and two weeks (gray) after infection. We compared bile acid tolerance to disease score at each time point by calculating the linear regression (solid lines, with 95% confidence interval in the corresponding shaded region), * $p < 0.05$. ns = not significant. O.D. = optical density (600 nm).