In vivo pathogenicity and sporulation patterns of *Clostridium difficile*

by

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For mom and dad

Para ma y pa

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ABSTRACT

Clostridium difficile infection (CDI) is one of the most commonly reported hospital-associated infections in the U.S., causing to around 500,000 infections and 29,000 deaths each year. This dissertation describes the results of three different studies exploring the role of the host and pathogen on CDI severity.

For the first study, 200 successive CDI positive stool samples were collected from the Microbiology Laboratory at Saint Joseph Mercy Hospital. Fecal toxin, spore, and *Candida albicans* levels were quantified to characterize the *in vivo* association between toxin and spore levels and whether this association was modified by *C. albicans*. Results indicate a strong association between toxin and spore levels within the human CDI host, with the association varying by ribotype. *C. albicans* overgrowth did not modify this association.

We next performed a case-control study comparing 120 toxin positive CDI and 91 toxin negative PCR positive CDI hospitalized patients. We described clinical and epidemiological differences between CDI patients according to their laboratory diagnosis. In a population with 92% CDI treatment rate, we confirmed a milder CDI presentation and lower 30-days risk of mortality among toxin negative PCR positive patients. Furthermore, spore levels were associated with CDI severity. These results highlight a major limitation of using PCR in the CDI laboratory algorithm and the importance of the severity of the patient's

clinical presentation when considering treatment for a patient testing as toxin negative PCR positive.

Lastly, we used an in-host mathematical model to characterize the role of sporulation/germination patterns on risk of recurrence and compare the effectiveness of several current tapered/pulsed vancomycin regimens on risk of recurrence by specific ribotypes. Our results confirm the importance of *C. difficile* ribotype in explaining CDI recurrence rates and the effectiveness of treatment. All of the evaluated treatment regimens for repeated CDI were effective; further, regimens with reduced duration or dosage of treatment were still highly effective, suggesting that vancomycin regimens may be modified to a lower level that better protects gut microbiota while preventing CDI recurrence.

CHAPTER I

Introduction

Clostridium difficile infection (CDI) is currently the most prevalent healthcare associated infection (HAI) in the United States (US) (1). Every year approximately half a million infections and 29,000 deaths are attributed to *C.difficile* (2). Due to its high incidence and mortality rate, and its reported associated excess healthcare cost of 4.8 billions dollars, hospital-associated CDI rates are now used as an indicator of healthcare quality (3). As a further indication of its public health significance, in March 2015 the White House set a national goal to reduce CDI by 50% by 2020 (4).

Although much has been learned about the pathogenicity and transmission of CDI, managing, controlling, and preventing CDI in healthcare settings remains a challenge. One such challenge includes the lack of an accurate diagnosis of CDI symptomatic patients in order to correctly differentiate them from *C. difficile* carriers and to customize their management, infection, and control practices accordingly. This dissertation addresses this challenge, particularly identifying differences between CDI positive patients based on according to the laboratory algorithm used for diagnosis, including differences in their pathogenicity and sporulation patterns *in vivo*.

The remaining chapters of the dissertation are as follows: Chapter 2 describes the epidemiology of CDI, and highlights its significance as a public

health problem. Chapter 3 presents the results of a laboratory analysis of stool samples from CDI cases aimed at characterizing the *in vivo* correlation of *C. difficile* spore and toxin production, and testing whether this correlation is mediated by *Candida albicans*. In Chapter 4 a compartmental in-host mathematical model is used to analyze the ribotype-specific effectiveness of recommended tapered/pulsed vancomycin regimens, to gain insight into the importance of varying sporulation patterns across ribotypes in the risk of CDI recurrence. Chapter 5 describes differences in risk factors, severity, and clinical outcomes between toxin positive and toxin negative/PCR positive CDI patients. This chapter also identifies hosts' risk factors associated with spore production *in vivo*. Chapter 6 concludes the dissertation with a discussion of the implications of the results for future research, treatment, and infection control.

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CHAPTER II

Background and Significance

Clostridium difficile is an anaerobic, spore-forming, Gram-positive bacillus found in nature as either toxigenic or non-toxigenic, based on the presence or absence respectively of the pathogenicity locus (PaLoc) that encodes for genes associated with toxin production (1, 2). Although approximately 5% of healthy adults and 30-70% of healthy infants carry toxigenic *C. difficile* asymptomatically (3), toxigenic strains are also associated with *Clostridium difficile* infection (CDI), a broad-spectrum toxin-mediated intestinal disease (4, 5).

CDI causes significant morbidity and mortality, greatly increasing healthcare costs. In March 2015 the White House included CDI reduction in its national goals (6). This chapter consists of a comprehensive literature review of the CDI's epidemiology, pathogenicity and transmission, and its public health significance.

2.1.0. Epidemiology

2.1.1. *Clostridium difficile* Infection (CDI) burden

CDI was initially described in 1978 (7). While at first CDI was considered a rare infection, today 12.1% of all reported nosocomial infections are CDI,

making *C. difficile* the most commonly reported nosocomial pathogen (8). Between 2001 and 2010, CDI incidence nearly doubled in the United States (US) (6), reaching almost half of a million infections and 29,000 associated deaths in 2011 (9). However, possibly due to increased awareness, during the last five years, CDI incidence has started to plateau and even decrease. In 2015, Centers for Disease Control and Prevention (CDC) reported the number of CDI cases in US hospitals decreased 10% between 2012 and 2013 (10).

The high CDI incidence and associated morbidity and mortality (11) places a significant financial burden on the US healthcare system. In acute-care settings alone, excess healthcare costs associated with CDI total an estimated 4.8 billion dollars every year (5, 12, 13). Each CDI episode is estimated to cost up to $7,000 (14), increasing hospital costs for a CDI patient by 54% (15). In addition, length of hospital stay may increase up to 12 days (9, 16). Furthermore, institution of proper infection control procedures including patient isolation, use of contact precautions and personal protective equipment can reduce incidence and spread within hospitals; therefore, hospital-associated CDI rates are currently used as an indicator of healthcare quality (17).

2.1.2. Epidemiological classification of CDI cases

The change in CDI epidemiology over time has also affected populations previously considered low-risk for CDI. An increasing number of cases, including severe ones, have been reported in pregnant women and healthy community members (18). To facilitate CDI surveillance and research, the CDC recommends classifying CDI cases by previous healthcare exposure and time of onset of CDI

symptoms as follows: 1) *Healthcare facility (HCF)-onset and HCF–associated* when the onset of symptoms occurred more than 48 hours after admission to a HCF, 2) *Community-onset, HCF-associated* when either the patient's symptoms started 48 hours or less after admission, or in the community but less than 4 weeks after being discharged from a HCF, 3) *Community-associated* when either the patient's symptoms started 48 hours or less after admission, or in the community, but after more than 12 weeks after discharge from a HCF, 4) *Indeterminate* when exposure setting is hard to establish, for example if the patient was discharged from a HCF between 4 and 12 weeks before onset of symptoms, and finally, 5) *Unknown disease* when data are not available to define exposure (19).

Based on this classification, Lessa et al. (2015) have estimated a total of 159,700 community-associated CDI cases, in comparison to 293,300 hospital-associated CDI cases in the US for 2011. Of the hospital-associated CDI cases, 37% had a hospital-onset, 36% had a nursing home onset, and 28% had a community-onset (9).

2.1.3. Prevalent strains

Several different strains of *C. difficile* have been identified, which vary in pathogenicity, virulence and transmissibility. Currently in the US, the most prevalent strains are North American pulsed-field (NAP) 1, NAP4, NAP6, NAP7, NAP11, which represent PCR ribotypes 027, 014-020, 002, 078, and 106 respectively (9).

Of greatest public health concern is ribotype 027. Since 2003, ribotype 027 (NAP1, Toxinotype III, Restriction endonuclease analysis (REA) B1) has caused several outbreaks of severe CDI in Canada, Europe, and the US (1). Over time, ribotype 027 isolates have become increasingly virulent compared to historical 027 strains or other *C. difficile* strains (1). The increases in pathogenicity and transmissibility are attributed to: 1) higher toxin production, 2) resistance to fluoroquinolones and erythromycin that complicates treatment and favors its selection in healthcare settings, and 3) greater amount of spores produced (1, 13, 20-23).

2.1.4. Risk Factors

As the number of *C. difficile* carriers increases, it is essential to recognize that colonization with bacteria is not sufficient for development of CDI. Virulence factors from the infecting *C. difficile* strain, the host's gut microbiota composition, and other host-associated factors play a role in the initiation of symptoms (4). Prior antibiotic use is the leading risk factor for CDI (24). Individuals have a 7 to 10-fold increase risk of developing CDI while on antibiotic therapy or within one month of completing the regimen (25). The antibiotics most commonly associated with CDI are fluoroquinolones, third- and fourth-generation cephalosporins, ampicillin, and clindamycin (24). Other risk factors include advanced age, exposure to the healthcare system (including outpatient facilities), immunosuppressant therapy, use of proton-pump inhibitors (PPIs), gastrointestinal tract surgery, inflammatory bowel disease, and enteral tube feeding (1, 9, 24).

Antibiotic treatment is associated with CDI development because it disrupts the gut's microbiota community structure. Changes in diversity and composition of the healthy fecal microbiota create an environment that permits *C. difficile* overgrowth. Bile metabolism and food competition have been reported as two essential mechanisms leading to colonization resistance or lack thereof (26). Shaninas et al. (2012) showed that a healthy balance of Bacteriodetes and Firmicutes is necessary to avoid *C. difficile* from proliferating (27). Likewise, highly infectious colonized individuals known as supershedders have reportedly a microbiota mostly composed of *Escherichia coli*, *Proteus mirabilis, Klebsiella pneumonia*, and *Enterococcus faecalis* (28). Interestingly, Skraban et al. (2013) found no difference in fungal diversity in the gut between *C. difficile* colonized and non-colonized individuals (29). However, this same study found that CDI patients colonized with ribotype 027 were more likely to be colonized with *Candida albicans* and *Candida glabrata* than healthy individuals (29).

Candida spp. is considered a gut commensal. Its colonization frequency ranges from 4-88% in healthy individuals but is generally at low levels ($\leq 10^4$ CFU/mL) (30). Supporting the conclusions of Skraban et al. (2013) (29), some researchers suggest the prevalence of *Candida* spp. is increased among CDI positive patients, and that colonization might positively or negatively mediate risk of CDI symptoms (31-33). In a double-blind, randomized, phase III clinical trial in multiple hospitals in the US and Canada, Nerandzic et al. (2012) reported 16% *Candida* spp. positivity among 301 CDI patients before initiation of CDI treatment. *Manian* et al. (2013) reported 17% *Candida* spp. overgrowth among 60 American CDI cases (31, 32). In contrast, Raponi et al. (2014) identified 83%

Candida spp. colonization among 40 CDI positive patients at hospital Policlinico "Umberto I" in Rome (33).

In 2014, Raponi and colleagues described a positive association between *Candida* spp. and CDI, particularly with *C. albican*s. In that study, 61% of CDI positive patients (n=24) were colonized with the yeast in contrast to 34% of CDI negative patients (n=34) (33). By contrast, Manian et al. (2013) observed a protective association between *C. albicans* and CDI, where overgrowth of *C. albicans* was significantly less likely among CDI positive patients (10 of 60) than CDI negative patients (103 of 338) (31). One question for the current study is to investigate whether the presence of *C. albicans* is associated with *C. difficile* spore and toxin production, which will provide further evidence to evaluate the association between both microorganisms in the gut.

2.2.0. Transmission

C. difficile transmission occurs through the fecal-oral route. *C. difficile* spores are the bacteria's transmissible vehicle (3). Based upon mice experiments, 7 spores per cm^2 is estimated to be sufficient to cause transmission (1). Spores are acquired from the environment when shed by colonized or infected hosts (4). Approximately 50% of recovered patients continue to shed spores into the environment 1-4 weeks after CDI treatment (34).

Spores can withstand cleaning and desiccation, and can be shed at high rates. Therefore, in the absence of proper hygiene and use of personal protective equipment, healthcare workers can act as vectors transmitting CDI within

hospitals. Up to 38% of CDI cases in healthcare settings have been attributed to healthcare worker transmission (34). With the purpose of reducing this role, in 2014 the Society of Healthcare Epidemiology of America (SHEA) recommended healthcare workers follow a set of dress code guidelines, which include the use of short-sleeves, no neckties, jewelry or wristbands while in clinical practices (35). Farrington et al. (2010) observed that a "bare below the elbows" approach improved hand hygiene effectiveness (36). Although no evidence demonstrates the effectiveness of not using neckties in reducing nosocomial pathogen transmission, studies have isolated pathogens such as *Staphylococcus aureus* and Gram negative bacillus from physicians' neckties (37).

In addition to hospital settings, *C. difficile* has been isolated from soil, hay, sand, or feces from cows, horses, dogs, cats, and rodents (38) CDI has been reported particularly in horses but also in pigs and calves (2). The distributions of prevalent strains among human and animal populations overlap, suggesting that strains can transfer between humans and other animals (39, 40). The prevalence in farm animals and recent isolation of *C. difficile* toxigenic strains from retail food products suggests that foodborne acquisition may be an important additional mode of transmission explaining community-associated infection where there are no other known risk factors of exposure (40).

Spores are resistant to the acidic environment in the stomach. Following exposure to the appropriate germinant, they germinate into vegetative cells in the small intestine (3, 4). The spore's outer surface is smooth during its dormant state but during germination it develops filamentous projections that attach to the colonic microvilli (1). The signaling pathway that triggers *C. difficile* sporulation and its environmental cues are still not well understood. In mice,

sporulation starts around 15 hours after infection (41), while *in vivo* transcriptome analysis in monoxenic mice showed that sporulation genes are upregulated as early as 4 hours after infection (42). The transcription factor Spo0A is known as the master regulator of sporulation after its activation by several histidine kinases. Spo0A then activates downstream regulators associated with sporulation while repressing others related to vegetative functions (43). Starvation, aerobic environments, or quorum sensing are also suspected to play a role in *C. difficile* sporulation (43, 44).

Subsequently, *C. difficile* spore germination is induced by the presence of taurocholate and glycine (43). Taurocholate is abundant in human bile and is hydrolyzed in the gut into secondary bile salts (eg. chenodeoxycholate), which are considered inhibitors of spore germination (5, 43). However, mice experiments have shown that antibiotic treatment leads to a higher proportion of primary bile salts than secondary bile salts in the gut promoting *C. difficile* spore germination and vegetative growth leading to CDI (43).

2.3.0. Pathogenesis

Following germination in the small intestine, vegetative cells travel to the colon where they multiply and colonize the gut's mucosa. However, in susceptible individuals, *C. difficile* is able to penetrate the mucus layer and adhere to the enterocytes (1, 4, 5). Susceptibility could be mediated by the presence of a protective microbiota, inactivation of germinant molecules by chenodeoxycholate or its analogs (45), production of molecules that inhibit *C.*

difficile (46), competitive exclusion from the microenvironment by non-toxigenic strains(47), and occlusion of host receptors used by *C. difficile* (5).

Toxin synthesis usually occurs in response to nutrient limitation, but stress and catabolite repression can also enhance toxin production (48). For most *C. difficile* ribotypes, when vegetative cells reach the stationary growth phase, they start the synthesis and secretion of toxins (4, 49). By contrast, ribotype 027 starts producing toxin in exponential and stationary growth phases (22). Intestinal damage from the toxin occurs only when there is direct contact of the vegetative cells with the enterocytes (4).

Toxigenic strains contain a pathogenicity locus (PaLoc) that carries the genes necessary for production and regulation of toxin A and B, both of which have been associated with CDI (1). Toxin A (TcdA) is an enterotoxin, while toxin B (TcdB) is a cytotoxin (1). Both alter the function of GTPases from the Ras superfamily, which leads to cell shrinking, disruption of cell-signaling pathways and cytoskeleton integrity, and eventually cell death (1, 4). The *tcdC* gene in the PaLoc encodes an anti-sigma factor that regulates TcdA and TcdB. Some *C. difficile* ribotype 027 strains have a missense mutation in the *tcdC* gene, which is believed to enhance the strain's toxin production (5).

Intoxication leads to fluid accumulation and increased vascular permeability (4). Toxins also stimulate the release of tumor necrosis factor and cytokines that induce an significant inflammation response (1). Tissue damage is characterized by epithelial cell loss and significant neutrophil infiltration that contribute to local and systemic manifestations of CDI (4).

Some *C. difficile* strains, including ribotype 027, also produce a binary toxin, also known as *C. difficile* transferase (CDT) (5, 34). CDT is found in

approximately 6-12.5% of *C. difficile* strains (1). CDT is an ADP-ribosylating toxin that affects the cell's cytoskeleton, leading to fluid loss, rounding of the cell, and cell death (1). CDT also forms long protrusions that improve adhesion of *C. difficile* vegetative cells to the epithelium (34). The presence of CDT is associated with a greater virulence and mortality (5).

2.4.0. Clinical Manifestations

2.4.1. Asymptomatic carriers

Approximately 5% of healthy adults and 30-70% of healthy infants carry *C. difficile* asymptomatically (3). Protection of adults with asymptomatic colonization may stem from the host's antibody response against toxin A (7). In the case of healthy infants, the protection may be related to the absence of toxin receptors or poorly developed cell-signaling pathways in the gut mucosa (50). Although there is no formal definition, at least one author suggests that asymptomatic colonization with *C. difficile* occurs when the bacteria is present in stool of an individual without CDI symptoms over a period of 7 days (51).

Asymptomatic carriage is assumed to be more likely among those in healthcare than community settings. An estimated 10% of hospitalized patients in acute-care facilities and up to 15% of patients in long-term care facilities asymptomatically carry *C. difficile* at admission (52, 53). Furthermore, up to 21% of inpatients may become colonized during their hospitalization, most of which

remain asymptomatic. This group is suspected to play an important role in ongoing healthcare transmission (3, 22).

2.4.2. *Clostridium difficile* infection (CDI)

The Infectious Diseases Society of America and Society for Healthcare of Epidemiology of America (IDSA/SHEA) defines CDI as the passage of three or more unformed stools in 24 hours combined with a positive stool sample for toxigenic *C. difficile*, or its toxins, and/or histopathological findings associated with pseudomembranous colitis (51). However, CDI can present with a broad spectrum of clinical manifestations ranging from mild diarrhea to more severe disease. Severe manifestations include toxic megacolon, pseudomembranous colitis, sepsis, and death (13). Up to 25% of antibiotic-associated diarrheal cases are attributed to *C. difficile* (12).

2.4.3. CDI severity

Although it seems biologically plausible, the question remains as to whether toxin levels are directly correlated with severity of symptoms and disease. In 2006, Akerlund et al. reported a positive correlation among 164 patients between diarrhea severity and fecal toxin levels detected by Toxin Enzyme Immunoassay (EIA) (54). Moreover, Planche et al. (2013) reported a significantly higher mortality among 435 patients with detectable fecal levels of toxin using Cell Cytotoxicity Assay (CCTA) versus patients only positive through

Cytotoxigenic Culture (CC) (n=207) or CDI negative patients (n=5880) (55). By contrast, Anikst et al. (2015) observed no significant difference in mean fecal toxin concentrations between patients with (n=59) or without (n=59) clinically significant diarrhea (three or more episodes in 24 hours) with toxin detected using a quantitative Toxin A and B ELISA and a qualitative toxin EIA (56). Similarly, Rao et al. (2015) recently reported that among 1144 CDI cases (37.2% EIA Toxin positive) detectable toxin was not predictive of CDI severity and mortality (57). Differences in observed results may be due to differences on tests' sensitivities, study power, CDI prevalence in the study population, and outcome definitions.

There is also limited evidence associating spore levels with CDI severity. Carlson et al. (2013) compared *in vitro* sporulation patterns from 106 *C. difficile* clinical isolates. Although the authors were unable to detect a significant association between spore levels and ribotype, they observed significantly higher sporulation among isolates associated with severe CDI (n= 34) versus those from non-severe cases (n= 72) (58). Furthermore, Deakin et al. demonstrated that *spo0A* gene is essential in CDI persistent disease, in addition to *C. difficile* transmission (59).

Other studies have focused on the relationship between *C. difficile* burden in the gut and CDI severity. If CDI severity is truly correlated with gut levels of toxin, there should also be an observable correlation between levels of *C. difficile* vegetative cells in the gut and disease severity, assuming all vegetative cells are producing toxins equally and simultaneously. To quantify the number of vegetative cells in the gut, several studies have used a Real-Time PCR (RT-PCR) assay that detects the gene of Toxin B (tcdB)(Cepheid Xpert). Baker et al. (2013)

observed that the PCR cycle threshold (CT) values from EIA Toxin positive patients (n=42) were usually strong (<30); however, EIA negative patients (n=87) tended to have a wide range of CT values (60). These results suggest that a high burden of *C. difficile* vegetative cells correlates with detectable levels of toxin in stool; however, the converse was not observed for EIA negative patients. Furthermore, Rao et al. (2015) reported no association between CT values and CDI severity and mortality among 718 PCR positive patients (57). Anisk et al. (2015) were also unable to find a significant difference in CT values between patients with or without significant diarrhea (p=0.25) (56). By contrast, Reigadas (2016) et al. recently reported that a low PCR CT value appeared to be strong predictor of poor CDI outcome, defined as recurrent CDI, treatment failure or advancement to severe complications. The authors proposed a cut-off of less than 23.5 toxin B CT value for prediction of poor outcome with a diagnostic accuracy of 88.1% (61).

Studies linking specific ribotypes to CDI severity have also presented conflicting evidence. Walk et al. (2012) performed a cross-sectional study comparing 310 CDI cases (11% of which were classified as severe cases). Although ribotype 027 and 078 were found to be good predictors of severity, this did not hold after adjusting for confounders (62). By contrast, Miller et al. (2010) reported a strong association between ribotype 027 and CDI severity. Among 1008 patients from the Canadian Nosocomial Infection Surveillance Program, 12.5% of all patients infected with a NAP1 strain (n=311) developed a severe outcome in comparison to only 5.9% of patients infected with another type (63). Similarly, Walker et al. (2013) reported that *C. difficile* genotype was a good predictor of CDI severity. This study observed that patients with ribotype 078

(Clade 5) showed a 25% (16 of 63) 14-day mortality, while ribotype 027 (Clade 2) was 20% (111 of 560), both significantly higher than clade 1 with only 12% (137 of 1168) (64). Rao et al. (2015) also reported an association between ribotype 027 and CDI severity/mortality even after adjusting for age (57). Similar results have been described when comparing ribotype 027 with other ribotypes (015 or 106) (65, 66). These differences in results could potentially be explained by differences in study populations, timing of CDI diagnosis, circulating strain types, and administered treatment (67).

2.5.0. Diagnosis

Presence of watery diarrhea, fever, leukocytes, loss of appetite, nausea, and abdominal pain and tenderness are the most common symptoms associated with CDI (68); however, as they are not specific to CDI, laboratory confirmation is required. To minimize risk of misdiagnosis due to asymptomatic carriage, accurate *C. difficile* laboratory diagnosis depends on detecting the presence or production of Toxin A and Toxin B by toxigenic strains (69). To further minimize potential for misdiagnosis, most laboratories only test soft or liquid stools for CDI (17).

IDSA/SHEA recommends a two or three-step algorithm for CDI diagnosis (69, 70). This algorithm uses glutamate dehydrogenase (GDH) detection as a screening test, followed by or/and in conjunction with a method detecting either toxigenic *C. difficile* and/or *C. difficile* toxins as a confirmatory test (17).

GDH is a cell wall antigen found in both toxigenic and non-toxigenic *C. difficile* strains. The GDH detection test cross-reacts with other intestinal microorganisms, affecting its specificity, but it has a high sensitivity (87-90%) and negative predictive value (above 99%), making it a good screening test in symptomatic populations (17, 69). If samples are positive for GDH, several confirmatory tests could follow; however, it is essential to understand the differences in the principle and target of each specific test, as they have the potential to impact clinical and infection control decisions.

SHEA/IDSA recommends the use of cytotoxigenic culture (CC) or cell cytotoxicity assay (CCTA) as confirmatory tests (70). CCTA is based on the neutralization of the toxins' cytopathic effect in cell culture in the presence of antitoxin antibodies (71). By contrast, CC relies on the culture of *C. difficile* spores in stool. After culture, detection of toxins either by CCTA or a toxin enzyme immunoassay (EIA) is necessary to rule out non-toxigenic strains (72). CCTA and CC are considered reference laboratory methods for CDI detection as they are very sensitive and specific; however, they are labor-intensive, and time-consuming with a slow turn around (69). In addition, they require special expertise and technology not always available in clinical settings.

An alternative is to use EIA to detect the presence of *C. difficile* toxins. Toxin A/B EIA has moderate sensitivity (25-86%) and specificity (75-100%) compared to reference methods. However, due to its moderate sensitivity, EIA Toxin tests are recommended only as part of a two or three step algorithm (71, 73). Other available confirmatory tests are nucleic acid amplification tests (NAAT), including real-time PCR, helicase-dependent amplification, and loop-mediated isothermal amplification, all of which have a greater sensitivity (73-

100%) than EIAs compared to CC, and shorter turnaround time in comparison to CCTA (17). Using PCR as the third or second step in the lab algorithm is recommended (17, 73).

At the University of Michigan Health System and the St. Joseph Mercy Hospital in Ann Arbor, a two-step-algorithm is usually used for clinical diagnosis, composed of a GDH/Toxin EIA as an initial test followed by PCR. This algorithm is estimated to have 100% (95%CI 78-100%) sensitivity and 97% specificity (95%CI 93-99%) (17).

Nonetheless, NAATs only detect *C. difficile* toxin genes (*tcdA, tcdB,* and/ or *tcdC*) and thus cannot identify the functionality or production of that toxin (69). Because of this, even though PCR improves both the specificity and sensitivity of the laboratory diagnosis, its introduction has raised several questions regarding its clinical applicability because PCR tests cannot distinguish between colonization and disease states (17, 74). Knowing that an estimated 10% of hospitalized patients are colonized with *C. difficile* (52, 53) and that most cases of nosocomial diarrhea are not associated with CDI (75), it is likely that some patients with diarrhea are simultaneously also *C. difficile* carriers and will be mistakenly diagnosed as CDI positive patients by PCR (76). When compared to CCTA, 50-80% more positives are detected using NAATs (71). In these cases, PCR may result in overdiagnosis and overtreatment (17, 76).

Some studies report no significant difference between clinical and laboratory characteristics between CD toxin positive or toxin negative/PCR positive patients(57, 77); however, among 53 toxin negative/PCR positive patients in comparison to 24 toxin positive patients, Baker et al. (2013) found a significantly lower 14 days all-cause mortality and a smaller proportion with

prolonged diarrhea (>5 days) (60). In this same study, 97% of toxin negative/PCR positive patients resolved their diarrhea within 14 days without receiving CDI treatment (60). Similarly, Polage et al. (2015) reported that toxin negative/PCR positive patients (n=162) had comparable outcomes with CDI negative patients (n= 1123) (78).

2.6.0. CDI Recurrence

The current recommended treatment for CDI is oral metronidazole or vancomycin; however, CDI recurs in 5-35% of patients following appropriate treatment (5, 58, 79). A recurrent case is defined as an episode of CDI that occurs 8 weeks or less after a previous CDI case has been resolved (19). First-time recurrences are more common among hospital-associated cases (21%) than among community-associated ones (14%) (9). In addition, approximately 12% of CDI patients have two recurrences, while 6% have more than two (67).

Clinically, it is not possible to determine if a recurrent case is a relapse of the same infection, a new infection with a different strain, or a new infection with the same strain (80). However, after strain fingerprinting using both serotyping and PCR-ribotyping, Barbut et al. (2000) reported that 45 out of 93 (48.4%) recurrences in his study population were due to a new infection, reporting 42 days as a mean of time between the two cases (81). By contrast, Figueroa et al. (2012) using restriction endonuclease analysis (REA) concluded that relapses were more common than reinfections in both early (1-14 days after treatment) and late (15-31 days) recurrences (80), where only 13.3% or 23.3% recurrences

respectively were due to new strains. Thus, relapses are considered to occur on average 14.5 days after the initial CDI case, while a reinfection occurs on average after 42.5 days (82).

Risk factors associated with CDI development -- antibiotic resistance, weakened immune response, and disturbed gut microbiota (5, 82, 83) -- are also associated with CDI recurrence. In addition, initial CDI infection with ribotype 027 is considered as a risk factor of recurrence (84). In North America, *C. difficile* strains from group REA B1, which includes ribotype 027 are responsible for 47% of CDI recurrences (80).

Tapered-pulsed vancomycin treatment is recommended for treating CDI recurrence, particularly following a second recurrence (85). This usually consists of a regimen of lower doses of vancomycin for 7-10 days, followed by several extra doses every 48 or 72 hours for up to 8 weeks (85). This regimen is expected to reduce antibiotic levels in the gut just enough for the remaining spores to germinate into vegetative cells, a state when vancomycin is expected to be effective. Furthermore, a tapered/pulsed regimen is assumed to provide a chance for the healthy gut microbiota to recover while *C. difficile* is suppressed (85-87). For further recurrences, other treatments have been suggested such as Fecal Microbiota Transplant, probiotics, immunotherapy, or newer drugs such as fidaxomicin (82).

2.7.0. Toxin-spore correlation

Finally, another important issue that may potentially shed some light into the pathogenicity and transmission patterns of *C. difficile* is the association between its toxin and spore production. Toxin and spore production requires an important amount of energy from the bacteria; therefore, it is biologically plausible that the bacteria trade offs one process for the other during its life cycle. Evidence of this trade-off (or its absence) would provide valuable insights to inform CDI management, treatment, and control and prevention for both symptomatic and asymptomatic carriers.

Positive and negative correlations have been reported between spore and toxin production. Underwood, et al. (2009) demonstrated that inactivation of a known spore regulator, Spo0A, negatively affected both spore and toxin production in the *C. difficile* 630 strain, but this result was not confirmed by Mackin et al. (2013) using the same strain (88, 89). However, Mackin et al. (2013) also showed that SpoA negatively regulates toxin A and B levels in ribotype 027 (89). Akerlund et al. (2006) compared toxin yield and spore levels among 13 clinical isolates *in vitro* and observed an inverse correlation (54). By contrast, spores and toxins were produced simultaneously 24 hours after infection in a mouse model (N=30), suggesting a positive correlation between fecal toxin and spores levels (41).

2.8.0. Public Health Significance

If a correlation between spores and toxins is identified *in vivo,* toxin levels, which may be measured using current laboratory techniques, could be used as "a proxy" of fecal spore levels. Doing so would allow us to identify patients who are likely to shed a large number of spores during their hospital stay and who should be given further attention from an infection control perspective. Similarly, the correlation would provide further support to a re-analysis of the need to identify *C.difficile* carriers in healthcare settings and determine whether they should be under isolation precautions while hospitalized.

Moreover, identifying a positive *in vivo* correlation between spores and toxin levels will provide further evidence to determine the clinical applicability of molecular tests for CDI diagnosis, treatment, and infection control. It is essential to understand the role played by both Toxin positive and Toxin negative PCR positive individuals in CDI transmission, in order to improve CDI control and prevention guidelines not only in acute-care settings but also in outpatient facilities and community settings.

Finally, increasing our knowledge about the impact of host factors on spore and toxin levels will provide us with more clear guidelines to tailor infection control practices toward reducing the spread of *C.difficile* within healthcare settings. Particularly, improved understanding of the effect of *Candida* spp. on *C.difficile* spore and toxin levels will inform the treatment and management of both CDI and Candidiasis patients to avoid further medical complications.

2.9.0. References

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CHAPTER III

Clostridium difficile shows no trade-off between toxin and spore production within its human host

3.0.0. Abstract

Clostridium difficile is the most commonly reported microorganism associated with nosocomial infections in the US Healthcare system. Toxin and spore levels are associated with severity of *C. difficile* infection (CDI) suggesting that toxin and spore production may be positively correlated. While both positive and negative correlations have been reported in the literature, no studies have described the correlation within an infected human host. Our study aimed to fill this gap by directly measuring *C. difficile* spore and toxin levels in CDI positive stool samples. We also assessed whether overgrowth of *Candida albicans* modified this association, as *C. albicans* has been suggested as both a risk and a protective factor for *C. difficile*. We measured toxin, spore, and *C. albicans* levels among 200 successively collected stool samples testing positive for CDI, and PCR ribotyped the recovered *C. difficile* isolates. Growth of ≥5 logs of *C. albicans* CFU/g of stool was defined as overgrowth. *C. difficile* toxin and spore levels were positively associated ($p<0.001$); this association did not vary by C. *albicans* overgrowth. However, ribotypes 027/078 were significantly associated with high levels of toxin and of spores and *C. albicans* overgrowth. Although on average spore levels

were higher in toxin positive samples than in toxin negative/PCR positive samples, spores were found in almost all toxin negative samples. The ubiquity of spore production emphasizes the importance of strictly following already establish CDI control and prevention measures in healthcare settings. Furthermore, our study highlights the importance of considering the asymptomatic state when implementing CDI diagnosis, treatment, and control and prevention measures.

3.1.0. Introduction

Clostridium difficile infection (CDI) is one of the most important healthcare-associated infections (HAIs). In the United States (US), CDI accounts for 12% of HAIs, leading to almost 500,000 infections and approximately 29,000 deaths per year (1-3). The associated costs are significant: CDI costs an estimated 4.8 billion dollars per year among US acute care facilities (4).

CDI is defined by the presence of diarrhea and other symptoms, and one of the following: a stool test positive for *C. difficile* toxins or *C. difficile* toxin genes or the presence of pseudomembranous colitis (5). Symptomatic CDI is attributed to toxin production. Therefore, laboratory diagnosis may follow a multi-step algorithm that includes an initial test for a *C. difficile* highly conserved cell wall-associated enzyme, glutamate dehydrogenase (GDH), followed by a test for toxin (6). If negative for detectable toxin but positive for GDH, the sample is tested for the presence of toxin genes using a PCR assay (6). This multi-step algorithm assumes that the presence of toxin genes is indicative of disease – even in the

absence of detectable toxin. However, diarrheal symptoms can be caused by many different pathogens, and *C. difficile* is found among an estimated 5% of the general population (7) and 8% of the hospitalized population without symptoms (8).

In addition, there are many different *C. difficile* ribotypes. Hypervirulent ribotypes reportedly produce higher levels of toxin and spores. Warny et al. (2005) reported ribotype 027 (Toxinotype III) (n=15) produced an average of 16 to 23 times more toxin *in vitro* than 25 *C. difficile* isolates from Toxinotype 0 (9). By contrast, Merrigan et al. (2010) reported 4 hypervirulent strains (ribotype 027) did not produce significantly greater amounts of toxins than 4 non-hypervirulent strains (10). However, this same study observed a significant difference in spore production among the hypervirulent strains (10).

Severity of CDI symptoms is expected to increase with toxin levels (11), as CDI is considered a toxin-mediated intestinal disease. Whether symptom severity increases with spore levels is less certain. Carlson et al. (2013) reported that cultured isolates from CDI severe cases (n=34) produced more spores after 24 hours than isolates from non-severe CDI cases (n=72) (12). Spores germinate into vegetative cells, so if there are more spores there are probably more vegetative cells producing toxin -- resulting in more severe disease (12). For the latter to be true, toxin and spore production must be directly correlated.

Underwood et al. (2009) demonstrated that inactivation of the spore regulator, Spo0A, negatively affected both spore and toxin production in the *C. difficile* 630 strain, but this result was not confirmed by Mackin et al. (2013) using the same strain but different methods (13, 14). However, in the same study Mackin and colleagues showed that SpoA negatively regulates toxin A and

B levels in ribotype 027 (14). Akerlund et al. (2006) compared toxin yield and spore levels among 13 clinical isolates *in vitro* and observed an inverse correlation (11). By contrast, spores and toxins were produced simultaneously 24 hours after infection in a mice model (N=30), suggesting a positive correlation between fecal toxin and spores levels (15). Thus, the correlation between toxin and spore levels in humans remains to be confirmed. The magnitude and direction of this correlation may have significant implications for infection prevention and control.

Although toxin production is essential for pathogenicity, it is not sufficient. Toxin can be detected in the absence of symptoms, suggesting that other factors modify the effect of the toxin on the host (16). One factor we hypothesize to potentially mediate the effect of toxin is the presence of *Candida* spp. in the gut microbiota. Estimated prevalence of *Candida* spp. in the gut ranges from 16% to 83% among CDI patients (17-19) and 23-76% among healthy individuals (20) (but only 10% specifically for *C. albicans* (21)). Although some studies have reported a positive or a negative association between CDI and *Candida* spp. (18, 19), whether if the presence of *Candida* spp. is associated with *C. difficile* spore and toxin production has not been investigated.

Our study aimed to fill gaps in our understanding of the *in vivo* correlation of *C. difficile* spore and toxin levels and their association with the overgrowth of *C. albicans* by directly testing clinical samples.

3.2.0 Methods

3.2.1. Sample collection

We collected 200 successive CDI positive stool samples from the microbiology laboratory at Saint Joseph Mercy Hospital (SJMH) in Ann Arbor, Michigan (MI)- a reference laboratory for several hospitals, outpatient healthcare centers, and urgent care facilities within the Saint Joseph Mercy Health System. The Institutional Review Boards at SJMH and the University of Michigan (IRB Health Sciences and Behavioral Sciences) approved our study protocol.

We included all samples submitted for CDI testing between February 1st and July 20th, 2015 that were determined to be positive using the SJMH laboratory algorithm and had sufficient volume left for our testing protocol. The SJMH laboratory algorithm includes an initial GDH/Toxins AB screening using C.DIFF QUIK CHEK COMPLETE® (Alere, Waltham, MA./Techlab, Blacksburg, VA.). In case of indeterminate results, samples are tested using a *tcdB* PCR assay (Cepheid Gene Xpert®, Sunnyvale, CA.) for presence of the toxin genes (Figure 3.1). We recorded the age, gender, and location code (inpatient vs. outpatient) of the individual providing the sample, and qualitatively assessed the physical consistency of the study stool samples.

Samples were classified as 1) formed/semi-formed, 2) soft and unformed, 3) liquid, and 4) mucoid. As diarrheic samples were the focus of our study, we considered all hard or soft samples that retained their shape in their collection cup to be formed or semi-formed. Stools that were loose and took the shape of

the collection cup were identified as soft and unformed, while samples that could be pipetted and resembled the consistency of water were defined as liquid. Soft or liquid samples that contained significant amount of mucous were considered mucoid (Modified from (22)).

3.2.2. Determination of toxin levels

Following determination of positivity by the SJMH laboratory, we measured *C. difficile* toxin AB levels using the C. DIFFICILE TOX A/B II™ Immunoassay (Techlab, Blacksburg, VA). We quantitated the color intensity of the reaction by measuring the optical density at 450 nm using an ELISA plate reader (PerkinElmer, 2030 Multilabel Reader, Waltham, MA.) according to the kit's manufacturer instructions (23). Due to the known variability of this assay, we normalized each sample value by subtracting its corresponding negative control and dividing this value by its positive control, also corrected by subtracting its negative control value. Therefore, we report the ratio of toxin levels in each sample relative to that in the positive control. In addition, due to the high intra-assay coefficient of variation (10% across fecal specimens (23)), we categorized our toxin levels based on quantiles.

3.2.3. Determination of spore levels

Following a heat treatment (65^{o}C for 20 minutes), spore levels were quantified by culturing serial dilutions of the fecal specimen on Cycloserine

Cefoxitin Fructose Agar with Horse Blood and Taurocholate (Anaerobe Systems, Morgan Hill, CA.) for 24 hours at 37°C under anaerobic conditions. Spore levels were reported as colony forming units (CFU) per gram of feces.

3.2.4. Detection of *Candida albicans*

DNA was extracted in duplicate from 200-250uL of stool using the PowerMag® Soil Isolation Kit (Mo Bio, Carlsbad, CA) optimized for epMotion® (Mo Bio, Carlsbad, CA). *C. albicans* levels were quantified in triplicate for each extraction using RT-PCR (BioRad, CFX96 Realtime System, Hercules, CA) with conditions and primers previously described (24). Because stool is not a microbiologically and chemically homogeneous sample, we analyzed 6 replicates of each sample: if at least 2 out of the 6 replicates were positive the sample was considered positive and an average level of *C. albicans* was calculated from the detected estimates. *C. albicans* overgrowth was defined as the growth of ≥5 logs of CFU/g of stool as described by Raponi et al. (2014) (25).

3.2.5. Ribotyping of *C.difficile* isolates

A fluorescent PCR ribotyping technique using capillary gel electrophoresis was performed to ribotype the *C. difficile* isolates recovered from our study samples as previously described (26).

3.2.6. Determination of dry weight of samples

Samples from severe cases are expected to be more diarrheic, which may dilute spore and toxin levels resulting in an underestimation of toxin and spore production especially in more severe cases. To determine if this bias occurred, we desiccated the 159 samples with sufficient material remaining in a vacuum concentrator (eppendorf Vacufuge™ AG 22331, Hamburg, Germany) at 60^{o}C and 1400 revolutions per minute for 3 hours. The wet gram weight of each stool sample used for toxin and spore quantification was adjusted to quantity per dry gram weight. For the remaining 41 specimens without sufficient sample, we imputed water content, dry spore, and dry toxin levels, using SAS multiple imputation. Water content results also were used to validate our qualitative classification of stool consistency.

3.2.7. Statistical analysis

To test if the distribution of spores was significantly different across toxin categories for both wet and dry samples we used ANOVA, linear regression, and Test of trend. Kruskal Wallis test or Mantel-Haenszel was used to compare toxin levels between groups of different age, gender, location, stool consistency, *C. difficile* ribotype and *Candida* growth. T-test or ANOVA was used to compare log-transformed spore levels between the different groups including laboratory diagnosis at SJMH (toxin positive or toxin negative/PCR positive) and water content. Finally, univariate and multivariate ordered logistic regression and linear regression were used to estimate the measures of association linked to

toxin or spore (log-transformed) levels with/without adjustments for confounders. SAS 9.4 (Cary, NC.) and STATA 14 (College Station, TX.) were used to perform all analysis and graphics.

3.3.0 Results

Of the 200 stool samples, 90 were from inpatients and 110 from outpatients (including ER patients). Most samples were from females (60.5%) and half (50.5%) from individuals aged 65 or older. Stool sample consistencies were primarily soft (40.5%), liquid (32.0%), mucoid (in addition to liquid or soft) (19.5%), or semi-formed/formed (8.0%).

There were a total of 37 different ribotypes among our collected isolates (Table 3.1, Appendix 3.1). The most common ribotypes were 027 (19%) and 014-020 (16%). Ribotype 078-126 occurred in 5 samples; this and ribotype 027 are considered hypervirulent (27, 28). Therefore, they were combined for further analysis. The median toxin level after normalization was 0.01 (range -0.06 to 3.94), but this varied by ribotype (Table 3.1). The median number of spores detected was 1.6×10^4 CFU/g (range 0 to 5.5×10^6), and also varied by ribotype (Table 3.1).

There was a significantly different distribution of log-transformed spore levels by toxin category ($p<0.001$) (Figure 3.2). The ANOVA results describing differences between spore levels by toxin categories did not change after correcting for dry weights ($p<0.001$). Similarly, estimates obtained from a linear regression that modeled log-transformed spores as the outcome and either wet

toxin levels or those corrected for dry weight as the independent variable were not statistically significantly different (not shown).

We observed a strong positive association between toxin and spore production that remained after controlling for age, gender, location, stool consistency, and ribotype (Linear regression, $p<0.001$). Moreover, a strong positive trend was observed between toxin and spores levels (Test of trend, $p<0.001$). When we stratified by ribotype, the strong positive trend remained. However, when the analysis was restricted to 014-020 isolates, the observed increase in spore production with toxin level was not statistically significant (Test of trend, $p=0.10$) (Figure 3.2).

Toxin negative/PCR positive stool samples had significantly fewer spores *in vivo* than toxin positive samples (T test, $p<0.001$) (Figure 3.3). When stratified by ribotype, a significant difference in spore level was still observed by diagnostic test for ribotype 027/78 and 014-020 (Figure 3.4).

Toxin levels did not significantly differ by age ($p=0.51$) or gender ($p=0.89$). Outpatients were 1.70 (95%CI 1.00-2.90) times more likely than inpatients to have higher toxin levels; however, after adjusting for age, gender, stool consistency, and ribotype in a multivariate ordered logistic regression the association was no longer statistically significant (OR=1.61 (95%CI 0.88-2.93)). Stool samples infected with ribotypes 027 or 078 compared to other ribotypes (excluding 014-020) were 2.19 (95% CI 0.99-4.84) times more likely to have high levels of toxin. This association remained strong after adjusting by age, gender, location, and stool consistency (OR=2.24 (95% CI 0.99-5.05)). By contrast, those with ribotype 014-020 compared to other ribotypes (excluding 027 and 078) had lower toxin levels even after controlling by host variables (OR=0.11 (95%CI 0.01-

0.84). Mucoid samples were 3.03 (95% CI 1.47-6.20) times more likely to have higher toxin levels than soft samples (Appendix 3.2). This association became stronger after controlling for gender, age, patient location, and ribotype (OR=3.9 (95% CI 1.69-8.97).

Spore production did not differ by gender (p=0.38) or patient location (p=0.64). However, patients 65 or older had higher average levels of spores than younger patients (p=0.07), but this appears to be attributed to differences in ribotype rather than location, stool consistency, or gender. Mucoid samples also tended to have higher numbers of spores than samples with a soft consistency (p=0.07); this association remained after adjusting for age, gender, location, and ribotype (p=0.05) (Appendix 3.3). Only ribotype 014-020 was significantly associated with spore levels when compared to other ribotypes (excluding 027/078), and the association was negative (p=0.008) and remained after adjustment for age, gender, location, and stool consistency (0.19 unit change of log-transformed spores from ribotype 014-020 to other ribotypes, p=0.008).

Liquid stool was more common among inpatients, while soft stool was more common among outpatients (p=0.02). Although water content was significantly associated with stool consistency, (which validates our qualitative assessment (p<0.001, Appendix 3.4)), there was no statistically significant difference between water content and patient location (p=0.64). Stool consistency was distributed differently by diagnostic test (p=0.04): toxin positive cases were more likely to have mucoid stool than PCR positive patients (OR=5.21(95%CI=1.82-14.94)) after adjustment for host variables and ribotype. Furthermore, stool samples infected with ribotypes 027/078 were 4.55 (OR

95%CI 1.59-13.04) times more likely to be mucoid than soft after controlling for age, gender, and location.

C. albicans could be quantified for 166 samples (83%) and was detected in 18% of samples. No significant differences by gender, age, location, or stool consistency were identified between these samples and those that could not be quantified (due to lack of sample). We did not identify any significant difference in *C. albicans* levels by age (p=0.80), gender (p=0.40), stool consistency (p=0.80), or spore levels (p=0.63). We observed a slight difference by toxin levels (p=0.10); however no linear trend was observed (p=0.76) between both variables. However, outpatients have significantly higher average levels of *C. albicans* than inpatients (p=0.01). This relationship remained after controlling for age, gender, stool consistency and *C. difficile* ribotype in a multivariate linear regression model. An increase of 3.97 CFU log of *C. albicans* was observed between inpatient and outpatient category (p=0.05).

High levels ("overgrowth", defined as 5 or more logs of *C. albicans*) were detected in 13% (n=22). *C. albicans* overgrowth did not vary by age (p=0.61) or stool consistency (p=0.33). Although outpatients were 2.4 times more likely to have *C. albicans* overgrowth than inpatients (95%CI 1.0-5.8), this association was explained by *C. difficile* ribotype. There were no significant differences between the distributions of toxin (p=0.12) or spore levels (p=0.21) between patients with *C. albicans* overgrowth compared to those without it. The association between spore and toxin levels was not affected by *C. albicans* overgrowth, and was still significant among the subset of samples with *C. albicans* overgrowth (ANOVA, p<0.001) and among the subset without it (ANOVA p=0.04). However, stool samples with ribotypes 027/078 were 3.46 (OR 95% CI 1.18-10.17) times

more likely than other ribotypes (excluding 014-020) to have *C. albicans* overgrowth, and this association remained after adjustment by host variables (OR 4.32 (95%CI 1.38-13.45).

3.4.0. Discussion

We observed a strong, positive trend between *in vivo* levels of *C. difficile* toxin and spore production among 200 *C. difficile* positive stool samples from in- and outpatients. This trend remained even after stratifying by ribotype. Toxin and spore levels were not associated with *C. albicans* overgrowth, although samples with ribotypes 027/078 were more likely to have overgrowth. Contrary to a previous *in vitro* finding (11), our results do not support a trade-off between spore and toxin production within the human CDI host. This suggests that spore production may enhance colonization as well as transmission. While results of a mouse model previously suggested a positive association between *C. difficile* toxin and spore levels (15), we found no similar reports in humans.

Surprisingly, we observed a significant overlap in the distribution of spore levels among toxin positive and toxin negative/PCR positive samples (Figure 3.3), which persisted after stratifying by ribotype (Figure 3.4 and Figure 3.5). This confirms previous reports of inter-strain variation of sporulation and germination patterns across ribotypes (29-31). This observed overlap raises the question of whether the same cell population produces both toxin and spores, or if the cell population is separated into toxin and spore producers. Saujet et al. (2011) described an alternative sigma factor SigH in *C. difficile* that has an

inverse correlation with toxin production but a direct one with spore regulation (32). This supports the hypothesis of separate populations (33). However, in a related species, *Clostridium perfringes*, spores and enterotoxin (CPE) are released simultaneously (34). Further studies are required to better elucidate how spore and toxin production might be linked in *C. difficile* and to identify bacterial and host signals regulating their productions *in vivo*.

The strong toxin-spore association suggests a reexamination of the interpretation of the PCR test. Approximately 10% of hospitalized patients are colonized with *C. difficile*; thus it is likely that some patients with diarrhea of a different etiology also carry *C. difficile* (8, 35, 36). In these cases, lab algorithms that include PCR testing may result in overdiagnosis and overtreatment (6, 35). The increased sensitivity of detection and corresponding increase in treatment may not improve prognosis. Polage et al. (2015) reported that 162 toxin negative/PCR positive patients (among whom only 41% received CDI treatment) had outcomes comparable to those CDI negative by either test (36). Similarly, Baker et al. (2013) reported that the symptoms of 97% of toxin negative/PCR positive patients (n=53) resolved after 14 days without CDI treatment (37).

In order to reduce overdiagnosis, the Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America (SHEA/IDSA) guidelines recommend against CDI testing of formed stools or test of cure (5), yet our sample contained 8% (n=16) formed/semi-formed stool samples. Although a small number of non-diarrheic samples may be specifically requested to be tested by the physician, this higher than expected percent of formed/semi-formed stools in our study population suggests there may be underreporting--particularly of semi-formed stools (soft but still formed)-- in

clinical settings. Interestingly, we found clinically relevant levels of toxin and spore production in these samples. These findings underscore the importance of considering the full clinical picture before testing for *C. difficile,* as once it is found it can be difficult to ignore. Notably, antibiotic therapy is a primary risk factor for CDI and treating asymptomatic carriage may lead to CDI and transmission to others (38, 39). However, our results also highlight that patients with no or milder symptoms (formed/semi-formed stools) still may merit contact precautions as they may be shedding spores at significant levels.

Our results confirm the hypervirulence previously described for ribotypes 027 and 078 (11, 27, 28). These ribotypes were strongly associated with toxin positive cases and higher levels of fecal toxin and spores even after controlling for age, gender, location, and stool consistency. However, our study was unable to link these ribotypes to CDI severity, a point still under debate (26, 40).

The prevalence of *C. albicans* in our collection (18%) is similar to previous reports among CDI patients. In a double-blinded, randomized, phase III clinical trial in multiple hospitals in the US and Canada, Nerandzic et al. (2012) reported 16% *Candida* spp. positivity among 301 CDI patients before initiation of treatment (17). *Manian* et al. (2013) reported 17% *Candida* spp. overgrowth among 60 American CDI cases (18). In addition, only one previous study found a positive association between *C. albicans* and CDI (25), however, Manian and colleagues (18) also reported the contrary association. In this study, we did not observe an association between *C. albicans* overgrowth and *C. difficile* toxin and spore fecal levels. As far as we know, this specific association has not been described elsewhere. Nerandzic et al. (2012) (17) described that vancomycin treatment can favor *Candida* spp. acquisition; however, our samples were

collected based on the hospital protocol before CDI treatment was given for the current CDI episode (although we did not take into account whether this was a first or recurrent episode). Thus, future research should investigate if *Candida* spp. affects *in vivo* spore-toxin correlation during or after CDI treatment, when overgrowth of this yeast may be more prevalent. Similar to Raponi et al. (2014), we found a high prevalence of *C. albicans* overgrowth among cases infected with ribotype 027. This association merits further research using longitudinal studies.

Our study was somewhat limited by the moderate sensitivity of our toxin test (95% CI 87-95 according to the manufacturer (23)). We chose this test because our purpose was to identify the total toxin A and B produced in the stool, and other, more sensitive tests such as a cytotoxicity assay, only detect active toxin. However, our sample size was large, and the association between spore and toxin production was strong – even after controlling for confounders. Further, this test assured that if we were able to identify a toxin-spore correlation, the results could be applied to infection control practices in healthcare settings, as it can easily be incorporated in clinical laboratories as a proxy of spore levels.

In conclusion, *in vivo* toxin and spore production were positively and significantly correlated among clinical CDI samples, even after stratifying by ribotype. Therefore – as is already standard –contact precautions are in order for all individuals with CDI. Nearly all of our patients with no detectable toxin production shed spores, sometimes at significant levels. This highlights the complexity of diagnosis and prevention of a condition with an asymptomatic state and underscores the need to identify alternative therapies and control

measures that better address not only the health of an infected individual but the risk to others.

Table 3.1. Distribution of toxin and spore levels by ribotype in order of prevalence across 200 consecutive CDI positive stool samples collected from the Microbiology Reference Laboratory of the Saint Joseph Mercy Health System, Ann Arbor, MI., from February 1st to July 20th, 2015.

Ribotype	n (%)	Normalized toxin levels Median (Range)	Spore levels (CFU/g) Median (Range)
027	31(19.0)	0.17(-0.06-3.94)	$3.8x10^4$($2.0x10^1$ -$5.5x10^6$)
014-020	26(16.0)	0.00(-0.01-0.51)	$3.0x10^3$($6.0x10^1$-$4.0x10^5$)
002	9(5.5)	0.01 (-0.05-0.90)	$4.0x10^4$ ($2.0x10^1$–$9.4x10^5$)
053-163	8(4.9)	0.02(-0.00-2.33)	$6.2x10^4$($3.5x10^2$-$2.2x10^6$)
078-126	5(3.1)	0.32(-0.02-0.96)	$7.5x10^4$($7.5x10^1$-$1.3x10^5$)
015	5(3.1)	0.00(-0.01-0.06)	$2.6x10^4$ ($1.5x10^2$-$1.5x10^4$)
012	2(1.2)	0.01(0.00-0.01)	$1.8x10^5$($3.8x10^4$-$3.8x10^5$)
001	1(0.6)	0.01(-)	1.5x104(-)
003	1(0.6)	1.48(-)	$8.1x10^5$(-)
017	1(0.6)	0.00(-)	$3.5x10^3$(-)
Others*	74(45.4)	0.01(-0.06-1.96)	$3.3x10^4$ ($5.0x10^0$-$2.3x10^6$)
No ribotype data**	37(18.5)	0.00(-0.04-1.96)	$3.5x10^3$(0-$1.4x10^6$)

* The category "Others" includes all isolates that could not be matched to a reference isolate. More detail on this category can be found in the appendix (Table S1).
** The category referred as "No ribotype data" includes stool samples from which *C.difficile* could not be cultured and isolates that could not be ribotyped.

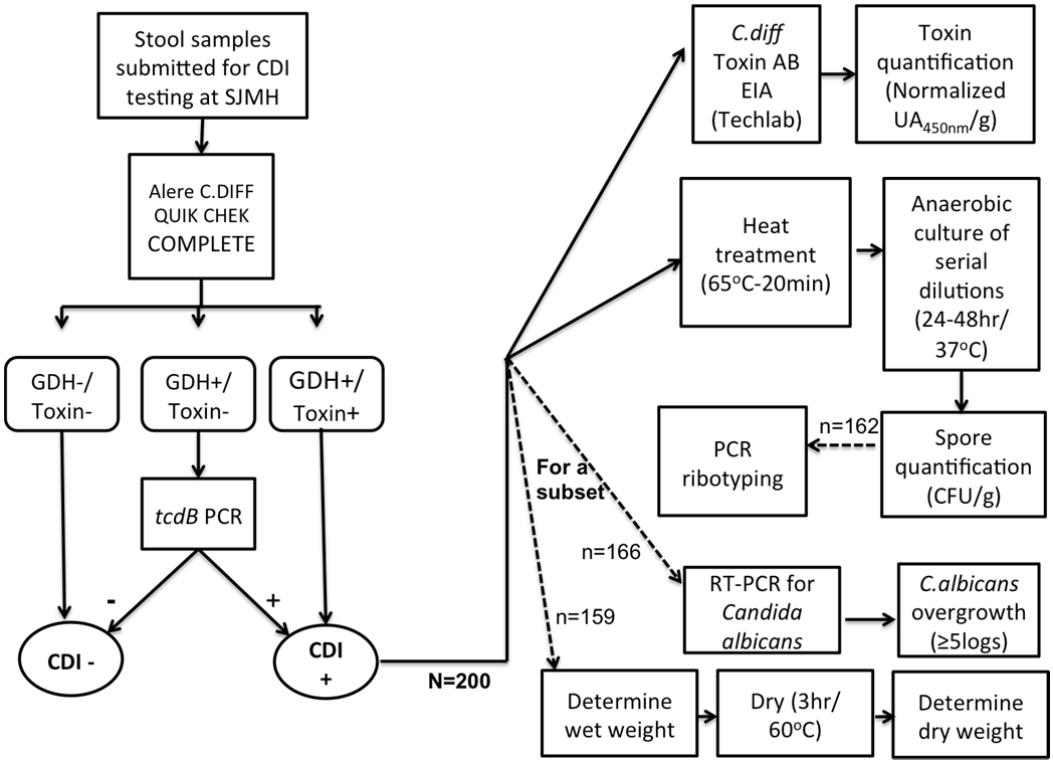
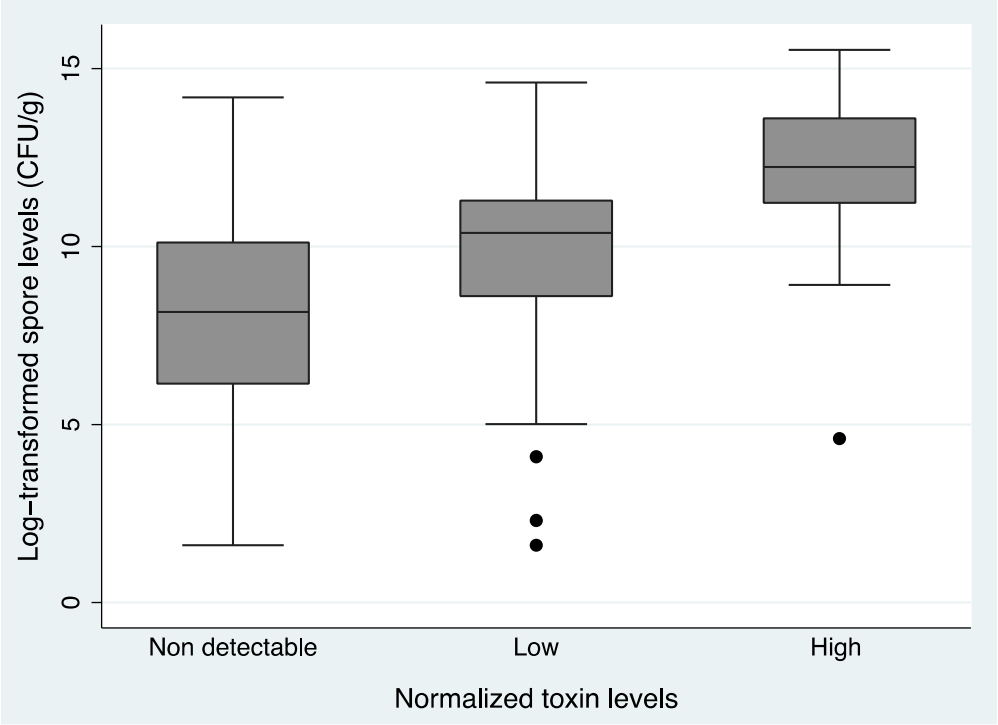


Figure 3.1. Laboratory algorithm used throughout this study.

A.


Log–transformed spore levels (CFU/g)
0
5
10
15
Non detectable
Low
High
Normalized toxin levels

B.

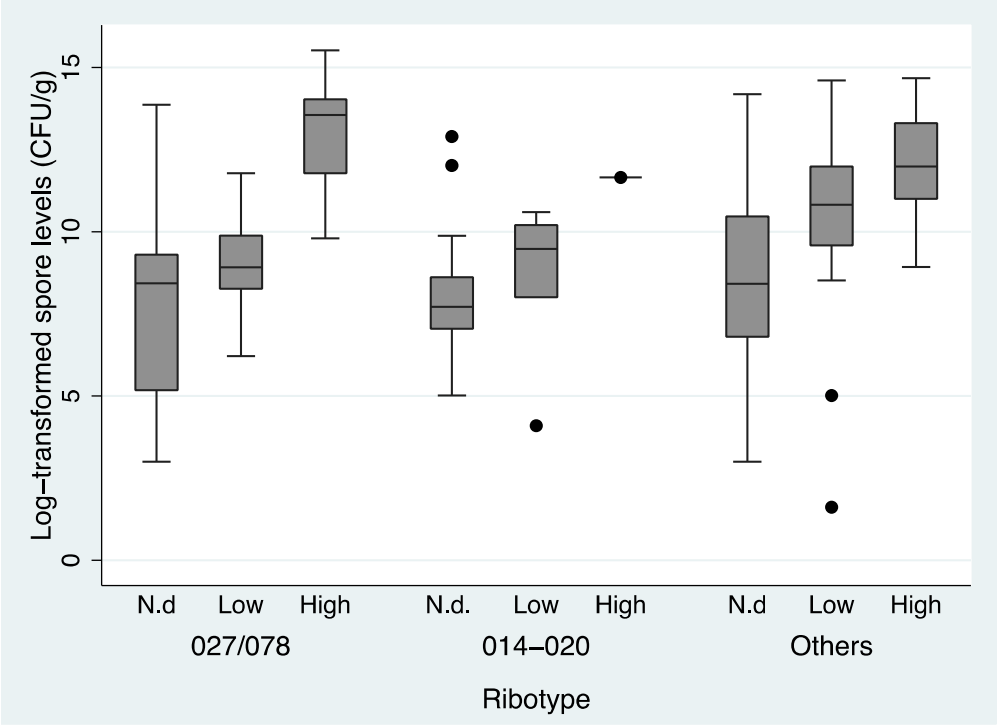

Log–transformed spore levels (CFU/g)
0
5
10
15
N.d
Low
High
N.d.
Low
High
N.d
Low
High
027/078
014–020
Others
Ribotype

Figure 3.2. Distribution of spore levels by toxin levels and ribotype across 200 consecutive CDI positive stool samples collected from the Microbiology Reference Laboratory of the Saint Joseph Mercy Health System, Ann Arbor, MI., from February 1st to July 20th, 2015. Panel A shows distribution of spore levels by categories of increasing toxin levels: Non-detectable levels (≤0.007 UA/mL), low levels (0.008-0.367) and high levels (0.368-3.941) (Test of trend, $p<0.001$). Panel B shows distribution of spore levels across categories of increasing toxin levels and by ribotype: 027/048 (Test of trend, $p<0.001$), 014-020 (Test of trend, $p=0.10$), and other ribotypes (Test of trend, $p<0.001$).

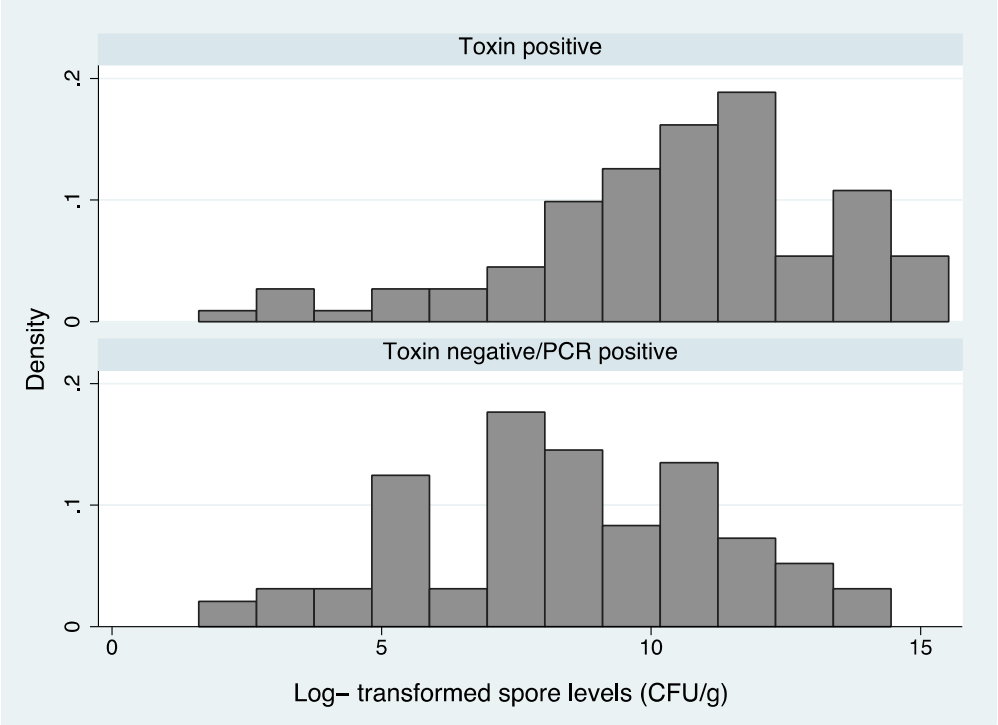
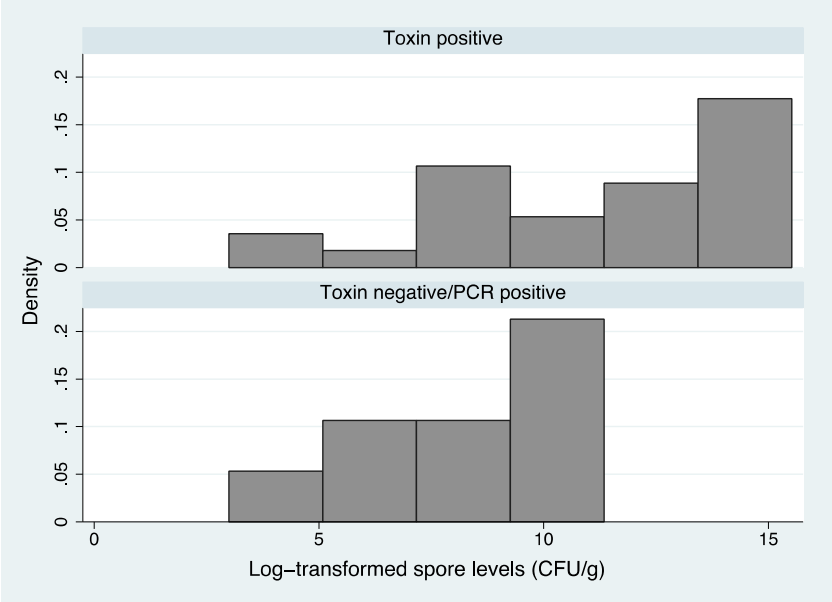
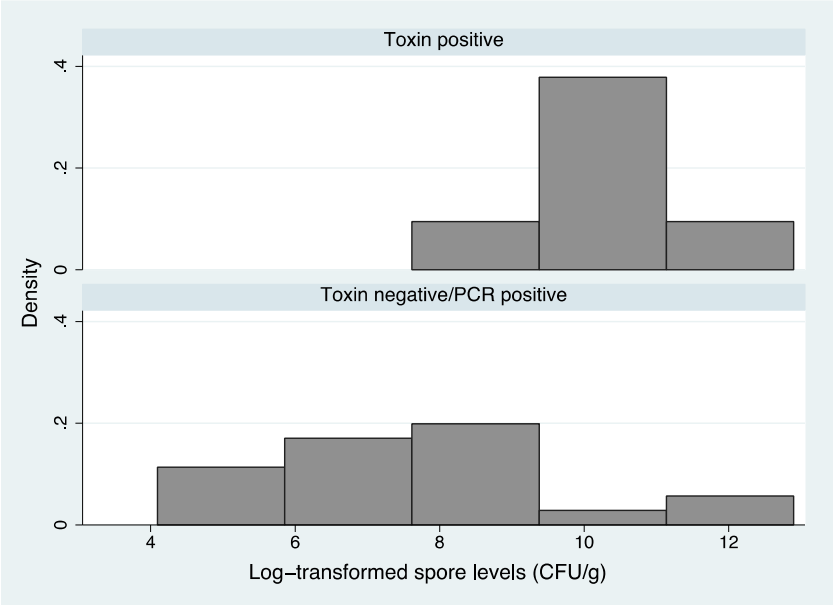


Figure 3.3. Difference in the distribution of spore levels across 200 consecutive CDI positive stool samples by CDI laboratory diagnosis (toxin Positive (N=109)) and toxin negative/PCR positive (N= 91)), collected from the Microbiology Reference Laboratory of the Saint Joseph Mercy Health System, Ann Arbor, MI., from February 1st to July 20th, 2015 (t-test, $p<0.001$).

A.


Toxin positive
Toxin negative/PCR positive
Density
0
.05
.1
.15
.2
0
5
10
15
Log–transformed spore levels (CFU/g)

B.


Toxin positive
Toxin negative/PCR positive
Density
0
.2
.4
4
6
8
10
12
Log–transformed spore levels (CFU/g)

C.

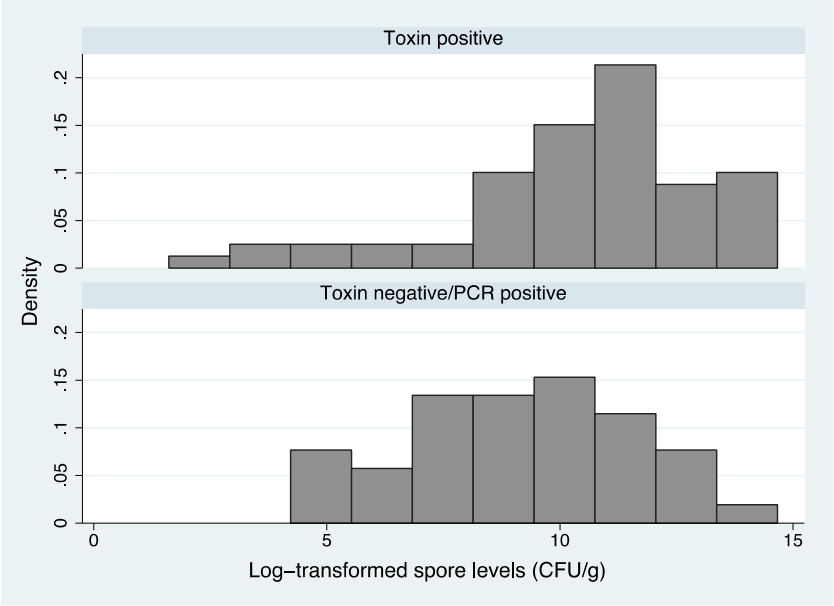


Figure 3.4. Difference in the distribution of spore levels by CDI laboratory diagnosis and ribotype across 200 consecutive CDI positive stool samples, collected from the Microbiology Reference Laboratory of the Saint Joseph Mercy Health System, Ann Arbor, MI., collected from February 1st to July 20th, 2015. Panel A includes ribotypes 027/078 (t-test, p=0.04), panel B includes ribotype 014-020 (t-test, p=0.01), and panel C includes all other ribotypes (t-test, p=0.35).

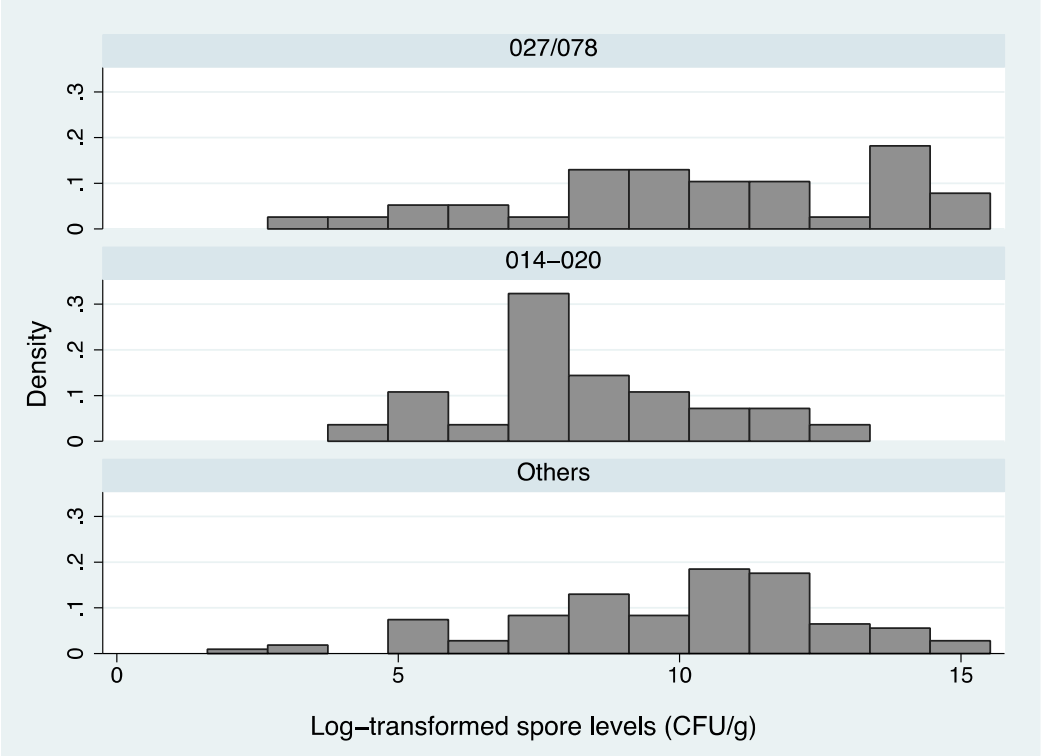


Figure 3.5. Distribution of spores by ribotype across 200 consecutive CDI positive stool samples collected from the Microbiology Reference Laboratory of the Saint Joseph Mercy Health System, Ann Arbor, MI., from February 1st to July 20th, 2015. Top panel includes ribotypes 027/078 (n=36), the middle panel includes ribotype 014-020 (n=26), and the bottom panel includes the rest of the ribotypes in our population (n=101) (ANOVA, p=0.002)

3.5.0. References

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Appendix 3.1. Distribution of toxin and spore levels by ribotype across 200 consecutive CDI positive stool samples collected from the Microbiology Reference Laboratory of the Saint Joseph Mercy Health System, Ann Arbor, MI., from February 1st to July 20th, 2015.

Ribotype	N	Normalized toxin levels Median (Range)	Spore levels (CFU/g) Median (Range)
027	31	0.17(-0.06-3.94)	$6.2x10^4$($3.5x10^2$-$2.2x10^6$)
014-020	26	0.00(-0.01-0.51)	$3.0x10^3$($6.0x10^1$-$4.0x10^5$)
002	9	0.01 (-0.05-0.90)	$4.0x10^4$ ($2.0x10^1$–$9.4x10^5$)
SJ485	9	0.10(-0.00-0.83)	$1.9x10^4$($9.9x10^2$-$4.2x10^5$)
053-163	8	0.02(-0.00-2.33)	$6.2x10^4$($3.5x10^2$-$2.2x10^6$)
SJ420	7	0.28(-0.00-1.35)	$1.3x10^5$($7.5x10^2$-$1.4x10^6$)
SJ308	6	0.00(-0.00-0.29)	$6.8x10^4$($7.5x10^1$-$2.2x10^6$)
SJ467	6	1.16(-0.00-1.58)	$3.3x10^5$($1.4x10^4$-$2.3x10^6$)
078-126	5	0.32(-0.02-0.96)	$7.5x10^4$($7.5x10^1$-$1.3x10^5$)
015	5	0.00(-0.01-0.06)	$2.6x10^4$ ($1.5x10^2$-$1.5x10^4$)
SJ311	5	0.41(-0.01-1.96)	$3.5x10^4$($3.0x10^3$-$2.6x10^6$)
SJ406	4	0.09(-0.01-1.15)	$1.1x10^5$($1.2x10^3$-$8.5x10^5$)
SJ435	4	-0.01(-0.04-0.01)	$1.2x10^4$($1.5x10^2$-$4.2x10^4$)
SJ312	3	0.00(-0.06-0.01)	$8.5x10^3$($3.5x10^1$-$1.5x10^6$)
SJ402	3	0.62(0.00-0.91)	$2.5x10^4$($2.0x10^3$-$6.9x10^4$)
SJ423	3	0.00(-0.00-1.35)	$4.3x10^4$($6.0x10^3$-$6.0x10^4$)
012	2	0.01(0.00-0.01)	$1.8x10^5$($3.8x10^4$-$3.8x10^5$)
SJ310	2	0.00(0.00-0.00)	$1.2x10^4$($1.8x10^3$-$2.2x10^4$)
SJ409	2	0.14(0.01-0.27)	$3.1x10^3$($1.5x10^2$-$6.0x10^3$)
SJ413	2	0.93(-0.00-1.86)	$1.1x10^4$($4.5x10^3$-$2.2x10^5$)
SJ428	2	0.00(0.00-0.00)	$1.5x10^4$($1.5x10^2$-$4.2x10^4$)
SJ443	2	0.58(0.00-1.15)	$6.5x10^4$($1.5x10^4$-$1.2x10^5$)
SJ451	2	1.41(1.24-1.58)	$1.4x10^5$($6.0x10^4$-$2.2x10^5$)
001	1	0.01(-)	$1.5x10^4$(-)
003	1	1.48(-)	$8.1x10^5$(-)
017	1	0.00(-)	$3.5x10^3$(-)
SJ313	1	0.00(-)	$3.1x10^4$(-)
SJ316	1	0.00(-)	$5.0x10^3$(-)
SJ318	1	1.59(-)	$2.6x10^5$(-)
SJ405	1	0.12(-)	$3.6x10^5$(-)
SJ407	1	0.00(-)	$5.1x10^3$(-)
SJ412	1	0.02(-)	$1.2x10^5$(-)
SJ442	1	1.82(-)	$1.6x10^4$(-)
SJ448	1	0.00(-)	$2.5x10^2$(-)
SJ459	1	0.01(-)	$5.0x10^0$(-)
SJ464	1	0.00(-)	$1.5x10^2$(-)
SJ468	1	0.02(-)	$3.8x10^4$(-)
SJ494	1	0.80(-)	$1.0x10^4$(-)
No ribotype data**	37	0.00(-0.04-1.96)	$3.5x10^3$(0-$1.4x10^6$)

Appendix 3. 2. Median levels of toxin by stool consistency across 200 consecutive CDI positive stool samples collected from the Microbiology Reference Laboratory of the Saint Joseph Mercy Health System, Ann Arbor, MI. from February 1st to July 20th, 2015..

Stool consistency	N	Normalized toxin levels Median (Range)	OR (95% CI)*	OR_{Adj} (95% CI)**
Soft	81	0.00(-0.06-3.94)	1.00(REF)	1.00(REF)
Formed/ Semi-formed	16	0.02(-0.02-1.96)	1.64(0.60-4.45)	1.04(0.30-3.60)
Mucoid	39	0.28(-0.05-2.33)	3.03(1.47-6.24)	3.90(1.69-8.97)
Liquid	64	0.00(-0.03-1.52)	0.78(0.38-1.60)	0.78(0.38-1.59)

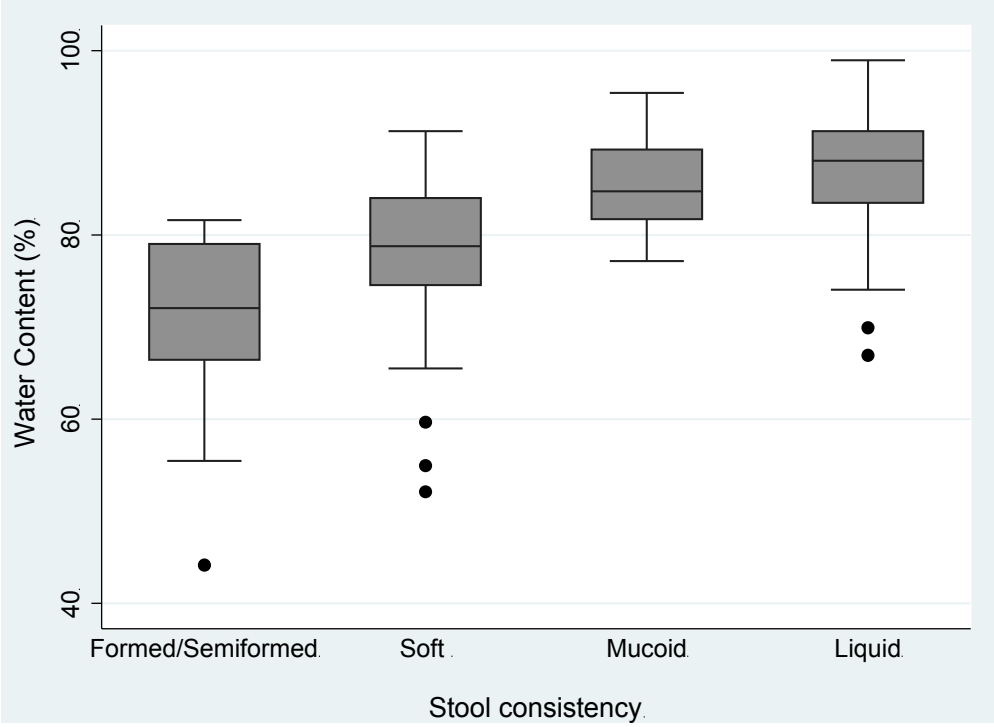
** Estimate adjusted for age, gender, location and ribotype.

Appendix 3. 3. Median levels of spores by stool consistency across 200 consecutive CDI positive stool samples collected from the Microbiology Reference Laboratory of the Saint Joseph Mercy Health System, Ann Arbor, MI, from February 1st to July 20th, 2015.

Stool consistency	N	Spores levels (CFU/g) Median (Range)	Unit change in spore levels*	p value	Adj. unit change in spore levels**	p value
Soft Formed/Semi	81	2.5×10^4(0-4.0×10^6)	REF		REF	
-formed	16	7.0×10^3(0-2.4×10^6)	2.35	0.32	1.76	0.54
Mucoid	39	6.2×10^4(0-5.5×10^6)	2.84	0.07	3.30	0.05
Liquid	64	7.5×10^3(0-2.8×10^6)	0.38	0.25	0.63	0.38

*Unit change in spore levels compared to soft stool consistency.
** Estimate adjusted for age, gender, location and ribotype.

Appendix 3.4. Water content (%) distributed across stool consistency across 153 consecutive CDI positive stool samples collected from the Microbiology Reference Laboratory of the Saint Joseph Mercy Health System, Ann Arbor, MI., from February 1st to July 20th, 2015. Stool consistency was classified in the following four categories: formed/semi-formed, soft, mucoid, and liquid (Kruskal Wallis Test, $p<0.001$).



CHAPTER IV

Clinical and epidemiological characteristics of toxin negative/PCR positive and toxin positive *C. difficile* infection patients.

4.0.0. Abstract

Clostridium difficile is associated with a well–known toxin-mediated intestinal disease, *C. difficile* infection (CDI); however, the bacterium is also carried asymptomatically in an estimated 5% of healthy individuals and in an even higher percentage of hospitalized patients. Although its clinical applicability is still controversial, PCR is used by ~44% of acute-care hospitals in the US as part of their CDI laboratory algorithm. A limited number of previous studies suggest that, in comparison to toxin positive individuals, toxin negative/PCR positive patients have a milder CDI presentation that resolves without CDI treatment. This study aimed to determine if these differences could be observed among a population with high CDI treatment rates. In addition, as toxin and spore levels are correlated, we sought to identify host-associated factors with fecal spore levels in CDI patients. In a population with 92% CDI treatment rate, we observed a milder CDI presentation among the 91 toxin negative/PCR positive compared to 120 toxin positive patients. There was a strong association between toxin positivity and white race, and CDI severity and

spore levels. These results will help improve CDI diagnosis and infection and control measures.

4.1.0. Introduction

Each year, *Clostridium difficile* causes an estimated half of a million infections and 29,000 deaths in the United States (US) (1). *C. difficile* infection (CDI) is characterized by a wide range of clinical manifestations, ranging from mild diarrhea to more severe disease including toxic megacolon, pseudomembranous colitis, sepsis, and death (2). The bacterium is carried asymptomatically among an estimated 5% of healthy individuals, 10% of hospitalized patients in acute care settings, and 15% of patients in long-term care facilities (3-5).

As nosocomial diarrhea can have multiple etiologies, and *C. difficile* infection does not necessarily result in symptoms, the diagnosis can be complicated. Thus, the laboratory has become an important player in CDI diagnosis. However, particularly in the US, there is not one agreed upon laboratory algorithm recommended for clinical settings (6).

The Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America (SHEA/IDSA) and the American College of Gastroenterology recommend a multi-step laboratory algorithm starting with the detection of glutamate dehydrogenase (GDH), followed by either a confirmatory test for detection of toxin using an Enzyme Immunoassay (EIA) or a Cell Cytotoxicity Assay (CCTA)), or detection of the toxigenic bacteria through PCR or

cytotoxigenic culture (CC) (7-9). CCTA and CC are considered *C. difficile* reference laboratory methods; however, they are labor-intensive and have a slow-turn around (10), and as such are not suitable for CDI clinical diagnosis. Due to their limited sensitivity, toxin EIA techniques are not considered appropriate as sole diagnostic tests (8). By contrast, PCR is considered suitable as a confirmatory CDI test, as it has both high sensitivity and specificity (8). However, the PCR test targets the toxins' genes rather than the toxins themselves, which does not allow differentiation between CDI symptomatic and asymptomatic individuals (11, 12).

In 2014, 44% of acute care hospitals participating in the National Healthcare Safety Network (NHSN) reported using molecular tests (PCR) alone or in combination with other tests for diagnosis of CDI (13). The implementation of this more sensitive test has led to increases of between 50 and 100% in CDI reported rates (13). Considering that colonization with *C. difficile* is 5-10 times more common than CDI, and that *C. difficile* is responsible for only ~20% of all nosocomial diarrheas, it is likely that some patients diagnosed as CDI positive using a PCR test have diarrhea of a different etiologic origin (13, 14).

A limited number of studies have compared the clinical and epidemiological differences between CDI patients by laboratory diagnostic criteria. While not consistent across studies (15), most previous studies observed clinical and epidemiological differences between patients with detectable levels of toxin (through EIA or CCTA) and those diagnosed only through the detection of toxigenic *C. difficile* strains (PCR or CC) (12, 15, 16). Baker et al. (2013) reported that toxin negative/PCR positive (toxin-/PCR+) patients (n=53) had significantly lower 14 days-all-cause mortality than toxin

positive (toxin+) patients (n=24) (16). Moreover, even though only 15% of the toxin-/PCR+ patients received CDI treatment, 97% of them had resolved their diarrhea after 14 days (16). Similarly, Polage et al. (2015) reported toxin-/PCR+ patients (n= 162) had similar outcomes as CDI negative patients (n=1123), even though only 41% of toxin-/PCR+ patients received CDI treatment (13). Longtin et al. (2013) also observed that only 3% of PCR+ (n=85) developed CDI complications in comparison to 39% toxin+ patients (n=56) (17). This study also reported similar associations with CDI recurrence and 30-days mortality (17).

Longtin and colleagues did not specify the percent of PCR positive patients who were treated with vancomycin or metronidazole (17). However, as their routine CDI laboratory algorithm used solely PCR, we can assume their study population consisted of a higher percent of treated PCR+ patients than in the previously mentioned studies where the PCR was not part of the regular clinical algorithm (13, 16). However, as CDI treatment may obscure or exacerbate differences in clinical severity, our study aimed to determine if the previously described clinical and epidemiological differences between toxin-/PCR+ and toxin+ CDI patients were observed among a population with high CDI treatment rates.

As we also reported in Chapter III of this dissertation, toxin production is correlated with spore production; however, both toxin+ and toxin-/PCR+ CDI patient can shed spores (15, 18). We further investigated the host-associated characteristics of spore production. As far as we know, this is the first study specifically investigating host-associated factors impacting spore stool levels in CDI patients.

4.2.0. Methods

Study population

We conducted a case-control study of CDI patients receiving care at Saint Joseph Mercy Hospital (SJMH), an acute care hospital in Ann Arbor, MI. The Institutional Review Boards at SJMH and the University of Michigan (IRB Health Sciences and Behavioral Sciences) approved our study protocol.

Our study is composed of two sets of populations. First, we included all hospitalized patients aged 18 and older who were laboratory diagnosed with CDI based on SJMH laboratory guidelines from January 1st through June 30th, 2014. To research the association between spore levels and disease severity, we included all patients with the same characteristics who were hospitalized between February 1st and July 20th 2015, for whom we possessed a positive CDI stool sample from a previous study (Figure 4.6).

The SJMH CDI laboratory algorithm includes an initial GDH/Toxins AB screening using C.DIFF QUIK CHEK COMPLETE® (Alere, Waltham, MA./Techlab, Blacksburg, VA.). In case of indeterminate results, samples are further tested for presence of the toxin B gene using a *tcdB* PCR assay (Cepheid Gene Xpert®, Sunnyvale, CA.). A positive CDI result is reported in case of two positive test combinations: 1) GDH positive and toxin positive (toxin+), or 2) GDH positive, toxin negative and PCR positive (toxin-/PCR+). Based on this classification, we defined cases as toxin+ patients and toxin-/PCR+ patients as controls. In case of successive tests of the same individual, we included only their first CDI positive case during the study period.

Data collection

We gathered several epidemiological, clinical, laboratory, and treatment variables from the medical record. Variables included details and dates/times of CDI test results and CDI treatment; use of antibiotics, H2 receptors antagonists (H2 blockers), proton pump inhibitors (PPI), corticosteroids, and chemotherapy during the 90 days before sample collection; use of laxatives in the week before sample collection; and history of hospitalization, surgery, or CDI diagnoses in the 90 days before sample collection. CDI severity was evaluated based on age, latest levels of white blood cells (WBC) and serum creatinine before initiation of CDI treatment (compared to baseline levels in the last year), and ICU stay due to CDI during the current episode. We used the SHEA/IDSA guidelines to classify CDI severity as: mild, moderate, severe, and severe and complicated (7).

Through an electronic data abstraction, we collected demographic characteristics (age, gender, race based on self-reporting), length of stay, Charlson Comorbidity Score, an institution-specific retrospective 30-days mortality risk (19, 20). Charlson Comorbidity Score is a validated estimator of risk of death from comorbid disease: the higher the score the higher the risk (21). By contrast, the institutional-specific 30-days mortality risk uses the lowest stratum (ei. risk stratum 1) to indicate the highest mortality risk and the highest stratum (ei. risk stratum 5) to indicate the lowest risk of mortality (19, 20). In addition, we recorded all-cause and CDI-associated readmission within 30 days from discharge, and all-cause and CDI-associated mortality within 30-days of CDI diagnosis (utilizing the Michigan Death Index).

Determination of spore levels

Spore levels were quantified in stool samples available for a subset of our study population (Figure 4.6). Following a heat treatment (65^{o}C for 20 minutes), spore levels were quantified by culturing serial dilutions of the fecal specimen on Cycloserine Cefoxitin Fructose Agar with Horse Blood and Taurocholate (Anaerobe Systems, Morgan Hill, CA.) for 24 hours at 37^{o}C under anaerobic conditions. Spore levels were reported as colony-forming units (CFU) per gram of feces.

Ribotyping of *C.difficile* isolates

A fluorescent PCR ribotyping technique using capillary gel electrophoresis was performed to ribotype the *C. difficile* isolates recovered from our study samples as previously described (22).

Statistical analysis

Chi-square or the Mann Whitney test was performed to determine the presence of significant differences between cases and controls with respect to demographic characteristics, CDI severity, CDI risk factors, CDI treatment and clinical outcome. Cochran-Armitage Trend Test was used to evaluate linear treads across the variables and frequency of cases. We further fitted univariate and multivariate logistic regression models to estimate the strength of the

associations between selected variables and toxin+ versus toxin-/PCR+ patients. For the subset of the study population (n=77) where spore levels were known, we used univariate and multivariate linear regression to model the log-transformed average level of spores in relation to known CDI risk factors and other epidemiological and clinical characteristics. Stepwise regression (with a significance level of entry and stay equaled to 0.30 and 0.35 respectively) and previous knowledge from the literature informed the selection of variables for the multivariate models. SAS 9.4 (Cary, NC.) and STATA 14 (College Station, TX.) were used to perform all analysis and graphics.

4.3.0. Results

Of 211 CDI hospitalized patients, 120 (56.9%) were toxin+ and 193 (91.5%) had receipt of CDI treatment recorded in their medical record. Toxin+ patients were slightly older than toxin-/PCR+ patients (OR= 1.02, 95% CI 1.00-1.04), but gender distributions were similar (Table 4.2). Whites were twice as likely as blacks or multiracial patients to be toxin+ (OR=2.11 (95%CI 1.00-4.46). This association remained after adjustment for age, creatinine and WBC levels, CDI severity, previous use of antibiotics, PPI, corticosteroids, laxatives and chemotherapy, previous surgery and CDI episode, and 30-days all-cause mortality (OR= 2.12; 95% CI 0.92-4.88) (Table 4.3).

Using SHEA/IDSA guidelines to classify severity, toxin+ patients had a more severe CDI presentation than toxin-/PCR+ patients. Although not statistically significant, severe and complicated CDI cases were more likely to be

toxin+ (OR=2.20, 95% CI (0.68-7.13). Further, as CDI severity increased, the number of toxin+ patients increased (test for trend p=0.11) (Figure 4.7).

Toxin+ patients also tended to be more ill than toxin-/PCR+ patients, having a slightly higher Charlson Comorbidity Score (OR=1.30 (95% CI 0.93-1.36). In addition, the proportion toxin+ cases increased with the comorbidity score (test of trend p=0.22) (Figure 4.7). Further, toxin+ patients had a higher risk of 30-days mortality (lower strata indicates higher risk of mortality) (OR=0.79 (95% CI 0.62-1.00). As mortality risk increased, the percent of toxin+ patients increased (test of trend p=0.06) (Figure 4.7). Although we did not observe a significant difference in all-cause 30-days readmission or mortality by toxin status, all four patients with a CDI-associated death were toxin+.

In regards to CDI risk factors, we did not find a strong association between previous antibiotic history and toxin+ patients (OR=1.40 (95% CI (0.69-2.84)). However, users of macrolides during the 3 months prior to sample collection were 1.93 (95% CI 0.84-4.45) times more likely to be a toxin+, whereas penicillin (OR=0.53; 95% CI: 0.28, 1.01) and carbapenems users (OR=0.40; 95% CI: 0.13-1.23) were less common in toxin+ than toxin-/PCR+ patients (Table 4.2). Previous use of H2 blockers, hospitalization, and surgery also tended to be more common among toxin+ than toxin-/PCR+ patients (Table 4.2). Although only recorded for a subset of our population (n=77), enteral tube use was less common among toxin+ patients (p=0.17).

Among the subset of our study population (n=77), where spore levels in stool were available, we identified a strong positive association between spore levels and history of hospitalization (Table 4.4.). Spore levels tended to increase with history of antibiotic use during the previous 90 days, but the association

was not statistically significant. Use of lincosamides (ei. clindamycin) (p=0.05) was negatively associated with spore levels, as was a history of previous chemotherapy (p=0.004). History of hospitalization (p=0.06) and Charlson Comorbidity Score (p=0.05) remained associated to spore levels even after adjusting for gender, Charlson Comorbidity Score, 30-days mortality risk, complicated CDI severity, use of enteral tube, history of antibiotics, PPI, chemotherapy, hospitalization as necessary (Table 4.4).

We observed a strong association between spore levels and CDI severity, particularly among severe/complicated individuals (p=0.33) (Table 4.4, Figure 4.8). This association remained significant after adjusting for confounders. However, when we stratified by ribotype, the association decreased and was no longer statistically significant, but we note that the sample size was also severely reduced (Figure 4.8).

4.4.0. Discussion

In a comparison of hospitalized CDI patients with and without a positive stool test for toxin, CDI severity, Charlson Comorbidity Score, 30-days mortality risk, and white race were strongly associated with detection of toxin. When we did not consider presence of toxin, we also observed an association of spore levels with CDI severity.

We found no previous reports confirming or refuting our observation that specifically increased fecal spore levels in clinical samples –omitting the *in vitro* analysis step - are associated with increased CDI severity and 30-days all cause

mortality. However, our finding is consistent with the hypothesis that greater spore production is likely to be associated with the presence of more vegetative cells (23)-- hence, greater toxin levels when produced. Consistent also with our results, in a study of 106 clinical isolates, Carlson et al. (2013) previously linked *in vitro* spore production to CDI severity (23). Furthermore, spore production was higher among individuals with known CDI risk factors: previous history of antibiotics, proton pump inhibitors, and previous hospitalization (24).

The association of CDI severity and toxin detection is consistent with previous studies (13, 16, 17, 25). However, unlike previous reports, toxin+ CDI cases were no more likely than toxin-/PCR+ CDI cases to be re-admitted within 30 days of discharge or to die within 30 days post CDI diagnosis (16, 17, 26). This may be due to differences in classification schemes (not all used toxin-/PCR+ as a reference (26)), outcome definitions, study population, or treatment regimen. Unlike previous studies (13, 16, 26), the majority of our participants received CDI treatment.

Similar to previous studies, toxin+ and toxin-/PCR+ CDI patients did not differ by age and gender (13, 14). However, we did observe a strong association between white race and Toxin+ CDI cases (OR= 2.11 (95% CI 1.00-4.46)). Although a positive association between white race and CDI occurrence has been reported previously (1, 27, 28), we found no previous reports of an association of race and toxin positivity.

It is most likely that white race is a marker for CDI risk factors. In the US, white individuals generally have better access and quality of healthcare than others (29), which make them more likely to have access to treatments that are CDI risk factors (30). Indeed, those of white race were more likely to report

previous use of H2 blockers, PPI, and chemotherapy--all previously described CDI risk factors (7, 24, 31, 32). However, the association between toxin positivity and white race in our study population remained after adjusting for several risk factors and case severity. This association deserves further scrutiny.

Our results add to growing evidence questioning the clinical applicability of including PCR testing as part of CDI laboratory analysis (6, 13). If toxin-/PCR+ patients have a milder CDI presentation and are able to resolve their diarrhea without further treatment (16), the question arises whether CDI treatment is advisable. Due to the disruption of the gut microbiota, treatment of asymptomatic carriers can increase spore shedding and symptomatic CDI occurrence (33-34). Further, anti-anaerobic therapies such as vancomycin, can promote the selection and spread of vancomycin-resistant enterococcus colonization (35, 36). Finally, as the PCR test cannot distinguish between colonizing isolates and disease, the number of diagnoses and associated treatment will increase. Indeed, following the introduction of molecular testing, the number of reported CDI cases has increased by as much as 100% in some hospitals (13, 17). Furthermore, CDI rates are now used as a healthcare quality indicator and as there is no single recommended CDI laboratory algorithm in the US, the use of PCR by some but not all laboratories may confound the ability to appropriately compare performance across healthcare settings (6). Nevertheless, the increased sensitivity of PCR testing can aid infection control; CDI spores are found in toxin-/PCR+ stools, although the average levels are significantly lower than for toxin+ CDI patient, as reported in a previous chapter.

To prevent overdiagnosis and overtreatment, the United Kingdom has implemented a different algorithm that might be considered elsewhere. The

algorithm recommends PCR as part of its CDI lab algorithm; however, it is used for screening purposes only. All stool samples are processed initially using PCR; then, a sensitive toxin EIA follows for PCR positive samples. Only if the EIA test for toxin is positive is the CDI positive result reported to the physician. Toxin-/PCR+ patients are reported only to the Infection Control Unit for further analysis (37).

In conclusion, in our study population, toxin -/ PCR + CDI patients were less ill, had lower 30-days mortality risk, and shed, on average, lower levels of spores. However, we did observe an increase in CDI severity with spore levels, particularly among severe and complicated CDI patients. These findings raise questions regarding use of the PCR test for clinical purposes as opposed to infection control. Alternative CDI diagnostic algorithms should be evaluated to compare the trade-offs in terms of patient risk and benefit, infection control, and cost.

Table 4.2. Distribution of toxin positive (n=120) and toxin negative/PCR positive CDI patients across demographic, epidemiological, severity, and clinical characteristics, Saint Joseph Mercy Hospital, Ann Arbor, MI., January 1st –July 30th, 2014/February 1st to July 20th, 2015.

	Group, n (%)/mean (SD)				Toxin Positive vs.	
	M	Toxin Positive N= 120	PCR Positive N= 91	p	OR	95% CI
Demographics						
Age (years)		69.13(15.86)	63.79(16.99)	0.03	1.02	1.00-1.04
Gender Female		67(55.83)	50(54.95)	0.90	1.04	0.60-1.79
Race White Black or Multiracial	1	105(88.24) 14(11.76)	71(78.02) 20(21.98)	0.10	2.11	1.00-4.46
Risk factors (90 days before CDI sample collection)						
Antibiotic history		101(84.17)	72(79.12)	0.34	1.40	0.69-2.84
Penicillins		23(19.17)	28(30.77)	0.05	0.53	0.28-1.01
Cephalosporins		81(67.50)	62(68.13)	0.92	0.97	0.54-1.74
Carbapenems		5(4.17)	9(9.89)	0.10	0.40	0.13-1.23
Macrolides		21(17.50)	9(9.89)	0.12	1.93	0.84-4.45
Quinolones		23(19.17)	19(20.88)	0.75	0.90	0.46-1.77
Sulfonamides		7(5.83)	4(4.40)	0.64	1.35	0.38-4.75
Tetracyclines		1(0.83)	0(0)	0.38		
Aminoglycosides		5(4.17)	4(4.40)	0.94	0.95	0.25-3.63
Lincosamides		18(15.00)	9(9.89)	0.27	1.61	0.69-3.77
Glycopeptides		62(51.67)	41(45.05)	0.34	1.30	0.76-2.25
Amebicides		45(37.50)	36(39.56)	0.76	0.92	0.52-1.60
H2 blockers		49(40.83)	28(30.77)	0.13	1.55	0.87-2.76
Proton pump inhibitor		57(47.50)	49(53.85)	0.36	0.78	0.45-1.34
Corticosteroids		40(33.33)	30(32.97)	0.96	1.02	0.57-1.81
Chemotherapy		9(7.50)	5(5.49)	0.56	1.40	0.45-4.31
Previous hospitalization		78(65.00)	52(57.14)	0.25	1.39	0.80-2.44
Previous surgery		43(35.83)	24(26.37)	0.14	1.56	0.86-2.83
Previous CDI episode		17(14.17)	10(10.99)	0.49	1.34	0.58-3.08
Use of laxatives (Previous week)		52(43.33)	30(32.97)	0.13	1.56	0.88-2.74
Enteral tube (Current encounter)**	70	1(2.56)	4(10.00)	0.17	0.24	0.03-2.22
Length of hospital stay before CDI test order (Current encounter)(days)		3.52(4.67)	2,83(6.32)	0.70	1.02	0.97-1.08
CDI severity						

WBC (thou/mcL)		13.48(7.76)	11.81(8.57)	0.02	1.03	0.99-1.07
Creatinine (mg/dL)		1.42(1.19)	1.92(2.36)	0.84	0.85	0.72-1.00
30-days Mortality risk		2.42(1.09)	2.72(1.18)	0.06	0.79	0.62-1.00
Charlson Comorbidity Score	2	2.17(1.53)	1.91(1.42)	0.19	1.13	0.93-1.36
CDI severity						
Mild		25(20.83)	24(26.37)	0.34	0.74	0.39-1.40
Moderate		38(31.67)	33(36.26)	0.48	0.81	0.46-1.45
Severe		46(38.33)	32(32.97)	0.42	1.26	0.71-2.22
Severe and complicated		11(9.17)	4(4.40)	0.18	2.20	0.68-7.13
Clinical outcomes						
Length of stay (days)		9.47(8.86)	8.2(7.81)	0.14	1.02	0.98-1.05
ICU stay	1	39(32.77)	25(27.47)	0.41	1.29	0.71-2.34
Total Cost (Dollars)	2	63,381(84,024)	52,307(60,508)	0.19		
Received CDI Treatment		111(92.50)	82(90.11)	0.54	1.35	0.52-3.56
Metronidazole❖		56(46.67)	43(47.25)	0.93	0.98	0.56-1.69
Vancomycin ❖		95(79.17)	63(69.23)	0.10	1.69	0.90-3.16
Readmission within 30 days of discharge (All cause)		26(21.67)	21(23.08)	0.81	0.92	0.48-1.77
Death within 30 days of CDI diagnosis (All cause)		13(10.83)	13(14.29)	0.45	0.73	0.32-1.66
Death-associated to CDI *	1	4(100%)	0(0)	0.04	4.05	

** Among a subset of our population (n=77).
* Among those individual who died 30 days after CDI diagnosis
❖Among those patients who received CDI treatment
M column represent missing values in each category

Table 4.3. Association between white race and demographic, epidemiological, clinical, and severity characteristics of CDI positive patients (N=211), Saint Joseph Mercy Hospital, Ann Arbor, MI., January 1st –July 30th, 2014/February 1st to July 20th, 2015.

	Odds Ratio	95% CI
Demographics		
Age	1.01	0.99-1.04
Gender	1.32	0.63-2.75
Risk factors (90 days before CDI sample collection)		
H2 Blockers	1.44	0.65-3.20
Proton Pump Inhibitor	1.77	0.56-2.46
Laxatives (previous week)	0.76	0.36-1.60
Corticosteroids	0.67	0.31-1.42
Chemotherapy	2.63	0.33-20.82
Previous hospitalization	0.87	0.40-1.86
Previous surgery	0.81	0.37-1.75
Previous CDI episode	0.83	0.29-2.36
Previous antibiotic history	0.56	0.18-1.69
Severity and clinical outcomes		
WBC (thou/mcL)	1.08	1.01-1.15
Creatinine (mg/dL)	0.82	0.69-0.97
30-days mortality risk	1.14	0.82-1.58
Charlson Comorbidity Score	0.84	0.66-1.07
CDI severity		
Mild	0.82	0.35-1.19
Moderate	0.42	0.97-0.33
Severe	0.66	0.31-1.38
Severe and complicated	2.85	0.36-22.42
Length of stay (days)	0.98	0.95-1.02
Readmission within 30 days of discharge (All cause)	0.93	0.39-2.21
Mortality within 30 days of CDI diagnosis (All-cause)	0.79	0.27-2.52

Table 4.4. Unit change of spore levels by demographic, epidemiological and severity and clinical characteristics (N=77) of CDI positive patients, Saint Joseph Mercy, Ann Arbor, MI., February 1st to July 20th, 2015.

	Univariate analysis		Multivariate analysis*	
	Unit change in spore levels by one unit change/compared to reference group	**p**	**Unit change in spore levels by one unit change/compared to reference group**	**p**
Demographics				
Age (years)	1.00	0.93		
Gender Female	0.45	0.28	0.52	0.41
Race White	0.96	0.97		
Risk factors (90 days before CDI sample collection)				
Antibiotic history	2.74	0.24	0.28	0.22
H2 Receptor Blocker use	1.69	0.50		
Proton Pump Inhibitor use	1.88	0.40	1.35	0.71
Corticosteroid use	1.64	0.51		
Chemotherapy	0.16	0.004	0.12	0.21
Previous hospitalization	8.12	0.73	5.47	0.06
Previous surgery	0.75	0.73		
Previous CDI episode	2.58	0.37	0.63	0.70
Laxatives (previous week)	1.37	0.68		
Enteral tube	5.82	0.24	4.10	0.36
Severity and clinical outcomes				
WBC (thou/mcL)	1.05	0.45		
Creatinine (mg/dL)	1.11	0.52		
30-days Mortality risk	0.60	0.12		
Charlson Comorbidity Score	2.22	0.01	1.91	0.05
CDI severity				
Mild	0.61	0.56		
Moderate	0.62	0.55		
Severe	1.65	0.52		
Severe and complicated	5.09	0.33	2.55	0.61
Length of stay (days)	1.05	0.30		
Readmission within 30 days of discharge (All cause)	1.31	0.76		
Death within 30 days of CDI diagnosis (All cause)	5.37	0.22	1.63	0.75

* Multivariate model included the following variables: gender, history of antibiotics, proton pump inhibitors, chemotherapy, hospitalization, and CDI, Charlson Comorbidity Score, complicated CDI severity, use of enteral tube during current encounter, and 30-days mortality.

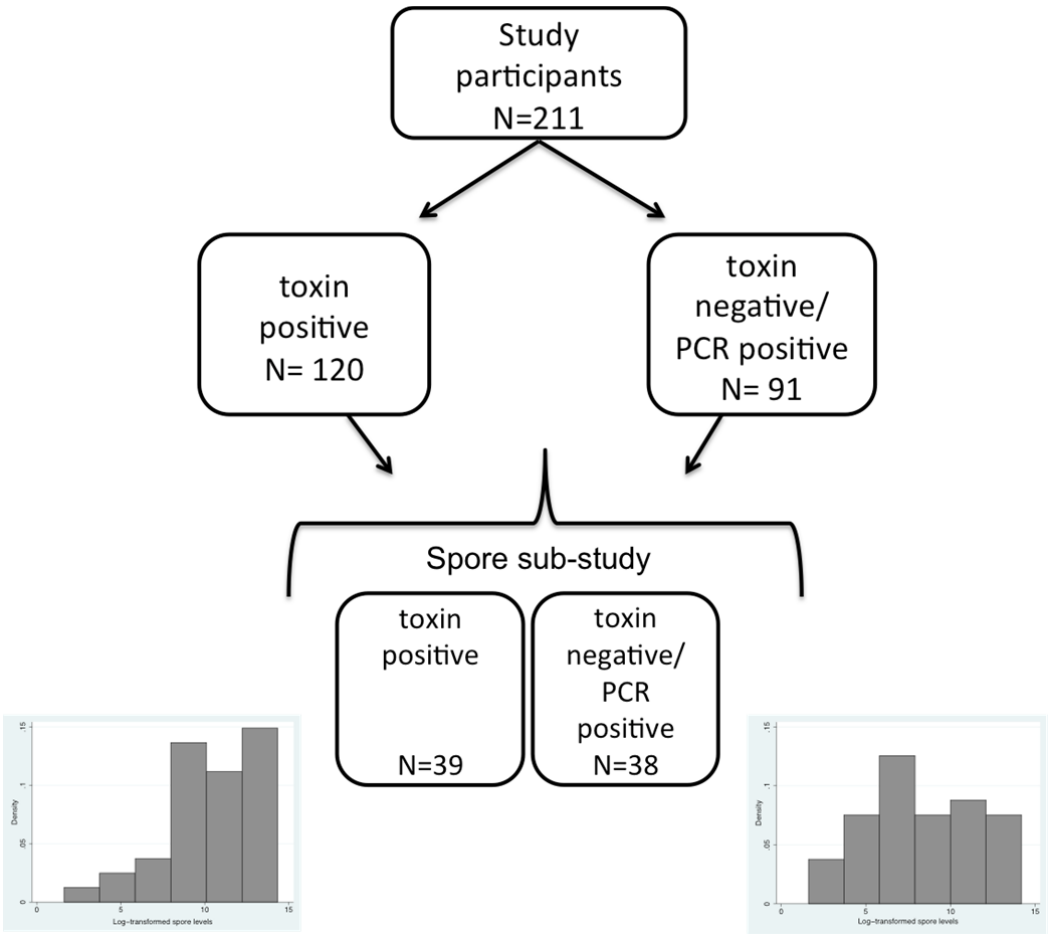
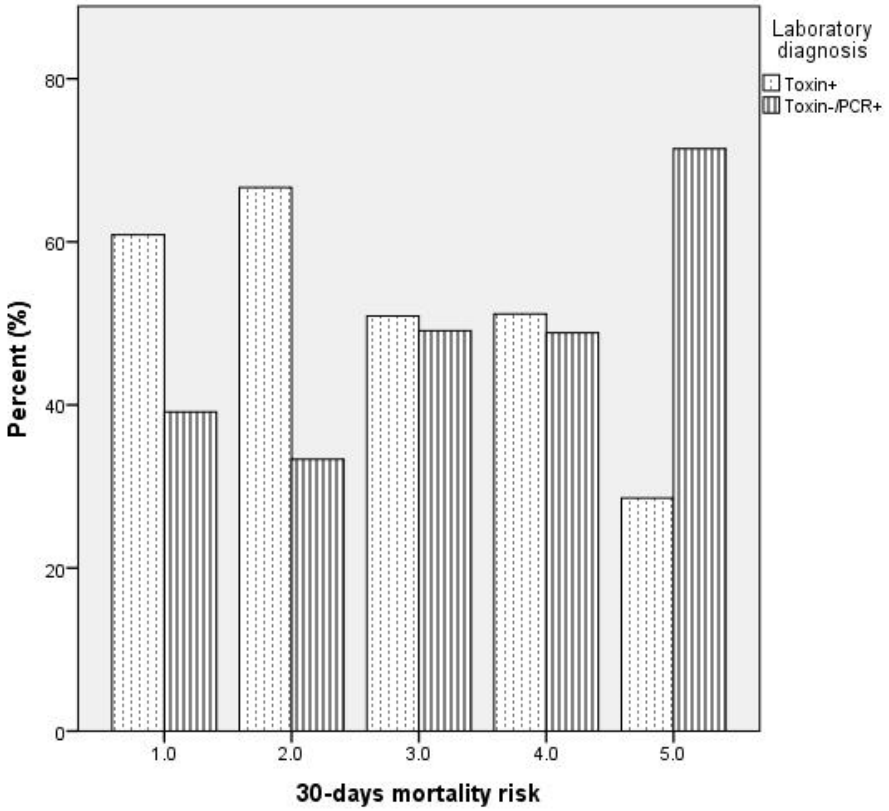
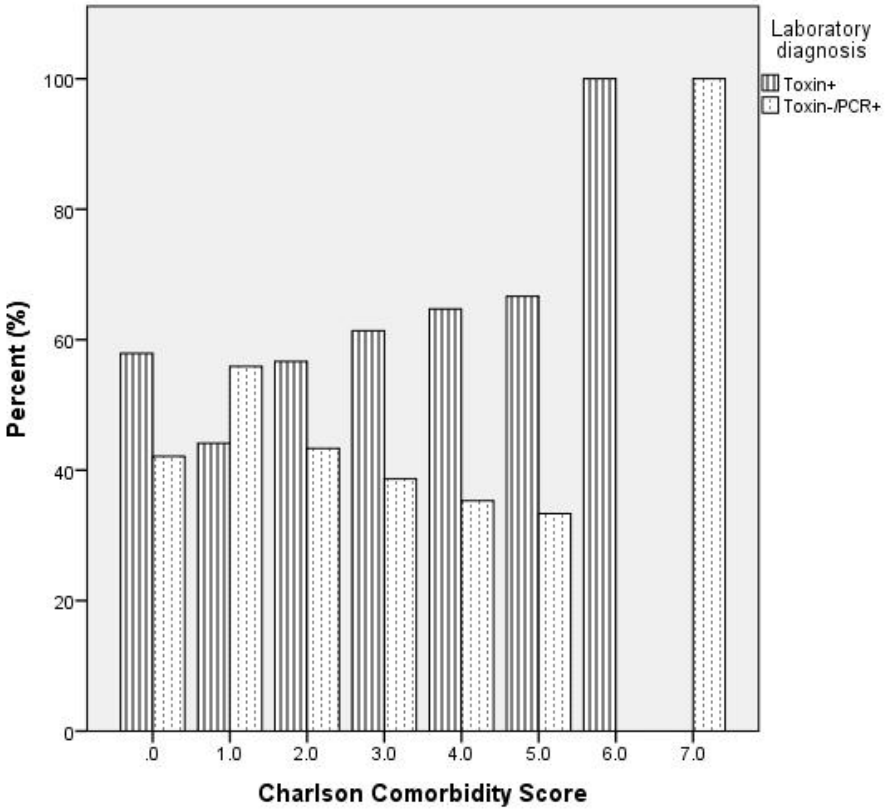


Figure 4.6. Distribution of study population across laboratory diagnosis and spore data availability.

A.


Laboratory diagnosis
Toxin+
Toxin-/PCR+
Percent (%)
0
20
40
60
80
1.0
2.0
3.0
4.0
5.0
30-days mortality risk

B.


Laboratory diagnosis
Toxin+
Toxin-/PCR+
Percent (%)
0
20
40
60
80
100
.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
Charlson Comorbidity Score

B.

C.

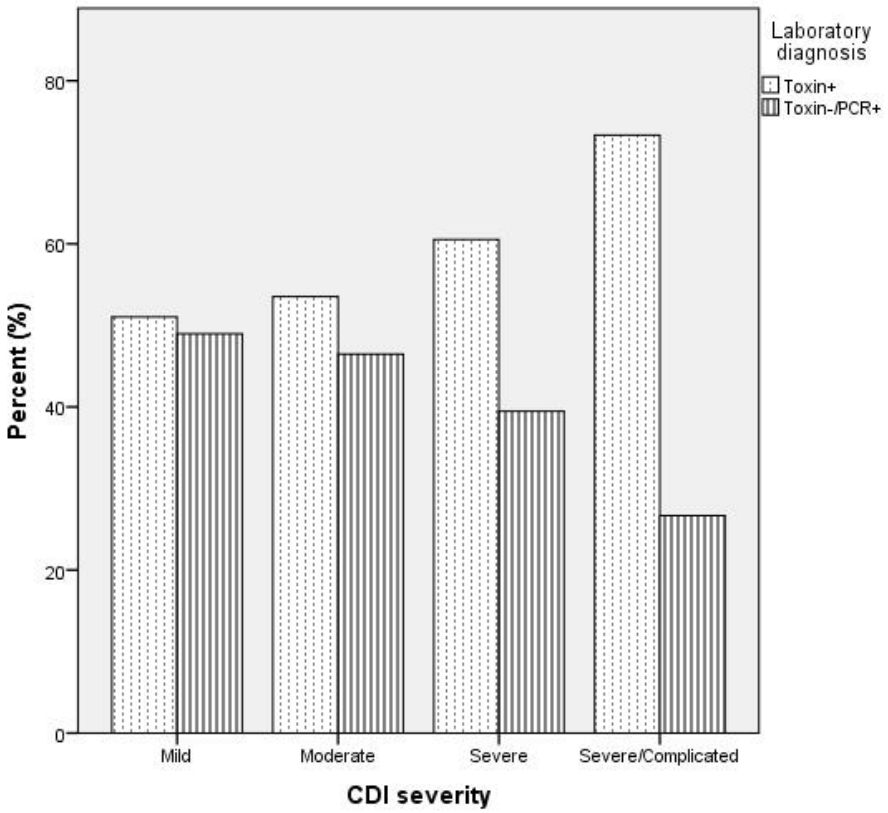
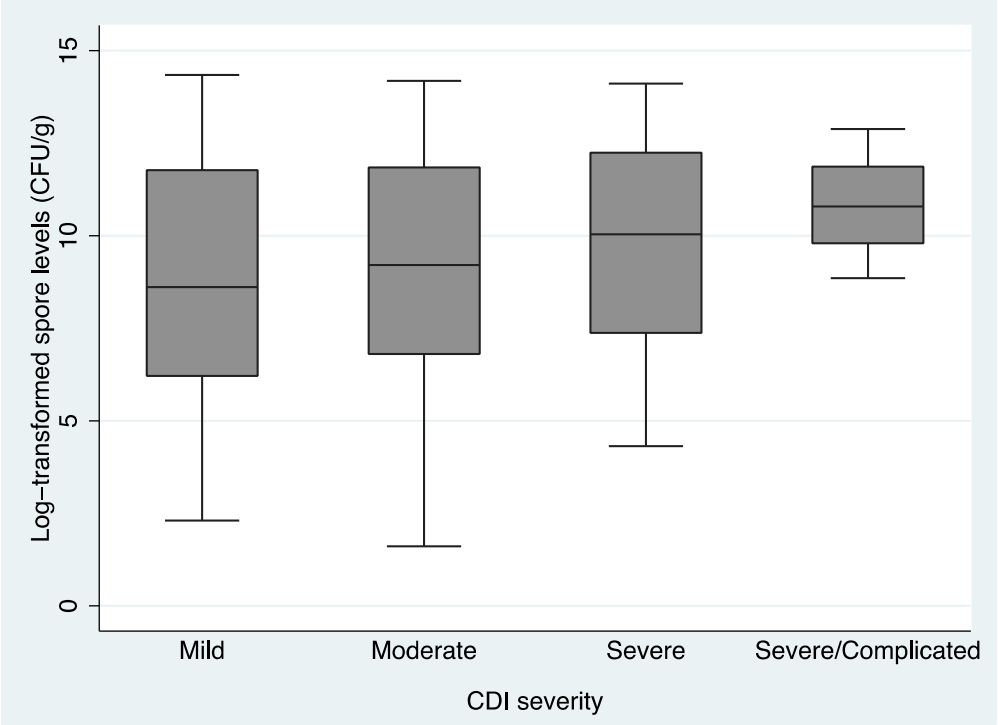


Figure 4.7. Distribution of study participants by laboratory diagnosis (percent): A) across categories of 30-days- mortality risk (lower strata indicates higher risk of mortality)(test of trend, p=0.06) B) across categories of Charlson Comorbidity Score (higher strata indicates more morbidity)(test of trend, p= 0.22), C) across categories of CDI severity (test of trend, p=0.11).

.A.


Log–transformed spore levels (CFU/g)
0
5
10
15
Mild
Moderate
Severe
Severe/Complicated
CDI severity

B.

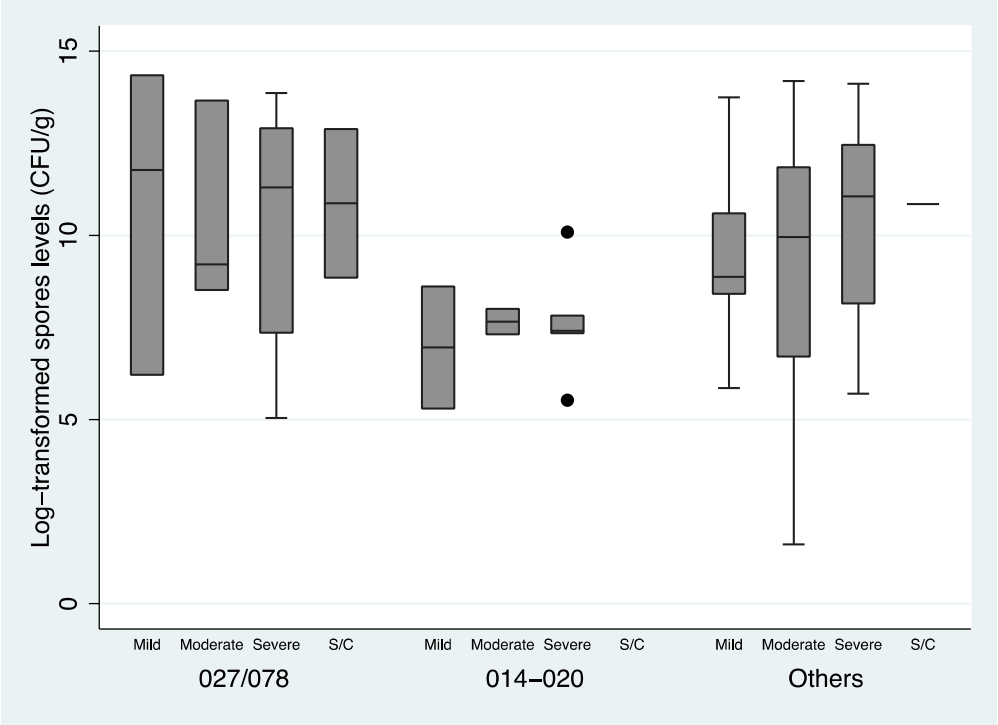

Log–transformed spores levels (CFU/g)
0
5
10
15
Mild
Moderate
Severe
S/C
027/078
014–020
Others

Figure 4.8. Distribution of log-transformed spores: A) across CDI severity (Test of trend p=0.26), and B) across CDI severity and *C.difficile* ribotypes: ribotype 027/078 (n=16, Test of trend, p=0.89), ribotype 014-020 (n=9, Test of trend p=0.85), and other ribotypes (n=39, Test of trend, p=0.25).

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CHAPTER V

An *in silico* evaluation of treatment regimens for recurrent *Clostridium difficile* infection

5.0.0. Abstract:

Clostridium difficile infection (CDI) is one the leading causes of nosocomial infection in the United States. Although effective treatment is available, up to 35% of infections recur. Risk of CDI varies by ribotype, with some of the variation in risk attributed to differences in sporulation and germination rates. However, whether sporulation and germination rates mediate effectiveness of treatment and risk of recurrence remains unclear. Recommended treatments for repeated recurrence include tapering or pulsing of oral vancomycin. Although clinical trials show tapered/pulsed vancomycin treatment to be more effective at reducing CDI recurrence than the standard longer and higher doses, no controlled data exist evaluating the relative effectiveness of these regimens. In the absence of a controlled trial, an *in silico* model can provide useful insights. Therefore, we developed a compartmental in-host mathematical model of CDI, composed of vegetative cells, toxins, and spores. We created both deterministic and stochastic versions to determine the role of sporulation/germination patterns in recurrence and the effectiveness of current tapered/pulsed vancomycin regimens by ribotype. Our models confirm the importance of sporulation/germination patterns for pathogenicity and transmission. Most of

the evaluated treatment regimens for repeated CDI were effective in reducing risk of an additional recurrence, with the effectiveness varying by ribotype. However, we could not detect a significant difference in treatment effectiveness between pulsing doses at 48 versus 72 hours. Furthermore, treatment effectiveness was maintained even when the duration or dosage of most of the recommended regimens was reduced.

5.1.0 Introduction

Clostridium difficile is an anaerobic, spore-forming, Gram-positive bacillus associated with the toxin-mediated intestinal disease known as *C. difficile* infection (CDI) (1, 2). CDI is one of the leading reported causes of healthcare-associated infections in the United States (US) (3). Although CDI was initially reported in 1978, its epidemiology worldwide has changed significantly in the last two decades (2). This change in epidemiology was initially attributed to the emergence of a *C. difficile* ribotype (ribotype 027) known to have higher rates of sporulation and toxin production (4). However, more recent studies have found no correlation between *C. difficile* ribotype and clinical disease (5-7).

Recommended CDI treatment is a course of oral metronidazole or vancomycin. However, CDI still recurs in 5-35% of patients following appropriate treatment (8-10). Recurrence is defined as a CDI that occurs within 8 weeks following resolution of the initial episode (11). Without appropriate laboratory testing, it is not possible to determine if a recurrent case is a relapse of the same infection, a new infection with a different strain, or a new infection

with the same strain (12). However, it has been estimated that on average half of recurrences are due to relapse of the original *C. difficile* strain (13, 14).

C. difficile spores that persist after treatment can germinate and lead to recurrence (15). Therefore, ribotypes with higher sporulation rates are expected to be associated with higher rates of recurrence. For example, ribotype 027, known to have a higher sporulation rate, has been reported as a risk factor for CDI recurrence (12, 16). Similarly, strains with high germination efficiency have been associated with severe and recurrent CDI (14, 17).

Tapering or pulsing of oral vancomycin is one of the recommended treatments for CDI recurrence, particularly for repeated recurrent CDI (18). By tapering or pulsing, it is assumed that time and a proper environment allow any spores still in the gut to germinate and then be killed in the vegetative cell state. Further, the regimen allows the microbiota to recover (19, 20). Although clinical trials show tapered/pulsed vancomycin treatment to be more effective at reducing CDI recurrence than the standard longer and higher doses (20), no controlled data exist evaluating the relative effectiveness of the specific regimens used for this purpose (21).

We used a mathematical simulation model to fill this gap. We simulated the *in vivo* levels of spores and vegetative cells within the CDI host by the four most common ribotypes in the U.S. (22) with the initial purpose of determining the importance of sporulation/germination patterns across ribotypes and their contribution to the observed differences in CDI recurrences. We also evaluated the effectiveness of current tapered/pulsed vancomycin regimens by ribotype.

5.2.0. Methods

5.2.1. Deterministic Ordinary Differential Equations (ODE) model

We developed a compartmental in-host mathematical model for CDI patients, composed of the major parts of the bacteria's life cycle within the human host: vegetative cells, toxins, and spores. As our study purpose was to determine CDI recurrence, our model simulated and measured: number of vegetative cells (C), viable spores (Spl), non-viable spores (Spd), and toxin (T) per mL of gut contents per day.

Due to the limited data available to inform our overall model, the lack of knowledge about the level of simplification needed for the model structure, and the need to streamline model development and parameter estimation, we used a forcing function approach (similar to that developed in (23)), in which the overall model was broken into two submodels. The two submodels were: (1) vegetative cells and (2) spores and toxin. As illustrated in Figure 5.9, vegetative cell data define the spore and toxin model, while vancomycin concentration and spore data define the vegetative cell model. We developed and fit each submodel separately, combining the submodels for final parameter estimation from data, as described further below.

Vegetative cell submodel

Vegetative cells are able to proliferate in the colon if conditions are permissible. A protective microbiota and other processes may inhibit colonization (8). When modeling the growth of *C. difficile* vegetative cells (C), we first considered the bacteria's growth rate (k) limited by their carrying capacity within the human gut (Cap). For the logistic growth term, we tested several exponents, and chose the lowest integer value that yielded a visually good fit (cubic power). In addition, we considered the formation of new cells due to the germination of available spores (k_{ger}). We also subtracted the loss of cells (k_{LC}), either because they sporulated (k_{sp}) or they were shed into the environment through feces (k_{ExC}). Due to identifiability issues, we were unable to separate the effects of k_{LC} at this point (where $k_{LC} = k_{sp} + k_{ExC}$).

Finally, we considered the loss of cells due to vancomycin treatment (k_{txt}). To better represent the vancomycin pharmacokinetics, we added an extra equation to this submodel. When using the standard regimen of 125mg/L four times a day, oral vancomycin is poorly absorbed, so stool concentrations significantly exceed the MIC_{90} of most *C. difficile* isolates (18, 24). The vancomycin concentration (V) was first fitted to vancomycin data (25) using sum of least of squares, then its parameter (k_v) was fixed for the remainder of the parameter fitting.

The following equations make up this first submodel:

$$\frac{dC}{dt} = kC\left(1 - \frac{C^3}{Cap^3}\right) + k_{ger}Spl - k_{txt}C - k_{LC}C, \quad where \quad k_{LC} = k_{sp} + k_{ExC}$$

$$\frac{dV}{dt} = u(t) - k_V V, \quad where \quad u(t) = vancomycin \quad input$$

Spores/toxin submodel

During its life cycle, *C. difficile* vegetative cells produce endospores (18). Spores are highly resistant to immune system cells, antibiotics, and harsh environmental conditions (1, 26). If ingested, spores survive the stomach's acid environment and germinate in the small intestines when stimulated by bile salts (1, 26). Vegetative cells of toxigenic strains produce several toxins. Toxin A and toxin B are most commonly associated with CDI. Toxin A (TcdA) is an enterotoxin, while toxin B (TdB) is a cytotoxin (1).

As vegetative cells produce toxins and spores within the gut, we modeled them together. In order to account for different degrees of spore viability across ribotypes, we separated spores into two compartments: viable (Spl) and non-viable (Spd). SpV represents the fraction of viable spores produced by ribotype. We further accounted for the *C. difficile* sporulation rate (k_{sp}) and the loss of spores (k_{LS}) either because they are shedded into the environment through feces (k_{ExSpl}/k_{ExSpd}) or they germinate into vegetative cells (k_{ger}).

Similarly, in the toxin compartment, we accounted for the toxin production rate by vegetative cells (k_{tox}) and exit of toxin (k_{ExT}), either because it was used up, lost through feces, or decayed. We also incorporated a toxin delay, by a set of slots (n) through which the toxin had to pass before exiting the toxin compartment. The latter allowed us to account for the slow decay of the toxin in the gut/feces (27). The in-host interaction between spores and toxin production is described in the following equations:

$$\frac{dSpd}{dt} = (1 - SpV)(k_{sp}C) - (k_{ExSpd}Spd)$$
$$\frac{dSpl}{dt} = SpV(k_{sp}C) - k_{LS}Spl, \qquad where \quad k_{LS} = k_{ger} + k_{ExSpl}$$
$$\frac{dT_1}{dt} = k_{tox}C - k_{ExT}T_1$$
$$\frac{dT_2}{dt} = k_{ExT}(T_1 - T_2)$$
$$\vdots$$
$$\frac{dT_n}{dt} = k_{ExT}(T_{n-1} - T_n)$$

Submodel Parameter Estimation

Both submodels were fitted using *C. difficile* ribotype 027 data from a gut model reported by Baines et al. 2009 (25), using sum of least of squares. The parameters within the vegetative cells submodel were specifically fitted to the vegetative cells data, using the spore data as inputs. Similarly, the spore/toxin model was fitted to the spore and toxin data, using the vegetative cell data from (25) as an input. For the model inputs, we used linear splines to interpolate between data points, generating a continuous input to each submodel.

Overall model

After individually fitting each submodel, we combined them to generate the overall model representing a CDI host (Figure 5.9). The overall model is described with the following equations (parameter details for ribotype 027 can be found in Table 5.5):

$$\frac{dC}{dt} = kC\left(1 - \frac{C^3}{Cap^3}\right) + k_{ger}Spl - k_{txt}C - (k_{ExC} + k_{sp})C$$
$$\frac{dSpd}{dt} = (1 - SpV)(k_{sp}C) - (k_{ExSpd}Spd)$$
$$\frac{dSpl}{dt} = SpV(k_{sp}C) - (k_{ExSpl} + k_{ger})Spl$$
$$\frac{dT_1}{dt} = k_{tox}C - k_{ExT}T_1$$
$$\frac{dT_2}{dt} = k_{ExT}(T_1 - T_2)$$
$$\frac{dV}{dt} = u(t) - k_V V, \qquad where \quad u(t) = vancomycin \quad input$$

5.2.2. Stochastic Model

As an ODE model can only provide an average of recurrence by ribotype and stochasticity may play an important role during extinction or recurrence of an infection. Thus, we created a stochastic model to estimate the probability of recurrence by ribotype. We focused on three compartments of the overall ODE model: 1) vegetative cells, 2) non-viable spores, and 3) viable spores. As vancomycin concentrations are continuous values and unlikely to be stochastic at the scale we are considering, we used the same ODE representation for vancomycin treatment as in the deterministic model. We simulated the vancomycin concentration by treatment regimen ahead of time using a simple model, which only included the vancomycin equation. The resulting datasets were used to feed our stochastic model.

Due to the large number of vegetative cells and spores that made up our system, we simulated our model using the Tau-leaping method (28). This method is similar to the Gillespie algorithm, but it determines the probability of each event to happen at each pre-specified interval of time, in our case every 2.4

hours (10 times a day). The model estimated the probability of occurrence of 5 specific events: 1) vegetative cell growth, 2) germination of viable spores, 3) exit or death of vegetative cells, 4) sporulation of vegetative cells, and 5) exit of viable and non-viable spores. After each evaluation, the model added or subtracted vegetative cells or spores from their specific compartment as needed. At the end of the simulation, we compiled the total number of vegetative cells and spores (CFU/mL per day).

5.2.3. Simulations

Effect of sporulation rates and viability of spores in CDI recurrence

The most prevalent ribotypes currently circulating in the US are ribotype 027, 002, 014-020, and 106 (22). Thus, we based our simulations on these four ribotypes. Using data from the literature and the fitted parameters of our overall model of ribotype 027 (Table 5.5), we estimated an average rate of sporulation and a fraction of viable spores for each ribotype. For example, using *in vitro* data from the literature, we estimated an average number of spores produced per day per mL of 40,295 for ribotype 002, while for 027 we estimated 109,054 spores per day per mL. As we knew the sporulation rate of 027 from our model fit, we were able to solve for the sporulation rate of ribotype 002 ($k_{sp002}=k_{sp027}/(109{,}054/40{,}295)$. Similarly, we solved for the k_{sp} of the other ribotypes. The fractions of the viable spores per ribotype were averaged from the literature (5, 7, 17, 29-32). The parameters are described in Table 5.6.

Due to the nature of our ODE model, any fraction of vegetative cells or spores (even if much less than a single cell or spore) would lead to microbiological recurrence given enough time to proliferate, which is not consistent with nature. To conservatively control for this issue, we modified our model to indicate that if the vegetative cell or spore compartment had equal or less than 0.01% of one cell or one spore, the particular compartment was equal to zero.

We ran both of our models for a total of 200 days. All model parameters remained the same as in Table 5.5, except for sporulation rates and spore viability, which we modified by ribotype (Table 5.6). On day 13, we added the regular vancomycin treatment (125mg/L four times a day for 10 or 14 days) to our simulation. The stochastic model was run 500 times for each ribotype.

Vancomycin tapered/pulsed-treatment effectiveness against microbiological recurrence

In this case, on day 13, we simulated three commonly used tapered/pulsed vancomycin treatments recommended by the Infectious Diseases Society of America/ Society for Healthcare Epidemiology of America (IDSA/SHEA), and the College of Gastroenterology (ACG). In addition, we included an alternate regimen based on clinical expertise (33) (Figure 5.10).

IDSA/SHEA 2010 recommendations for recurrent CDI include oral vancomycin 125mg/L four times daily for 10-14 days (we simulated 14 days instead of 10, in order to be more conservative), followed by vancomycin twice a day for a week, then vancomycin once daily for one week, concluding with

vancomycin every 48-72 hours for 2-8 weeks (18). Similarly, ACG recommends 125 mg/L 4 times a day for 10 days followed by every 72 hours for ten doses (21). Finally, a recent published review recommends based on expert opinion an alternate regimen described as follows: 125mg/L four times daily for 1-2 weeks, then 125mg/L three times a day for 1 week, followed by 125mg/L twice a day for 1 week, then 125mg/L once daily for 1 week, next 125mg/L every 48 hours for 1 week, and concluding with 125mg/L every 72 hours for 1 week (33) (Figure 5.10).

In addition, we tested the SHEA/IDSA recommended regimens, omitting the initial regular treatment (125 mg/L four times a day for 10-14 days). Similarly, we experimented with the alternate regimen removing the initial regular treatment of oral vancomycin four times a day for 1-2 weeks. We further tested a modified ACG regimen by 1) reducing the number of pulsed doses and 2) reducing the vancomycin dose from 125mg/L 4 times a day to twice a day (Figure 5.10).

Similarly to previous simulations, we modified our ODE model to indicate the same minimal threshold for the vegetative cell or spore compartments. The stochastic model was run 500 times for each ribotype per regimen.

Sensitivity analysis

The recommended vancomycin regimen of 125mg/L four times a day achieves vancomycin fecal levels several hundred times higher than the vancomycin MIC_{90} for *C. difficile* (24). Thus, our model's ability to fit the vancomycin-killing rate is limited, as the vancomycin regimen kills all *C. difficile*

vegetative cells very quickly. In order to assure a proper fitting of this parameter, we ran our stochastic model using an upper (1.8) and lower threshold (1.3) of k_{txt} values. We chose these thresholds because they were the largest and smallest values that yielded a visually nearly indistinguishable fit to the data. We ran our simulations 1000 times with these values to validate our model and guarantee its agreement with available literature on recurrence rates.

5.3.0. Results

5.3.1. Overall Model

Our overall model simulates the pathogenicity and transmission patterns of *C. difficile* within its human host (Figure 5.9 and 5.11). The final model parameters are described in Table 5.5 and 5.6.

5.3.2. Simulations

Effect of sporulation rates and viability of spores on CDI recurrence

As expected, sporulation rates and viability of spores affected pathogenicity and transmission patterns *in vivo* (Figure 5.12). Using the deterministic model, all ribotypes but 014-020 recurred after 10 days of 125 mg/L four times a day. Ribotype 014-020 was able to cause a first CDI episode, but treatment was enough to control the bacteria within the gut and avoid a

recurrence. By contrast, ribotypes (027, 106, 002) were able to recur approximately 30-55 days after initial CDI treatment. Notably, the levels of spores were quite diverse. For example at day 55, spore levels ranged from 626 to $4.4x10^4$ CFU/mL. However, when we extended the regular treatment to 14 days, ribotype 002 was also unable to recur.

In our stochastic model, all of the ribotypes recurred in at least one simulation after 10 days of treatment. However, the probability of recurrence varied by ribotype: ribotype 106 (18.8%), ribotype 027 (12.2%), ribotype 002 (6.8%), and 014-020 (1.0%) (Figure 5.12). This matches the more binary results seen in the deterministic model, with a more nuanced outcome. Similarly, when we extended treatment 4 extra days (14 days total), the probability of recurrence was reduced even further: ribotype 106 (4.0%), ribotype 027 (4.0%), ribotype 002 (1.2%), and 014-020 (0%) (Figure 5.12).

Vancomycin tapered/pulsed-treatment effectiveness against microbiological recurrence

All of the recommended vancomycin tapered/pulsed regimens for treating repeated CDI were effective in avoiding recurrence from all ribotypes. Moreover, 2 weeks of vancomycin pulses (either at 48 or 72 hours) as part of the SHEA/IDSA regimen were enough to avoid recurrence. Similarly, one week of the regular oral vancomycin regimen (125mg/L four times a day) at the beginning of the simulated alternate regimen was sufficient to avoid future relapses (Table 5.7).

We proceeded to perform further modifications to the recommended regimens. For the SHEA/IDSA regimen, when we completely skipped the initial regular treatment (125mg/L four times a day for 14 days), we observed the regimen was totally effective for all ribotypes after only four weeks of pulsed doses. However, this modification with only two weeks of pulsing every 48 hours was effective for all but ribotype 027, which recurred 0.2% of the time. Interestingly, if instead we pulsed every 72 hours for 2 weeks, both ribotype 027 and ribotype 106 recurred but also at low levels (0.2-0.6%). Similarly in the alternate tested regimen, when we eliminated the 2 initial weeks of 125mg/L of oral vancomycin four times daily, we still did not observe any recurrences for any of the ribotypes. When we reduced the ACG pulsed doses to 7 (every 72hours), the regimen remained effective at avoiding recurrence from all ribotypes. Furthermore, when we reduced the dose to 125mg/L twice a day for 10 days followed by pulses every 72 hours for 10 doses; the treatment was still effective, with only ribotype 27 recurring, and at a very low rate (0.2% recurrence) (Table 5.7).

Sensitivity analysis

To assess the sensitivity of our assessments, we ran the same simulations but modified the killing rate by an upper or lower threshold, which yielded nearly identical results (recurrence rates with the alternate values were all within 2.5% of the recurrence rates using the fitted value of k_{txt}).

5.4.0. Discussion

We used a compartmental in-host mathematical model for CDI patients to simulate *in vivo* toxin and spore rates for the most prevalent *C. difficile* ribotypes and evaluate whether ribotype-specific sporulation/germinations patterns affect CDI recurrence and effectiveness of treatment regimens for reducing risk of repeated recurrence. All the recommended treatment regimens were effective in reducing risk of an additional recurrence. Furthermore, reducing the duration or dosage of most of the assessed regimens did not change effectiveness.

Our simulations confirm that differences in sporulation/germination patterns across *C. difficile* ribotypes are risk factors for recurrence. Ribotype 014-020 has higher spore viability but lower sporulation rates than the other ribotypes evaluated, and recurred up to 1.0 % of the time following initial CDI treatment with vancomycin, much less frequently than other ribotypes. Indeed, although this ribotype does recur, it accounts for only 5% of CDI recurrences in North America (34). Moreover, these results suggest that the CDI recurrence rate can be explained by differences in sporulation and germination patterns by ribotype, which also validates the model's predictive ability. In addition, our model highlights the benefit of a longer initial CDI treatment, that is 14 days instead of 10 days, in order to reduce the likelihood of an initial CDI recurrence.

If we consider the reported distribution of ribotypes found in hospital and community-associated CDI cases (22) and our recurrence rates, ribotypes 027, 002, 014-020, and 106 would lead to a combined recurrence rate of 3-11% (depending on the duration of initial treatment), which overlaps with the recurrence range of 5-35% reported in the literature (8-10). Nevertheless, the 5-

35% range includes relapses and reinfections by the same or different strains, and our estimate includes only relapses from the same strain. Relapses reportedly account for 25 to 87.5% of all recurrences (14) (i.e. there is a ~1-31% rate of relapse among initial CDI cases), which is also consistent with our results.

In our models, all the recommended treatment regimens for repeated CDI recurrence were highly effective in avoiding an additional recurrence, which is validated by the fact that only up to 6% of CDI cases recurred 2 or more times (35). Our models also suggest that there is no significant difference in treatment efficacy between pulsing periods of 48 or 72 hours when applied to the regimens as they are currently recommended. However, the regimens could be reduced in duration or in dosage and still be highly effective.

This finding has potentially important clinical implications, as vancomycin therapy is not without risk. Vancomycin treatment suppresses *Bacteriodes* spp., a marker of normal gut microbiota (36), and a healthy gut microbiota prevents the introduction or colonization of pathogens, including the reemergence of *C. difficile* (37). Furthermore, vancomycin and similar anti-anaerobic therapy can promote the selection and spread of vancomycin-resistant enterococcus colonization (37, 38). Our results suggest that vancomycin regimens might be further modified to a level that better protects gut microbiota while preventing CDI recurrence, although this can only be definitely answered using appropriate controlled clinical trials. In addition, studies examining the role of probiotics in conjunction with tapered/pulsed vancomycin may uncover potential regimens to reduce the vancomycin dosage and duration even further.

Similarly, our model results support the potential of tailoring initial CDI treatment based on the causative *C.difficile* ribotype. Patients diagnosed as

having CDI due to ribotype 027 are more susceptible to recur; thus, physicians may decide to follow these patients more closely to early identify CDI recurrence. Moreover, these patients may benefit from an initial tapered/pulse oral vancomycin treatment instead of the regular higher dosed treatment usually given as CDI initial treatment. However, it is essential to consider host risk factors and biological markers in conjunction with CDI ribotype to better customize CDI treatment.

As with any simulation study, our model is limited by the current state of knowledge and data available to inform the model. *C. difficile* vegetative cells, spores and even toxin are found among asymptomatic individuals (39); thus our model could not distinguish between *C. difficile* colonization and symptomatic CDI recurrence. We did not include the effects of host factors that might directly influence *C. difficile* sporulation and toxin production in our model, as there is limited *in vivo* human data available. Similarly, our model does not account for biofilm formation within the human gut, which could potentially decrease CDI treatment effectiveness and increase CDI recurrence (40). In addition, our models do not account for inter-strain variation of sporulation and germination patterns across ribotypes, which have been observed in *in vitro* and gut models (7, 25, 30, 31, 41, 42). Finally, this study does not include other potential recommended treatments for recurrent CDI, such as fidaxomicin (33).

In conclusion, we developed a compartmental in-host mathematical model of CDI. Our results highlight the importance of sporulation/germination patterns across *C. difficile* ribotypes on CDI pathogenicity and transmission, which directly affects CDI treatment and infection control. Current CDI vancomycin regimens particularly for treating recurrent cases should be further

studied to better balance their associated risks and benefits. More broadly, this model provides a tool to explore the role of the human host in *C. difficile* colonization, infection, recurrence and transmission.

Table 5.5. Final overall model parameters for ribotype 027. These parameters were used to feed our simulations for all ribotypes, the sporulation rates (k_{sp}) and fraction of viable spores (SpV) were the only parameters that were modified based on the ribotype across our model simulations.

Model Parameter	Description	Value
N	Number of toxin delay compartments	2
K	*C. difficile* growth rate (cells/day)	1.1953
Cap	Carriage capacity (cells/day)	1.2241×10^6
k_{sp}	Sporulation rate (1/day)	0.0072
SpV	Fraction of viable spores (1/day)	0.534050
k_{ger}	Germination rate (1/day)	0.0006
k_{tox}	Toxin production rate (1/day)	0.0043
k_{ExC}	Exit rate of vegetative cells (1/day)	0.0352
k_{ExSpd}	Exit rate of dead spores (1/day)	0.3577
k_{ExSpl}	Exit rate of alive spores (1/day)	0.3197
K_{ExT}	Exit rate of toxin (1/day)	0.3164
K_{txt}	Treatment killing rate (vc killed/day)	1.5811
k_v	Exit rate of vancomycin (1/day)	1.3116
u(t)	Vancomycin input	Simulation dependent

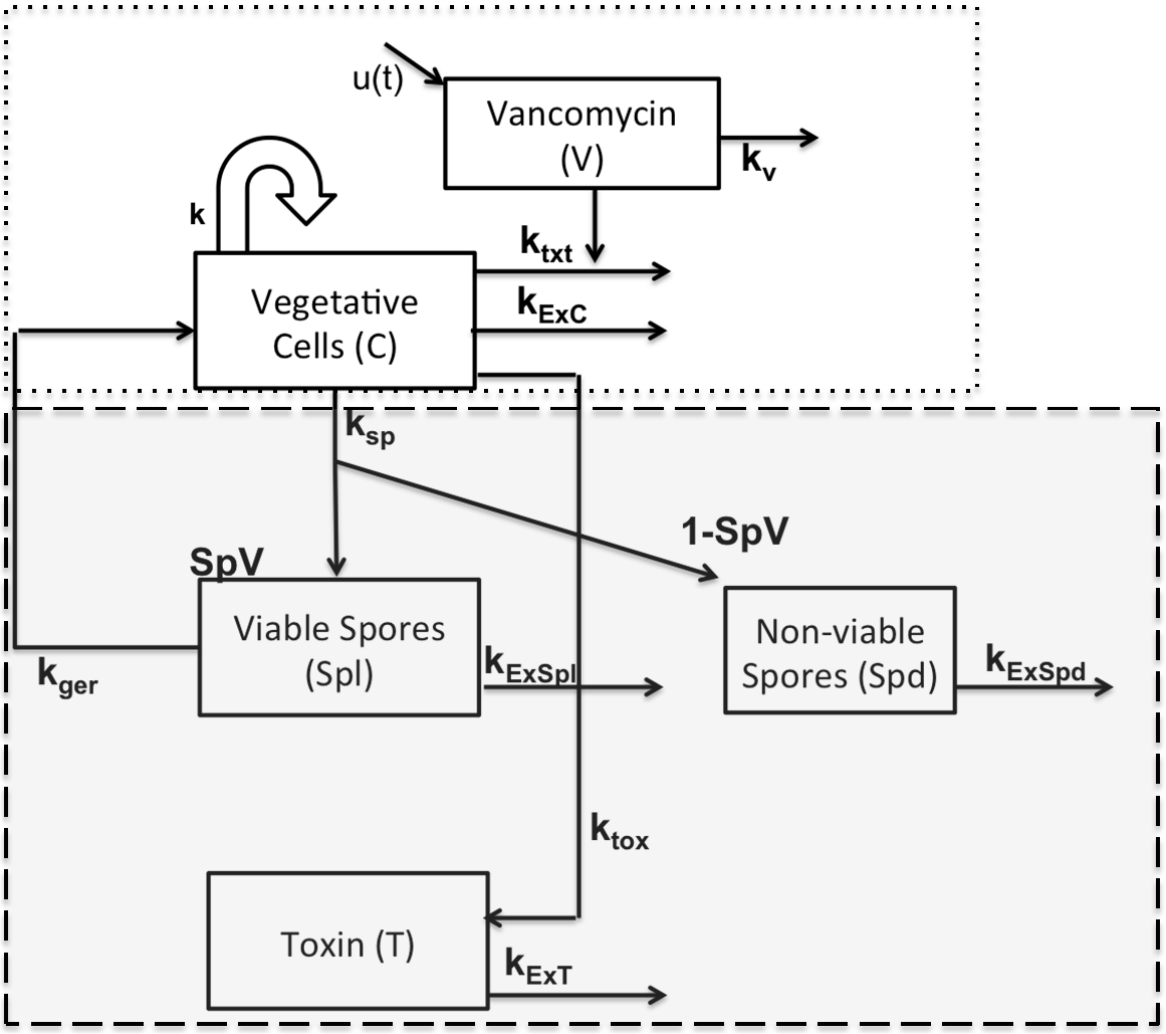
Table 5.6. Sporulation rates, spore viability, and data source by selected ribotypes used to inform the in-host model of *Clostridium difficile* infection

Simulation Parameters			
Sporulation rates by ribotype	Spores/day per mL	Sporulation rate ((spores/day)/mL)	Sources used
027	109,054		(5, 7, 29, 30, 32)
002	40,295	0.002665654	(7, 30)
106	184,555	0.012208954	(7, 30)
014-020	2,773	0.000183444	(5)
Spore viability by ribotype	Fraction of spore viability		Sources used
027	0.5341		(5, 17, 31)
002	0.4700		(31)
106	0.4151		(17)
014-020	0.6276		(5, 31)

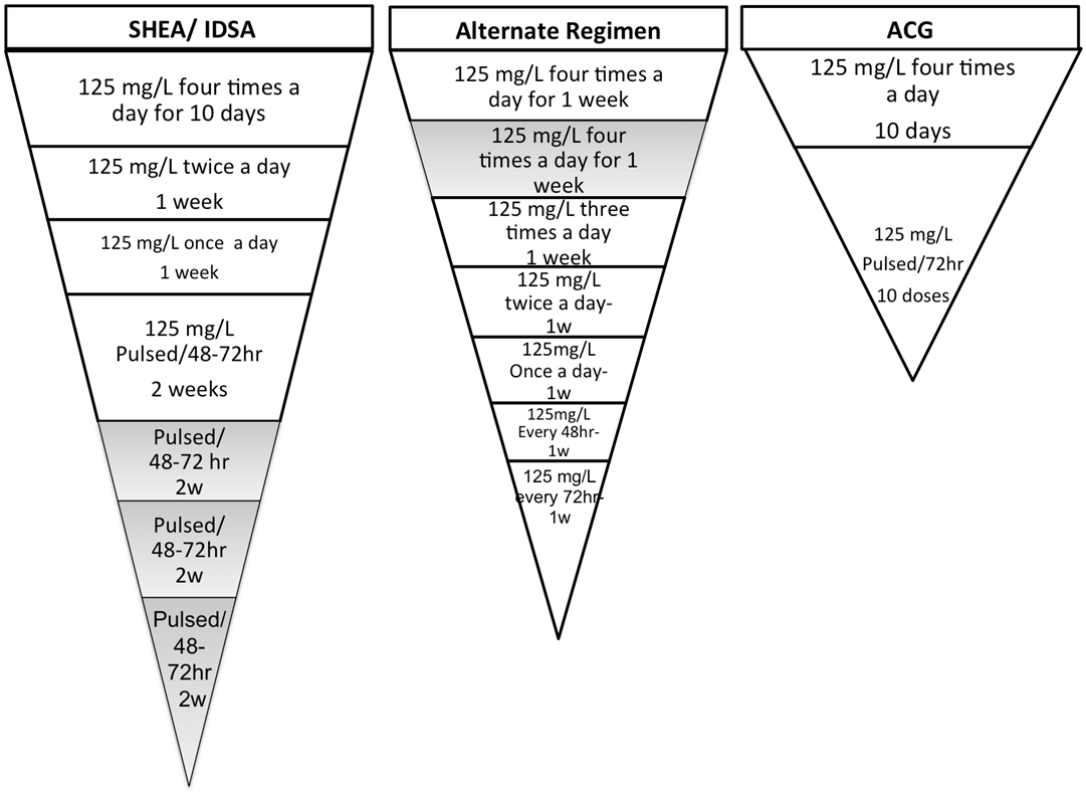
Table 5.7. Recurrence rate by ribotype after receiving recommended or modified oral vancomycin tapered/pulsed regimens.

Regimen	Ribotypes			
	106	027	002	014-020
Commonly used regimens				
SHEA/IDSA: pulses every 48h for 2 weeks	0%	0%	0%	0%
Alternate regimen: pulses every 72hr for 2 weeks	0%	0%	0%	0%
Alternate regimen: with only 1 week of 125 mg/L four times a day	0%	0%	0%	0%
ACG: Full regimen	0%	0%	0%	0%
Modifications				
SHEA/IDSA: without initial regular txt + 72hr pulses for-2weeks	0.6%	0.2%	0%	0%
SHEA/IDSA: without initial regular txt + 48hr pulses for 2 weeks	0%	0.2%	0%	0%
SHEA/IDSA: without initial regular txt + 72hr pulses for 4 weeks	0%	0%	0%	0%
SHEA/IDSA: without initial regular txt + 48hr pulses for 4 weeks	0%	0%	0%	0%
Alternate regimen: without initial regular treatment (125 mg/L four times a day for 2 weeks)	0%	0%	0%	0%
ACG: 125mg/L twice a day+10 pulsed doses	0%	0.2%	0%	0%
ACG: 125 mg/L 4 times a day + only 7 pulsed doses	0%	0%	0%	0%

Figure 5.9. Graphical representation of the overall in-host compartmental CDI model within its human host. Upper rectangle (dotted-lines) represents the vegetative cells submodel. Lower rectangle (dashed-lines) represents the spore/toxin submodel.



A. Recommended regimens



B. Modified regimens

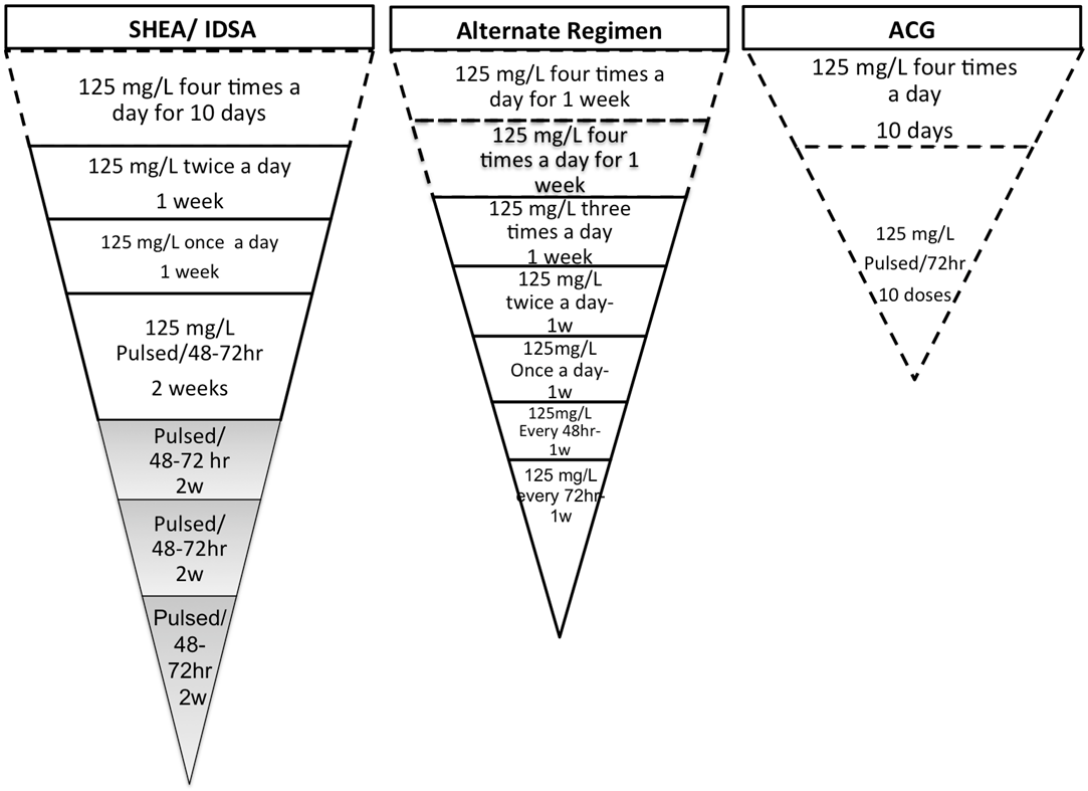


Figure 5.10. Graphical representation of tested A) recommended tapered/pulsed oral vancomycin regimens and B) modified recommended regimens. We tested three specific regimens: one recommended by The Society for Healthcare Epidemiology of America (IDSA/SHEA), another by the American College of Gastroenterology (ACG), and the third one is an alternate regimen based on expert opinion (33). The gray-colored blocks represent optional steps, and the dotted-lined blocks represent the steps that were eliminated or modified for the purposes of this study.

Abbreviations: w=week, hr= hour

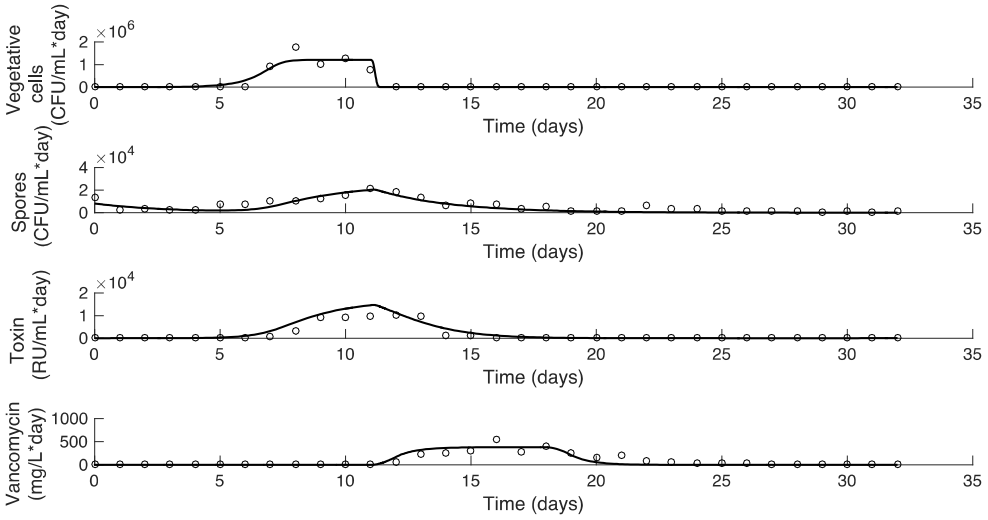
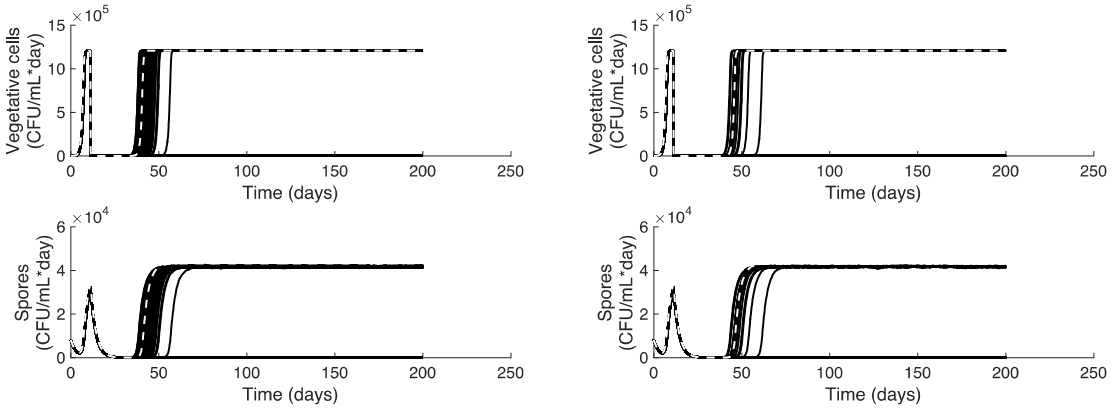


Figure 5.11. Fitting of overall in-host compartmental model of CDI within the symptomatic host (Ribotype 027). Circles indicate the data used for fitting (25) and solid line is the fitted model.

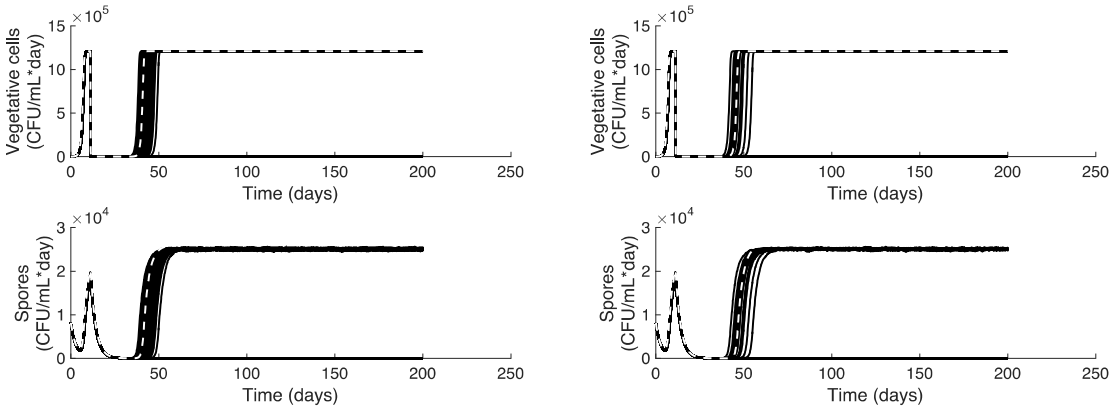
Treatment duration

10 days **14 days**

A. Ribotype 106 (4.0-18.8% recurrence)



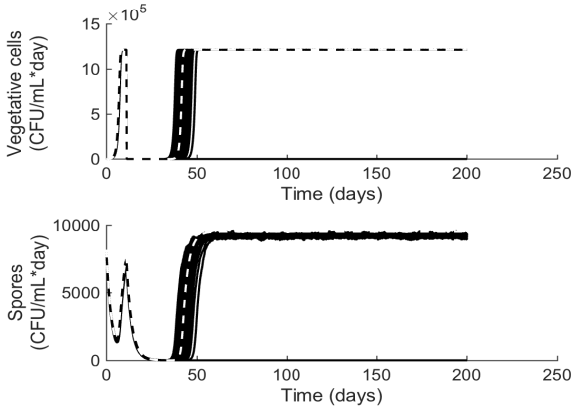
B. Ribotype 027 (4.0-12.2% recurrence)

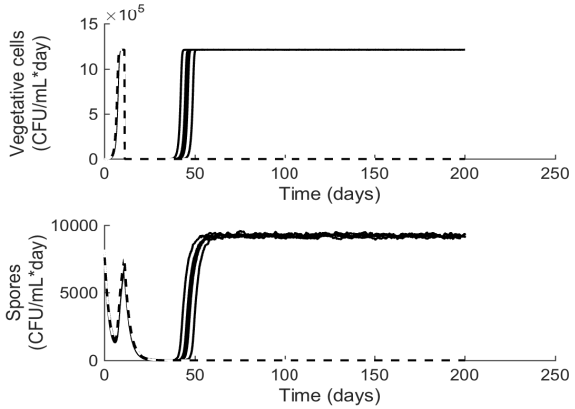


Treatment duration

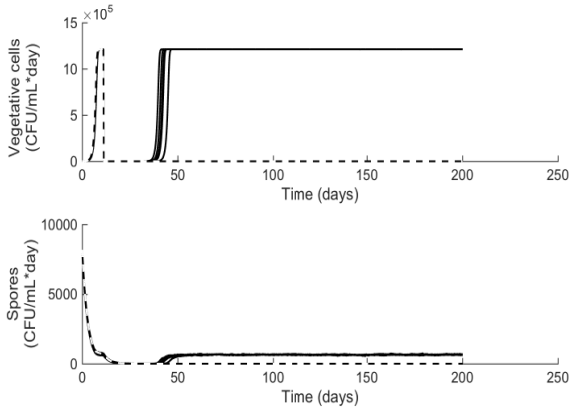
10 days **14 days**

C. Ribotype 002 (1.2-6.8% recurrence)





D. Ribotype 014-020 (0-1.0% recurrence)



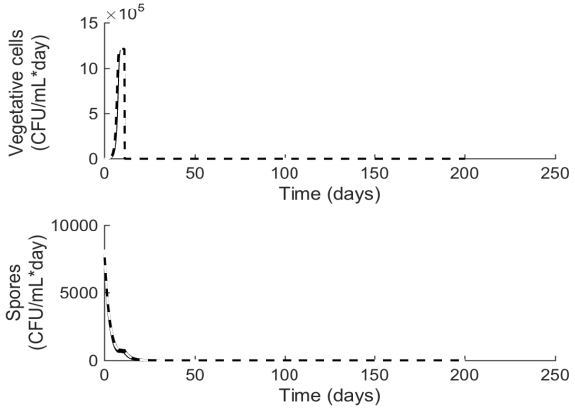


Figure 5.12. Simulated recurrence rate by ribotype: differences on sporualation rates and fraction of viable spores per ribotype are reflected on differences on recurrence rates. On day 13 post infection, we added to the model the regular vancomycin CDI treatment :125mg/L four times a day for 10 days (left) or 125mg/L four times a day for 14 days (right). The black solid lines represent each of the 500 runs of the stochastic model, and the white slashed-line represents the results of our deterministic model.

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CHAPTER VI

Conclusions

Clostridium difficile is indeed a difficult organism. Although the last decades have increased understanding of its biology, pathogenicity, and transmission, *C. difficile* remains one of the most reported causes of nosocomial infection in the United States and around the world. Furthermore, *C. difficile* infection (CDI) has spread to community settings and now infects individuals previously considered low-risk.

This dissertation provides new insights into the pathogenicity and transmission patterns of *C. difficile* within its human host. Although much research has been done on CDI, much of the research has used animal or gut models. Research using clinical samples directly without the intermediate '*in vitro*' component to approximate the sporulation and toxin production of *C. difficile* isolates is less common. The primary goal of this dissertation was to provide *in vivo* CDI insights and to apply them to three specific areas: 1) infection control and prevention, 2) diagnosis, and 3) treatment.

The main goal of our first study was to characterize the *in vivo* association of *C. difficile* spore and toxin production and their association with the presence of *Candida albicans*. We observed a strong positive association between *C. difficile* toxin and spore levels, with the association varying slightly by ribotype. Consistent with this finding, toxin negative/PCR positive stool samples had

significantly fewer spores than toxin positive CDI ones. However, contrary to our hypothesis, the presence of *C. albicans* did not modify these associations in our study population. Nevertheless, ribotypes 027 and 078 were significantly associated with high levels of toxin and spores and *C. albicans* overgrowth in the gut.

The absence of a *C. difficile* spore-toxin trade off within its human host suggests that spore production may enhance pathogenicity as well as transmission. This conclusion has important implications for CDI infection control and prevention. A spore-toxin association implies that more severe cases, which are expected--although this is still controversial-- to have larger amounts of toxin, will also be shedding larger amounts of spores. This highlights the importance of timely CDI patient isolation. Additionally, it suggests that extra precautions should be taken when dealing with CDI patients in the ICU or other high-risk settings.

Our second study aimed to better understand the clinical and epidemiological differences between CDI patients according to their laboratory diagnosis. We investigated if the differences that previous authors had reported were maintained among a population with high CDI treatment rates. Like previous investigators, we observed that toxin negative PCR positive patients had a less severe CDI presentation than toxin positive patients. In addition, we identified a strong association between white race and a toxin positive CDI diagnosis, which was maintained even after controlling for known CDI risk factors. Providing further evidence to support these differences by CDI laboratory diagnosis confirms the need to revise and standardize the current laboratory algorithms in the US.

In this same study, we also sought to identify host-risk factors associated with fecal spore levels. Spore levels were strongly associated with CDI severity and known CDI risk factors such as previous history of antibiotics, proton pump inhibitors, and previous hospitalization. These results support the conclusions from our first study, that the more severe CDI presentation, the more spores are shed. Healthcare workers should be aware that these patients are more infectious and that already established precautions should be followed strictly and even heightened when dealing with patients with severe CDI.

In our third study, we aimed to develop an in-host mathematical model to simulate the *in vivo* toxin and spore levels within its human host. We created both a deterministic and stochastic version of a compartmental model to determine the role of sporulation and germination patterns in recurrence and the effectiveness of current tapered/pulsed vancomycin regimens by ribotype. All of the evaluated treatment regimens for repeated CDI were effective in reducing risk of an additional recurrence, with the effectiveness varying by ribotype, and thus by their sporulation and germination patterns. Furthermore, treatment effectiveness was maintained even when the duration or dosage of most of the assessed regimens was reduced. These results highlight that tapered/pulsed vancomycin regimens might be further modified to a level that better protects gut microbiota while preventing CDI recurrence.

These results suggest several lines for future research. Future laboratory studies should elucidate how spore and toxin production are linked in *C. difficile* and identify bacterial and host signals regulating their productions *in vivo*. Clinical studies are needed to improve our understanding of the role played by asymptomatic carriers in healthcare and community settings. Prospective

longitudinal studies are warranted to clarify the significance of asymptomatic carriers in CDI transmission. Our in-host mathematical model could be modified to further elucidate the role of asymptomatic carriers. In addition, more investigation is necessary to identify if there is a racial/genetic predisposition to CDI or if race is acting as a proxy of a group of other independents CDI predictors. A larger, more representative study that incorporates racial groups other than whites and blacks, and includes further data such as ethnicity and nativity, will be required. Furthermore, a cost-effectiveness analysis of the use of molecular tests as part of the CDI laboratory diagnosis should be performed. This study is essential for estimating the economic, social, and clinical impact of including PCR as part of the diagnosis. Finally, further controlled clinical trials are necessary to improve CDI treatment regimens to minimize CDI recurrence and to lessen gut microbiota disturbance.

Collectively this dissertation demonstrates the importance of considering the pathogen and the human host together when researching CDI. In our first study, we demonstrated that the toxin-spore correlation *in vivo* is different than the one previously observed *in vitro*. Similarly, in our second study, we identified the importance of the patient's clinical presentation when receiving a toxin negative PCR positive result. Finally, in our third study we demonstrated the importance of *C. difficile* sporulation and germination patterns for CDI recurrence and thus, for the effectiveness of CDI treatment. Thinking of CDI as a multifactorial disease promises to be an effective path towards managing, and controlling and preventing CDI.