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Determining effective cycle thresholds for evaluating colonization versus true infection of *Clostridium difficile* using Xpert assay

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Abstract

Clostridioides difficile is an anaerobic, spore-producing bacterium and is the most common cause of infectious diarrhea in healthcare settings (Lee, 2019). All strains of *C. difficile* carry the toxin gene; however, only some actually produce toxins, making detection via PCR challenging to differentiate between asymptomatic colonization and symptomatic disease. Therefore, the objective of this study was to explore a current PCR technique, Xpert *C. difficile* assay, to determine a more precise protocol for doctors to predict true *Clostridioides difficile* infection (CDI) in a clinical setting. Using previously collected data from 45 PCR- positive CDI patients over spring, 2019 from a New York metropolitan hospital, PCR products of *C. difficile* toxins A and B were analyzed and compared to their respective patient's symptoms, which were assessed for severity. Overall, these severity scores showed statistically significant differences, as demonstrated by their associated amplification of the toxin genes, for each of the three conditions (unlikely, mild, and severe infection). Interestingly, a significant relationship was also found between the level of one specific indicator, binary toxin, and severity of infection, corroborating the work of previous research that associated the NAP1/ribotype 027 strain of *C. difficile* specifically with binary toxin. Overall, our results point to quantifiable statistically significant distinctions via a methodology that uses measured PCR cycle thresholds to distinguish between colonization and true infection. Ultimately, timely and accurate diagnoses of true CDI may lead to more efficient treatments as well as the potential mitigation of antibiotic resistance to *Clostridioides difficile*.

Keywords: Cycle threshold values, binary toxin

Introduction

In 2017, 223,900 patients with *Clostridioides difficile* infection (CDI) were hospitalized, resulting in 12,800 deaths in America (Clostridioides, 2019). Moreover, 82% of reported cases are diagnosed as community-acquired CDI each year (Clostridioides, 2019), meaning the patient has symptoms within 48 hours of admission to a hospital or 12 or more weeks after discharge (Kim & Zhu, 2017). Thus, CDI is a continuous, large-scale threat to the healthcare system around the globe. Additionally, *C. difficile* is one of the leading causes of both morbidity and mortality in several countries, including America (Balsells et al., 2019). It is estimated that over 40,000 cases go

undiagnosed in Europe every year (Balsells et al., 2019).

C. difficile is an anaerobic, spore-producing bacterium, which is the most common cause of infectious diarrhea in healthcare settings (Lee, 2019). *C. difficile* strains are harmful to the gut microbiome once they begin producing toxins, including toxin A, toxin B, or binary toxin. Binary toxin is regulated by different toxin gene(s) "cdt" and is therefore not a combination of toxins A and B. However, some strains of *C. difficile* may be carried in negligible amounts, and not produce a toxin by-product, henceforth not indicating true infection. The issue is that common tests used for diagnosis, such as Polymerase Chain Reaction (PCR), are often not able to differentiate between colonization (having *C. difficile* bacteria which do not produce toxins at all) versus true infection (toxin production).

CDI is currently detected by a wide array of techniques, including PCR which isolates bacterial DNA, rapid diagnostic tests based on antigen detection (RDTs), as well as immunoenzyme assays (EIAs) that indicate overall bacterial presence. PCR detects bacterial DNA for the toxin genes, RDTs detect *C. difficile* proteins, and EIAs detect toxins. However, these tests can be either over or under sensitive, leading to overdiagnosis or underdiagnosis of CDI (Polage et al., 2015). Additionally, these inaccurate tests can be costly and waste time. Thus, this current study will specifically utilize PCR, to amplify the toxin gene, which can reflect bacterial load as well, of the patient's sample to hopefully find a correlation between the toxin production in the stool and the severity of the patient's symptoms prior to testing.

One PCR-based diagnostic tool is a Xpert *C. difficile* assay. When amplifying the toxin B gene using Xpert *C. difficile* assay, labs obtain a cycle threshold value, a quantitative value that identifies the number of times the PCR had run to identify a patient's stool sample as positive for CDI. Cycle threshold values are important because they indicate the quantity of the toxin B gene in proportion to the time necessary to pass the threshold for the PCR test to come back positive for *C. difficile* bacterium. Therefore, the less time it takes for the PCR machine to amplify the toxin gene, the greater amount of toxin present in the sample and subsequently, the lower the cycle threshold value. Therefore, we will be investigating these cycle threshold values by comparing them to the patient's respective symptoms to better understand how to make a conclusive CDI diagnosis. The end goal is to establish quantifiable data to define specific cycle threshold values that correspond to true infection.

In addition to testing, symptoms are assessed for diagnosis of true infection. Indicators for CDI may include clinically significant diarrhea, cramping, fever and nausea; however, many of these potential symptoms are shared between CDI and other gut microbiome infections such as *E. coli* and bacterial diarrhea caused by parasites in developing countries (Hodges & Gill, 2010). Utilizing symptoms and PCR allows for a diagnosis to be made, yet potential CDI patients are not always tested for infection.

Overall, the goal of the study was to correlate symptoms of unlikely, mild, and severe CDI with the respective patient's cycle threshold values. This will enable us to attempt to establish a site-specific cycle threshold cutoff value, clearly allowing physicians to diagnose CDI.

Review of Literature

When a patient contracts *C. difficile* and the bacteria produce toxins, a wide range of symptoms may occur, such as abdominal pains, diarrhea, fever, cramping, ileus (obstruction of the ileum), megacolon (abnormal dilation of colon), sepsis, septic shock, an increased white blood cell, and an increased blood creatinine level (Lee, 2016). In 2017 alone, an estimated 223,900 CDI patients were hospitalized (Clostridioides, 2019). Further, CDI extends inpatient hospital stays by 2.3 to 12 days, increasing the overall financial burden from $2,454 to $27,160 per case (Clostridium, 2017). If a patient develops symptoms, it is important they be tested for CDI as quickly as possible, to insure effective treatment.

Symptoms become prevalent once *C. difficile* bacteria produce toxins. The literature has shown when patients exhibit only toxin A in stool samples, symptoms often do not occur (Carter, Rood & Lyras, 2009). However, if a patient begins to produce toxin B, they may display indicators of true infection (Carter, Rood & Lyras, 2009). In fact, if a patient produces binary toxin, they display the greatest number of symptoms as well as the most severe infection (Cowardin, 2016). However, there are issues with diagnostic tests as tests may be over-sensitive and lack precision.

Faults of Current Tests

There may be several reasons why someone might not be accurately diagnosed. One reason may be due to the nature of test results; Xpert *C. difficile* amplifies toxin genes not the actual toxins, which in turn may not indicate gene expression (Babady et al., 2010). Thus, if patients are not truly positive for a virulent infection misdiagnosis can have detrimental outcomes. First, treatment drugs could destroy the healthy microbiome and secondly, misuse of antibiotics may prohibit further use of these drugs again (Zhang & Chen, 2019).

There are many issues with testing (PCR Troubleshooting Guide, N.d.). First, patients do not get tested when they should. One example of a lack of testing may be when a patient is asymptomatic and left untreated. Second, those who are tested may be overdiagnosed. For example, a patient may be diagnosed with CDI when negligible amounts of *C. difficile* bacteria, which do not produce toxins, are in the patient's gut. The reason why this problem can occur is because PCR amplified the toxin gene (Babady et al., 2010). This current study will explore the use of PCR in a hospital in New York City.

Need for Site-Specific Threshold Values

Ideally, a site-specific hospital threshold can be developed (Reigadas et al., 2016). This value can be obtained by comparing individual threshold values in respect to the symptoms necessary to indicate true CDI amongst patients admitted to the hospital who have the infection. In this current study, cycle threshold values will be compared in respect to patients' symptoms, which indicate varying levels of severity. Ultimately our study will help to establish a statistically significant way to obtain diagnoses that are reflective of the patient's cycle threshold values and symptoms, culminating in a research path for future scientists to obtain a site-specific threshold value at NYU Langone Hospital.

Diagnostic Situations

The cycle threshold value established in this study must be site-specific because hospitals are known to handle diagnostic situations differently from one another (Freeman et al., 2010). Slight changes in diagnostic situations can be the temperature samples are stored at (36-46 degrees Fahrenheit) and for how long these samples are stored for (overnight versus hours after collection) (Clostridium difficile, N/d). It is imperative to note these varying conditions as they are small differences that may affect the accuracy of PCR assays and other CDI tests.

Overdiagnosis and Underdiagnosis of CDI

According to Polage et. al in 2015, out of the 21% of adults who were hospitalized due to a positive PCR test result for CDI, only 44.7% of these patients had toxins detected via a molecular test. Hence, to reduce overdiagnosis (colonization) or underdiagnosis (true infection) of CDI, distinct cycle threshold cutoff values need to be determined for individual hospitals and long-term care facilities (Polage et al., 2015).

Rationale of Study

Firstly, 45 de-identified patients admitted in the spring of 2019 for potential CDI at a leading hospital will be sorted into three categories, "severe *C. difficile*", "mild *C. difficile*", and "unlikely *C. difficile*", based on their symptoms prior to testing. Secondly, a patient's diagnosis will be compared to Xpert *C. difficile* assay's cycle threshold values to determine relationships between these values and severity scores. Overall, this study aims to create a site-specific cycle threshold cutoff value that can potentially accurately detect true CDI. If a specified unique value can be determined that is able to predict true infections, then diagnostic testing for *C. difficile* can be more precise and accurate at this

specific hospital and the protocol can then be implemented by other specific locations.

Objectives

This study has four objectives. The first one is to utilize a risk stratification chart and categorize patients into three categories: "unlikely *C. difficile*", "mild *C. difficile*", or "severe *C. difficile*". The next one is to categorize patients based on cycle threshold values of Xpert *C. difficile* assay, to later compare if each diagnostic group has a statistically significant mean cycle threshold value. The third one is to compare cycle threshold values and the severity of true infection indicated by patient's symptoms. The fourth and final one is to compare overall symptoms and diagnostic category of patients with each level of infection.

Hypotheses

H_0: There will be no significant difference between the cycle threshold values of patients with likely CDI (severe and mild) and unlikely CDI.

H_1: A more accurate diagnosis of severe *C. difficile* infection can be made by comparing PCR cycle thresholds for patients with symptoms highly compatible with *C. difficile* infection versus those who do not have significant symptoms.

Methodology

Subjects

Data came from 45 subjects who were recruited from 14 different NYU laboratories or hospitals. Testing was initially performed on these patients as they either demonstrated signs and symptoms of CDI or if they didn't have new diarrhea, had inflammatory bowel disease (ulcerative colitis and Crohn's disease), because CDI positivity has prognostic significance. Not all patients had data for each of the symptoms. Therefore, for an unknown symptom a value of zero was imputed into their chart.

Risk Stratification

In order to characterize patients as either having severe CDI, mild CDI, or unlikely for CDI, symptoms were evaluated and scored utilizing a four-step method in the form of a risk stratification chart. Point values zero to three were added for each respective symptom (Figure 1). A three indicated that the symptom is a strong indication of infection, a two indicated the symptom is essential in the diagnosis of infection, and a zero indicated the symptom most probably did not influence the diagnosis of an infection.

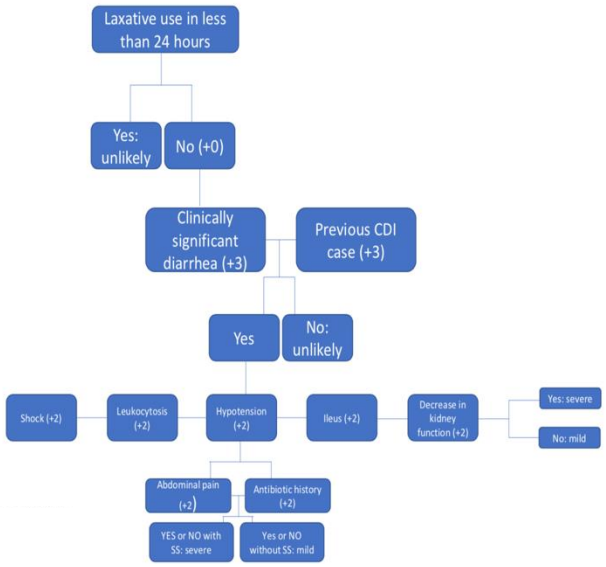

FIGURE 1. Risk stratification chart.

Firstly, there was an evaluation determining if a patients had taken a laxative within 24 hours of testing. The second step of this method was to evaluate if a patient had clinically significant diarrhea (liquid stool documentation and/or three bowel movements in less than 24 hours of testing) and/or if the patient had a past record of CDI. If either of these two questions pertained to the patient, they were automatically characterized as likely for CDI and three points were added to a patient's severity score for each of these symptoms. The third step was to identify if a patient had either sepsis, shock, hypotension, a white blood cell count greater than 15,000, and/or a blood creatinine value greater than 1.5. For each of these symptoms, another two points were added to the patient's severity score. Additionally, if a patient displayed any of these five symptoms in addition to clinically significant diarrhea, they were assumed to be severely infected. Thus, if a patient solely had clinically

significant diarrhea and/or recurrence, they were assumed to be mildly infected.

The fourth, and final step of this method was to evaluate if a patient had a past antibiotic history and/or if abdominal imaging was performed. For each of these indicators of true CDI, another two points were added to the patient's severity score. If these two symptoms were displayed without the five mentioned in the previous paragraph and the patient had clinically significant diarrhea and/or recurrence, then the patient was characterized as mildly infected. Conversely, if these symptoms were in union with clinically significant diarrhea and/or recurrence and at least one of the five symptoms mentioned in the previous paragraph, then the patient was characterized as severely infected. For instance, a patient with clinically severe diarrhea and sepsis would have a severity score of five. For statistical analyses, alpha was set at 0.05.

Results

An average difference between the cycle threshold values of patients with likely CDI and unlikely CDI was identified (Table 1). There was no statistically significant difference among the three groups (unlikely, mild, severe) for their respective cycle threshold values ($p=.382$). Thus, we fail to reject our null hypothesis. However, it was observed that mean cycle threshold values per category increases as severity of infection decreases (Table 1). A T-test was conducted to compare levels of binary toxin in patients who had likely infection (severe or mild) compared to those with unlikely infection (Table 2). In this case, there was a statistically significant difference in the amount of binary toxin in these two patient groupings ($p<0.05$), indicating that patients in the likely groups had a greater average mean binary toxin levels. Further, T-tests were run to compare severity scores (including and not including abdominal imaging) for likely infected patients compared to unlikely infected patients. The reason why some patients had abdominal imaging performed and others did not is because this procedure is not routinely recommended. Abdominal imaging is usually recommended if there is concern for severe disease, as it can identify ileus and megacolon. Statistically significant relationships were found between the severity groupings and severity score. In simpler terms, the greater severity scores a patient has, the more severe their infection is.

ANOVAs were run across the three categories (unlikely, mild, and severe) including and not including abdominal imaging ($p<0.05$) (Table 3). T-tests were also conducted to compare cycle threshold values and each individual severe symptom (such as a raised white blood cell count and septic shock) (Table 4). No statistically significant relationships were found between a certain severe symptom and its correlation to a more severe infection ($p>0.05$).

TABLE 1. Cycle threshold values for each of the three categories of truly infected patients.

Group	Average	Variance	Significance (p Value)
Unlikely (n=9)	28.022	15.069	
Mild (n=22)	27.295	22.511	
Severe (n=14)	25.679	12.817	
			p = 0.382

TABLE 2. Levels of binary toxin in patients who are categorized into severe for CDI compared to unlikely.

Group	Average	Significance (p Value)
Unlikely (n=9)	0	
Likely (n=36)	27.717	
		p=0.014

TABLE 3. Severity scores for each of the three categories of truly infected patients.

Group	Severity score including abdominal imaging or not	Significance (p Value)
Unlikely (n=9) compared to severe (n=14)	Not including	p=4.932E-14
Unlikely (n=9) compared to severe (n=14)	Including	p=7.797E-07
Unlikely (n=9) compared to mild (n=22) compared to severe (n=14)	Not including	p=6.814E-09
Unlikely (n=9) compared to mild (n=22) compared to severe (n=14)	Including	p=1.166E-09

TABLE 4. Severe symptoms compared to cycle threshold values.

Symptom	Significance (p Value)
White blood cell count > 15,000	p=0.936
Blood creatinine > 1.5	p=0.308
Recurrence	p=0.616
Ileus	p=0.985
Hypotension	p=0.583

Discussion

Our data shows that binary toxin production is associated with patients who are truly infected by CDI. Many statistically significant relationships among the severity groupings and severity scores were discovered. However, because our data suggested but did not have a statistically significant relationship between CT values and severity groupings, we are unable to reject our null hypothesis. Therefore, at this time we are unable to set a site-specific cycle threshold value to differentiate between the severity levels of infection. We are cognizant of the limitations of our study.

Limitations

There were several shortcomings of this study. We utilized a small patient cohort of 45 adults. Since the association between CDI and children is not well understood (Shim, 2014), it was determined that solely investigating adults would better reflect the knowledge of researchers. Additionally, because of the lack of complete patient charts during the chart review process, certain symptoms such as abdominal imaging and white blood cell count were unknown for some patients. The final limitation that should be considered is the lack of patients who were negative for CDI. It is dire to note that these negative patients could have had altered cycle threshold values. Future research can examine the difference between the cycle threshold values amongst patients with no symptoms who test positive via Xpert *C. difficile* Assay compared to those who have no symptoms who test negative via Xpert *C. difficile* Assay.

As for the statistical significance we did achieve with regards to binary toxin and a more severe infection, results reflect a continuation in the literature, and is not a novel finding (Cowardin et al., 2016). As for the statistical significance achieved by finding a relationship between severity scores and severity grouping, results demonstrate a novel method that can be used to diagnose patients with preliminary CDI. This novel method is using a nine-factor indicator scale with corresponding point values to predict true CDI.

Binary Toxin

Only patients who demonstrated at least one symptom of CDI had binary toxin in their stool sample. Therefore, the statistical significance observed in Table 2, where a relationship is shown between binary toxin levels and severity of infection, supports the literature's conclusion that binary toxin increases the virulence of CDI (Cowardin et al., 2016). Since the bacterium is producing two strains of toxin rather than one, a more severe infection is predicted.

Severity Scores

In our study, we used a method of a preliminary protocol to hope to flag certain symptoms as indicative of CDI, hoping to increase

the diagnostic differences between colonization and true infection. If patients display symptoms of CDI, meaning having a higher severity score, we hypothesized they would have a lower CT value. While this hypothesis could not be supported by our data, our results suggest our working hypothesis. Firstly, because each of the severe symptoms did not display significant relationships between their respective cycle threshold values, certain severity symptoms should not be prioritized over others when making a diagnosis of CDI. This means that just because a patient may have one indicator or another, a proper diagnosis cannot be concluded. On a separate note, it should be acknowledged that no patients in this study displayed septic shock. This fact is important to keep in mind as statistical relationships were not able to be concluded between this symptom and cycle threshold values. Therefore, this study should be repeated with a patient cohort that displays each of the nine indicators to determine any significant relationships between a specific symptom and true infection.

However, there were statistically significant correlations between all three categories (unlikely, mild, and severe) as well as between unlikely and likely infections, including and not including abdominal imaging, when comparing severity scores. Yet, this method of using severity scores should be used with precaution and care as symptoms can overlap between CDI, other gut microbiome infections, and pneumonia (Pneumonia, 2020). Future research should look for a statistically significant relationship between CT values and severity scores as our data suggests that a correlation is prevalent.

Conclusion

This study demonstrates the use of a novel severity score method statistically demonstrated to properly divide patients into groups of likely versus unlikely for CDI as well as a method to further divide a patient cohort into three subsections for risk: unlikely, mild, and severe. Additionally, our conclusions support the literature in that patients who have a binary toxin level recorded, have a statistically more severe infection. These findings are important because they can lead to the accurate prescription of medication, such as fidaxomicin and vancomycin (Louie et al., 2011).

Data from past literature suggest that once improved hygienic procedures are established, infection and transmission rates may decrease. During a pandemic, with less patients moving around units, fewer people in each room, and greater hand hygiene, reduced rates of CDI have been reported (Ponce-Alonso et al., 2020). With this newfound evidence, it is predicted that a similar outcome would ensue if hospitals implemented more hygienic procedures. This finding is imperative as it suggests that *C. difficile* would remain stable due to these precautions, even though antibiotic prescription has decreased during the pandemic (King et al., 2020).

Our study has the potential to reduce the time to make a diagnosis by the establishment of severity scores, which were found to statistically indicate infection. Cycle threshold values are indicative of the severity of the case of CDI and can be found on the commercially available Xpert *C. difficile* assay (Kamboj, 2018), making these methods and results attainable across the globe. With the appropriate medication, it is with hope and desire that patients can seek the treatments needed for their health as quickly as possible.

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