CLINICAL SYMPTOMS AND RISK FACTORS TO REFINE PERFORMANCE CHARACTERISTICS OF LABORATORY ASSAYS FOR *CLOSTRIDIUM DIFFICILE* INFECTIONS

by

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Submitted to the Graduate Faculty of

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Public Health

University of Pittsburgh

2012

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

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University of Pittsburgh, 2012

Current laboratory diagnostic assays for *Clostridium difficile* infection (CDI) vary in their performance characteristics, timeliness, and cost. The gold standard diagnostic assay, toxigenic culture, has a turnaround time of 4-7 days which makes it impractical for routine clinical use. Furthermore, There is no widely accepted standard for the laboratory diagnosis of CDI, and a variety of FDA-approved diagnostic assays are used at the discretion of hospital laboratories. Therefore, this study was conducted during November 2011-February 2012 on 524 stool samples from 427 patients to examine the performance characteristics (sensitivity, specificity, positive predictive value, negative predictive value) of five diagnostic assays including newer molecular assays. Toxigenic culture was used as the gold standard. To examine the possibility that the newer generation of molecular assays are identifying asymptomatic carriers, a retrospective chart review was conducted for patients with discrepant test patterns.

In the absence of clinical data, molecular nucleic acid based assays (RT-PCR, iLAMP) possess superior performance characteristics. Additionally, there are no significant differences between mean *C. difficile* likelihood scores when comparing discrepant groups. Further, when examining presence of three main clinical factors including diarrhea, inpatient exposure, and antibiotic usage, there was no significant difference in the percentage of patients expressing these factors between each discrepant result group. Therefore, it is unlikely to refine the performance

characteristics when incorporating clinical data. When examining semi-quantitative toxigenic culture which indicated *C. difficile* concentration in colony forming units per gram, there was no significant difference in scores between groups, indicating that *C. difficile* patients were likely to have CDI regardless of concentration. Similarly, the percentage of positive results on each toxin-detecting assay did not vary significantly. Furthermore, 54-100% of patients in all semi-quantitative culture groups possessed all three clinical factors, indicating no relationship between presence of these factors and concentration.

Public Health Implications: *Clostridium difficile* infection (CDI) is one of the most common healthcare-associated infections. Since its discovery in 1978 as the cause of antibiotic associated pseudomembranous colitis, its incidence has increased significantly affecting a greater number of populations. Determining a diagnostic assay for use in clinical setting which possesses excellent performance characteristics allows for the reduction of disease burden.

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ACKNOWLEDGEMENTS

I would first like to thank Dr. Charles Rinaldo, Dr. William Pasculle, and Dr. Scott Curry for allowing me the opportunity to participate in this study, consequently allowing for my professional development. This experience has improved my research skills, and allowed me to further explore an area of interest. I would also like to recognize Dr. Jeremy Martinson for his suggestions and guidance during thesis development. Additionally, I would like to recognize the contributions of Lloyd Clarke. Without his contributions as an honest broker, much of this study would not have been possible. Finally, I would like to thank Diana Pakstis, for her input in applying for institutional review board approval, Kathleen Shutt for her database expertise, Dr. Jessi Thommandru for the processing of laboratory samples, and Dr. Karina Soares for providing assistance when I had various questions.

1.0 INTRODUCTION

1.1 HEALTHCARE-ASSOCIATED INFECTIONS

Healthcare-associated infections (HAI) are endemic infections which occur in hospitals and long-term care facilities, with the potential for outbreaks. HAIs are typically defined as those with symptom onset at least 72 hours after admission and are not present or incubating during admission. This definition also includes infections that appear after discharge from the healthcare institution. These infections are caused by a variety of causative agents, including bacteria, viruses, parasites and fungi. Examples include methicillin resistant *Staphylococcus aureus* (MRSA), Vancomycin-resistant *Enterococcus* (VRE), urinary tract infection, hospital-acquired pneumonia, and *Clostridium difficile* infections (CDI). A number of factors promote the development of nosocomial infections. Inadequate infection control techniques including incorrect usage of isolation gowns, gloves, and mask/respirators are an additional risk factor. Inadequate environmental sanitation characterized by improper disinfectant usage, inadequate frequency and effectiveness of sanitation is a major source of nosocomial infections. [1]

1.2 *CLOSTRIDIUM DIFFICILE*

Clostridium difficile infection (CDI) is an example of a nosocomial infection. This infection is caused by the causative organism, *Clostridium difficile*. First identified in 1935 by Hall and O'Toole in gut flora of healthy neonates, *C. difficile* is an anaerobic gram-positive spore-forming bacillus (Figure 1).[2] Asymptomatic carriage is of toxin-producing *C.* difficile is present in 5-15% of healthy, non-hospitalized and up to 50% of asymptomatic patients with a length of hospital stay longer than two weeks.[3,4] In 1978, *Clostridium difficile* was implicated as the cause of antibiotic-associated diarrhea and pseudomembranous colitis.[5]

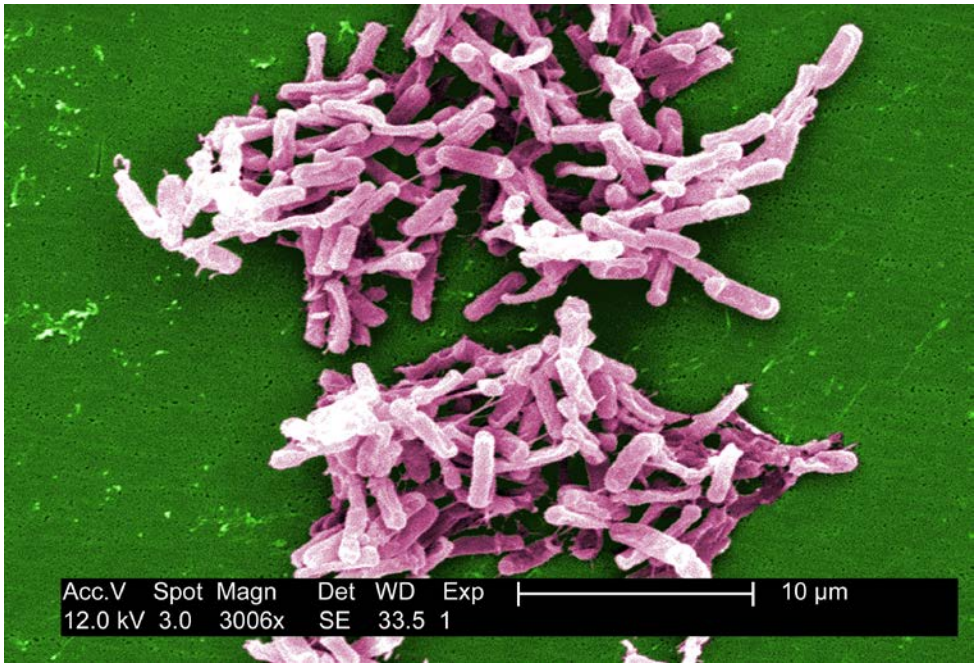

Figure 1. Scanning electron microscopy of *Clostridium difficile* bacilli (Centers for Disease Control and Prevention)

C. difficile infections present with a wide spectrum of severity. Levels of severity range from asymptomatic carriage to fulminant pseudomembranous colitis, and toxic megacolon. Septic shock and death can occur in the most severe cases.[6] The most common presentation involves voluminous watery diarrhea and abdominal cramps. C. *difficile* can be difficult to treat due to high occurrence to relapse, with an estimated 25 percent of patients treated for one CDI episode experiencing at least one relapse.[7,8,9] The chance of relapse increases further as the number of CDI episodes increases, with estimates of relapse reaching 50-65 percent.[10]

Fecal-oral transmission of this anaerobe is facilitated by the spore forming nature of the organism. *C. difficile* spores are resistant to heat, quaternary ammonium disinfectants, and ethanol allowing survival in harsh environmental condition and rendering the organism resistant to common hand sanitizers and hospital disinfectants.[11]

1.2.1 Pathogenesis

As with other *Clostridium* species, toxigenic *C. difficile* strains produce several toxins which are responsible for the infection. The toxin genes are located in the 19.6 kb pathogenicity locus (PaLoc) (Figure 2a).[12,13] The PaLoc is highly conserved across all toxigenic strains. The PaLoc is composed of genes *tcdA, tcdB, tcdC, tcdE* and *tcdR.* Two of the most well characterized toxins, Toxin A and Toxin B and are encoded by *tcdA* and *tcdB,* respectively. Toxin A is an enterotoxin which has demonstrated the ability to inactivate Rho GTPases. Toxin B is a potent cytotoxin, and depolymerizes actin within a wide variety of cell lines through an as-yet-unknown receptor. A holin protein encoded by *tcdE* may be responsible for the release of toxin from the vegetative cell. Supplemental genes, including positive and negative regulators are present on the

PaLoc. Gene *tcdR* is a positive regulator of *tcdA* and *tcdB* through its action as a σ-factor, while *tcdC* is a negative regulator of toxin production preventing transcription of the PaLoc.[5,12,13] Another toxin, binary toxin is encoded by the CDT locus 4.3 kb (CdtLoc) (Figure 2b). This particular toxin is only found in up to 5-12% of strains. There are two subunits *cdtA* and *cdtB*, and both are required for pathogenicity. This toxin disrupts the structural integrity of the cell via loss of cytoskeleton, and cell contents. Binary toxin is important as it has been found in many epidemic strains implicated in outbreaks, however, it has not been found to cause disease in animal models of CDI when PaLoc genes are inactivated. There are 28 toxinotypes of *C. difficile.* Most strains of *C. difficile* possess toxinotypes with functional *tcdA* and *tcdB* genes. However, an increasing number of strains are being isolated from patients which lack functional tcdA toxin due to mutations in the gene.[5,14]

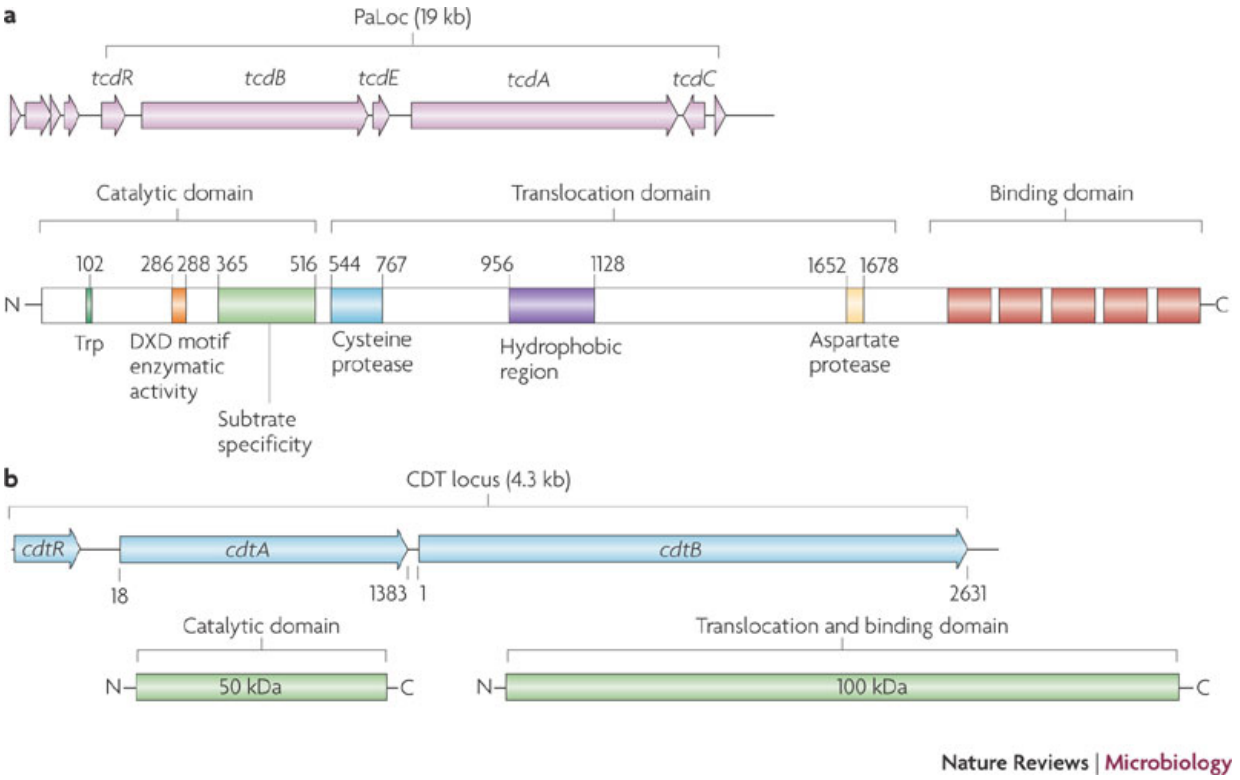

Figure 2. Pathogenicity Locus of *Clostridium difficile*

a) The pathogenicity locus (PaLoc) is a 19 kb stretch of DNA composed of five genes, with the center holin gene tcdE which is flanked by two toxin genes *tcdB* and *tcdA* and is capped by the positive and negative regulators, *tcdR and tcdC*, respectively. b) The binary toxin called *C. difficile* transferase is encoded by the CDT locus containing two subunits *cdtA* and *cdtB* and is 4.3 kb in length of DNA. In some strains of *C. difficile* it is a component of pathogenicity. Reprinted with permission from Macmillan Publishers Ltd: *Nature Reviews Microbiology*, Rupnik M, et al. 7:532 copyright 2009 (116).

C. difficile is transmitted through the fecal-oral route in spore form and is able to withstand the acidic environment of the stomach. Upon advancement through the digestive tract, the spores germinate into vegetative cells, reproduce, mature and produce toxins affecting the colon leading to development of disease.

The current hypothesis for successful development of CDI involves three major key events. The first event is antimicrobial exposure which leads to alteration of normal gut flora.

This event creates a niche environment for *C. difficile* to flourish. The second event involves toxigenic *C. difficile* acquisition before or after the administration of antimicrobials in the healthcare setting. Lastly, there must be a failure of the host to mount an anamnestic IgG antibody response to Toxin A.[14] This is a key prerequisite event to development of CDI. If the preceding two events occur, and the individual mounts an anamnestic IgG response, the individual will not develop disease and is likely to in a state of asymptomatic *C. difficile* colonization (Figure 3).[5]

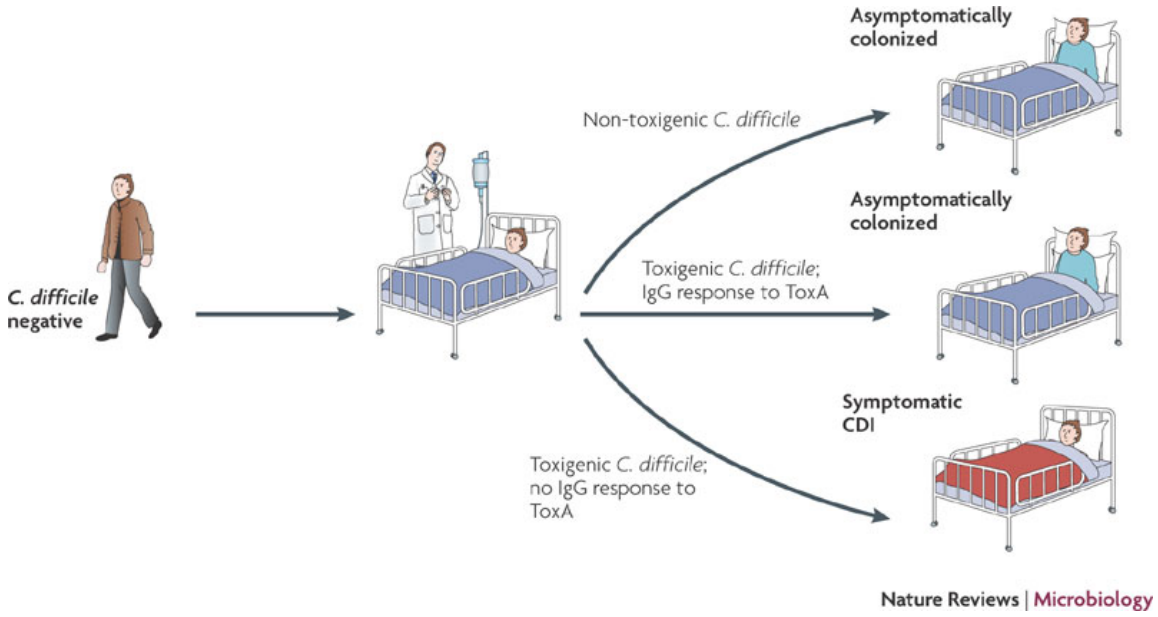

Figure 3. Proposed model for acquisition of *Clostridium difficile* infection in healthcare settings.

The proposed model of CDI in the healthcare setting is believed to occur when a previously CDI negative individual visits a medical facility and acquires *C. difficile* spores through the fecal-oral route, antimicrobials administered either pre- or post-exposure disrupt the gut flora allowing a niche environment to form for the organism. If the organism acquired is non-toxigenic, the patient is said to be colonized and patient will not exhibit symptoms. If the patient acquires a toxigenic *C. difficile* and fails to acquire an anamnestic immunological response IgG to toxin A then the patient will present with CDI, otherwise the patient will likely be asymptomatically colonized. Reprinted with permission from Macmillan Publishers Ltd: *Nature Reviews Microbiology*, Rupnik M, et al. 7:532 copyright 2009 (116).

1.2.2 Risk Factors

A wide variety of risk factors exist for CDI. The main risk factor for disease is the use of broad-spectrum antimicrobials including clindamycin, broad spectrum beta-lactams, cephalosporins and quinolones. However, almost all antimicrobials have been observed to have an associated increased risk of development of disease.[15] Recovery of normal gut flora can take up to three months after consumption of a course of antibiotics, leading to greater time for vulnerability and exploitation by the *C. difficile* pathogen. The factors that increase risk of CDI include and are not limited to advanced age, medical co-morbidities, immunosuppression, recent CDI and healthcare exposure. Other risk factors for development of CDI are listed below (Table I). These include highly invasive medical procedures such as surgical procedures involving the abdomen, which allow *C. difficile* spores to enter, colonize, and cause disease. As previously mentioned, HAIs transmission is enhanced through various tubes and drains which breach or bypass the patient host defenses and *Clostridium difficile* is no exception in taking advantage of these breaches. One example is nasogastric intubation increase the risk by providing a means for spores to enter the abdomen. [16,17,18,19,20,21,22,23] Reduced gastrointestinal pH due to the use of pharmaceutical agents, mainly proton pump inhibitors and H_2-receptor antagonists, has been shown to be a risk factor.[24,25]

Table 1. Risk Factors for development of *Clostridium difficile* Infection

Advanced age	Reduced gastric pH due to use of Proton Pump Inhibitors (PPI) and/or H_2-receptor antagonists
Invasive medical procedures	Intubation
Antimicrobial usage	Medical Co-morbidities
Immunosuppression	Recent healthcare exposure- Inpatient, Outpatient, or Skilled Nursing Facility
Recent CDI episode	

1.2.3 Treatment

A variety of treatment options are available to combat CDI. Accompanying any treatment involves the discontinuation of the offending antimicrobials that led to the development of CDI episode. Currently, there are two established efficacious antimicrobial agents which are used to treat CDI. These agents are metronidazole (Flagyl) and vancomycin (Vancocin). Metronidazole is indicated for mild-moderate cases of CDI. Typical dosage is 500mg three times daily, oral administration for 10-14 days. Vancomycin is indicated for severe CDI episodes, with dosages of 125mg four times daily, oral administration for 10-14 days. In some severe cases, vancomycin is administered, rectally via enema form.[26] Newer antimicrobial agents such as fidaxomicin, have recently been approved by the Food and Drug Administration as of 2011 to treat CDI and is being investigated as a possible routine treatment.[27] In patients with hypogammaglobulinemia, intravenous immunoglobulin (IVIG) is used as another treatment to confer passive immunity. In the most severe cases, surgical treatment may be required.[24] The surgical procedure typically

performed is a colectomy which is the removal of the colon. However, other newer surgical procedures have been explored including minimally invasive temporary loop ileostomy with vancomycin lavage.[28] Other alternative treatments, such as fecal microbiota transplantation (FMT), which involves the restoration of beneficial gut flora, are sometimes performed.[29]

1.2.4 Prevention

Because *C. difficile* can be difficult to treat due to high relapse rates and the overall robust nature of the organism, prevention plays an important role in combating disease burden. There are a variety of prevention strategies can be used to reduce the burden of disease. Ensuring that each category of prevention is properly executed is essential. Surveillance within the healthcare institution via infection control personnel precludes any form of prevention. A number of clinical practice guidelines for prevention have been identified by experts including the Centers for Disease Control (CDC), Society for Healthcare Epidemiology of America (SHEA) and Infectious Diseases Society of America (IDSA).

Effective sanitation in the healthcare setting is an absolute necessity. Because of the spore forming nature of the organism, few agents are effective in disinfection. Three established disinfectant agents which have been shown to be effective are minimum concentrations of 10% sodium hypochlorite (NaOCl), 10% hydrogen peroxide (H_2O_2) and 2% alkaline glutaraldehyde. Other disinfectants such as ethanol and quaternary ammonium compounds, which are highly effective against other pathogens, have been shown to be ineffective and in some cases promote sporulation. Using effective agents on all surfaces in the healthcare setting, especially patient rooms is necessary, no matter how challenging and time consuming this can be. In some cases,

the practicality of this is problematic and utilization of disposable healthcare items instead of reusable items is preferable. One example is the use of disposable rectal thermometers. The practice has been shown to reduce the incidence of CDI.[10]

Proper contact precautions can aid in providing a barrier to propagation of the pathogen, and have been demonstrated to be effective in prevention. Ensuring proper compliance among healthcare workers with current hand-hygiene protocols emphasizing appropriate time intervals, duration of wash, and use of soap and water is one aspect. Proper agitation during hand washing facilitates spore removal from contaminated hands. Expanding this protocol to include visitors during outbreaks is advisable. Wearing gloves prevents contamination of the hands with spores and should supplement hand hygiene protocol. Wearing isolation gowns prevents deposition of spores on clothing when caring for patients and therefore aids in prevention of CDI. Patient placement is important, ensuring that unaffected patients are not placed in close proximity to known *C. difficile* patients. One study suggested that when a patient is placed in a room with a prior CDI occupant, the patient faces an 11% risk of developing disease, while those who were placed in rooms with patients who were not affected by CDI had only a 4.6% risk.[30]

One of the most important prevention strategies is the proper use of antimicrobials. Because antimicrobial usage is involved in the pathogenesis of CDI, ensuring antimicrobials are only used when necessary is key. It is currently estimated that 50% of all antimicrobial use is of inappropriate nature. Therefore, an excess of patients receiving care are exposed to antimicrobials and their gut flora is altered, leading to increased risk of CDI. One way to combat this problem within the healthcare institution is the implementation of an antimicrobial stewardship program which monitors overall antimicrobial usage and restricts usage of broad-spectrum, high-risk antimicrobials, to cases which are only necessary.[31]

Other strategies are being researched but have not been adapted by SHEA, IDSA, or CDC. One such strategy is to ensure proper use of proton pump inhibitors (PPI) and H_2 receptor antagonists in the patient population. As previously mentioned, the use of these medications has been identified as a risk factor of CDI. The results of a study have demonstrated that a large proportion (63%) of patients who developed CDI did not present valid indications for usage of these medications.[32] The use of prophylactic probiotic therapy is another prevention strategy currently being explored, but is not recommended at this time, due to mixed results. Identification of asymptomatic carriers within patient populations has been explored, but this is not recommended as it has not been shown to be effective.

1.2.5 Diagnosis

A timely diagnosis of CDI is critical in all situations; especially during outbreaks within the healthcare setting. An accurate diagnosis allows for a number of events to take place including the initiation of therapy, discontinuation of offending antimicrobials, initiation of contact precautions, and adjustment of hand-hygiene practices amongst other institutional procedures and protocols. Furthermore, an accurate diagnosis allows for discontinuation of other non-antimicrobial pharmaceutical agents such as laxatives and stool softeners, which may exacerbate symptoms. An accurate diagnosis of CDI is made through a combination of clinical assessments, interpretation of results on diagnostic assays, and assessment of risk factors by a qualified medical professional. Improper conclusions regarding the presence of CDI can be reached if information from clinical assessments, risk factors, or diagnostic assays is excluded. Therefore,

in order for accurate diagnosis to be reached, all relevant medical data regarding possible CDI must be considered.

1.2.5.1 Clinical Assessments

A number of clinical assessments can cause the clinician to raise the suspicion of possible CDI. The gastrointestinal (GI) assessment is one extremely important aspect. It is important to note, however, many clinical data which are observed are shared with other gastrointestinal illnesses with varying etiology. These include and are not limited to *Salmonella, Shigella, Campylobacter, Giardia,* and *Cryptosporidium*. The most common presentation in symptomatic infections is frequent (>3) watery diarrhea episodes. A characteristic foul odor emanating from the stool is often observed by the clinician. Blood and mucus may be observed in the stool in more severe instances. Other common symptoms during a GI assessment are abdominal cramps, nausea and vomiting. Clinical signs of tenderness with possible abdominal distention which could suggest fulminant pseudomembranous colitis. In severe cases, toxic megacolon may be present. In the overall general clinical assessment, a variety of data may be observed. These include varying grades of fever; presence of malaise, dehydration, and anorexia. Clinical laboratory assessments such as complete blood count with differential, may present results indicating leukocytosis defined as leukocyte count greater than 10,000 units/mL, bandemia defined as bands greater than 5%, and hypoalbunemia defined as albumin level <3 mg/dL. The clinician must differentiate clinical presentations as there are other causes of antibiotic-associated diarrhea. These other cases of antibiotic-associated diarrhea usually are less severe and often resolve without treatment when compared to *C. difficile*.[32,33]

1.2.5.2 Laboratory Diagnostic Methods

Diagnostic assays are the major contributory factor in determining whether the causative *Clostridium difficile* agent is present in the patient. A number of types of assays are available and are based on different principles (Table 2). Each of these assays varies in the level of their effectiveness, possessing different performance characteristics such as values of sensitivity, specificity, positive and negative predictive values. In addition, these assays possess their own advantages and disadvantages in regards to economics. Timeliness or the overall turnaround time to obtain results varies significantly as well. Unfortunately, SHEA and IDSA do not recommend a single assay which possesses the attributes of timeliness, cost-effectiveness, and accuracy. Therefore, there is a lack of guidance in the medical community as to which assay should be used. In many cases, healthcare institutions must continually evaluate different laboratory assays, along with clinical protocols, to determine the best diagnostic approach.

Two main assays exist to determine the existence of the causative organism, but these methods provide limited data on toxin production. The first assay is the anaerobic stool culture with subsequent confirmation of toxin production, also known as toxigenic culture. This assay is considered to be a gold standard assay as it is the one of the most sensitive assays available. Estimated sensitivity levels are 89-100% based on a meta-analysis.[15] However, this particular assay lacks timeliness, as it takes 4-7 days to obtain results, and therefore it cannot be practically incorporated into patient care settings. Additionally, protocols vary significantly among institutions, and therefore, can provide varying accuracy. Most importantly, this assay cannot distinguish the different toxin-producing strains. The Glutamate Dehydrogenase or GDH antigen assay detects the metabolic enzyme antigen of *C. difficile* in a stool sample through antibodies. These allow for rapid detection of *C. difficile* in a matter of hours. However, the positive result

GDH antigen assay does not provide information differentiating between a toxigenic and non-toxigenic strain. For this reason, GDH is used as part of two-step algorithm, where another class of assays, which detect toxin, is performed after a positive GDH result. Sensitivity levels for this assay are estimated to be 71-100%, while specificity levels are estimated to be 76-98%.[14]

Another group of assays is used to determine the presence of and distinguish toxin produced by *C. difficile* within the stool sample. The first is the cell culture based cytotoxin assay (CCNA). The principle is based on cytopathic effects (CPE) which are observed when stool filtrate is inoculated with a monolayer of cells, usually Human Foreskin Fibroblast. In addition to CPE, the neutralization of toxin after incubation of cells with antitoxin is observed in a positive culture. Like the toxigenic culture, the disadvantages to this assay are the slow turnaround times around 1-2 days. Furthermore, this assay is sensitive to technique, and experienced laboratory technologist personnel are required. Sensitivity levels have been estimated to be in the range of 67-86%, and specificity levels are estimated to be 97-100%.[14] Another method, is the enzyme immunoassay (EIA) which detects toxins A and B through the use of antibodies. This assay is highly rapid and results can be observed in a matter of hours. However, the levels of sensitivity and specificity are highly variable with sensitivity ranging from 31-99%, and specificity ranging from 84-100%.[14] The Infectious Diseases Society of America have taken the position stating this diagnostic approach is suboptimal for routine use, due to low sensitivity levels.

A final group of newer molecular diagnostic assays, utilize nucleic acid amplification to determine presence of *C. difficile.* These assays are highly promising, with accuracy levels surpassing other methods, with the exception of toxigenic culture. One example is the real-time polymerase chain reaction (PCR) for qualitative detection of toxigenic *Clostridium difficile* toxin

genes, specifically the *tcdB* region of the PaLoc. PCR appears to be rapid, sensitive, and specific. Sensitivity levels have been observed to be 77-92%, while specificity levels have been observed to be 95-99% for one commercial manufacturer's kit.[14] Other newer molecular diagnostic assays include isothermal Loop Mediated Isothermal Amplication (iLAMP) technology. Unlike other nucleic acid amplification methods, iLAMP targets a region of *tcdA*. Estimated Sensitivity ranges from 83-99% while estimated specificity ranges from 96-100%. The biggest advantage is the quick turnaround time with iLAMP technology promising turnaround times in less than one hour. [34,35,36]

Regardless of the diagnostic assay used, IDSA and SHEA have made the following recommendations. First, testing for *C. difficile* and its accompanying toxins should be done on samples that are unformed diarrheal stool samples. Second, repeating testing on samples is usually unhelpful and unproductive and therefore is discouraged. Additionally, routine testing on asymptomatic patients should not receive testing in routine patient care except in cases when an epidemiological study is being performed.

Table 2. Summary of *Clostridium difficile* diagnostic methods

Diagnostic Assay	Processing Time	Sensitivity	Specificity	Advantages	Disadvantages
Toxigenic Culture	Minimum 72 hours	89%-100%	N/A	Gold standard	Does not distinguish toxins, unstandardized protocol, not timely
Cell Cytotoxicity Neutralization Assay	Minimum 48 hours	67%-86%	97%-100%	Detects toxin producing strains	Technique Sensitive, not timely
Glutamate Dehydrogenase (GDH) common assay	30-60 minutes	71-100%**	76-98%**	Easy to use, allows negative results to be obtained quickly	Part of two-step regimen to detect toxigenic strains
Enzyme Immunoassay (EIA)	2 hours	31-99%**	84-100%**	Easy to use, timely	Low sensitivity
Real-Time Polymerase Chain Reaction (RT-PCR)*	3 hours	77-92%**	95-99%**	High sensitivity and specificity superior to other methods, timely	Unfavorable, economics
Isothermal loop mediated amplification (iLAMP)	15-30 minutes	83-99%***	96-100%***	Timely, high sensitivity and specificity**	Requires proprietary instrumentation
Endoscopy	1-2 hours	51%	N/A	Allows for visualization pseudomembranes	Low sensitivity

* RT-PCR sensitivity and specificity levels apply to Prodesse ProGastro™ assay

**Sensitivity and specificity based on a meta-analysis of comparisons utilizing various reference standards including toxigenic culture, CCNA or a composite gold standard.[14]

***Sensitivity and specificity based on a meta-analysis utilizing toxigenic culture as a reference standard[34.35,36]

1.2.5.3 Other Diagnostic Methods

Other diagnostic methods are sometimes used in the overall diagnosis of CDI including endoscopy to observe presence of pseudomembranes and radiographic methods such as abdominal computerized tomography (CT) scans to observe colonic thickening and free air which are characteristic of CDI. However, sensitivity and specificity are not sufficient as compared to laboratory diagnostic assays with estimated sensitivity levels of 51% for endoscopy. Therefore, these diagnostic tools are not accurate and should not be relied upon to make a definitive diagnosis of CDI.

1.3 PUBLIC HEATH SIGNIFICANCE

The public health significance of *Clostridium difficile* infection is demonstrated through increased incidence, mortality rates, and attributable healthcare costs. Furthermore, populations previously at low risk are increasingly becoming vulnerable. The public health impact is forecasted to increase further. One problem encountered with quantifying CDI is the varying requirements for reporting of CDI. At a national level, surveillance of CDI is a patient safety component of the National Healthcare Safety Network (NHSN) overseen by the CDC. The NHSN is internet based surveillance system with voluntary reporting by healthcare institutions. Only institutions from 27 states have reported to this database as of 2010. Additionally, reporting requirements to territorial and state health departments on the incidence of hospital acquired infections vary by state. However, more surveillance programs are incorporating CDI. For

instance, CDI is also part of the emerging infections program (EIP) which is part of the CDC, however, data from this program has not been released at this time. Because of this, the ability to obtain complete and truly accurate surveillance data on CDI at the national level is hampered. Nevertheless, there is evidence to support an increase in the public health burden through various initiatives. These include initiatives and campaigns through insurance companies, and state health departments. Based on current data, there is an interest in combating this preventable nosocomial infection.

According to the Healthcare Cost and Utilization Project (HCUP), part of the Agency for Healthcare Research and Quality (AHRQ), an increasing trend has been observed in the incidence CDI being classified as a primary or secondary diagnosis during hospital stays. There has been an increase from 85,700 hospital stays in 1993 to 336,600 hospital stays in 2009 (Figure 4). Restated, there has been an increase from 86,000 hospital discharges involving CDI in 1993 to 337,000 hospital discharges in 2009. Elderly patients over the age of 85 years, who are among highest at risk are disproportionately affected and have had incidence rates increase to 1,089 per 100,000 patients. This contrasts to all other age groups with a combined rate of 603 per 100,000 patients.[37] Additionally, peripartum women, previously at low risk have seen infection rates increase.[5] The number of deaths due to CDI has increased over time as well. During the time period 2000-2007 deaths related to CDI have increased 400% (Figure 5). As of 2010, 14,000 deaths were attributable to this disease.[38] Ninety percent of those deaths occurred in individuals 65 and older. According to the CDC, comparing the time periods 1991-1997 and 2004-2009, there has been a 12 fold increase in incidence of disease in children from 2.6 cases per 100,000 to 32.6 cases per 100,000.[39]

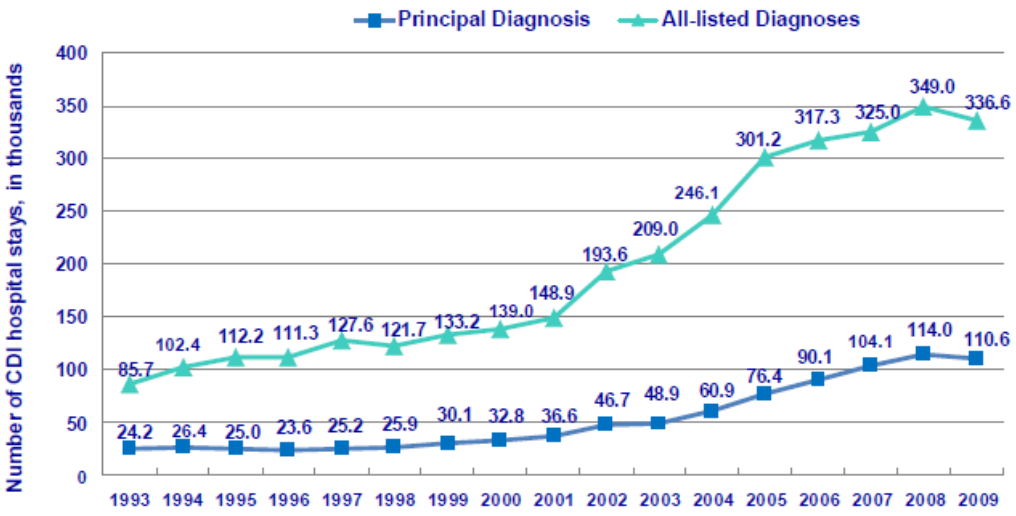

Figure 4. Trends in hospital admissions from 1993-2009, with a primary or secondary *Clostridium difficile* infection (CDI) (Agency for Healthcare Research and Quality)

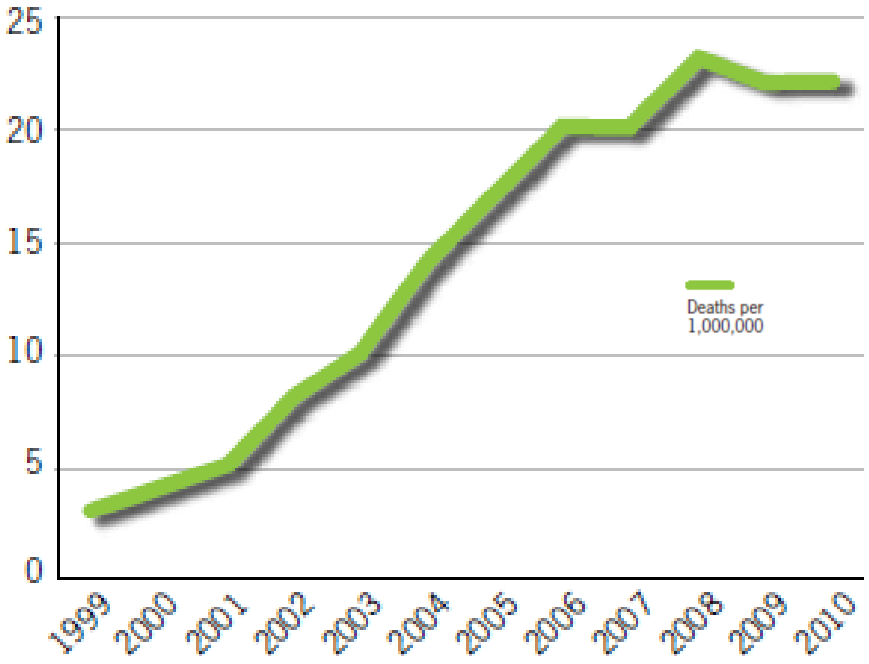

Figure 5. Age-Adjusted rates of deaths attributable to CDI (primary cause) (CDC National Center for Health Statistics, 2012)

Economic costs attributable to CDI are estimated to be $8.2 billion annually as of 2009. Because of the disproportionate effect on the elderly population, Medicare is more financially impacted bearing 67.9 percent of all *C. difficile* financial costs covered. The average cost for a hospital stay attributable to CDI is $24,400 as of 2009. Considering the high potential for relapse the economic costs have a potential to increase further. Additionally, not taking into account indirect economic costs, such as loss of economic productivity, total economic costs could be much higher.[33]

The increased public health burden is partly attributable to newer, more virulent Clostridium *difficile* strains, mainly the group BI restriction endonuclease, North American pulse-field-type 1 characterized by pulse-field gel electrophoresis and ribotype 027 (BI/NAP1/027) strain. This strain is characterized by possession of CDT binary toxin and 18 bp deletion of the negative regulator *tcdC* in the PaLoc. This strain has increased in prevalence due to the increased use of fluoroquinolones. This new more virulent strain as of 2010 has spread to 40 states in the US, seven Canadian providences, parts of Asia, Europe, and England. Notable outbreaks included severe outbreak of disease in the University of Pittsburgh Medical Center starting January 2000 ending April 2001, and severe disease outbreaks in hospitals throughout Quebec Canada starting in 2002 and ending 2006.[5, 26]

The current 2013 national prevention target set by the Department of Health and Human services is a 30% reduction in hospitalizations with primary or secondary healthcare associated CDI based on the 2008 baseline measurement of 11.7 hospitalizations diagnoses per 1,000 discharges.[40]

2.0 THESIS AIMS

There are multiple approaches to achieve reduction of *C. difficile* infection. The main approaches include continual research in prevention strategies, treatment strategies, and diagnostics. Arguably, the most important approach is through the continual evaluation and adjustment of current diagnostic capabilities and protocols in the healthcare institution. This strategy is crucial as accurate, timely, and cost-effective diagnostics allow for effective surveillance, especially for reporting to state and federal disease surveillance programs. Diagnostics allow for the identification of any trends in the incidence of disease. Without identification of disease trends, the case for a focus on disease prevention and development of better therapies cannot be supported. Furthermore, improving diagnostics allow for quicker initiation of treatment, and allows for isolation and contact procedures to be implemented. Therefore, continual evaluation and necessary modification of current diagnostic protocols is essential. Because of a lack of general consensus on a single assay which is timely, accurate, cost-effective within the medical community, it is up to the medical institution to routinely evaluate current practices and compare to other currently available diagnostic methods to determine a routine assay which provides the best fit for the institution and the patient.

Numerous research studies involving the comparison of commercially available *Clostridium difficile* diagnostic assay kits have been published. While this is the case, many of

these studies have limitations. Few studies have used the current gold standard within the medical community, anaerobic stool culture for toxin-producing *Clostridium difficile* (toxigenic culture), rather these studies have used either CCNA or a composite gold standard, consisting of positive result on multiple assays within the study as a gold standard.[41,42,43] Furthermore, the most important deficiency of these and many other studies is lack of incorporation of clinical data to further assess the assay. A diagnosis of CDI is made in conjunction with clinical data, such as patient symptoms, and assessment of risk factors. Asymptomatic CDI carriers could be detected with their diarrhea being caused by other factors such as laxative and stool softener use, or other gastrointestinal infection. If the clinician did not take these clinical aspects into account, it would cause the wrong conclusion to be made about performance characteristics of the assay being evaluated. Few studies have incorporated clinical data, and the need for further studies is therefore warranted.

Understanding shortcomings of the previous studies presents opportunities for improvement in which the study design corrects errors to perform an effective comparison of *Clostridium difficile* assays providing better data for the medical institution and the medical community which translates to better clinical care and overall outcomes.

The purpose of this research study was to evaluate the performance characteristics (sensitivity, specificity, positive predictive value, and negative predictive value) of existing *C. difficile* assays (e.g. GDH, EIA CCNA, anaerobic toxigenic culture) and of newer generation molecular diagnostic assays (e.g. RT-PCR, iLAMP) through a comparative evaluation utilizing semi-quantitative toxigenic culture as a reference standard. Further, there is an interest to determine if it is possible to refine the estimate of the performance characteristics through the incorporation of risk factors, laboratory data and clinical symptoms from the medical record of

patients who undergo testing for *Clostridium difficile.* Based on the current literature, we hypothesize that performance characteristics of newer generation molecular assays will be better than their older counterparts. Additionally, we hypothesize that the performance characteristics of each assay can be refined through the through the use of clinical data. Lastly, we hypothesize that discrepant positive results on molecular nucleic acid amplification assays are asymptomatic carriers.

This research study is divided into three specific aims:

AIM #1: Explore performance characteristics of various diagnostic assays for CDI using toxigenic culture as a reference standard.

AIM #2: Explore potential refinement of performance characteristics through incorporation of clinical data into the results.

AIM #3: Explore potential associations between individual risk factors, symptoms and results on semi-quantitative toxigenic culture.

3.0 MATERIALS AND METHODS

3.1 STUDY DESIGN

The study design was composed of two components, a lab-based component which involved collection and testing of patient stool samples, and an epidemiologic component which involved retrospective review of medical records to review patient symptoms and risk factors. The University of Pittsburgh Institutional Review Board reviewed and approved the study protocol. During the study period-November 2011 through February 2012- 524 consecutive stool samples were tested from 427 patients using specimens from tests ordered by physicians for *Clostridium difficile* stool toxin. Samples were collected from either UPMC Presbyterian or UPMC Shadyside campuses including their respective hospital-based outpatient clinics and were sent to the clinical microbiology laboratory at UPMC Presbyterian. Specimens were collected daily Sunday-Thursday and stored overnight at 4°C for further processing. Only stool specimens which were unformed and had total volume greater than or equal to 20cc were included which ensured enough volume for all five diagnostic assays within the study. Physicians were not made aware of the results of assays other than the existing CCNA assay routinely reported.

3.2 DIAGNOSTIC ASSAYS

3.2.1 Anaerobic Toxigenic culture with subsequent confirmation of toxin production (toxigenic culture)

Toxigenic culture was performed to determine the presence of either non-toxigenic or toxigenic *C. difficile*. In a biological safety cabinet, 10 µl of stool was planted on cefoxitin cycloserine mannitol agar with 0.1% taurocholic acid and lysozyme (CCMA-TAL) using four-quadrant technique. An additional 10 µl of stool was broth-enriched in 5 ml cefoxitin cycloserine mannitol broth with taurocholate and lysozyme (CCMB-TAL) (Anaerobe Systems, Morgan Hill, CA). CCMA-TAL and CCMB-TAL media were transferred after inoculation to an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) and incubated at 37°C for three days. *C. difficile* was identified by morphology and yellow fermentation on CCMA-TAL and characterized as rare, light, moderate, or heavy growth depending on the number of quadrants in which it appeared. If the no *C. difficile* was observed on the directly plated specimen, the CCMB-TAL broth-enrichment specimen was sub-cultured to trypticase soy agar with 5% sheep blood (SBA, Becton Dickinson, Franklin Lakes, NJ) if the broth showed a color shift from red to yellow. All suspected *C. difficile* colonies were sub-cultured to SBA plates to ensure purity and confirmed as *C. difficile* using L-prolineaminopeptidase activity (PRO disk, Remel, Lenexa, KS). Isolates were store in chopped meat broth (Anaerobe Systems) and regrowtn on BHIYT plates for DNA extraction by the NucliSens easyMag system (Biomerieux, Durham, NC). Isolates underwent

tdcC and MLVA genotyping. All isolates with a *tcdC* genotype were inferred to be toxigenic. Any isolate with no amplification of *tcdC* was tested with lok1/lok3 primer set to confirm that it was non-toxigenic *C. difficile.*

3.2.2 Glutamate Dehydrogenase assay (GDH) and Enzyme Immunoassay (EIA) assay

Stools were assayed for the glutamate dehydrogenase antigen using the Alere™ QUIK CHEK COMPLETE (Techlab, Blacksburg, VA) following manufacturer's instructions. In addition to determining the presence of GDH common antigen, the assay includes an enzyme linked immunoassay for toxins A and B. Accordingly this kit gave two separate results, one for GDH and one for EIA.

3.2.3 Cell cytotoxicity neutralization assay (CCNA)

Stool samples were stored at 2-8°C and processed within 24 hours of collection to ensure integrity of toxin within the sample. A 1 ml fecal sample aliquot was centrifuged at 10,000g for 10 minutes and supernatant was collected, filtered using a 0.22µm syringe filter (Millipore, Billerica, MA). Supernatant was diluted to 1:40 with M4 medium then added to human foreskin fibroblast (Hs27, Diagnostic Hybrids, Athens OH) in a 96 well plate. A control was prepared with specimen supernatant and 50ul of *C. difficile* goat derived antitoxin (Techlab Inc., Blacksburg, VA). After incubation at 37°C for 24 hours, cells where checked for cytopathic

effects which was defined as CPE in 50% or greater of the monolayer and anti-toxin confirmation defined by no cytopathic effects in control cells incubated with *C. difficile* antitoxin. This test is the current clinical test at UPMC Presbyterian and was reported in the clinical record.

3.3 MOLECULAR DIAGNOSTIC ASSAYS

3.3.1 Isothermal loop mediated amplication (iLAMP)

The Meridian Illumigene™ molecular diagnostic assay (Meridian Bioscience, Cincinnati, OH) which utilized isothermal loop mediated amplification was utilized as one means of molecular diagnostics for *C. difficile*. The system targets the *tcdA* gene for amplification. The assay was performed as directed by the manufacturer. The results of the assay were read using the illumipro-10 instrument (Meridian Bioscience, Cincinnati, OH) and results were interpreted according to manufacturer's protocol as positive, negative, or indeterminate.

3.3.2 Real-time polymerase chain reaction (RT-PCR)

The Prodesse Progastro Cd real-time polymerase chain reaction (RT-PCR) assay (Gen-Probe Prodesse, Waukesha, WI) targets and amplifies the *Clostridium* difficile toxin B gene *(tcdB)*. The RT-PCR assay was modified to run on ABI 7500 Fast thermal cycler (Applied Biosystems, Foster City, CA). The marker of *Clostridium difficile* burden was the cycle threshold value (Ct). It is defined as the PCR cycle where enough amplification of the nucleic acid amplification has occurred to generate a fluorescent signal which can be detected. In our study, a Ct value ≤35 was considered positive.

3.4 RETROSPECTIVE CHART REVIEW OF CLINICAL DATA

A retrospective chart review was conducted on a subset of the medical records of 427 patients in the study who had discrepant results on iLAMP, PCR, and CCNA assays. The medical records were accessed by an honest broker and anonymized. Demographic data, history and physical data, laboratory data, radiology reports, progress reports, operative and pathology reports, pharmacological data, and microbiology reports were reviewed.

A CDI likelihood score was assigned based on a number of factors found in previous studies to be associated with CDI in case-control studies (Table 3).[44] Each factor was assigned a numerical value and the sum of applicable numerical values determined the likelihood score. Factors which favored CDI included prior history of CDI, prior outpatient and inpatient medical exposure over the course of 12 weeks, medical co-morbidities, intubation, antimicrobial

exposure in previous 12 weeks, H_2 and PPI use in prior 12 weeks were risk factors which favored CDI. Symptoms which favored CDI diagnosis included diarrhea, abdominal pain, fever greater than 37.9°C, elevated white blood cell count, and hypoalbuminemia. Other clinical assessments which favored CDI include colonoscopic evidence of pseudomembranous colitis, and pathological and radiological evidence suggesting colitis. However, clinical features which suggested alternative diagnoses were considered. These included a diagnosis of other gastrointestinal illnesses with infectious origins, use of laxatives, lack of diarrhea/presence of formed stools/constipation accompanying positive *Clostridium difficile* toxin result. Presence of alternative pathologies involving the gastrointestinal tract such as Crohn's disease, ulcerative colitis, and irritable bowel syndrome were assigned negative numerical values and lowered the CDI likelihood score.

Measurements of medical co-morbidities involved the use of Horn's index. The Horn's index is a four-tiered index of disease severity and is assigned based on clinical judgment. Previous studies have incorporated the Horn index to predict *C. difficile* outcomes. Horn scores of 1 indicate typically a single mild acute illness with higher tiered scores indicating numerous chronic diseases with the potential for fatal outcomes.

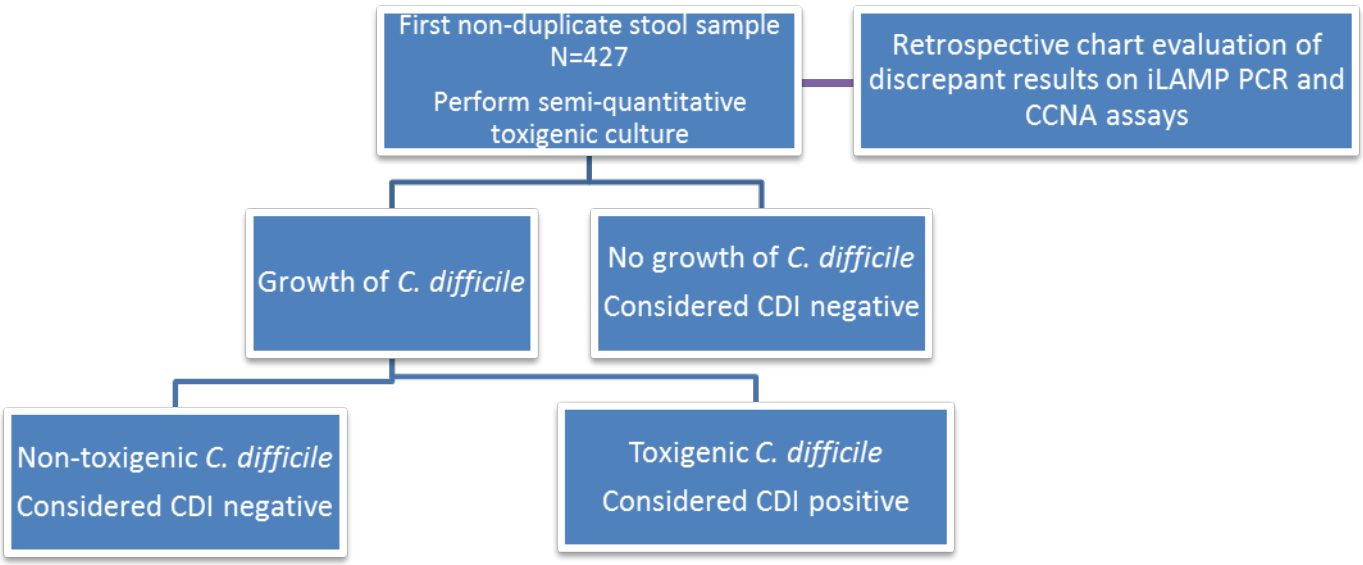

Figure 6. Summary Diagram of Study Design.

Semi-quantitative toxigenic culture was performed on the first 427 non-duplicate stool samples. If *C. difficile* growth was not observed, the patient was classified as not having CDI. If growth of *C. difficile* was observed, genotyping was performed. If an isolate possessed a *tdcC* genotype the strain was inferred to be toxigenic. Otherwise the *C. difficile* sample was not considered toxigenic and the patient was considered CDI negative. These results were used as a basis for evaluation of the other assays A retrospective chart evaluation was performed on the discrepant results with positive and negative controls.

Table 3. Scoring criteria for *Clostridium difficile* likelihood score

Features favoring CDI	**Points**	**Comments**
Prior history of CDI	1	
Prior outpatient healthcare exposure (<12 weeks)	1	Only (highest-scoring) category per patient
Prior inpatient (hospital or Skilled Nursing Facility) exposure (<12 weeks prior) with length of stay <1 week	1	
Prior inpatient (hospital or Skilled Nursing Facility) exposure (<12 weeks prior) with length of stay 1-2 weeks	2	
Prior inpatient (hospital or Skilled Nursing Facility) exposure (<12 weeks prior) with length of stay >2weeks	3	
Medical co-morbidity index: Horn score of 1	0	Only one score
Medical co-morbidity index: Horn score of 2	1	
Medical co-morbidity index: Horn score of 3	2	
Medical co-morbidity index: Horn score of 4	3	
Tube feedings (nasogastric, gastric, or duodenal)	1	
Diarrhea (any mention in notes -5 to +5 days from 1st CDIFT sent)	2	
Abdominal pain/cramping	2	
Fever > 37.9 °C (-5 to +5 days from first CDIFT sent)	1	
Peripheral WBC count >10K (max +/- 5 days from 1st CDIFT)	1	Only highest score
Peripheral WBC count >15K (max +/- 5 days from 1st CDIFT)	2	
Peripheral WBC count >20K/Band % >5% (max +/- 5 days from 1st CDIFT)	3	
Colonoscopic evidence of pseudomembranous colitis	2	
Albumin < 3 mg/dL (min +/- 5 days from 1st CDIFT)	2	
Antimicrobial exposure in 12 weeks prior to testing: quinolones/ceftriaxone/clindamycin	3	
Antimicrobial exposure in 12 weeks prior to testing: all other antibiotics	2	
Pathological evidence of pseudomembranous colitis	2	
Radiological evidence of colitis (colonic thickening, free air, pneumatosis coli)	2	
H_2 blocker or PPI use in 12 weeks prior to testing	1	
Features favoring alternative diagnosis		
Diagnosis of alternate infection (*Salmonella, Shigella, Campylobacter*, *Giardia, Cryptosporidium*, other OI in HIV+ patient)	- 15	
Use of laxatives (colace, senna, PEG, Mg-citrate, lactulose) +/- 5 days from CDIFT testing	-5	
Mention of formed stools/constipation/"no diarrhea" in clinical notes -3 to 0 days from first positive CDIFT	-5	
Alternative pathological diagnosis (e.g. ischemic colitis)	-10	
Mention of Crohn's disease, ulcerative colitis, Irritable bowel syndrome	-1	

3.5 STATISTICAL ANALYSIS

Statistical analysis was performed using SAS 9.3 (SAS Institute Inc, Cary, NC). 95% confidence intervals for performance characteristics of tests were generated using Clopper-Pearson exact binomial intervals.

4.0 RESULTS

4.1 AIM #1: EXPLORE PERFORMANCE CHARACTERISTICS OF VARIOUS DIAGNOSTIC ASSAYS FOR CDI USING TOXIGENIC CULTURE AS A REFERENCE STANDARD

4.1.1 Toxigenic Culture

The current gold standard assay, toxigenic culture, was performed on the first non-duplicate sample submitted from all 427 patients, out of 524 samples within the study period. Results from the toxigenic culture were categorized semi-quantitatively as being a non-toxigenic or toxigenic strain (Table 4). A semi-quantitative result of *C. difficile* from broth only indicated growth of *C. difficile* in CCMB-TAL broth only. Rare, light, moderate, and heavy *C. difficile* result indicated growth in one, two, three, or all four quadrants of the CCMA-TAL directly plated specimen, respectively. Three hundred thirty-nine samples yielded no *C. difficile* when toxigenic culture was performed. Growth of non-toxigenic strains of *C. difficile* occurred in 31 samples and growth of *C. difficile* toxigenic strains were present in 57 patient samples. These 57 patients with toxigenic *C. difficile* served as the primary focus for further evaluation throughout the study. For

purposes of this study, the 339 specimens with no growth of *C. difficile* and 31 non-toxigenic results were defined as negative by the gold standard.

Table 4. Classification of Toxigenic culture by semi-quantitative growth and toxin production

Semi-quantitative result	Non-toxigenic	Toxigenic
No *C. difficile*	339	
C. difficile from broth only	9	6
Rare *C. difficile*	5	22
Light *C. difficile*	5	5
Moderate *C. difficile*	2	7
Heavy *C. difficile*	10	17
Total	31	57

4.1.2 Performance characteristics of Enzyme Immunoassay (EIA) defined by toxigenic culture

An evaluation of EIA based on the toxigenic culture defining true disease status was performed. Out of 427 patient samples of which both EIA assay and toxigenic culture were performed, there were 370 concordant negative results, and 16 concordant positive results (Table 5). The performance characteristics of the EIA based assay demonstrated low sensitivity. The sensitivity of the EIA assay was found to be lower than previously reported results at 28.1%, 95% CI (17.0, 41.6). The specificity of the EIA assay was excellent and was observed to be 100%, 95% CI (99.0, 100.0). Additionally, the positive predictive value was excellent and found to be 100%, 95% CI (79.4, 100.0) while the negative predictive value was found to be 90%, 95% CI (86.7, 92.6) (Tables 5 and 10).

Table 5. Performance characteristics of Enzyme Immunoassay defined by toxigenic culture

Alere™ QUIK CHECK EIA			
Toxigenic Culture	**Positive**	**Negative**	**Total**
Positive	16	41	57
Negative	0	370	370
Total	16	411	427
		95% CI	
Sensitivity	16/57=28.1%	(17.0-41.6)	
Specificity	370/370=100%	(99.0-100.0)	
Positive Predictive Value	16/16=100%	(79.4-100.0)	
Negative Predictive Value	370/411=90.0%	(86.7-92.6)	

4.1.3 Performance characteristics of cell cytotoxicity neutralization assay defined by toxigenic culture

Of 427 samples, there were 26 concordant positive results and 367 concordant negative results based on the toxigenic culture defining true disease status (Table 6). Sensitivity of the CCNA assay was observed to be below previously reported values at 45.6%, 95% CI (32.4, 59.3). Specificity was excellent with a high rate at 99.2%, 95% CI (97.7, 99.8). Positive predictive value was observed to be 89.7%, 95% CI (72.7, 97.8) and negative predictive value was observed to be 92.2%, 95% CI (89.1, 94.7) (Tables 6 and 10).

Table 6. Performance characteristics of cell cytotoxicity assay (CCNA) defined by toxigenic culture

Cytotoxicity assay (CCNA)			
Toxigenic Culture	**Positive**	**Negative**	**Total**
Positive	26	31	57
Negative	3	367	370
Total	29	398	427
		95% CI	
Sensitivity	26/57=45.6%	(32.4-59.3)	
Specificity	367/370=99.2%	(97.7-99.8)	
Positive Predictive Value	26/29=89.7%	(72.7-97.8)	
Negative Predictive Value	367/398=92.2%	(89.1-94.7)	

4.1.4 Performance characteristics of isothermal loop mediated amplification (iLAMP) assay defined by toxigenic culture

An evaluation of a newer-generation iLAMP molecular nucleic acid amplification assay, Meridian illumigene™, was performed, with toxigenic culture defining presence of *C. difficile* (Table 7). Of 427 samples, there were 35 concordant positive results and 366 concordant negative results. Sensitivity was found to be 61.4%, 95% CI (47.5, 74.0). Specificity on the other hand, was found to be in agreement with previously reported values with a rate of 98.9% , 95% CI(97.7, 99.7). Positive predictive value was found to be 89.7%, 95% CI (75.8, 97.1). The negative predictive values was found to be 94.3%, 95% CI (91.5, 96.4) (Tables 7 and 10).

Table 7. Performance characteristics of isothermal loop mediated amplification (iLAMP) assay defined by toxigenic culture

Isothermal loop mediated amplification (iLAMP)			
Toxigenic Culture	**Positive**	**Negative**	**Total**
Positive	35	22	57
Negative	4	366	370
Total	39	388	427
		95% CI	
Sensitivity	35/57=61.4%	(47.5-74.0)	
Specificity	366/370=98.9%	(97.7-99.7)	
Positive Predictive Value	35/39=89.7%	(75.8-97.1)	
Negative Predictive Value	366/388=94.3%	(91.5-96.4)	

4.1.5 Performance characteristics of Prodesse Progastro Cd™ Real-Time Polymerase Chain Reaction (RT-PCR) assay defined by toxigenic culture

An evaluation of a second molecular nucleic acid amplification assay, RT-PCR, was performed specifically the Prodesse Progastro Cd™ assay. This assay targeted the *tcdB* gene. As with all other evaluations, toxigenic culture defined presence of *C. difficile* (Table 8). All Ct values were considered positive. Of 427 samples, there were 35 concordant positive results and 366 concordant negative results. Sensitivity was found to be 57.9%, 95% CI (44.2, 70.9), in the range of previously reported sensitivity values. Specificity also was found to be in the range of previously reported values with 99.7%, 95% CI (98.5, 100). Positive predictive value was found

to be 97.1%, 95% CI (84.7, 99.9). The negative predictive value were found to be 93.9%, 95% CI (91.1, 96.1) (Tables 8 and 10).

Table 8. Performance characteristics of Prodesse Progastro Cd™ Real-Time Polymerase Chain Reaction (RT-PCR) defined by toxigenic culture

Prodesse Progastro Cd™ RT-PCR			
Toxigenic Culture	**Positive**	**Negative**	**Total**
Positive	33	24	57
Negative	1	369	370
Total	34	393	427
		95% CI	
Sensitivity	33/57=57.9%	(44.2-70.9)	
Specificity	369/370=99.7%	(98.5-100)	
Positive Predictive Value	33/34=97.1%	(84.7-99.9)	
Negative Predictive Value	369/393=93.9%	(91.1-96.1)	

4.1.6 Performance characteristics of Glutamate Dehydrogenase (GDH) assay defined by toxigenic culture

An evaluation of an assay the common metabolic enzyme antigen, Glutamate Dehydrogenase (GDH), specifically the Alere™ QUIK CHECK COMPLETE assay was performed. As with all other evaluations, toxigenic culture defined presence of *C. difficile* (Table 9). Of 427 samples, there were 45 concordant positive results and 347 concordant negative results. Sensitivity was

found to be 79.0%, 95% CI (66.1, 88.6), in the range of previously reported sensitivity values. Specificity also was found to be in the range of previously reported values at 93.8%, 95% CI (90.8, 96.0). Positive predictive value was found to be 66.2%, 95% CI (53.7, 77.2). The negative predictive values were found to be 96.7%, 95% CI (94.2, 98.3) (Tables 9 and 10).

Table 9. Performance characteristics of Glutamate Dehydrogenase (GDH) defined by toxigenic culture

Alere™ QUIK CHECK COMPLETE Glutamate Dehydrogenase (GDH)			
Toxigenic Culture	**Positive**	**Negative**	**Total**
Positive	45	12	57
Negative	23	347	370
Total	68	359	427
		95% CI	
Sensitivity	45/57=79.0%	(66.1-88.6)	
Specificity	347/370=93.8%	(90.8-96.0)	
Positive Predictive Value	45/68=66.2%	(53.7-77.2)	
Negative Predictive Value	347/359=96.7%	(94.2-98.3)	

Table 10. Performance characteristics of all assays evaluated defined by toxigenic culture

	CCNA	GDH	EIA	RT-PCR	iLAMP
Sensitivity	45.6%	79.0%	28.1%	57.9%	61.4%
Specificity	99.2%	93.8%	100%	99.7%	98.9%
Positive Predictive Value	89.7%	66.2%	100%	97.1%	89.7%
Negative Predictive Value	92.2%	96.7%	90.0%	93.9%	94.3%

4.2 AIM #2 EXPLORE POTENTIAL REFINEMENT OF PERFORMANCE CHARACTERISTICS THROUGH INCORPORATION OF CLINICAL DATA

In order to investigate the hypothesis that the new molecular assays are detecting patients who are asymptomatically colonized, the charts of all patients with discrepant results were reviewed. Serving as a negative and positive control, the charts of patients with concordant positive results (iLAMP, PCR, and CCNA) charts and concordant negative charts were reviewed. Concordant negative results had toxigenic culture results stratified to examine the *C. difficile* likelihood score. This was done by examining the mean, median and standard deviation of the assigned *C. difficile* likelihood score among these groups. Risk factors for CDI, defined as presence of diarrhea, use of antibiotics, and inpatient exposures were also examined for the same patients.

These factors are considered by many as the *sine qua non* of CDI and therefore are of interest. Between all nine groups, it was found that there was no significant difference between the mean or median *C. difficile* likelihood scores (Table 11)**.** Furthermore, a high proportion of patients had all three factors for disease, particularly antibiotics, with exceptions being group five which had lacked diarrhea according to chart review although having unformed stool samples for inclusion within the study. Specimens being submitted for CDI testing at this clinical microbiology laboratory have a high likelihood of CDI, and therefore negative tests within the discrepant categories are highly likely to represent false negative results.

Table 11. Comparison of *C. difficile* likelihood score and patient factors between discrepant results

Group	N=	Combinations of Results				*C.difficile* Likelihood Score			Patient Risk Factors			
		Prodesse RT-PCR	Illumigene™ iLAMP	CCNA	Toxigenic Culture	Mean	Median	Standard Deviation	Diarrhea None	Antibiotics Exposure		Inpatient
1	11	Positive	Positive	Positive	N/A	13	13.5	2.5	82%	90%	82%	0%
2	17	Positive	Positive	Negative	N/A	8.8	8	3.8	76%	76%	65%	0%
3	4	Positive	Negative	Positive	N/A	11	10	3.1	75%	100%	75%	0%
4	3	Positive	Negative	Negative	N/A	9.3	9	5.5	66%	100%	33%	0%
5	1	Negative	Positive	Positive	N/A	9	9	N/A	0%	100%	100%	0%
6	2	Negative	Positive	Negative	N/A	12	12	5.2	100%	100%	100%	0%
7	5	Negative	Negative	Positive	N/A	10	11	4	80%	80%	60%	0%
8	4	Negative	Negative	Negative	Positive	9.4	10	2.6	100%	100%	75%	0
9	9	Negative	Negative	Negative	Negative	8.9	9.5	5.0	60%	80%	60%	10%

4.3 AIM#3 EXPLORE POTENTIAL ASSOCIATIONS BETWEEN RISK FACTORS, SYMPTOMS, AND RESULTS ON SEMI-QUANTITATIVE TOXIGENIC CULTURE

The final analysis explored a potential association between semi-quantitative results on toxigenic culture and clinical characteristics, defined by the *C. difficile* likelihood score and individual risk factors. A potential association between positive results on toxin detecting assays and semi-quantitative toxigenic culture was investigated (Tables 12 and 13). No observable trend was observed between results of semi-quantitative culture and the percentage of positive results on any assay. Additionally, there was no correlation between the frequency of individual factors and results on semi-quantitative culture (Table 13). Further, there was no association between the factors and results on semi-quantitative culture. A large proportion of patients are observed in almost all cases to possess all three exposures, including those who tested negative on the semi-quantitative assay.

Table 12. Comparison of positive results on toxin detecting assays and *C. difficile* likelihood scores between semi-quantitative result

Semi-quantitative result	N	*C. difficile* likelihood score			Positive Results			
		Mean	Median	Standard Deviation	EIA	CCNA	Molecular iLAMP	Molecular Prodesse RT-PCR
(heavy)	M	12.5	12.5	2.5	5/12.5= 42%	7/12.5=58%	10/12.5=83%	10/12.5=83%
(moderate)	3	12	13	3.6	2/3= 66%	3/3= 100%	2/3= 66%	2/3= 66%
(light)	5	9.8	9	2.6	0%	2/5= 40%	4/5= 80%	5/5= 100%
(rare)	14	8.9	8	5.0	3/14= 21%	5/14=35.7%	9/14= 64%	10/14= 71%
(broth only)	4	8	8.5	3.6	0%	1/4= 25%	2/4= 50%	3/4= 75%
(negative)	13	9.2	9	5.9	0%	1/13=7.6%	1/13= 7.6%	1/13=7.6%

Table 13. Comparison of rates of patient exposures, symptoms and results on Semi-quantitative culture

Semi-quantitative result	N	Patient Factors			
		Diarrhea	Antibiotics	Inpatient	None
(heavy)	12	92%	100%	92%	0%
(moderate)	3	66%	100%	66%	0%
(light)	5	100%	60%	80%	0%
(rare)	14	79%	79%	43%	0%
(broth only)	4	100%	100%	75%	0%
(negative)	13	54%	85%	62%	0%

5.0 DISCUSSION

Clostridium difficile is a problematic infection routinely encountered by healthcare professionals and patients in the hospital setting. Once a rare, nuisance infection with few severe outcomes, the disease is increasingly affecting a variety of populations due to changes in risk factors and clinical practices. These include the indiscriminate use of antimicrobials, proton pump inhibitor/H_2 blocker, and poor infection control practices.

At the same time, there are other issues with *C. difficile* surveillance and diagnosis. There is limited surveillance data as reporting is voluntary and state laws vary in regards to reporting requirements. Only recently, other countries have started to establish surveillance programs for *C. difficile*, mainly in Western Europe as a consequence of increasing incidence. Potentially hampering the surveillance is the medical community lacking a cost-effective, rapid, and perhaps most importantly, accurate assay to help the clinician make the diagnosis of *Clostridium difficile*. Having a rapid assay allows for treatment initiation at an earlier time, leading to better outcomes, as the patient will not be allowed to have disease progress. Having an accurate assay, particularly one with high sensitivity, ensures, that patients are appropriately treated and isolated within hospital, potentially translating to fewer patients shedding spores in the healthcare setting. Ensuring the assay possesses high specificity is of importance as well. The greater number of false positive patients leads to increased use of antimicrobials, leading

increased treatment costs, and potential for development of other organisms resistant to the antimicrobial agents, particularly vancomycin.

The purpose of this study was to examine the performance characteristics independently of the other attributes of the assay, mainly the processing time, and cost-effectiveness. While these attributes are of importance to other parties including the patient and the medical institution, the results of this study will be used in conjunction with those attributes to determine which assay is best for our medical institution. A notable aspect of this study was its incorporation of clinical data through development of a *C. difficile* likelihood score in an attempt to refine the performance characteristics of assays, in particular the newer molecular assays which employ nucleic acid amplification. Additionally, there was an interest in determining if there was relationship between semi-quantitative result on a toxigenic culture and *C. difficile* likelihood score as well as individual clinical factors.

When examining the comparison of assays without clinical data, it was found that enzyme immunoassay had poor sensitivity, which supports the recommendation made by SHEA and IDSA that this as a method as less desirable. It was also found that the CCNA had high specificity and positive predictive value, but its sensitivity and negative predictive values were surprisingly low compared to some prior studies. One reason for this observation is the increasing skill of labs performing toxigenic culture, which included broth enrichment which may increase sensitivity. The GDH common antigen assay yielded fair performance characteristics. However, this assay did not have a sufficient negative predictive value to serve as a good screening test. Neither of the two molecular assays (Prodesse Progastro™ RT-PCR and illumigene™ iLAMP) yielded performance characteristics superior to the other, and compared to the gold standard toxigenic culture they miss up to 40% of patients with toxin-producing *C.*

difficile in their stools. However, both methods are superior to the currently used CCNA assay. Applying these results to clinical practice, treating CDI in context of a high pre-test probability is advisable based on these results. When interpreting the results of these molecular assays it is important to note that the gene target of each of these individual assays is different. The Prodesse Progastro assay utilizes the tcdB gene, while the illumigene iLAMP amplifies the tcdA gene. At the current time the most prevalent strains are tcdA+/tcdB+, with the small minority of strains possessing tcdA-/tcdB+ genotype. Therefore, continual evaluation of the strains present within the environment is essential to ensure that the performance characteristics are affected by a change in the genotype. Further, it is important to note that our study used the current gold-standard toxigenic culture as reference standard. Some previous studies within the meta-analysis listed in table 2 utilize a reference standard such as the previous gold standard assay, CCNA, or a composite gold standard composed of a positive test result on multiple assays. These reference standards could have provided results with demonstrated higher performance characteristics. The overall conclusion that molecular assays are better assays in comparison to their older counterparts is in agreement with other studies.[45]

In the second aim of this study, the resulting mean *C. difficile* score in this study was compared between various groups with different combinations of positive results on assays. Relationships between individual clinical factors and combinations of results on assays were examined as well. It was found that there was no significant difference between the mean clinical score and the combinations of outcomes. Of particular interest were differences between groups 2, 4, and 6, which had at least one positive result on at least one other assay than the cell cytotoxicity neutralization assay currently in use. When examining those three groups no significant difference was observed. Furthermore, when stratifying toxigenic culture results in

the negative control group no significant difference was found. This could be due to inaccurate results from toxigenic culture, or other antibiotic-associated diarrhea. Therefore, refinement using our scoring system is unlikely. This contrasts to other literature which has reported refinements in performance characteristics through incorporation of patient data.[46]

Because of the lack of significant differences among the group and the high proportions of patients possessing the three required factors to meet the threshold as having CDI, refinement of performance characteristics of the assays is not possible and therefore rejects the hypothesis that the assays are simply picking up patients colonized with *C. difficile*. The majority of patients tested had diarrhea, as well as the two main risk factors for CDI which are antimicrobial use and inpatient exposure within the previous twelve weeks. Notably, there was one discrepancy with one patient who lacked diarrhea in retrospective chart review (Table 10), who should have had an unformed stool for processing. It is possible to also make the conclusion that clinicians are properly utilizing *C. difficile* toxin testing when indicated and submitting only when risk factors and symptoms are present. A potential bias exists within this study however, exists, in that a requirement in this study was the processing of unformed stools which could have led to a higher number of CDI patients.

The third aim of this study sought to examine a potential relationship between semi-quantitative culture results and clinical *C. difficile* likelihood score, and individual patient factors. No significant differences were observed between the likelihood score among all the semi-quantitative culture. It was also found that a significant proportion of patients possessed the three clinical factors for *C. difficile.* Further, there were no observable trends between increasing colony forming units indicated by a higher category and being positive on any one assay. Therefore, the concentration of the organism isolated would not be a good predictor of

disease likelihood with the scoring system used in this study. Therefore, one would not be able to differentiate between asymptomatic and diseased individuals by semi-quantitative results. Previously reported data have indicated that asymptomatic carriers have a lower mean colony forming units per gram as compared to symptomatic patients.[47] This result contrasts to our study which suggests that all concentrations of *C. difficile* forming units would likely correspond to patients with true CDI.

Noteworthy, independent of the clinical data, is the fact that there was a greater percentage of positive assays for newer molecular assays at all semi-quantitative levels compared to both EIA or CCNA assays with at least 50% positive results. This could be due to the ability of the newer molecular methods to potentially better detect *C. difficile* as it amplifies the toxin B gene. Nevertheless, in this study we rejected the hypothesis that excess asymptomatic carriers were detected.

Based on only attributes of performance characteristics alone, at this point it is advisable that real-time polymerase chain reaction assay be used for routine clinical care as it amplifies the *tcd*B gene, which has the is less likely to possess mutations which do not allow for detection. Furthermore, considering that this assay is relatively new with a FDA approval in July 2010. Further studies on the ability of iLAMP assays to detect known toxigenic tcdA-/tcdB+ strains need to be performed to predict the performance characteristics if an outbreak were to occur within the healthcare institution. If future data suggests that iLAMP assays targeting *tcdA* are highly effective, a new recommendation could be made in conjunction with cost and timeliness.

5.1 FUTURE DIRECTIONS

While the data obtained from this study on performance characteristics independent of clinical data could assist in the selection of a new assay for routine use, and render any need for future work unnecessary, other evaluations could be performed in an attempt to refine and build upon this study. The *C. difficile* likelihood score used in this experiment was a novel scoring system which had not been previously used, therefore, its use as a predictor of *C. difficile* had not been validated in a large cohort of patients. Any resulting adjustments in the scoring system from a validation study would warrant further study as it relates to performance characteristics. Additionally, the development of other scoring systems should be explored. Continual evaluation will need to be performed on all assays to ensure that these assays possess the highest levels of accuracy over time. This is especially true when examining performance characteristics of molecular assays, as the distribution of toxinotypes within the medical institution could change favoring A-/B+ toxinotypes. This would be problematic if the target of the molecular assay is the *tcdA* gene. Because of this fact, further studies need to be performed to test the ability of iLAMP assays to detect known A-/B+ strains.

APPENDIX A: GLOSSARY OF TERMS

ANAEROBIC: Term used to describe conditions with an absence of oxygen.

ANAMNESTIC: A term relating to rapid enhanced immune response upon encounter with previously encountered antigen.

ANTIBODY: A protein molecule which binds to an antigen and neutralizes them or prepare for further elimination by phagocytes. Antibodies are produced by B-lymphocytes.

ANTIMICROBIAL: Substances with active ingredients which kill pathogens including bacteria and fungi.

ASSAY: A procedure in molecular biology and laboratory medicine to quantitatively assess the presence of a substance or organism.

BACILLUS: A rod-shaped bacterium.

BANDEMIA: An excess of band cells or immature lymphocytes in the blood released by bone marrow usually during periods of infection or inflammation. Bandemia is an indicator of possible CDI.

COLECTOMY: A surgical procedure involving resection of the colon.

COMORBIDITIES: A medical term used to describe secondary diseases in addition to the primary disease.

CYCLE THRESHOLD VALUE (CT): Defined as the PCR cycle where enough amplification of the nucleic acid amplification has occurred to generate a fluorescent signal which can be detected.

CYTOTOXIN: A substance which has toxic effects on cells.

DISINFECTANTS: Substances used to kill or inhibit growth of microorganisms on inanimate objects (e.g. Fomites).

ENDEMIC: A term used in epidemiology to describe a continual presence of disease within the population.

ENTEROTOXIN: A protein toxin released by a microorganism which primarily affects the intestine.

EPIDEMIC: A term used in epidemiology to describe presence of disease in with incidence levels greater than expected.

ELECTRON MICROSCOPY: An advanced form of microscopy which utilizes a beam of electrons to produce a highly magnified image.

ENDOSCOPY: A procedure where a medical instrument (endoscope) used to image inside the body cavity.

FULMINANT: Disease which with sudden, rapid onset with ability to be lethal.

ILEOSTOMY: Surgical procedure involving routing the ileum portion of the small intestine to the surface of the skin, allowing waste to collect to an external pouch. This procedure allows bypass of the large intestine (colon).

IMMUNITY: A term used to describe ability of an organism to resistance infection or disease.

IMMUNOSUPPRESSION: A term used to describe a state with decreased resistance to infection or disease (immunity).

INCUBATION: **U**sed in the medical context to describe time between exposure and time patient starts exhibiting symptoms.

INVASIVE: A medical procedure involving a breach of the skin.

LEUKOCYTOSIS: A medical term used to describe an increase of leukocytes (white blood cells) above the normal range in the blood.

LOCUS: A term used in the context of genetics to describe the position of a gene on a chromosome.

MEGACOLON: Abnormal dilation of colon, accompanied by paralysis, and tumor like masses called fecalomas.

NEGATIVE PREDICTIVE VALUE: A statistical measure used in epidemiology defined by the number of true negatives divided by the number of negative results defined by a diagnostic assay. In other words, negative predictive value is defined as the number of true negatives divided by the sum of true negatives and false negatives. This measure is typically used to measure performance of a binary diagnostic assay.

NOSOCOMIAL: A term meaning hospital origin, usually used in context of hospital acquired infections.

PATHOGENECITY: A term used to describe ability of pathogen to cause infectious disease.

PATHOGENESIS: A mechanism by which a disease in the host is caused by a pathogen.

POSITIVE PREDICTIVE VALUE: a statistical measure used in epidemiology defined by the number of true positives divided by the number of positive results defined by a diagnostic assay. In other words, positive predictive value is defined as the number of true positives divided by the sum of true positives and false positives. This measure is typically used to measure performance of a binary diagnostic assay.

PROBIOTICS: Live microorganism which provide positive benefits to the host organism.

RESERVOIR: Conditions where the infectious agent lives, grows and multiplies.

RETROSPECTIVE: A term used in medicine and epidemiology to describe the review of previously occurring medical history.

SANITATION: Means of improving and promoting health through improved hygiene and prevention measures.

SENSITIVITY: A measure to measure the performance characteristics of a binary diagnostic assay. Sensitivity rate is defined the number of true positives divided by the sum of number of true positives and number of false negatives. Stated differently, it is the probability of a positive assay given the presence of the condition being examined.

SPECIFICITY: A measure is to measure the performance characteristics of a binary diagnostic assay. Specificity is defined as the number of true negatives divided by the sum of the number of true negatives and false positives. State differently, it is the probability of a negative assay given the presence of the condition being examined.

SPORULATION: Term used to describe the active production of spores.

APPENDIX B: ACRONYMS

CCNA: Cell cytotoxicity neutralization assay

CDI : *Clostridium difficile* associated diarrhea

CDC: Centers for Disease Control

CdtA: *Clostridium difficile* transferase subunit A

CdtB: *Clostridium difficile* transferase subunit B

CPE: Cytopathic effects

CT: Computerized tomography

Ct Value: Cycle threshold value

EIA: Enzyme immunoassay

FMT: Fecal microbiota transplant

GDH: Glutamate dehydrogenase

HAI: Hospital acquired infections

IDSA: Infectious Diseases Society of America

iLAMP: Isothermal loop mediated amplification

IVIG: Intravenous immunoglobulin

MRSA: Methicillin resistant *Staphylococcus aureus*

PPI: Proton pump inhibitors

PCR: Polymerase chain reaction

RT-PCR: Real-time polymerase chain reaction

SHEA: Society for Healthcare Epidemiology of America

tcdA: *Clostridium difficile* toxin A

tcdB: *Clostridium difficile* toxin B

tcdC: *Clostridium difficile* toxin C

tcdE: *Clostridium difficile* toxin E

tcdR: *Clostridium difficile* toxin R

UPMC: University of Pittsburgh Medical Center

VRE: Vancomycin resistant *Enterococcus*

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