Comparison of recurrent and sporadic *Clostridium difficile* infection and microbiological investigation of response of recurrent *Clostridium difficile* infection to fecal microbiota transplantation

by

Connor Richardson

A Thesis

Presented to

The of Guelph

University

In partial fulfillment of requirements

for the degree of

Master of Science

in Clinical Studies

Guelph, Ontario, Canada

© Connor Richardson, April, 2014
ABSTRACT

COMPARISON OF RECURRENT AND SPORADIC CLOSTRIDIUM DIFFICILE INFECTION AND MICROBIOLOGICAL INVESTIGATION OF RESPONSE OF RECURRENT CLOSTRIDIUM DIFFICILE INFECTION TO FECAL MICROBIOTA TRANSPLANTATION

Connor Richardson
University of Guelph, 2013

Advisors:
Dr. J. S. Weese
Dr. A. Bersenas

This thesis investigates the strain distribution and characteristics of Clostridium difficile recovered from patients with sporadic and recurrent diarrhea. Samples were collected from patients with both recurrent and sporadic disease and ribotyping was conducted to determine the strain distribution, in particular examining the prevalence of NAP1/027. Antimicrobial susceptibility to vancomycin and metronidazole was determined using E-tests. It was found that both gender (female) and NAP1/027 were positively associated with recurrent disease. For patients undergoing human biotherapy for treatment of recurrent disease, pre and post-treatment samples were ribotyped. It was found that NAP1/027 was present in high proportions in individuals after clinical cure, however, no association was determined between the presence of C. difficile and response to fecal microbiota transplantation (FMT) treatment.
ACKNOWLEDGEMENTS

I would like to express my appreciation to my advisors Dr. Scott Weese and Dr. Alexa Bersenas for their support and advice throughout this project. I would also like to thank Dr. Peter Kim and Dr. Christine Lee for their contributions, and for making this project possible.

I would also like to thank Joyce Rousseau for her endless patience in the bacteriology laboratory, and for her invaluable advice.

Thanks go to my parents Dr. Carole Richardson and Dr. Warnie Richardson, as well as my brothers Lachlan and Brennan. I also need to thank Jessica Pask and Matt McSweeney for sticking with me through the ups and downs of graduate research, and all of my other wonderful friends who were so supportive throughout this whole process.

In addition, I would like to thank my granny Elsie MacLachlan, who was so supportive both emotionally and financially through this whole process, and who passed away in October 2013.

I would also like to thank the Canadian Institutes of Health Research (CIHR) and the Natural Sciences and Engineering Research Council (NSERC) Collaborative Health Research Projects (project 413548-2012) for their financial support of this research project.
DECLARATION OF WORK PERFORMED

I declare that the work reported in this thesis was performed by Connor Richardson.

Statistical analyses were performed with the assistance of Dr. Scott Weese.
TABLE OF CONTENTS

ABSTRACT

ACKNOWLEDGEMENTS ......................................................................................... iii

DECLARATION OF WORK PERFORMED ............................................................... iv

TABLE OF CONTENTS ......................................................................................... v

LIST OF TABLES .................................................................................................... viii

LIST OF FIGURES .................................................................................................. x

1.0 INTRODUCTION ............................................................................................ 1

1.1 GOALS OF THE STUDY ................................................................................ 3

1.2 Literature Review .......................................................................................... 4

  1.2.1. Introduction ............................................................................................. 4

  1.2.2. Microbiology of C. difficile ................................................................. 4

  1.2.3. Pathophysiology .................................................................................... 5

  1.2.4. Colonization vs. Infection ................................................................. 8

  1.2.5. Clinical Disease .................................................................................... 8

  1.2.6. Community Associated Disease vs. Hospital Associated Disease .......... 9

  1.2.7. Risk Factors .......................................................................................... 12

  1.2.8. Diagnosis of CDI .................................................................................. 13

  1.2.9. Isolation of C. difficile ......................................................................... 16

  1.2.10. Identification ....................................................................................... 18

  1.2.11 Characterization ................................................................................... 19
3.0 Examination of the association between *Clostridium difficile* strain type and response to treatment with Fecal Microbiota Transplants (FMT) in patients with recurrent disease

3.1 Abstract

3.1 Introduction

3.2 Materials and Methods
   3.2.1 Sample conditions
   3.2.2 Culture conditions
   3.2.3 Characterization of isolates
   3.2.4 Data Analyses

3.3 Results

3.4 Discussion

3.5 Conclusion

3.6 Acknowledgements

3.7 References

4.0 DISCUSSION

5.0 MASTER REFERENCE LIST
# LIST OF TABLES

## 1.2 Literature Review

<table>
<thead>
<tr>
<th>Table 1.1: Success rates of FMT in studies from 2010 to 2014.</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35</td>
</tr>
</tbody>
</table>

## 2.0 Manuscript COMPARISON OF THE DISTRIBUTION OF CLOSTRIDIUM DIFFICILE STRAINS AMONG SPORADIC AND RECURRENT CASES

<table>
<thead>
<tr>
<th>Table 2.1: Primers used in the multiplex toxin PCR, adapted from (Persson, Torpdahl, &amp; Olsen, 2008)</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>73</td>
</tr>
</tbody>
</table>

Table 2.2: Descriptive data comparing patients with recurrent *Clostridium difficile* infection (CDI) (n=27) and those with sporadic CDI (n=33)

<table>
<thead>
<tr>
<th>Table 2.3: Descriptive data comparing ribotypes found among patients with recurrent CDI (n=27) and sporadic CDI (n=33)</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>75</td>
</tr>
</tbody>
</table>

Table 2.4: Results of logistic regression analysis examining gender, age, and presence of 027 in patients with sporadic and recurrent CDI

<table>
<thead>
<tr>
<th>Table 2.5: MICs for metronidazole and vancomycin for patients with recurrent disease at both 24 and 48 hours.</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>77</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2.6: The association between ribotype 027 and MIC.</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>78</td>
</tr>
</tbody>
</table>

## 3.0 Manuscript EXAMINATION OF THE ASSOCIATION BETWEEN CLOSTRIDIUM DIFFICILE STRAIN TYPE AND RESPONSE TO TREATMENT WITH FECAL MICROBIOTA TRANSPLANTATION (FMT) IN PATIENTS WITH RECURRENT DISEASE

<table>
<thead>
<tr>
<th>Table 3.1: Primers used in the multiplex toxin PCR, adapted from (Persson et al., 2010)</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>101</td>
</tr>
</tbody>
</table>
Table 3.2: Isolation of *C. difficile* and *C. difficile* ribotype 027 from 28 patients with recurrent *C. difficile* infection

<table>
<thead>
<tr>
<th>Table 3.2: Isolation of <em>C. difficile</em> and <em>C. difficile</em> ribotype 027 from 28 patients with recurrent <em>C. difficile</em> infection</th>
<th>102</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.3: Post FMT treatment clinical cure rates</td>
<td>103</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>1.2 Literature Review</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1. Diagram of the Pathogenicity Locus</td>
<td>36</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2.0 Manuscript COMPARISON OF THE DISTRIBUTION OF CLOSTRIDIUM DIFFICILE STRAINS AMONG SPORADIC AND RECURRENT CASES</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2.1. <em>C. difficile</em> growth on chromogenic culture media.</td>
<td>79</td>
</tr>
<tr>
<td>Figure 2.2. Blood agar with 5% sheep blood (Oxoid) stamped with Etests for both vancomycin and metronidazole</td>
<td>80</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3.0 Manuscript EXAMINATION OF THE ASSOCIATION BETWEEN CLOSTRIDIUM DIFFICILE STRAIN TYPE AND RESPONSE TO TREATMENT WITH FECAL MICROBIOTA TRANSPLANTATION (FMT) IN PATIENTS WITH RECURRENT DISEASE</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 3.1. Cumulative response rate of patients (n=91) to consecutive FMT treatments</td>
<td>104</td>
</tr>
<tr>
<td>Figure 3.2. Cumulative post-treatment sample results of patients with resolution of gastrointestinal symptoms after FMT treatments, separated into those with <em>C. difficile</em> present after cure, those without <em>C. difficile</em> present after cure, and those with no post-treatment sample.</td>
<td>105</td>
</tr>
</tbody>
</table>
1.0 INTRODUCTION

_Clostridium difficile_, a spore-forming anaerobe responsible for a large proportion of hospital-acquired diarrhea, is of increasing concern for individuals in long-term care facilities, hospitals, and the general community (Kelly, C., & Kyne, L., 2011; Khanna et al., 2012; Ricciardi et al., 2007). Instances of _Clostridium difficile_ infection (CDI) are growing globally, and infections are increasing in severity as well as recurrence (Heslop et al., 2013; Khanna, S., & Pardi, D., 2012; Muto et al., 2005).

Of particular concern are so-called ‘outbreak strains’ that appear to be responsible for hospital-wide outbreaks. One such strain is known as North American Pulsotype 1 (NAP1)/ribotype 027. NAP1/027 has been documented in hospital outbreaks in North America and Europe, and more recently, has been identified in Latin America and Asia (López-Ureña et al., 2014; Pépin et al., 2004; Wang et al., 2014). It has been theorized that the increased virulence of this strain can be attributed to the presence of an additional toxin, known as binary toxin (Bacci et al., 2011; Goorhuis et al., 2008).

Individuals at particular risk, including those of advanced age (65 and older) and individuals who have experienced prolonged hospital stays, can experience symptoms ranging from diarrhea to sepsis, and even death; however, the greatest risk factor for all populations is exposure to broad-spectrum antibiotics (Khanna et al., 2012; Louie et al., 2013; Owens et al., 2008). This presumably relates to the intestinal microbiota, the complex microbial population that inhabits the intestinal tract and plays a critical role in protection from opportunistic enteropathogens such as _C. difficile_. Exposure to broad-spectrum antibiotics can result in the alteration of native microbiota, which then allows opportunistic _C. difficile_ bacteria to proliferate. Although discontinuation of the initial
antibiotic may result in resolution of gastrointestinal symptoms, sometimes antimicrobial treatment with metronidazole or vancomycin is required (Louie et al., 2013) and in some cases, severe disease that is refractory to treatment may develop.

Recurrent CDI is an increasing problem. After an initial episode of CDI, 15-35% of patients may develop recurrent disease (McFarland, L., Elmer, G., & Surawicz, C., 2002). Many patients with recurrent CDI respond poorly or fail to respond whatsoever to conventional therapy. In response to this, and based on the premise that gastrointestinal microbiota disruption is at the basis of recurrent disease, there has been increasing interest in fecal microbiota transplantation (FMT). This procedure, which involves administration of feces from a healthy donor, has proven to be effective, resolving up to 92% of cases (Brandt et al., 2012; van Nood et al., 2013; Gough et al., 2011). This treatment method involves the transfer of a fecal sample from a healthy donor via nasogastric tube, enema, or more recently, oral capsules. The FMT operates on the theory that the native intestinal microbiota of the patient is replenished, and therefore competes with the *C. difficile* bacteria, preventing *C. difficile* infection (CDI) (Buffie et al., 2012; McDonald et al., 2005). However, whether this treatment eliminates *C. difficile* completely or just causes a bacterial population decrease sufficient to eliminate the harmful effects of *C. difficile* colonization is not entirely known. Understanding the pathophysiology of recurrent CDI and the mechanisms of FMT are important for better understanding this important disease, understanding the true basis of this treatment and for development of novel treatment and preventive methods.
1.1 GOALS OF THE STUDY

This study was conducted at the University of Guelph, Ontario Veterinary College with the following objectives:

(i) to determine whether certain strains of *Clostridium difficile* are associated with recurrent CDI

(ii) to investigate strain-associated risk factors associated with recurrent disease

(iii) to determine whether *Clostridium difficile* remains present in the gut after clinical cure following FMT

(iv) to determine whether presence of certain strains are associated with treatment failure or persistence of colonization after FMT
1.2 LITERATURE REVIEW AND HYPOTHESIS

1.2.1. Introduction

_Clostridium difficile_ is an anaerobic, spore-forming bacillus that has been established as a major causative agent of antimicrobial associated diarrhea and pseudomembranous colitis in humans (Aas, J., Gessert, C., & Bakken, J., 2003). Although it is classically associated with endemic disease in healthcare facilities, recent increases in the incidence of outbreaks and disease severity (Kelly, C., & LaMont, J., 2008), along with identification of community-associated infection (Khanna, S., et al., 2012) have renewed interest in the mechanisms and pathophysiology of this pathogen.

1.2.2. Microbiology of _C. difficile_

_Clostridium difficile_, a member of the _Clostridium_ genus, is a gram-positive endospore-forming obligate anaerobe that resides in the intestinal tract of humans and various animal species (Bartlett, J., & Gerding, D., 2008). Generally, the normal intestinal microbiota present in the human gastrointestinal tract inhibits the growth of _C. difficile_ to either prevent its establishment in the intestinal tract or prevent it from causing disease. However, when the microbiota is disrupted, as is often the case with broad-spectrum antibiotic administration, _C. difficile_ may be able to proliferate and cause gastrointestinal disease (Owens et al., 2008).

An important feature of _C. difficile_ is its ability to produce environmentally hardy spores that are shed in feces. These spores are the primary infective form, and being resistant to environmental effects and many conventional disinfectants, can remain viable for weeks or even years (Poutanen, S., & Simor, A., 2004). Transmission occurs through
the fecal-oral route, and is a particular issue in healthcare settings such as hospitals and long-term care facilities.

1.2.3. Pathophysiology

*Clostridium difficile* can be characterized as either toxigenic or non-toxigenic based on its ability to produce one or more of three recognized toxins. Non-toxigenic strains lack a functional pathogenicity locus (PaLoc), the portion of the *C. difficile* chromosome on which the genes encoding the expression of toxins A (*tcdA*) and B (*tcdB*) reside (Cohen, S., Tang, Y., & Silva, J., 2000; Sun, X., Savidge, T., & Feng, H., 2010), and are assumed to be clinically irrelevant. Toxigenic strains can generate any of 3 toxins; two large clostridial toxins, Toxin A (TcdA, 308 kDa), Toxin B (TcdB, 270 kDa), and CDT (binary toxin) and are able to produce clinical disease; however, the specific roles of the various toxins have not been entirely defined (Sun et al., 2010) and toxigenic strains can be present in the intestinal tract in the absence of disease. In particular, the specific function of CDT (binary toxin) requires further investigation and its role in disease is currently unclear. In addition to the clinical effects of the toxins, toxins A and B or the genes encoding them are the primary markers for diagnosis of *C. difficile* in clinical settings (Cohen et al., 2010).

The aforementioned toxins A and B are encoded by the genes *tcdA* and *tcdB*, respectively. These genes are found on the 19.6 kb PaLoc on large open reading frames (Voth, D., & Ballard, J., 2005) (Figure 1.1). In addition to the toxin genes, regulatory genes are also located on the PaLoc. The gene *tcdD* serves to up-regulate toxin production, while *tcdE* functions as a release mediator (Tan, K., Wee, B., & Song, K., 2001). Most notably, *tcdC*, located downstream of the *tcdA* and *tcdB* genes, functions as a
down-regulator of toxin production (Dupuy et al., 2008; Voth, D., & Ballard, J., 2005), although its role in modulating toxin production has recently been questioned (Murray et al., 2009). Alterations in tcdC have been implicated in the pathogenesis of CDI, due to the fact that certain strains containing tcdC deletions were initially identified as hypervirulent (Carter et al., 2011). Originally, it was believed that, in strains with tcdC deletions, both toxin A and B production was substantially increased, indicating the importance of tcdC in regulating toxin production (Warny et al, 2005). However, recent studies have indicated that while tcdC deletions or mutations may be prevalent in strains that are associated with increased virulence (Verdoorn et al., 2010), there is not necessarily a significant difference in toxin production among those strains and tcdC-associated variations in toxin production may not be a defining factor of virulence (Bakker et al., 2012; Murray et al., 2009).

Toxin A is categorized as an enterotoxin. It was initially identified as the primary toxin responsible for the gastrointestinal symptoms associated with C. difficile infection (CDI) due to the ability of the toxin to induce disease in laboratory animals (Dove et al., 1990).

Toxin B is categorized as a cytotoxin, and was not initially believed to produce enterotoxic effects independently of Toxin A. In a 1985 study, hamsters were given purified Toxin B intragastrically, and without the synergistic effects of Toxin A or previous cecal disruption, no enterotoxic effects were observed (Lyerly et al., 1985). Subsequent research has since indicated that TcdB is capable of causing CDI, and has a larger role in the disease process than was initially suspected (Sun et al., 2010). Further, toxin A-negative, B-positive virulent strains are recognized causes of CDI in humans.
These strains can elicit disease similar to A-positive B-positive strains, offering further proof that Toxin B also plays a key role in disease (Alfa et al., 2000; Voth D., & Ballard, J., 2005). It is currently accepted that both toxins A and B are enterotoxic and proinflammatory in the human intestine (Sun et al., 2010).

The binary toxin (CDT) was discovered more recently than toxins A and B (Carter et al., 2007), and therefore much less is known about its function. Initially, it was reported that the binary toxin had no effects on clinical disease and virulence; however, more recently, the importance of the binary toxin has been re-addressed. The binary toxin is a two-component toxin encoded by \textit{cdtA} and \textit{cdtB}, which are located outside of the PaLoc region. These genes encode two proteins, CDTa and CDTb (Rupnik, M., Grabnar, M., & Geric, B., 2003). While most CDT-positive strains possess both \textit{tcdA} and \textit{tcdB}, the position of the encoding gene allows for A-negative B-negative strains to encode for the binary toxin and these have been identified, albeit very rarely (Geric et al., 2003). In some of these instances, these strains have still proven to be pathogenic, implicating the CDT as a potential factor in clinical disease (Geric et al., 2006, 2003). Some of the concern regarding CDT has related to its presence in the outbreak strain ribotype 027/NAP1, a strain that is associated with hospital-outbreaks and increased disease severity (Bacci et al., 2011; Warny et al., 2005). However, the true role of this toxin in disease is still elusive because of numerous other differences that are present in purported hypervirulent strains in addition to CDT production.
1.2.4. Colonization vs. Infection

An individual can have *C. difficile* present in their gastrointestinal tract and be either colonized or infected. Colonization refers to the presence of *C. difficile*, living and reproducing in the intestinal tract in the absence of disease. This can occur with both non-toxigenic and toxigenic strains of the bacterium. It occurs in approximately 2-3% of adults in the community and 10%-25% of hospitalized individuals (Bartlett, J., 1994; Simor, et al., 2002), although good colonization data are lacking for many populations and there are few recent studies. The implications of colonization are poorly described, and it is unknown whether colonization predisposes to infection, has no impact or is a protective factor. However, colonized individuals are a potential source of *C. difficile* transmission to susceptible individuals.

While colonization rates of healthy individuals in the community are typically low, colonization of infants is common. One study found infants from 0-2 years had colonization rates ranging from 25.8% to 72.2% and *C. difficile* is not considered to be pathogenic to infants, potentially because of a lack of toxin receptors (Rousseau et al., 2011; Larson, H., Barclay, F., Honour, P., & Hill, I., 1982).

1.2.5. Clinical Disease

The clinical signs and symptoms of *C. difficile* range in severity, from mild diarrhea to pseudomembranous colitis, and death (Cohen et al., 2010). Complications resulting from *C. difficile* infection can include toxic megacolon, bowel perforation, and sepsis (Cohen et al., 2010). Although the symptoms tend to be more extreme when present in individuals of advanced age, severe disease can occur in any individual, including those typically considered at low risk of CDI (Khanna et al., 2012; Wilcox et al., 2008).
example, a 22-year-old female in Jamaica presented to physicians after receiving clindamycin following a dental procedure. She had a five-day history of diarrhea, and was treated with metronidazole. Despite treatment, complications of the infection resulted in toxic megacolon and bowel perforation and she died within 10 days of presentation (Heslop et al., 2013). In another case, a previously healthy pregnant thirty one year old patient (who had not visited a healthcare facility for an extended period, nor been on antibiotics), presented with C. difficile infection. The disease progressed and ultimately the individual underwent a subtotal colectomy and died (Baron, E., & Tenover, F., 2010).

Although neither of the above individuals would have been identified as “at-risk,” both presented with severe community-acquired disease.

1.2.6. Community Associated Disease vs. Hospital Associated Disease

*Clostridium difficile* infection is generally classified as either healthcare associated or community associated, and an increase in community-associated disease has also been observed in recent years (Khanna, S., & Pardi, D., 2012; Ricciardi et al., 2007).

1.2.6.1. Community Associated CDI

Community-associated disease has been defined, both by the European Centre for Disease Control and Prevention, and it’s American counterpart, as “the onset of symptoms which occurred while the patient was outside a health care facility and the patient had not been discharged from a health care facility within 12 weeks before symptom onset (‘community onset’, ‘community acquired’); or the onset of symptoms which occurred within 48 hours after admission to a health care facility and the patient had no prior stay in a health care facility within the 12 weeks before symptom onset.
Community associated CDI has not been investigated as thoroughly as hospital associated disease, and many cases can be traced back to hospital acquired infections that have spread to those in close contact in the community. Diagnosis of community-associated \textit{C. difficile} infection (CA-CDI) may be complicated due to the fact that healthcare professionals do not often suspect \textit{C. difficile} in cases where the patient does not have traditional risk factors. This is of particular concern because of the apparent increase in CDI in people without known risk factors. In low risk individuals, mild symptoms may never be classified, and the actual incidence of community-acquired disease could be higher than originally believed (Wilcox et al., 2008). Multiple studies have attempted to establish the incidence of CA-CDI, and numbers have varied. A study of North Carolina Veteran’s Affairs hospitals found that approximately 20% of CDI identified in a hospital were community-associated, and a similar finding was noted at hospitals in Manitoba, Canada (Kutty et al., 2010). In Sweden, it was reported that 22-28\% of infections were community-associated (Karlström et al., 1998; Norén et al., 2004), and, similarly, in the UK a study suggested rates of approximately 20 per 100 000 (Wilcox et al., 2008). However, community surveillance of \textit{C. difficile} is lacking, and establishing the incidence of CA-CDI is difficult.

Although research is limited, a recent study examined the risk factors and comorbidities of community-associated CDI in children relative to hospital-associated CDI, and found that although CA-CDI presented with fewer comorbidities, there were increased incidences of septic shock, recurrences, and complications (Tschudin-Sutter et
1.2.6. Hospital Associated

*Clostridium difficile* is the leading cause of hospital-associated diarrhea, and infections can be classified as either sporadic or epidemic (outbreak) (Bacci et al., 2011; Rupnik et al., 2003). The persistence of *C. difficile* in hospitals is due to a multitude of reasons, including the presence of infecting spores, an at-risk population, and widespread exposure to antimicrobials. All of these factors contribute to the increased manifestation of *C. difficile* in healthcare settings relative to the presence of disease in the general population. More recently, an increased incidence and severity of CDI has been partially attributed to the emergence of the hypervirulent strain North American Pulsed Field type 1 (NAP1), or PCR ribotype strain 027 (Hookman, P., & Barkin, J., 2009). In 2005, 30 hospitals in Quebec reported rates of disease five times greater than the historical average, and the Centre Hospitalier Universitaire de Sherbrooke, specifically, reported that the proportion of patients who died within 30 days of diagnosis of CDI rose from 4.7% in 1992 to 13.8% in 2003. At this same hospital, for individuals over 65 years of age, an increase from 102 deaths in 1992 to 866 deaths in 2003 was reported (Pépin et al., 2004). In the United States, mortality rates reportedly increased from 5.7 per million population in 1999 to 23.7 per million population in 2004 (Carter, G., Rood, J., & Lyras, D., 2010). Many of the same circumstances exist in long-term care facilities. Moreover, cases of NAP1/027 have presented globally; recently, cases have been documented in both Asia and Latin America, where it had been previously undocumented (López-Ureña et al., 2014; Wang et al., 2014). As previously mentioned, issues with infection prevention and control, the presence of an at-risk population (including
immunosuppressed individuals) and exposure to antimicrobials all contribute to the presence of disease.

1.2.7. Risk Factors

Major risk factors of CDI include advanced age (60 years of age and older (Louie et al., 2013)), and exposure to antimicrobials (Owens et al., 2008; Pépin et al., 2005). In individuals 65 years of age or older, disease rates have been observed to be up to 20 fold higher than in younger patients (Loo et al., 2005; Pépin et al., 2005). However, infections have been observed in populations previously assumed to be low-risk, namely, peripartum women and young children (Crook et al., 2012). Recently, researchers described 10 pregnant women with severe CDI who had been healthy prior to their pregnancies. Of the 10, only 3 had a history of hospital admission (Rouphael et al., 2008).

Virtually any antimicrobial can predispose to development of CDI, but certain antimicrobials are recognized as higher risk. In particular, exposure to broad-spectrum antimicrobials, including clindamycin, cephalosporins, beta-lactams, and more recently fluoroquinolones (Brown et al., 2013; Rodríguez-Pardo et al., 2013) has been documented to increase risk of disease by altering the normal intestinal microbiota, allowing for *C. difficile* to proliferate and potentially cause CDI.

Prolonged hospital or health care facility stay, immunosuppression, physical proximity to an individual infected with *C. difficile*, and underlying disease severity have also been suggested to increase the risk of *C. difficile* infection (Bartlett, J., & Gerding, D., 2008; Chang, V., & Nelson, K., 2000; Moshkowitz et al., 2007). The association of *C. difficile* with prolonged hospital stays can be attributed to the presence of spores in many clinical settings, with increased risk of exposure by patients who are at risk because
of compromised immunity. The prevalence of spores in the environment in healthcare facilities is relatively high, as they are difficult to eliminate using routine disinfection regimens (McFarland et al, 1989). Colonization of patients in hospital settings has been observed to be 10%-25%, and colonization of those in long-term care facilities has been observed to be anywhere from 4%-20%, while the colonization rate of the general public remains from 2%-3% (Bartlett, J., 1994; Simor et al., 2002).

1.2.8. Diagnosis of CDI

*Clostridium difficile* infection is most often suspected in patients with diarrhea who have recently had exposure to antimicrobials, or when diarrhea develops >72 hours after hospitalization (Siegel, D., 1990); however, it is becoming clear that CDI occurs in the absence of those risk factors, so broader testing is indicated in both hospitals and the community. Stool is usually watery and generally has a characteristic odour, and fever and abdominal cramps are also common, (Bartlett, J., 2002) yet clinical signs are not adequate to diagnose CDI. Diarrhea may also be absent, either initially or throughout infection in some individuals with CA-CDI (Garg et al., 2013), one reason the name *C. difficile* infection is now used in place of the older term “*C. difficile*-associated diarrhea (CDAD)”. Suspicion of CDI is typically heightened in individuals with additional risk factors, including advanced age (>65) and residency in a long-term care facility.

There are various methods utilized in the diagnosis of CDI; however, the cost, specificity and sensitivity vary between methods and there is still debate over the optimal diagnostic procedure. A definitive diagnosis requires laboratory confirmation, typically utilizing PCR detection of *tcdB* in feces, a method that has replaced the previous clinical standard of detection of TcdA and/or TcdB. Additional confirmation of
pseudomembranous colitis via endoscopic examination and visualization is also useful (Kawamoto, S., Horton, K., & Fishman, E., 1999). This can be done by colonoscopy, sigmoidoscopy, or CT scan and allows physicians to examine the colon for areas of inflammation or pseudomembranes (Bartlett, J., & Gerding, D., 2008). Although these techniques can be unpleasant, expensive, and are relatively insensitive, they allow for immediate results. In addition, in cases when there are possible confounding conditions, they can prove especially useful.

The Gold Standard for diagnosis was historically detection of toxin B in feces by cell cytotoxicity assay. The cell cytotoxicity assay functions by facilitating and indicating the cytopathic effects of the toxins, and is able to describe the presence of *C. difficile* toxins at picogram levels (Drudy et al., 2007). This method involves the addition of a sterile fecal specimen, both with and without a specific (anti-toxin B) antiserum, to a cultured monolayer of cells. These cells are then incubated for 24 hours and monitored for cytopathic effects (Alfa et al., 2002; Huang et al., 2009). The test has been found to have varying sensitivity and specificity, with one trial estimating the sensitivity and specificity to be 86.4% and 99.2%, respectively (Eastwood et al., 2009). Other studies have indicated specificities as low as 76.7% (Peterson et al., 2007) and as high as 98% (O’Connor et al., 2001). Unfortunately, clinical use of this test is limited because it is expensive, time consuming (24-48 hrs), and technically demanding, (Huang et al., 2009; Luna et al., 2011); it is rarely (if ever) used as a clinical diagnostic tool. Commercial kits for enzyme-linked immunoassays (ELISAs) are a convenient alternative, allowing for a fast (2-6 hrs) and inexpensive diagnosis.

In clinical settings, Enzyme Immunoassays (EIAs) were previously often used to
confirm the presence of *C. difficile* toxins (She, R., Durrant, R., & Petti, C., 2008). These tests have a rapid turnaround time and a high specificity, allowing them to be used efficiently in hospital settings (Monaghan, T., Boswell, T., & Mahida, Y., 2009). However, sensitivity can be variable and is often limited. An additional EIA targeting glutamate dehydrogenase (GDH; also known as common antigen) is also available and is considered highly sensitive. Yet, it detects non-toxigenic strains and cross-reacts with some other bacteria, so it can lack specificity. Combination of GDH and toxin ELISA can be used either together or in a stepwise fashion to improve overall sensitivity and specificity (Ticehurst et al., 2006).

Due to superior sensitivity, hospitals have begun to move towards using real-time polymerase chain reaction (PCR) as a diagnostic method. This technique involves extraction of DNA from a stool sample, and the targeted amplification of DNA, typically *tcdB*. A fluorophore molecule acts as an indicator, allowing the rate of the generation of amplified DNA produced to be measured at each cycle, as opposed to at the end of the PCR process (Stamper et al., 2009). Real-time PCR is able to target both toxin genes *tcdA* and *tcdB* (Luna et al., 2011) rapidly. With proper tests and quality control, sensitivity and specificity can be excellent. A study in a pediatric hospital found a sensitivity of 95%, and a specificity of 100%, (Luna et al., 2011), while another study determined real time PCR had a sensitivity of 97%, and a specificity of 100% (Bélanger et al., 2003). Due to the rapid nature (approximately 45 minutes) and high sensitivity, real-time PCR is becoming the method of choice for the diagnosis of CDI, especially in larger hospitals.

Although the aforementioned tests are useful in the diagnosis of *C. difficile*, they do not provide information about the types of *C. difficile* that are involved. While not
necessarily immediately useful in clinical diagnosis, these techniques are important in regards to outbreak strain identification and understanding of the epidemiology of CDI. Stool culture, which has a relatively long turnaround time, limiting its clinical relevance, allows for both strain identification and investigation into antibiotic resistance (Reller et al., 2007). Clinical application is hampered by the turnaround time, the need to do subsequent testing to differentiate toxigenic and non-toxigenic strains, and the limited anaerobic culture capability in most diagnostic laboratories.

1.2.9. Isolation of C. difficile

Isolation of C. difficile is not commonly performed clinically because it is time consuming and requires anaerobic culture facilities. Because subsequent differentiation of toxigenic and non-toxigenic isolates is necessary, culture is a critical tool for research but is not useful alone as a diagnostic method (Reller et al., 2007). Due to the fastidious nature of C. difficile vegetative cells, culture methods are typically directed at recovering highly tolerant spores. This often involves spore selection to eliminate competing vegetative bacteria, selective media to reduce overgrowth of other spore-formers, or a combination of the two (Marler et al., 1992). In order to select for C. difficile spores, viable vegetative bacteria from competing species can be eliminated from the stool samples using either an ethanol shock or a heat shock. This increases the likelihood of detection of C. difficile by decreasing the competing bacterial burden present in the sample (Carson et al., 2013). An ethanol shock requires 95% ethanol added in equal volume to a fecal sample, either directly to stool or to a sample in enrichment broth, for 1 hour. This essentially destroys all bacteria except for the resilient spore cells. A heat shock functions in much the same way: samples are heated to a specific temperature (80-
85 degrees Celsius) for 10 to 15 minutes (Perry et al., 2010; Rodriguez-Palacios, A., & Lejeune, J., 2011). However, the ethanol shock procedure has been found to be more effective in selecting for *C. difficile* and is therefore used more frequently (Perry et al., 2010). A culture medium is then inoculated with the resulting sample.

The composition of the culture medium can also play an important role in the success of isolation. Ideally, the culture provides an enriched medium for the growth of *C. difficile*, while inhibiting the growth of all other organisms. Antimicrobials are often added to media in order to inhibit the growth of competing organisms, and lysozyme or sodium taurocholate can be added to facilitate the germination of spores (George et al., 1979; Perry et al., 2010).

Cycloserine-cefoxitin fructose (CCF) media is a basic nutritive base supplemented with concentrations of the aforementioned antimicrobials that inhibit growth of the majority of fecal flora (Aspinall, S., & Hutchison, D., 1992). Historically, it was the most commonly used selective agar; however, the reportedly increased sensitivities of other growth mediums have resulted in a heightened use of other agars (Aspinall, S., & Hutchison, D., 1992; Carson et al., 2013; Perry et al., 2010).

*Clostridium difficile* moxalactam norfloxacin agar is another selective medium that has become increasingly popular due to increased sensitivity. This medium has been reported to isolate 20% more strains than CCF, as well as reducing contaminating agents by 30% (Perry et al., 2010).

Additionally, a chromogenic culture medium has been introduced as a selective agar. The specific components are currently undisclosed by the manufacturer; however, it effectively facilitates the growth and detection of *C. difficile* within 24 hrs of inoculation,
without requiring prior growth in enrichment broth or alcohol/heat shock. It also helps differentiate *C. difficile* from other organisms by virtue of the colour of *C. difficile* colonies (Perry et al., 2010). This significantly decreases the total time necessary to isolate *C. difficile*.

### 1.2.10. Identification

Gram staining is a technique that characterizes bacteria cells as either gram positive or gram negative in response to their structural makeup. Gram positive bacteria have a thick, impermeable cell wall comprised of peptidoglycan, which is resistant to discoulouration and stains purple (Beveridge, T., 2001). Gram negative bacteria have a thinner wall of peptidoglycan surrounded by membranes, and stain pink. *Clostridium difficile* is a Gram positive bacterium, and gram stain, along with cytological appearance can be used as a preliminary identification step, in concert with other identification techniques (Ravizzola et al., 1998; Shanholtzer et al., 1983).

Another laboratory indicator of the presence of *C. difficile* is the typical colony morphology the organisms display when cultured on agar. In addition to a distinctive odour, *C. difficile* is suspected when colonies are light gray, and opaque with irregular edges on blood agar (Bloedt et al., 2009; Delmée, M., 2001).

Additionally, there are biochemical tests available that indicate the presence of certain organisms. *Clostridium difficile* produces the enzyme L-proline-aminopeptidase, and tests that can be used to detect this enzyme have been developed. Using a peptidase reagent, these tests enact a colour change to indicate the presence of the enzyme (Fedorko, D., & Williams, E., 1997). However, these tests cannot be used as definitive identification because other organisms can also produce L-amino-peptidase. Used in
conjunction with other identification methods, these tests are an effective way to screen samples for *Clostridium difficile* (Fedorko, D., & Williams, E., 1997; Weese, J., Staempfli, H., & Prescott, J., 2000).

Various molecular targets can be used to confirm the identity of *C. difficile*, such as detection of triphosphate isomerase gene (*tpi*) (Persson, S., Torpdahl, M., & Olsen, K., 2008). This can be done alone or as a multiplex technique with toxin genes to both confirm the presence of *C. difficile* and determine its toxigenicity.

1.2.11 Characterization

1.2.11.1. Toxin and toxin gene detection

Since non-toxigenic strains of *C. difficile* can be encountered, in vitro identification of toxigenicity of any isolated *C. difficile* is required. This can be done by detection of *in vitro* production of toxins or inferred by detection of genes encoding toxin production.

In addition to testing feces, immunoassays can be used to identify toxin B production by isolates *in vitro*. The assay functions by detecting the presence of either or both toxins A and B (Shanholtzer et al., 1992). These assays are less sensitive than the cell cytotoxicity assay, but have been widely used as an effective clinical tool for diagnosis of *C. difficile*.

While detection of production of functional toxin is in many ways ideal, it is impractical in most situations because of the time, expense and technical demands of the cell cytotoxicity assay. Further, practical methods for detection of TcdA and CDT are not available. Therefore, determination of toxigenicity typically involves PCR detection of
toxin genes, something that has been widely described (Bidet et al., 1999; Cartwright et al., 1995; Collier et al., 1996).

1.2.11.2. Molecular Typing Methods

Evaluating the strain types of *C. difficile* present in clinical samples is an important factor in understanding the epidemiology of the disease. Knowledge of the various bacterial strain types facilitates understanding of *C. difficile* transmission and evaluation of clinical and epidemiological differences between different lineages and comparison of strains affecting different populations. It also facilitates outbreak investigation and tracking the presence and spread of strains across geographical regions.

Various typing methods are available; however, the method of choice depends on the specific type of bacteria and objective of the research. While technical ease and cost are important factors, it is also necessary to consider reproducibility, discriminatory power, and an ability to standardize protocols. Discriminatory power is of great importance because minor indistinguishable strain differences illustrated by one typing protocol may be easily discriminated by another (Rupnik et al., 2001). This is particularly important when tracking disease outbreaks.

Initial *C. difficile* typing methods were based on the observable characteristics of the organism. Polyacrylamide gel electrophoresis (PAGE) of cell surface components, prolisys mass spectrometry, and bacteriophage sensitivity testing were utilized, however, other methods have been proven to be faster, more discriminatory, and more reliably reproducible.
More commonly used methods now target the organism’s genome. Many of these utilize the polymerase chain reaction, or PCR. Examples of these include PCR ribotyping, toxinotyping, and pulsed-field gel electrophoresis, among others.

1.2.11.3. PCR Ribotyping

PCR ribotyping is increasingly being used to identify different strains of *C. difficile* because it is relatively technically uncomplicated, reproducible, and discriminatory. This reproducibility allows for reasonable inter-laboratory comparisons, as well as the creation of an international reference library consisting of a constantly increasing databank of *C. difficile* isolates (Arroyo, L., 2005).

Two primers that target the 5’ and 3’ ends of ribosomal RNA are used to amplify the 16S-23S regions of the operons. *Clostridium difficile* contains 14 rRNA alleles with varying lengths of these spacer regions, and the resulting product, run on an agarose gel electrophoresis, produces up to 12 distinct bands ranging from 500 bp to 1100 bp in size (Bidet et al., 2000; Cartwright et al., 1995). These unique banding patterns can then be compared with other isolates, as well as to reference strains, to determine their identity.

Although the possibility of inter-laboratory comparisons is an advantage, there can be variations in methods among laboratories and some degree of inter-laboratory variation even when using the same strains and methods. This can result in differences in the PCR ribotyping results, rendering some comparisons inaccurate. Additionally, when compared to some other genomic typing methods (PFGE and REA), PCR ribotyping has a lower discriminatory power. However, PCR ribotyping remains a widely used and acceptable method of molecular typing because of its ease, low cost and throughput. The individual interpretation of the gel agarose presents some difficulties in regards to
standardization, especially as laboratories do not always have access to a large library of reference strains (Janezic, S., & Rupnik, M., 2010). An international library of ribotypes is maintained and strains are designated numbers (e.g. 027). Initially developed in Cardiff, UK, this collection is now referred to as the Cardiff/ECDC collection, and a subset of the collection, containing the most common human strains, is available to researchers internationally to use as reference strains. This allows for more inter-laboratory comparison, but is restricted to a subset of strains. A potential solution to this is the development of a capillary-based ribotyping technique that allows for results to be standardized into an online database (Indra et al., 2008); however it is not currently widely used nor has it been extensively validated (Janezic, S., & Rupnik, M., 2010).

1.2.11.4. Pulsed-field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) is considered the gold standard for typing many bacteria; however, it is not widely used for C. difficile. This is because it is a time-consuming procedure (at least 4 days), and has limited throughput as well as being costly and sometimes requiring hazardous chemicals (Bidet et al., 2000). Additionally, it was previously unable to type certain strains, including the outbreak strain 001. However, the latter issue was recently addressed with a modified PFGE protocol (Gal, M., Northey, G., & Brazier, J., 2005). PFGE is standard in governmental laboratories in North America and as those laboratories started to type C. difficile they tended to use PFGE. Thus, this method is increasingly used, although still more in selected laboratories. With PFGE, banding patterns are typically evaluated to determine the degree of relatedness, and strains with 80% similarity, or higher, are considered to be a single pulsotyper. Strains are
referred to as North American Pulsotypes (NAP) followed by a numerical designation (NAP1, for example) (Janezic, S., & Rupnik, M., 2010).

PFGE functions by using a restriction enzyme to digest entire genomic DNA, producing up to 10 fragments. The products are run on a gel, the results of which are used to group isolates by the number of bands and molecular weight (Collier et al., 1996). Early issues with previously non-typeable strains involved early DNA degradation, therefore preventing the use of PFGE. However, the modified protocol prevents this degradation to facilitate the PFGE process (Gal et al., 2005). Although this resolves one of the issues associated with PFGE, typing C. difficile strains can be time-sensitive, especially in outbreak situations, and therefore PFGE is often impractical.

1.2.11.5. Toxinotyping

Toxinotyping is not generally used as an initial typing method, but is instead used to confirm results from other protocols. It has a lower discriminatory power than both PFGE and PCR ribotyping (Rupnik et al., 2001). The A3 and B1 segments of the PaLoc are amplified using two separate PCR reactions, and then the bands are analyzed to classify isolates into a toxinotype. This technique has high reproducibility; however, it is technically difficult and is not always successful (Rupnik et al., 2001).

1.2.11.6. Other Methods

Restriction endonuclease analysis, or REA, is a genomic form of molecular typing that involves the use of restriction enzymes to cut DNA at specific sites. These DNA segments are then separated by electrophoresis, and distinctive banding patterns can be used to differentiate strains (Wren, B., & Tabaqchali, S., 1987). This method is fairly
discriminatory (Killgore et al., 2008); however, it is not used very frequently due to the fact that it is time consuming and difficult to analyze.

Another infrequently used molecular typing method is known as multiple locus variable tandem repeat analysis, or MLVA. This method utilizes PCR to amplify well-characterized loci, following which the size of each of these loci is determined by capillary electrophoresis. This allows for the determination of the number of repeats at each locus, which can then be compared to a database (Van den Berg et al., 2007). However, this method is time-consuming and expensive, resulting in minimal use.

A relatively new technique that is becoming increasingly popular is whole genome sequencing. Using this technique, researchers are able to establish the complete DNA sequence of an organism’s genome, allowing for greater discrimination than with other typing techniques (Caporaso et al., 2011). A recent study examining modes of transmission in *C. difficile* compared whole genome sequencing to conventional multi-locus sequence typing. It was found that whole genome sequencing was able to rule out methods of transmission that multi-locus sequence typing previously established as feasible (Eyre et al., 2013). As this technology becomes more widely available and costs continue to decrease, it will likely become the main mode of typing all organisms, including *C. difficile*.

**1.2.12 Antimicrobial Susceptibility**

In clinical settings, antimicrobial susceptibility testing is not generally performed for *C. difficile* because anaerobic bacterial susceptibility testing can be challenging, and susceptibility to the drugs typically used for treatment (metronidazole and vancomycin) has generally been predictable. Further, culture is uncommonly performed; therefore
isolates are not available for testing. The timeframe involved in isolating and testing *C. difficile* is also problematic, as clinical decisions cannot typically await the time required for *C. difficile* isolation and testing. Nevertheless, recent decreases in clinical response to commonly used antimicrobials, particularly metronidazole (Erikstrup et al., 2012; Pituch et al., 2011) along with reports of metronidazole resistance (Lynch et al., 2013) have caused the value of these susceptibility tests to become more apparent. Unfortunately, the previous lack of testing has left a gap in the research regarding *C. difficile* and antimicrobial susceptibility, and this has served to complicate treatment and prevention. Unlike most other bacterial pathogens, *C. difficile* susceptibility testing is also useful for evaluation of potential inciting causes, since resistance to antimicrobials used for other purposes may be an important factor in the ability of *C. difficile* to proliferate and cause disease during treatment.

*Clostridium difficile* is generally assumed to be susceptible to metronidazole and vancomycin, with resistance to other antimicrobials varying among countries (Bishara et al., 2006; Huang, Wu, et al., 2009; Pituch et al., 2011). For example, resistance to erythromycin and moxifloxacin was noted to be 87.5% among UK isolates; however, isolates from Sweden were susceptible to erythromycin and only 7.1% were resistant to moxifloxacin. Currently, resistance to both metronidazole and vancomycin is rare. Additionally, fidaxomicin, a macrocyclic antibiotic, has been introduced as a possible treatment option for CDI (Louie et al., 2013), although limited susceptibility data are available.

There are various methods of determining the Minimum Inhibitory Concentration, or MIC of isolates. The MIC is defined as the “minimum concentration of an
antimicrobial that will inhibit visible growth of a microorganism” (Andrews, J., 2001). MIC50 and MIC90 are the antimicrobial concentrations at which 50% and 90% of bacterial growth is inhibited, respectively. These values are often used when describing MIC.

The gold standard is known as the agar dilution method, and involves the incorporation of incrementally increasing concentrations of the antimicrobial in question into a nutrient agar medium. Following this, the plates are inoculated with bacteria and incubated and the ability of the bacterium to grow in the presence of different antibiotic concentrations is noted. Similarly, the broth dilution method involves increasing concentrations of the antimicrobial incorporated into a liquid growth medium. The growth medium is then inoculated with bacteria and incubated (Andrews, J., 2001; Erikstrupp et al., 2012). In both cases, the lowest concentration of antimicrobial presence that inhibits visible bacterial growth is determined to be the MIC.

Another method for testing antimicrobial susceptibility is the disk diffusion method. In this method, a lawn of bacteria is created and a disk infused with the antimicrobial in question is placed on the agar. As the antimicrobial diffuses across the surface of the agar, a zone of inhibition is created around the disk. The highest concentration is closest to the disk; therefore large zones of inhibition indicate greater sensitivity to the antimicrobial than small zones of inhibition (Erikstrupp et al., 2012).

An additional method is known as the Etest. It is simpler and uses fewer materials than the agar dilution method, and unlike the disk diffusion method it provides an MIC. Etests can slightly underestimate the MIC when compared with those resulting from the agar dilution tests (Barbut et al., 1999). Much like the disk diffusion method, a lawn of
bacteria is grown and the Etests are placed on the agar. Etests are saturated with incremen tally increasing concentrations of antimicrobials, and the lowest concentration at which no bacterial growth is visible is considered the MIC. The standard “breakpoint”, or the MIC at which bacteria are considered resistant, is established by the Clinical and Laboratory Standards Institute (CLSI). For metronidazole on anaerobic bacteria, MICs of less than or equal to 8 µg/ml are considered resistant.

As previously stated, it is generally assumed that C. difficile is susceptible to both vancomycin and metronidazole; however, there have been reports of metronidazole resistance (Lynch et al., 2013; Pelaez et al., 2008). There are also varying levels of antimicrobial susceptibility. In categorizing bacteria, the term susceptible indicates inhibition by a concentration of antimicrobial that is associated with a high likelihood of clinical success (Rodloff et al., 2008). “Intermediate” indicates that the bacterium would potentially be susceptible in vivo if high drug levels could be achieved at the site of infection, through physiological concentration or increased dosing. The clinical relevance of this with CDI is unclear, in large part because of a poor understanding of drug levels that are achieved in the gut. “Resistant” indicates inhibition by a concentration that is associated with high likelihood of treatment failure (Rodloff et al., 2008).

Independent of typical antimicrobial resistance, there is also the possibility of heteroresistance. Heteroresistance refers to the presence, within a population of susceptible microorganisms, of a subpopulation of organisms with decreased susceptibility (Deresinski, S., 2009). Although it has been defined for other microorganisms, such as Staphylococcus aureus, the extent of heteroresistance in C. difficile is largely unknown. Heteroresistance has been reported in C. difficile isolates
(Pelaez et al., 2008); however, there has been less research on heteroresistance to vancomycin (Deresinski, S., 2009).

1.2.13 Treatment of Sporadic CDI

There are a variety of treatments available for CDI, however antibiotics are the preferred therapy. Historically, metronidazole, a nitroimidazole antibiotic directed at anaerobic bacteria and protozoa, has been the first line treatment, along with cessation of other antimicrobial therapy whenever possible (Louie et al., 2013). Metronidazole is especially effective in mild to moderate cases of CDI; however, response to metronidazole has decreased in recent years (Aslam, S., Hamill, R., & Musher, D., 2005; Zar et al., 2007). Vancomycin is another common treatment option for C. difficile, and generally has the same efficacy against CDI as metronidazole for mild to moderate infections (98% and 90%, respectively). However it has been reported that vancomycin is more effective in cases of severe infection than metronidazole (97% versus 75%) (Zar et al., 2007). The lower cost of metronidazole and relatively similar efficacy often means that it is the initial treatment for CDI.

There are disadvantages to these antibiotic treatments, as all anaerobic bacteria are targeted, which suppresses the growth of anaerobic intestinal microbiota that normally serve as a defense against the proliferation of Clostridium difficile. This disruption of the regular gut flora can allow the recurrence of C. difficile, often through germination of dormant spores, and may be partially responsible for recurrent disease (Kelly C., & LaMont, J., 1998; Kelly, C., Pothoulakis, C., & LaMont, J., 1994). Recurrence rates of CDI are approximately 20% for both metronidazole and vancomycin.
(Mcfarland, L., 2005; Zar et al., 2007). This has prompted research into possible alternative treatments, as concerns regarding antibiotic resistance are growing.

Recently, the antibiotic fidaxomicin has been suggested as an alternative antibiotic treatment for CDI, in part because fidaxomicin is more specific in its activity with apparently less disruption of the intestinal microbiota (Tannock et al., 2010). In 2011, it was approved by the FDA as a treatment option for *C. difficile* associated diarrhea; however, it has yet to become one of the mainstay treatments for CDI, in part because of cost. Bacitracin, fusidic acid, rifampin, teicoplanin, erythromycin and moxifloxacin are also antimicrobial treatments with relatively comparable cure rates (83%-96%) that have been evaluated for CDI (Wenisch et al., 1996; Wullt M., & Odenholt, I., 2004; Young et al., 1985), yet they are rarely (if ever) used.

In addition to antimicrobials, there are also other treatment approaches to consider. Discontinuation of the inciting broad-spectrum antibiotic is often enough to resolve mild CDI. The discontinuation presumably allows the regular intestinal microbiota to grow, and this in turn acts as a defense mechanism against the proliferation of *C. difficile* (Mcfarland, L., 2005). In conjunction with this, it is prudent to avoid antiperistaltic pharmaceuticals in order to enable full function of the intestines. Probiotics are also sometimes recommended in the hopes that they will assist in restoring normal intestinal flora. Specifically, it has been reported that administration of *Lactobacillus* and *Saccharomyces boulardii* reduce the occurrence of antibiotic-associated diarrhea when given in conjunction with antibiotics (Buts, J., 2009; McFarland, L., 2009), yet data have been conflicting and probiotics are not widely recommended as adjunct therapy. The effects of immune products and adsorbents such as ion-exchange resins have also been
investigated with regards to the treatment of CDI with varied results (McPherson et al., 2006; Taylor, N., & Bartlett, J., 1980).

1.2.14 Recurrence

Approximately 15% - 35% of C. difficile cases present with at least one recurrent episode (McFarland, L., Elmer, G., & Surawicz, C., 2002). This is thought to be a result of the presence of spores that were not eliminated by the antibiotic treatment, along with residual defects in the intestinal microbiota and/or immune system. After treatment, often the normal intestinal flora has not re-established itself and the previously dormant C. difficile spores germinate. The lack of competing flora allows for the proliferation of C. difficile and this results in a recurrence of disease. Treatment for a single recurrent episode is generally another course of antibiotics, and is often a prolonged treatment (Musher et al., 2005).

Up to 65% of those that experience treatment failure can exhibit a recurrent pattern of disease. Of these recurrent cases, 38%-56% are infected with the same strain as previous infection (Huebner, E., & Surawicz, C., 2005). Once a recurrent pattern emerges, the treatment protocol requires adjusting. Depending on the risk factors present in the patient, treatment may involve alternative methods in conjunction with the same antibiotic or a course of a different antibiotic (Cohen et al., 2010). Metronidazole is generally the first treatment, and metronidazole resistant strains do exist. Attempting to resolve the CDI with an alternative antibiotic is sometimes effective. However, if the recurrent episodes continue, or the patient is at considerable risk due to severe illness or other complicating conditions, human biotherapy can be very effective.
1.2.15 Outcome

Despite the increasing incidence and severity of *C. difficile* infections, limited data exist regarding the outcomes of CDI. This is likely because many factors contribute to the outcome of infection. *Clostridium difficile* is often contracted while individuals are already hospitalized, therefore age, severity of disease, immune status and strain can all be factors of concern. *Clostridium difficile* has recently caused outbreaks of increased severity and with higher rates of recurrence than have been historically observed (Abraham Goorhuis et al., 2008; Kuijper et al., 2006; Pépin et al., 2004). The presence of the internationally recognized outbreak strain NAP1/027 could be a possible explanation for the increased severity of *Clostridium difficile* infections, however there may also be other contributing factors.

Because of the aforementioned risk factors, response to treatment can vary. Most infections are initially treated with a course of metronidazole, however the response to metronidazole has been steadily decreasing. A 2005 study examined 207 patients with positive ELISAs for both toxins A and B, and who had been treated with metronidazole, and found that 103 had a complete clinical cure during the first treatment. Of the remainder, 58 initially appeared to recover fully but had a recurrence of symptoms within 90 days, and 47 of these were confirmed as a recurrence (the others either did not provide additional samples or tested negative). These patients were then treated with prolonged metronidazole therapy, which was successful in 15 of the cases. Since this study was published, the severity of infection has only increased and the effectiveness of antibiotic treatments has decreased. The clinical responses to vancomycin and fidaxomicin appear to be similar to metronidazole, however fidaxomicin has been reported to result in
significantly fewer recurrences (Musher, Aslam, Logan, Nallacheru, Bhaila, Borchert, & Jamil, 2005).

A factor of particular significance in examining outcomes of CDI is the age of the patient. Studies have demonstrated that risk of a negative outcome increases significantly with advancing age, and therefore extra care must be taken to prevent disease in the elderly. A 2013 study predicted a 17% lower clinical cure, 17% greater recurrence, and 13% lower sustained clinical response by advancing decade in those younger than 40 (Louie et al., 2013). Mortality rates due to CDI vary, and may be underrepresented. Studies have reported mortality rates range from 1.3% to as high as 24% in severely ill patients (Loo et al., 2005; Rubin, M., Bodenstein, L., & Kent, C., 1995; Warny et al., 2005). Mortality rates are higher in elderly patients and those with compromised immune systems (Louie et al., 2013).

1.2.16. Fecal Microbiota Transplantation (FMT)

When patients repeatedly fail to respond to conventional treatment, physicians are increasingly considering human biotherapy. Often these patients are of advanced age, or have comorbid diagnoses (Brandt et al., 2012) and have a low likelihood of responding to conventional treatments. Human biotherapy, also known as a fecal microbiota transplantation (FMT), stool transplantation, transfaunation, intestinal microbiota transplant (IMT), or fecal enema, involves the administration of stool from a healthy donor to a person with recurrent CDI by enema, colonoscopy, nasogastric tube or, more recently, by oral capsules (Brandt et al., 2012).

While antibiotic therapy aims to eliminate C. difficile, fecal transplant functions to restore the individuals’ natural intestinal bacteria. Healthy humans maintain
approximately 300-500 species of microorganisms in their gut, and these bacteria serve a variety of functions (Rafii, F., Sutherland, J., & Cerniglia, C., 2008). In addition to aiding in digestion, immune response, and energy storage, these microorganisms also function to prevent invasion of external pathogens (Rafii et al., 2008). When these organisms are disrupted, as in the case of broad-spectrum antibiotic administration, there is little intestinal defense against the proliferation of other bacteria, in this case, *C. difficile*. Fecal transplantation aims to restore the natural intestinal microorganism population using bacteria from a healthy donor (Brandt et al., 2012), thus allowing natural intestinal microbiota to compete with *C. difficile* and prevent infection.

Sometimes, physicians try to establish a donor with close contact with the patient, either a family member or spouse, however, stool donations can also come from healthy individuals from the general population. The latter may be increasingly common because of the time and expense required for pathogen screening of potential donors. In either case, the donor is screened to ensure absence of disease and the presence of appropriate intestinal bacteria. Specific protocols often vary with regards to quantity of stool and preparation of patients, however the stool is then generally homogenized, and then administered as previously described (Gough, E., Shaikh, H., & Manges, A., 2011).

This treatment has proven to be extremely effective, resolving 92% of recurrent cases, often with only one treatment. In some individuals, however, multiple treatments are necessary to resolve the diarrhea (Brandt et al., 2012; Gough et al., 2011). Until recently, the effects of FMT in extremely immunosuppressed individuals have not been widely explored due to concerns regarding other opportunistic infections. However, a case study involving 2 individuals who underwent whole organ transplants found that
after only 2 treatments each, both experienced resolution of *C. difficile* (Friedman-Moraco et al., 2014). A summary of the cure rates of previous FMT studies can be found in Table 1.

Unfortunately, some individuals are uncomfortable with this treatment and therefore researchers are currently developing a synthetic version of the stool sample. The method remains the same: normal intestinal flora is transplanted from a healthy donor to a patient, thus acting as a defense against *C. difficile* infection (Petrof et al., 2013).

Of the patients that recover after FMT, there is the question of clinical versus microbiological outcome. A patient may present as clinically cured with resolution of diarrhea and gastrointestinal issues, but the bacteria may still be present in their stool samples. This may not be an issue if the patient’s intestinal flora has re-colonized, however it may leave them vulnerable to a recurrence should they be exposed to broad-spectrum antibiotics in the future.

### 1.2.17. Conclusion

*Clostridium difficile* is a growing concern in healthcare. Recurrent and severe infections are becoming more frequent, and mortality rates attributed to *C. difficile* infections are increasing. Although antibiotic therapy remains the primary treatment method, novel treatments such as human biotherapy are being more frequently pursued as the number of cases unresponsive to conventional therapy increases. Further investigation into the various *C. difficile* strains, particularly those present in outbreak situations, is necessary to enable an examination of the specific traits of these strains and allow researchers to identify the characteristics of these strains which makes them so virulent.
### TABLE 1.1. Success rates of FMT in recurrent cases of CDI in studies from 2010 to 2014

<table>
<thead>
<tr>
<th>Reference</th>
<th>Number of Patients</th>
<th>Success rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arkkila et al., 2010</td>
<td>37</td>
<td>91.9%</td>
</tr>
<tr>
<td>Garborg et al., 2010</td>
<td>40</td>
<td>82.5%</td>
</tr>
<tr>
<td>Khoruts et al., 2010</td>
<td>1</td>
<td>100%</td>
</tr>
<tr>
<td>Rohlke, F., Surawics, C., &amp; Stollman, N., 2010</td>
<td>19</td>
<td>100%</td>
</tr>
<tr>
<td>Russell et al., 2010</td>
<td>1</td>
<td>100%</td>
</tr>
<tr>
<td>Silverman, M., Davis, I., &amp; Pillai, D., 2010</td>
<td>7</td>
<td>100%</td>
</tr>
<tr>
<td>Yoon, S., &amp; Brandt, L., 2010</td>
<td>12</td>
<td>100%</td>
</tr>
<tr>
<td>Girotra et al., 2010</td>
<td>3</td>
<td>100%</td>
</tr>
<tr>
<td>Mattila et al., 2011</td>
<td>70</td>
<td>94.3%</td>
</tr>
<tr>
<td>Mellow, M., &amp; Kanatzar, A., 2011</td>
<td>13</td>
<td>92.3%</td>
</tr>
<tr>
<td>Brandt et al., 2012</td>
<td>12</td>
<td>100%</td>
</tr>
<tr>
<td>Hamilton et al., 2012</td>
<td>43</td>
<td>95.3%</td>
</tr>
<tr>
<td>Kassam et al., 2012</td>
<td>27</td>
<td>92.6%</td>
</tr>
<tr>
<td>Kelly, C., De Leon, L., &amp; Jasutkar, N., 2012</td>
<td>26</td>
<td>92.3%</td>
</tr>
<tr>
<td>Petrof et al., 2013</td>
<td>2</td>
<td>100%</td>
</tr>
<tr>
<td>Friedman-Moraco et al., 2014</td>
<td>2</td>
<td>100%</td>
</tr>
<tr>
<td>Lee, C., et al, 2014</td>
<td>94</td>
<td>86%</td>
</tr>
</tbody>
</table>
Diagram of the pathogenicity Locus (PaLoc), the portion of the *C. difficile* chromosome on which the genes encoding the expression of toxins A (*tcdA*) and B (*tcdB*) reside. Adapted from Halsey, J., 2008.

**FIGURE 1.1.** Diagram of the pathogenicity Locus (PaLoc), the portion of the *C. difficile* chromosome on which the genes encoding the expression of toxins A (*tcdA*) and B (*tcdB*) reside. Adapted from Halsey, J., 2008.
1.2.18. References


Baron, E., & Tenover, F. (2010). The emerging threat of Clostridium difficile infection: new insights into diagnosis and disease management. CEPHEID.


*Clinical Infectious Diseases, 31*(3), 717–22. doi:10.1086/314030


hospital-acquired infection to long-term care facility-based infection. *Digestive Diseases and Sciences, 58*(12), 3407-3412.


Goorhuis, A., Bakker, D., Corver, J., Debast, S., Harmanus, C., Notermans, D., Bergwerff, A., Dekker, F., & Kuijper, E. (2008). Emergence of *Clostridium difficile* infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. *Clinical Infectious Diseases, 47*(9), 1162–70. doi:10.1086/592257


Janezic, S., & Rupnik, M. (2010). Clinical practice guidelines for *Clostridium difficile* infection in adults- 2010 update by the society for healthcare epidemiology of America (SHEA) and the infectious diseases society of America (IDSA). *Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA), 1–26.*


via retention enema. *European Journal of Clinical Microbiology and Infectious Diseases.*
doi:10.1007/s10096-014-2088-9

Loo, V., Poirier, L., Miller, M., Oughton, M., Libman, M., Michaud, S., Bourgault, A.,
Nguyen, T., Frenette, C., Kelly, M., Vibien, A., Brassard, P., Fenn, S., Dewar, K.,
clonal multi-institutional outbreak of *Clostridium difficile*–associated diarrhea with high

López-Ureña, D., Quesada-Gómez, C., Miranda, E., Fonseca, M., & Rodríguez-Cavallini,
E. (2014). Spread of epidemic *Clostridium difficile* NAP1/027 in Latin America: case
reports in Panama. *Journal of Medical Microbiology, 63*(2), 322-324.

Louie, T., Miller, M., Crook, D., Lentnek, A., Bernard, L., High, K., Shue, Y., &
Gorbach, S. (2013). Effect of age on treatment outcomes in *Clostridium difficile*

Luna, R., Boyanton, B., Mehta, S., Courtney, E., Webb, C., Revell, P., & Versalovic, J.
(2011). Rapid stool-based diagnosis of *Clostridium difficile* infection by real-time PCR in
doi:10.1128/JCM.01983-10

Lyerly, D., Saum, K., MacDonald, D., & Wilkins, T. (1985). Effects of *Clostridium
difficile* toxins given intragastrically to animals. *Infection and Immunity, 47*(2), 349–352.


Pépin, J., Saheb, N., Coulombe, M.-A., Alary, M.-E., Corriveau, M.-P., Authier, S.,
Leblanc, M., Rivard, G., Bettez, M., Primeau, V., Nguyen, M., Jacob, C.-E., & Lanthier,
L. (2005). Emergence of fluoroquinolones as the predominant risk factor for Clostridium
difficile-associated diarrhea: a cohort study during an epidemic in Quebec. Clinical
Infectious Diseases, 41(9), 1254–1260. doi:10.1086/496986

Pépin, J., Valiquette, L., Alary, M.-E., Villemure, P., Pelletier, A., Forget, K., Pepin, K.,
Journal, 171(5), 466–472.

Perry, J., Asir, K., Halimi, D., Orenga, S., Dale, J., Payne, M., Carlton, R., Evans, J.,
& Gould, F. (2010). Evaluation of a chromogenic culture medium for isolation of
Clostridium difficile within 24 hours. Journal of Clinical Microbiology, 48(11), 3852–
3858.

detection of Clostridium difficile toxin A (tcdA) and toxin B (tcdB) and the binary toxin
(cdtA/cdtB) genes applied to a Danish strain collection. Clinical Microbiology and

Peterson, L., Manson, R., Paule, S., Hacek, D., Robicsek, A., Thomson, R., & Kaul, K.
(2007). Detection of toxigenic Clostridium difficile in stool samples by real-time
polymerase chain reaction for the diagnosis of C. difficile-associated diarrhea. Clinical
Infectious Diseases, 45(9), 1152–1160. doi:10.1086/522185


Rupnik, M., Grabnar, M., & Geric, B. (2003). Binary toxin producing *Clostridium

Rupnik, M., Brazier, J., Duerden, B., Grabnar, M., & Stubbs, S. (2001). Comparison of
toxinotyping and PCR ribotyping of *Clostridium difficile* strains and description of novel

relapsing *Clostridium difficile* infection in a child: a proposed treatment protocol.
*Paediatrics, 126*, 239–242.

*Clostridium difficile* toxin B is an inflammatory enterotoxin in human intestine.
*Gastroenterology, 125*(2), 413–20.

Shanholtzer, C., Peterson, L., Olson, M., & Gerding, D. (1983). Prospective study of
gram-stained stool smears in diagnosis of *Clostridium difficile* colitis. *Journal of Clinical
Microbiology, 17*(5), 906–908.

Comparison of the VIDAS *Clostridium difficile* toxin A immunoassay with *C. difficile*
culture and cytotoxin and latex tests. *Journal of Clinical Microbiology, 30*(7), 1837–
1840.


Tannock, G., Munro, K., Taylor, C., Lawley, B., Young, W., Byrne, B., Emery, J., & Louie, T. (2010). A new macrocyclic antibiotic, fidaxomicin (OPT-80), causes less alteration to the bowel microbiota of *Clostridium difficile*-infected patients than does vancomycin. *Microbiology*, 156(11), 3354–3359. doi:10.1099/mic.0.042010-0


2.0 Comparison of the distribution of *Clostridium difficile* strains among sporadic and recurrent cases

2.1 ABSTRACT

Recurrent *Clostridium difficile* infection is an increasing concern and North American Pulsetype 1/ribotype 027 (NAP1/027) has been associated with recurrent disease, however the association between NAP1/027 has never been explicitly demonstrated. In this paper, stool samples were ribotyped from patients with both sporadic and recurrent disease and the strain distribution and antimicrobial susceptibility in both groups were described. It was found that both gender (female) and presence of NAP1/027 were associated with recurrent disease. These results suggest that female gender and presence of NAP1/027 predispose individuals to recurrent disease.

KEYWORDS

*Clostridium difficile*, sporadic, recurrent, PCR ribotypes, antimicrobial susceptibility
2.2 Introduction

_Clostridium difficile_ (C. difficile), an anaerobic, spore-forming, gram positive bacillus, is the most commonly diagnosed cause of hospital-associated diarrhea in developed countries (Kelly & Kyne, 2011). _Clostridium difficile_ infection (CDI) can result in a variety of signs and symptoms ranging from mild diarrhea to pseudomembranous colitis, sepsis, and death (Kelly & LaMont, 2008; Kyne et al., 1999). Commonly reported risk factors include advanced age (Louie et al., 2013), prolonged hospital stay, comorbid diagnoses, (Khanna & Pardi, 2012; Louie et al., 2013), and, in particular, antimicrobial exposure (Owens et al., 2008).

Three main _C. difficile_ toxins are recognized; toxin A (TcdA), toxin B (TcdB), and binary toxin (CDT). Most toxigenic strains produce both toxins A and B, but A-/B+ variants are also recognized causes of disease (Shin et al., 2008). The functions of both of these toxins are relatively well described (Cohen et al., 2000; Sun et al., 2010). Genes encoding TcdA and TcdB, _tcdA_ and _tcdB_, respectively, are located on a section of the _C. difficile_ chromosome called the pathogenicity locus, (PaLoc). Binary toxin (CDT, encoded by _cdtA_ and _cdtB_), is not encoded in the PaLoc and is less commonly found (Carter et al., 2007). While the role of CDT in disease is less clear than that of toxins A and B, it may be a factor in the increased virulence of certain strains (Bacci et al., 2011; Warny et al., 2005). It can be found in a range of strains, most commonly those that produce both toxins A and B (Geric et al., 2006; M. Rupnik et al., 2003).

In the early 2000s, a remarkable change in the epidemiology of CDI was noted in many regions. This consisted of an increased incidence of disease, including disease in younger individuals and those with few or no risk factors, along with increased severity
of disease, mortality and incidence of relapse (Heslop et al., 2013; Khanna et al., 2012; Muto et al., 2005; Pépin et al., 2004). This increase in virulence has been partially attributed to the emergence of the hypervirulent, internationally recognized outbreak strain designated North American Pulsedtipe 1 (NAP1) and ribotype 027 (McDonald et al., 2005). This strain possesses genes encoding CDT and an altered PaLoc (Geric et al., 2003; Gonçalves et al., 2004; Warny et al., 2005), has various metabolic and proteomic differences compared to other strains (Carter et al., 2007; Gonçalves et al., 2004), is commonly implicated in outbreaks (A Goorhuis et al., 2007; J. R. O’Connor, Johnson, & Gerding, 2009; Warny et al., 2005) and is responsible for higher morbidity and mortality than has been historically observed (Loo et al., 2005; Pépin et al., 2004; Walker et al., 2013).

In the majority of cases of CDI, cessation of the inciting antimicrobial, supportive therapy, and treatment with metronidazole and/or vancomycin are able to achieve clinical cure (Hedge et al., 2008; Louie et al., 2013). However, decreasing response to conventional therapy and increasing rates of recurrent CDI have been noted internationally (Aspevall et al., 2006; Musher et al., 2005). Recurrence may occur in 15-35% of C. difficile infections (McFarland et al., 2002), with treatment response rates decreasing further in patients that have previously failed treatment (Huebner & Surawicz, 2007). Recurrent CDI can be a debilitating disorder, with markedly reduced quality of life (Lee, Petukhova, & Kim, 2013) and potentially profound direct (healthcare cost) and indirect (e.g. inability to work) costs.

Despite the severity and increasing incidence of recurrent CDI, little is known about the pathophysiology of this disease. Much attention has been focused recently on
the role of the gut microbiota, particularly given the apparently high response rates to
fecal microbiota transplantation (FMT, also referred to as stool transplantation) (Shahinas
et al., 2012, Brandt et al., 2012; Kassam et al., 2013; Rohlke, Surawicz, & Stollman,
2010). This treatment approach is believed to re-establish the native intestinal microbiota,
which, in turn, serves to compete with *C. difficile* and prevent its proliferation (Brandt et
al., 2012; Rafii et al., 2008). However, the reason that recurrent CDI develops is currently
unclear. A high leukocyte count at the time of initial diagnosis and continuation of proton
pump inhibitor use after diagnosis have been reported as risk factors for recurrence
(Chitnis et al., 2013; Dolors Rodríguez-Pardo et al., 2013a). Attention has also focused
on strains, including a study that reported initial infection with ribotype 027 being
associated with increased risk of relapse (Marsh et al., 2012). However, other results have
been conflicting. One study did not find 027 in any case of recurrent CDI (Rodríguez-
Pardo et al., 2013), and another reported an association of the presence of a CDT-
possessing strain with recurrence, but no association with ribotype 027/NAP1 (Moore et
al., 2013; Stewart, Berg, & Hegarty, 2013).

The objective of this study was to compare *C. difficile* strains from patients with
recurrent CDI and those with sporadic CDI that responded to therapy.

2.3 Materials and Methods

2.3.1 Sample conditions

Fecal samples were obtained from patients with recurrent or sporadic CDI from St
Joseph’s Healthcare, Hamilton, Ontario, Canada. Clinical cure was defined as resolution
of gastrointestinal symptoms, with assessments performed at baseline, day 1, and day 12
(physical examination, hematology, and stool collection). The recurrent disease group
consisted of patients that had experienced one or more recurrences despite antimicrobial therapy, and were to undergo FMT. The sporadic disease group consisted of individuals that were diagnosed with CDI by fecal toxin B PCR or ELISA but which responded to initial antimicrobial therapy and who were symptom-free at the time of subsequent examination by the referring physician. All patients provided consent, and patient enrollment and sample collection were approved by the Hamilton Integrated Research Ethics Board (HIREB) (projects 12-3683 and 05-2477), and the Guelph Ethics Board (GEB) (project 12AU013).

2.3.2 Culture conditions

Samples were inoculated directly onto *C. difficile* chromogenic culture media (chromID difficile, BioMerieux Canada, St Laurent, QC) and incubated for 48 hours at 37˚ C in an anaerobic chamber. The plates that demonstrated a colour change (clear to black) and growth patterns indicative of *C. difficile* (see Figure 2.1) were sub-cultured onto Columbia blood agar with 5% sheep blood, (Oxoid) and incubated for 48 hours at 37˚ C in an anaerobic chamber.

Stool samples from which there was no growth on direct culture were inoculated into 9 ml of *C. difficile* moxalactam norfloxacin (CDMN) broth (Oxoid, Nepean, ON), and incubated at 37˚ C for 7 days. These tubes were subsequently vortexed, and 2 ml of the resulting solution was subjected to alcohol shock through the addition of 2ml anhydrous ethanol (2ml) (100%) and incubation at room temperature for one hour. The tubes were centrifuged and the pellet was inoculated onto CDMN agar and incubated as described above.
Isolates were presumptively confirmed as *C. difficile* by the distinctive odour produced when cultured on Columbia blood agar, and the production of l-aminopeptidase.

### 2.3.3 Characterization of isolates

DNA was extracted using a Chelex resin-based commercial DNA extraction kit (Bio-Rad Instagene Matrix). All isolates were ribotyped according to the adapted methods described by Bidet et al (Bidet et al, 1999). Amplification was performed using a 25 µl total volume comprised of 5 µl of DNA extract, 1.25 µl of 16S primer (5’-CTGGGGAAGTG CGTAACAAGG-3’), 1.25 µl of a 23 S primer (5’GGTCCTTAGATGTTCAGTT-3’), 12.5 µl of KAPA, and 5 µl of sterilized deionized water. Amplifications were performed using one 6-minute cycle at 94˚ C, followed by 35 cycles (1 minute at 94˚ C, 1 minute at 57˚ C, and 1 minute at 72˚ C constituting one cycle), and concluded by a single 7-minute cycle at 72˚ C. Amplification products were fractionated by gel electrophoresis on 1.5% agarose gel (Invitrogen) for 2 hours in 0.5 x TBE Buffer, and submerged in a Gel Red nucleic acid stain (Biotium, Hayward, CA) for a minimum of 30 minutes. Images were then acquired using GeneSnap software (SynGene, Cambridge, UK).

The images produced during the PCR ribotyping were examined and compared visually to isolates from the Cardiff/ECDC reference library (denoted by a number, e.g. 027) and an internal collection of isolates (denoted by a letter, e.g. CD70). Suspected matches were re-evaluated by re-running PCR products side-by-side to confirm strain identification.

Detection of *tcdA*, *tcdB*, *cdtA* and *cdtB* was performed using primers reported in
Table 2.1 and methods adapted from Persson et al (Persson et al., 2008).

Amplifications were performed using an initial 10 min at 94°C, followed by 35 cycles of 50 seconds at 94°C, 40 seconds at 54°C and 50 seconds at 72°C, concluded by a single cycle of 3 minutes at 72°C. Amplification products were fractionated by electrophoresis on agarose gel (Invitrogen, Life Technologies, Carlsbad, CA) for one hour in 0.5 x TBE Buffer, and submerged in a Gel Red nucleic acid stain (Biotium, Hayward, CA) for a minimum of 30 minutes. Images were then acquired using GeneSnap software. Positive and negative controls were included for each run.

2.3.4 Antimicrobial Susceptibility

The isolates were tested for susceptibility to vancomycin and metronidazole using Etests (bioMerieux, St Laurent, QC). A McFarland 1 suspension was prepared in brain heart infusion broth and inoculated diffusely onto Mueller Hinton agar with 5% sheep blood. The plates were allowed to dry for 10 minutes before being stamped with Etests for both vancomycin and metronidazole (see Figure 2.2).

They were incubated at 37°C in an anaerobic chamber and minimum inhibitory concentration (MIC) recorded after 24 and 48 hours. Susceptibility was determined using European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints, with ≤2 µg/ml indicating susceptibility for both antimicrobials (EUCAST, 2013).

2.3.5 Data Analyses

Categorical comparisons were performed using Fisher’s exact test. Logistic regression was used to evaluate the association between age and C. difficile strain.

Logistic regression was performed to model the impact of age, gender and the presence/absence of ribotype 027 on the likelihood of recurrent versus sporadic CDI. To
examine the relationship between MIC (24 and 48 hours) for both vancomycin and metronidazole, and recurrent/sporadic CDI. t-tests were performed. A P-value of <0.05 was considered significant for all comparisons. The statistical software JMP10 (SAS, Cary, North Carolina) was used.

2.4 Results

A total of 60 samples were analyzed, 27 (45%) from patients with recurrent CDI and 33 (55%) from patients with sporadic CDI (Table 2.2). In total, 27 distinct PCR ribotypes were found. Ribotype 027 was most common, accounting for 29/60 (48%) of isolates overall; 70.4% in the recurrent groups vs 30.3% in the sporadic group (P=0.0306). Six other recognized strains were identified; ribotypes 431, 464, 680, 1008, 73, and 30, albeit only accounting for single isolates each. Seventeen ribotypes that were not consistent with those in the reference library were identified, also only accounting for single samples each. Overall, 31/60 (51.7%) isolates possessed \( tcdA, tcdB, cdtA \) and \( cdtB \), while 29/60 (48.3%) possessed \( tcdA \) and \( tcdB \) but not \( cdtA \) and \( cdtB \). Ribotype 027 accounted for 29/31 (93.5%) isolates that possessed CDT genes, with single isolates of ribotypes 431 and 30 also identified. Strains that were found in more than one patient are described in Table 2.3.

Female gender, advancing age and the presence of ribotype 027 were identified as associated with recurrent CDI (Table 2.2). The results of logistic regression are presented in Table 2.4. MIC\(_{50}\) and MIC\(_{90}\), data are presented in Table 2.5.

Fifty-six (93%) isolates were susceptible to vancomycin at both 24 and 48h. Fifty-nine (98%) were susceptible to metronidazole at 24h and 58 (97%) at 48h. There was no difference in the prevalence of non-susceptibility between sporadic and recurrent isolates.
for either drug at either timepoint (all $P > 0.58$). There was also no association between ribotype 027 and non-susceptibility to vancomycin; however, there was an association between ribotype 027 (vs all other strains) and metronidazole non-susceptibility at 48h ($P < 0.0001$) (see Table 2.6).

### 2.5 Discussion

While there is frequent discussion of the role of ribotype 027 in recurrent CDI (Ciaran P. Kelly & LaMont, 2008; Sahil Khanna & Pardi, 2012), reported data to date have been limited and conflicting. This study indicates a significant association between the presence of ribotype 027 and recurrent CDI in this study population. This finding is consistent with the purported hypervirulence of NAP1/027 (Sahil Khanna & Pardi, 2012; J. R. O’Connor et al., 2009; Walker et al., 2013) and provides further evidence of the impact of this strain. However, multiple other strains were identified in individuals with recurrent CDI, as were patient-associated risk factors, supporting the notion that there are both microbial and host aspects that determine the likelihood of recurrence.

While ribotype 027 had the strongest association with recurrent CDI, gender was also a significant factor. Female gender has been associated with acute CDI in numerous studies (Fekety et al., 1997; Gash, Brown, & Pullyblank, 2010). Less information is available about recurrent CDI, although FMT studies have also reported a disproportionately high number of women (Brandt et al., 2012; Gough et al., 2011). Reasons for this have not been well established, although various factors have been suggested, such as a greater incidence of comorbidities (Gash et al., 2010). Females also tend to have longer life spans, and age has previously been an established risk factor for sporadic CDI (Louie et al., 2013; Rodríguez-Pardo et al., 2013), presumably because of
age-related changes in the immune system and intestinal microbiota, along with increased incidence of comorbidities. Age was not associated with CDI in the logistic regression model in this study, which was an unexpected finding. The relative impacts of age and gender on recurrence deserve further investigation.

Most isolates were susceptible to metronidazole or vancomycin, as was expected (Aspevall et al., 2006; Drummond, 2003). Resistance to metronidazole has been previously reported (Lynch et al., 2013; Musher et al, 2005), but this appears to be rare; and decreased response to treatment has not been clearly associated with resistance (Johnson, Sanchez, & Gerding, 2000). While significant differences in MIC were noted between groups in this study (metronidazole at 48 hours vs all others), and non-susceptible isolates were found, the MICs were within narrow ranges and the MICs were only slightly above the breakpoint. Further, these isolates would all be classified as susceptible to metronidazole if the Clinical and Laboratory Standards Institute (CLSI) susceptibility breakpoint of $\leq 8 \mu\text{g/ml}$ was used (Clinical and Laboratory Standards Institute, 2007). This finding is interesting but the relevance is unclear. It is possible that slight increases in MIC could be associated with decreased efficacy in the gut, since the relationship between MIC and efficacy in the intestinal lumen has not been established. Subtle increases in MIC could impact resistance if there is a narrow therapeutic window for metronidazole or vancomycin at the site of infection. Alternatively, it could simply indicate minor, and clinically irrelevant laboratory differences between isolates. Metronidazole and vancomycin exposure data were not available for all patients so this was not assessed further.
While there was no difference in metronidazole MIC amongst recurrent vs sporadic isolates, ribotype 027 had significantly higher metronidazole MICs after 48h of incubation, and a P value approaching significance (P=0.06) after 24h. The clinical relevance of this is unclear, as is discussed above, but deserves further study.

2.6 Conclusion

Female gender and the presence of NAP1/027 were associated with recurrent CDI in this study population. Non-susceptibility to both metronidazole and vancomycin was identified but was rare, and while there was an association between ribotype 027 and decreased metronidazole susceptibility, the clinical relevance of this requires further study. A better understanding of the pathophysiology and epidemiology of recurrent CDI is required to facilitate development of optimal prevention and treatment approaches.

2.7 Acknowledgements

The authors thank the Canadian Institutes of Health Research (CIHR) and the Natural Sciences and Engineering Research Council (NSERC) Collaborative Health Research Projects (project 413548-2012) for their financial support of this research project.
<table>
<thead>
<tr>
<th>Gene target</th>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tcdA</td>
<td>tcdA-F3345</td>
<td>GCATGATAAGGCAACTTCAGTGGTA</td>
</tr>
<tr>
<td></td>
<td>tcdA-R3969</td>
<td>AGTTCCCTCCTGCTCCATCAAATG</td>
</tr>
<tr>
<td>tcdB</td>
<td>tcdB-F5670</td>
<td>CCAAARTGGGAGTTACAAAAACAGGTA</td>
</tr>
<tr>
<td></td>
<td>tcdB-R6079A</td>
<td>GCATTTCCTCCATTCTCAGCAAAGTA</td>
</tr>
<tr>
<td></td>
<td>tcdB-R6079B</td>
<td>GCATTTCCTCCGTTTCAGCAAAGTA</td>
</tr>
<tr>
<td>cdtA</td>
<td>cdtA-F739A</td>
<td>GGGGAAGCAGTTATATACAGCAGAAC</td>
</tr>
<tr>
<td></td>
<td>cdtA-F739B</td>
<td>GGGAAACATTATATTAAGCAGAAC</td>
</tr>
<tr>
<td></td>
<td>cdtA-R958</td>
<td>CTGGGTTAGGATTATTTACTGGACCA</td>
</tr>
<tr>
<td>cdtB</td>
<td>cdtB-F617</td>
<td>TTGACCCAAGTTGATGTCTGATTG</td>
</tr>
<tr>
<td></td>
<td>cdtB-R878</td>
<td>CGGATCTCTGCTCAGTCTTTATAG</td>
</tr>
<tr>
<td>16S rDNA</td>
<td>PS13</td>
<td>GGAGGCACGAGTGAGAAAA</td>
</tr>
<tr>
<td></td>
<td>PS14</td>
<td>TGACGGCGGTGTTGATCAAAG</td>
</tr>
</tbody>
</table>
**TABLE 2.2.** Descriptive data comparing patients with recurrent *Clostridium difficile* infection (CDI) and those with sporadic CDI

<table>
<thead>
<tr>
<th></th>
<th>Recurrent n= 27</th>
<th>Sporadic n=33</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female gender</td>
<td>18 (66.7%)</td>
<td>13 (39.4%)</td>
<td>0.03</td>
</tr>
<tr>
<td>Age: mean (SD, range)</td>
<td>75.3 (SD 13.3, range 24-91)</td>
<td>66.11 (SD 16.2, range 34-90)</td>
<td>0.025</td>
</tr>
<tr>
<td>Ribotype 027</td>
<td>19 (70.4%)</td>
<td>10 (30.3%)</td>
<td>0.036</td>
</tr>
<tr>
<td>CDT+</td>
<td>20 (74.1%)</td>
<td>11 (33.3%)</td>
<td>0.021</td>
</tr>
</tbody>
</table>
**TABLE 2.3.** Descriptive data comparing ribotypes found among patients with recurrent CDI and sporadic CDI

<table>
<thead>
<tr>
<th>Ribotype (toxin genes)</th>
<th>Total n= 60</th>
<th>Recurrent n= 27</th>
<th>Sporadic n= 33</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>027 (A+/B+/CDT+)</td>
<td>29 (48%)</td>
<td>19 (70.4%)</td>
<td>10 (30.3%)</td>
<td>0.004</td>
</tr>
<tr>
<td>287 (A+/B+/CDT-)</td>
<td>4 (6.7%)</td>
<td>0%</td>
<td>4 (12.1%)</td>
<td>&gt;0.306</td>
</tr>
<tr>
<td>558 (A+/B+/CDT-)</td>
<td>2 (3.3%)</td>
<td>1 (3.7%)</td>
<td>1 (3%)</td>
<td>1.0</td>
</tr>
<tr>
<td>CD70 (A+/B+/CDT-)</td>
<td>2 (3.3%)</td>
<td>2 (7.4%)</td>
<td>0%</td>
<td>&gt;0.58</td>
</tr>
</tbody>
</table>

Only strains that were found in more than one patient are described.
TABLE 2.4. Results of logistic regression examining gender, age, and presence of 027 in patients with sporadic and recurrent CDI

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>3.3</td>
<td>1.02-11.5</td>
<td>0.047</td>
</tr>
<tr>
<td>Age</td>
<td>1.03</td>
<td>0.993-1.08</td>
<td>0.12</td>
</tr>
<tr>
<td>Ribotype 027</td>
<td>4.3</td>
<td>1.3-14.6</td>
<td>0.017</td>
</tr>
</tbody>
</table>
**TABLE 2.5.** MICs for metronidazole and vancomycin for recurrent patients at both 24 and 48 hours.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (µg/ml)</th>
<th>Range (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin (24h)</td>
<td>0.5</td>
<td>0.75</td>
<td>0.5-1</td>
</tr>
<tr>
<td>Metronidazole (24h)</td>
<td>1</td>
<td>1.5</td>
<td>0.38-6</td>
</tr>
<tr>
<td>Vancomycin (48h)</td>
<td>0.5</td>
<td>0.75</td>
<td>0.5-1</td>
</tr>
<tr>
<td>Metronidazole (48h)</td>
<td>1</td>
<td>2</td>
<td>0.25-3</td>
</tr>
</tbody>
</table>
**TABLE 2.6.** The association between ribotype 027 and MIC.

<table>
<thead>
<tr>
<th></th>
<th>Ribotype 027</th>
<th>Other ribotypes</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metronidazole MIC, 24h</td>
<td>1.1</td>
<td>0.69</td>
<td>0.06</td>
</tr>
<tr>
<td>Metronidazole MIC, 48h</td>
<td>1.1</td>
<td>0.53</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Vancomycin MIC, 24h</td>
<td>0.91</td>
<td>0.86</td>
<td>0.81</td>
</tr>
<tr>
<td>Vancomycin MIC, 48h</td>
<td>0.95</td>
<td>0.87</td>
<td>0.73</td>
</tr>
</tbody>
</table>
FIGURE 2.1. *C. difficile* growth on chromogenic culture media
FIGURE 2.2. Blood agar with 5% sheep blood (Oxoid) stamped with Etests for both vancomycin and metronidazole
2.8 References


Gash, K., Brown, E., & Pullyblank, A. (2010). Emergency subtotal colectomy for fulminant *Clostridium difficile* colitis-is a surgical solution considered for all patients?
doi:10.1308/003588410X12518836439164


Lee, C., Petukhova, T., & Kim, P. (2013). *Clostridium difficile* infection and health-related quality of life at baseline. Unpublished manuscript.


increased fluoroquinolone use. *Infection Control and Hospital Epidemiology*, 26(3), 273–280. doi:10.1086/502539


3.0 Examination of the association between *Clostridium difficile* strain type and response of recurrent patients to treatment with fecal microbiota transplantation (FMT)

**ABSTRACT**

Incidences of recurrent and severe *Clostridium difficile* infections (CDI) are becoming increasingly common. This has been partially attributed to the “international outbreak strain” North American Pulsetype 1/ribotype 027 (NAP1/027). In this paper, samples from individuals who contracted recurrent *C. difficile* undergoing fecal microbiota transplants (FMT) were ribotyped. Results showed no association between strain type and outcome to treatment, however a high proportion of NAP1/027 was found in this group of patients, and the majority of individuals remained colonized with *C. difficile* after clinical cure. While the results suggest that strain type is not a predictor of treatment outcome, further research is necessary to determine the role of intestinal microbiota in recurrent CDI.

**KEYWORDS**

*Clostridium difficile*, recurrent, PCR ribotypes, FMT
3.1. Introduction

*Clostridium difficile* (*C. difficile*) is an anaerobic spore-forming bacillus, and the primary cause of hospital-associated diarrhea in developed countries (Kelly & Kyne, 2011; Kelly & LaMont, 2008). Two to three percent of adults in the community, and 10-25% of hospitalized individuals can be colonized without symptomatic infection (Bartlett, 1994; Simor et al., 2002); however, for those who become ill, symptoms can range from mild diarrhea to pseudomembranous colitis (Kelly & LaMont, 2008). Severe *C. difficile* infection (CDI) can result in sepsis, and death (Kyne et al., 1999), with reported case-fatality rates of 6%-30% (Kuijper, E., Coignard, B, & Tüll, P., 2006; Miller et al., 2010).

In healthy individuals, the gut is colonized with a wide range of bacteria (the commensal microbiota), which is responsible for many different functions (Dethlefsen et al., 2006; Fujimura et al, 2010). However, when the population of native microbiota is disrupted, opportunistic pathogens can flourish, potentially resulting in development of disease, as is the case with CDI (Buffie et al., 2012). *Clostridium difficile* proliferation most often occurs due to exposure to broad-spectrum antimicrobial therapy, which eliminates populations of competing gut bacteria (Buffie et al., 2012; Owens et al., 2008).

In addition to antimicrobial exposure, established risk factors for CDI include advanced age (over 65 years) (Loo et al., 2005; Louie et al., 2013), prolonged hospital stay, and comorbid conditions (Bartlett, J., & Gerding, D., 2008; Chang, V., & Nelson, K., 2000). Other risk factors such as gender and strain type have also been implicated, however, conflicting data are available (Brandt et al., 2012; Marsh et al., 2012; Rodríguez-Pardo et al., 2013). Strain type is of particular importance because globally,
incidences of severe or recurrent disease have increased, and this has been partially attributed to the emergence of a hypervirulent strain known as North American Pulsetype 1 (NAP1) or ribotype 027 (NAP1/027) (Hookman, P., & Barkin, J., 2009; McDonald et al., 2007).

Toxigenic strains of *Clostridium difficile* produce three main toxins; toxin A (TcdA), toxin B (TcdB) and binary toxin (CDT) (Rupnik et al., 2003; Voth, D., & Ballard, J., 2005). Most toxigenic strains produce both toxins A and B, and their encoding genes (*tcdA* and *tcdB*, respectively) are located on a section of the *C. difficile* chromosome known as the pathogenicity locus (PaLoc) (Voth, D., & Ballard, J., 2005). The functions of these toxins are well-described, and both are established as able to cause disease (Cohen et al., 2000). Binary toxin (CDT), encoded by genes *cdtA* and *cdtB*, is less common among strains of *C. difficile* and is encoded outside of the PaLoc (Rupnik et al., 2003). Less is known about this toxin; however, it is suspected to be a factor in the increased virulence of some strains, particularly NAP1/ribotype 027 (Bacci et al., 2011).

Typically, discontinuation of the antimicrobial responsible for infection, and treatment with metronidazole and/or vancomycin is sufficient to resolve gastrointestinal symptoms caused by CDI (Louie et al., 2013). However, in 15-35% of cases the initial treatment is not successful or disease recurs after a variable period of clinical normalcy (McFarland et al., 2002). Fecal microbiota transplantation (FMT) (otherwise known as stool transplantation or human biotherapy (HBT) is now being used in individuals who have experienced a number of treatment failures or recurrences, on the premise that restoration of the intestinal microbiota is a critical aspect of successful outcome. FMT
involves the transplantation of fecal matter from a healthy screened donor, by enema, colonoscope, nasogastric tube or oral administration of capsules (Gough et al., 2011).

The high success rates associated with FMT, a procedure that does not directly target *C. difficile*, has emphasized the role of the microbiota in elimination of disease and prevention of recurrence. Indeed, data to date, while preliminary, support FMT as an effective treatment (Brandt et al., 2012; Gough et al., 2011), with reported response rates of up to 92% (Brandt et al., 2012; Gough et al., 2011). However, resolution of diarrhea does not necessarily indicate the absence of *C. difficile* from the patient, and limited study of *C. difficile* shedding has been reported in patients undergoing FMT. This is important for various reasons, including understanding the mechanism of FMT and recurrent CDI, along with the challenges of potential infection control posed by individuals that have recently responded to treatment. The objective of this study was to evaluate *C. difficile* shedding in patients after FMT and, if present, to characterize recovered isolates.

3.2. Materials and Methods

3.2.1. Sample conditions

Fecal samples were obtained from patients from St Joseph’s Healthcare, Hamilton, Ontario, Canada that were undergoing FMT as part of clinical management of recurrent CDI. Recurrent CDI was defined as complete resolution of diarrhea for at least 3 days followed by new onset of diarrhea, as determined by complete clinical and laboratory documentation by chart or telephone review.

All patients were evaluated clinically after FMT treatment. Fecal samples were collected, whenever possible, before treatment and after single or multiple rounds of FMT. Assessments (physical examination, hematology, and stool collection) were
performed at baseline (day 1) and day 12 after treatment. Clinical cure was defined as resolution of gastrointestinal signs by day 12 post FMT. All patients provided consent, and patient enrollment and sample collection were approved by the Hamilton Integrated Research Ethics Board (HIREB) (projects 12-3683 and 05-2477), and the University of Guelph Research Ethics Board (GEB) (project 12AU013).

3.2.2. Culture conditions

Stool samples were inoculated directly onto *C. difficile* chromogenic agar (chromID difficile, BioMerieux Canada, St Laurent, QC) and incubated for 48 hours at 37°C in an anaerobic chamber. Plates that demonstrated a colour change (clear to black) and growth patterns indicative of *C. difficile* were sub-cultured onto Columbia blood agar with 5% sheep blood, (Oxoid, Nepean, ON) and incubated for 48 hours at 37°C in an anaerobic chamber.

Stool samples from which there was no growth on direct culture were inoculated into 9 ml of *C. difficile* moxalactam norfloxacin (CDMN) broth (Oxoid, Nepean, ON), and incubated at 37°C for 7 days. These tubes were subsequently vortexed, and 2 ml of the resulting solution was subjected to alcohol shock through the addition of anhydrous ethanol (2ml) and incubation at room temperature for one hour. The tubes were centrifuged and the pellet was inoculated onto CDMN agar and incubated as described above.

Isolates were presumptively confirmed as *C. difficile* by the distinctive odour produced when cultured on Columbia blood agar, and the production of l-aminopeptidase.
3.2.3. Characterization of isolates

DNA was extracted using a Chelex resin-based commercial DNA extraction kit (Bio-Rad Instagene Matrix). All isolates were ribotyped according to the adapted methods described by Bidet et al (Bidet et al, 1999). Amplification was performed using a 25 µl total volume comprised of 5 µl of DNA extract, 1.25 µl of 16S primer (5' CTGGGGTGAACT CGTAAAGGGG-3'), 1.25 µl of a 23S primer (5'GGTACCTTAGATGTTTCAGTT-3'), 12.5 µl of KAPA, and 5 µl of sterilized de-ionized water. Amplifications were performed using one 6-minute cycle at 94˚ C, followed by 35 cycles (1 minute at 94˚ C, 1 minute at 57˚ C, and 1 minute at 72˚ C constituting one cycle), and concluded by a single 7-minute cycle at 72˚ C. Amplification products were fractionated by gel electrophoresis on 1.5% agarose gel (Invitrogen) for 2 hours in 0.5 x TBE Buffer, and submerged in a Gel Red nucleic acid stain (Biotium, Hayward, CA) for a minimum of 30 minutes. Images were then acquired using GeneSnap software (SynGene, Cambridge, UK).

The images produced during the PCR ribotyping were examined and compared visually to isolates from the Cardiff/ECDC reference library (denoted by a number, e.g. 027) and an internal collection of isolates (denoted by a letter or letter/number combination, e.g. CD70). Suspected matches were re-evaluated by re-running PCR products side-by-side to confirm strain identification.

Detection of tcdA, tcdB, cdtA and cdtB was performed by PCR using primers reported in Table 3.1 and methods adapted from Persson et al (Persson et al., 2008). Amplifications were performed using an initial 10 minutes at 94˚C, followed by 35 cycles of 50 seconds at 94˚C, 40 seconds at 54˚C and 50 seconds at 72˚C, concluded by a single cycle of 3 minutes at 72˚C. Amplification products were fractionated by electrophoresis
on agarose gel (Invitrogen, Life Technologies, Carlsbad, CA) for one hour in 0.5 x TBE Buffer, and submerged in a Gel Red nucleic acid stain (Biotium, Hayward, CA) for a minimum of 30 minutes. Images were then acquired using GeneSnap software. Positive and negative controls were included for each run.

3.2.4. Data Analyses
Categorical comparisons were performed using Fisher’s exact test. A P-value of <0.05 was considered significant for all comparisons. The statistical software JMP10 (SAS, Cary, North Carolina) was used.

3.3. Results
Twenty-eight patients were enrolled; all patients who underwent at least one round of FMT were enrolled. Nineteen (67.8%) were female and 9 (32.2%) male. The mean age at the time of first treatment was 73.9 y (SD 15.2, range 24-91) and 24 (85.7%) individuals were greater than 65 years of age.

Patients underwent a total of 88 FMT treatments (mean 3.1, median 2, range 1-15). Nineteen patients (67.8%) underwent multiple rounds of treatment, with treatments administered over periods ranging from 1 day to 27 weeks. The mean duration between FMT treatments was 11.1 days (range 1-105, median 7, SD 4.95). There was ultimate clinical resolution of diarrhea in 24/28 (85.7%) patients.

Ninety-one stool samples were available for testing, ranging from 1 to 7 per patient (mean 3.3, median 3). Forty samples from 23 (82%) patients were collected before the first treatment, 17 samples from 15 (53.6%) patients were collected after the initial treatment and 18 samples from 9 (32.1%) patients were collected after subsequent treatments. Twenty-four samples from 17 patients were available after the final treatment,
and of these, 8 samples from 7 patients were also included in the post-initial treatment group, as they resolved after a single treatment (see Tables 3.2 and 3.3).

Overall, *Clostridium difficile* was isolated from 74/91 (81.3%) samples. Twenty-four different ribotypes were identified. Ribotype 027 (*tcdA*+, *tcdB*+, *cdtA/B*+) was most common, accounting for 51/74 (68.9%) isolates. Ribotype CD70 (*tcdA*+, *tcdB*+) accounted for four (4.4%) isolates. All other ribotypes were only found once. Of those only found once, 18/19 (94.7%) strains were *tcdA*+,*tcdB*+,*cdtA/B*-, while 1/19 (5.3%) was *tcdA*+,*tcdB*+,*cdtA/B*+.

*Clostridium difficile* was isolated from 31/40 (78%) of pre-treatment samples from 19/23 (82.6%) patients. Of these, 24/31 (77.4%) isolates from 13 (46%) patients were ribotype 027, 3/31 (9.7%) from 2 (8.1%) patients were CD70, and the remainder were individual A+/B+/CDT- strains. Six patients had more than one positive sample prior to initial treatment, and that same strain was present in both samples in all but one case. In that case, both ribotypes 027 and 287 were found.

Thirty-five (87.5%) pre-treatment samples came from 21/23 (91.3%) patients who ultimately responded to treatment, while 5/40 (12.5%) came from two (8.7%) who did not respond. Ribotype 027 was present initially in 14/23 (61%) patients; 12/21 (57.1%) that responded versus, 2/2 (100%) that did not (*P*=1.0).

Samples were available after the initial treatment for 15 (53.6%) patients. *Clostridium difficile* was isolated from 13/15 (86.7%) patients; 5/7 (71.4%) that responded to that particular course of treatment and 8/8 (100%) that did not (*P*=1.0). Eight of 15 (53.3%) patients harboured ribotype 027, 2/7 (28.6%) that responded to that treatment and 6/8 (75%) that did not (*P*=0.16).
A further 11 samples from 5 patients were taken between FMT treatments, after unsuccessful treatment and prior to a subsequent successful course. *Clostridium difficile* was isolated from 11 (100%) samples, with ribotype 027 accounting for 10 (90.9%) isolates (see Figure 3.1).

Overall, *C. difficile* was isolated from 15/17 (88.2%) patients post FMT therapy that had resolution of clinical signs, compared to 4/4 (100%) patients after their final FMT treatment where diarrhea persisted (P=1.0). Ribotype 027 accounted for 9/15 (60%) of post-treatment isolates from patients with treatment response versus 3/4 (75%) from treatment failures (P=1.0) (see Figure 3.2).

### 3.4. Discussion

As is consistent with other reports (Brandt et al., 2012; Gough et al., 2011), the clinical cure rate following FMT was high (86%) in this study population, although multiple treatments were sometimes required. Results of this study indicate that microbiological cure (elimination of *C. difficile* shedding) occurs in a minority of patients with clinical cure after FMT, at least in the initial post-treatment period. This provides further information about the likely pathophysiology of recurrent CDI and the mechanism of FMT. The clinical response from this treatment is likely based on effects on the intestinal microbiota that suppress the impact of *C. difficile*, and not elimination of the pathogen. Previous antimicrobial failure may be attributed to their inability to ‘restore’ the normal gut microbiota and their disruption of the microbiota while trying to eliminate *C. difficile*, the opposite of the effect that is desired.

Not surprisingly, in this study, ribotype 027/NAP1 predominated. This strain has been identified as a common cause of recurrent CDI in other studies (Kelly, C.,
Pothoulakis, C., & LaMont, J., 1994; Khanna, S., & Pardi, D., 2012; Marsh et al., 2012) and is widespread in healthcare facilities in both endemic and epidemic situations. Although NAP1/027 has been associated with recurrent disease, questions remain about whether the presence of this ribotype is associated with poor treatment outcome. In addition, further information is needed regarding how frequently NAP1/027 remains in the intestinal tract after clinical cure. This study indicates no significant association between the presence of *C. difficile*, (both during and after FMT), and ultimate treatment resolution. Additionally, there was no apparent impact on the presence of ribotype 027 at baseline or after treatment and the likelihood of successful treatment. This finding is inconsistent with the purported (and clinically apparent) hypervirulence of NAP1/027 in acute CDI (Bacci et al., 2011; Warny et al., 2005). It is important to interpret the results of this study in the context of the study population, and this study cannot determine a role of 027/NAP1 in recurrent disease since a group of non-recurrent CDI patients was not included.

The samples included in this study were from 28 individual patients with varying complicating factors and in various stages of disease, necessitating a wide range of treatment regimens. Although each patient was diagnosed with recurrent disease, resolution for some of these patients occurred after a single treatment, while some were still unresolved after multiple treatments.

Samples were obtained as part of clinical care, and not all samples were available for each patient throughout the entirety of their treatment process. It would have been ideal to have pre- and post-treatment samples from all patients but this was not possible because of the sporadic nature of sample submission and the retrospective nature of the
study. Similarly, it would have been ideal to have a patient population that underwent identical treatment regimens and inter-treatment intervals. The sample size was also relatively small and while having lesser impact on the prevalence data, there was limited statistical power for many comparisons. This was particularly true for study of patients that did not ultimately respond to treatment because of the small size of that group. Therefore, care must be taken when interpreting the lack of significant differences. However, these issues reflect the clinical use of FMT and the results generated here may be applicable to similarly managed patient populations.

3.5. Conclusion

*Clostridium difficile* colonization commonly remains after resolution of recurrent CDI in patients treated with FMT further supporting the importance of the intestinal microbiota in treatment and prevention of CDI. There was no evidence that the presence or absence of *C. difficile* after treatment is a predictor of outcome. While common in this group of patients with recurrent CDI, the presence of ribotype 027/NAP1 did not appear to influence the likelihood of response to FMT or the persistence of *C. difficile* after treatment. Although this is an uncontrolled study, these data provide more information about the potential efficacy of FMT and its potential mechanism of action. In particular, these data highlight the likely importance of the intestinal microbiota in recurrent CDI.

3.6. Acknowledgements

The authors thank the Canadian Institutes of Health Research (CIHR) and the Natural Sciences and Engineering Research Council (NSERC) Collaborative Health Research Projects (project 413548-2012) for their financial support of this research project.
<table>
<thead>
<tr>
<th>Gene target</th>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tcdA</td>
<td>tcdA-F3345</td>
<td>GCATGATAAGGCAACTTCAGTGTA</td>
</tr>
<tr>
<td></td>
<td>tcdA-R3969</td>
<td>AGTTTCTCCTGCTCCATCAAATG</td>
</tr>
<tr>
<td>tcdB</td>
<td>tcdB-F5670</td>
<td>CCAARGGGAGTGTTACAAAAACAGGTG</td>
</tr>
<tr>
<td></td>
<td>tcdB-R6079A</td>
<td>GCATTTCTCCATTTCAGCAAAGTA</td>
</tr>
<tr>
<td></td>
<td>tcdB-R6079B</td>
<td>GCATTTCTCCGTTTTCAGCAAAGTA</td>
</tr>
<tr>
<td>cdtA</td>
<td>cdtA-F739A</td>
<td>GGGAAAGCAGTTATATTAAAGCAGAAGC</td>
</tr>
<tr>
<td></td>
<td>cdtA-F739B</td>
<td>GGGAAACATTATATATTAAAGCAGAAGC</td>
</tr>
<tr>
<td></td>
<td>cdtA-R958</td>
<td>CTGGGTAGGTTGATTTACTGGACCA</td>
</tr>
<tr>
<td>cdtB</td>
<td>cdtB-F617</td>
<td>TGGACCCAAGTTGATGTCTGATAG</td>
</tr>
<tr>
<td></td>
<td>cdtB-R878</td>
<td>CGGATCTCTTGTCTTGTACAAAG</td>
</tr>
<tr>
<td>16S rDNA</td>
<td>PS13</td>
<td>GGAGGACAGCTGGGGAATA</td>
</tr>
<tr>
<td></td>
<td>PS14</td>
<td>TGACGGCGGATGTGTACAAAG</td>
</tr>
</tbody>
</table>
**TABLE 3.2.** Isolation of *C. difficile* and *C. difficile* ribotype 027 from 28 patients with recurrent *C. difficile* infection

<table>
<thead>
<tr>
<th></th>
<th>C. difficile</th>
<th>Ribotype 027</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>28/28 (100%)</td>
<td>18/28 (64.3%)</td>
</tr>
<tr>
<td>Before initial treatment</td>
<td>19/23 (82.6%)</td>
<td>13/23 (56.5%)</td>
</tr>
<tr>
<td>After any treatment</td>
<td>21/23 (91.3%)</td>
<td>13/21 (61.9%)</td>
</tr>
<tr>
<td>After successful treatment</td>
<td>14/17 (82.3%)</td>
<td>9/14 (64.3%)</td>
</tr>
<tr>
<td>After ultimate treatment failure</td>
<td>4/4 (100%)</td>
<td>3/4 (75%)</td>
</tr>
</tbody>
</table>
### TABLE 3.3. Post FMT treatment clinical cure rates (total number of patients N=28)

<table>
<thead>
<tr>
<th>Number of treatments</th>
<th>Number of patients</th>
<th>Clinical cure</th>
<th>Cumulative number of patients with clinical cure</th>
<th>Microbiological Cure</th>
<th>Colonized after treatment</th>
<th>NAP1/027 after treatment</th>
<th>Fecal sample received and tested after clinical cure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 FMT</td>
<td>9</td>
<td>9</td>
<td>9/28 (32.1%)</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>2 FMT</td>
<td>5</td>
<td>4</td>
<td>13/28 (46.4%)</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>3 FMT</td>
<td>6</td>
<td>4</td>
<td>17/28 (60.7%)</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>4 FMT</td>
<td>6</td>
<td>5</td>
<td>22/28 (78.6%)</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>5+ FMT</td>
<td>2</td>
<td>1</td>
<td>23/28 (82.1%)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Cells for which data was not available are blank
FIGURE 3.1. Cumulative response rate of patients (resolution of gastrointestinal symptoms) to consecutive FMT treatments (n=91)
Number of patients are denoted in each column section.

**FIGURE 3.2.** Cumulative response rate of patients (resolution of gastrointestinal symptoms) to FMT treatments, separated into those *with C. difficile* present after cure, those *without C. difficile* present after cure, and those with no post-treatment sample.
3.7 References


Kelly, C., & Kyne, L. (2011). The host immune response to *Clostridium difficile*. *Journal of Medical Microbiology, 60*(8), 1070–1079. doi:10.1099/jmm.0.030015-0


unfavorable clinical outcomes: results of a hospital-based study in Barcelona, Spain. 


4.0 GENERAL DISCUSSION

This study explored the risk factors and treatment outcomes associated with recurrent *Clostridium difficile* infection. *Clostridium difficile* infections have increased in incidence, recurrence and severity in recent years, some of which has been attributed to the international outbreak strain NAP1/027. However, objective data investigating NAP1/027 have, been surprisingly limited. This study, therefore, had a particular focus on the role of this strain in recurrent *C. difficile* infection.

A much higher proportion of NAP1/027 was found among individuals suffering from recurrent disease, when compared to those with sporadic CDI. These results confirmed the pre-existing assumption that NAP1/027 is associated with recurrent disease, and indicated a need for further examination into the mechanisms of recurrent disease overall, and the mechanism behind the over-representation of this strain.

Like the majority of pathogen-borne illnesses, CDI is multifactorial and therefore other potential risk factors were also examined. Interestingly, age was not found to be associated with recurrent disease; however, this was likely the result of study population limitations, particularly the relatively advanced age of individuals in both groups studied (sporadic and recurrent patients). In this study population, the average age of the patients with sporadic and recurrent CDI was 66.1 and 75.3 years respectively, and a study with a greater age distribution may have yielded different results with regards to age and recurrent disease. Regardless, while these data are consistent with various reports of CDI being predominantly a disease of older individuals, there was no evidence that age played a role in the likelihood of recurrence. Conversely, female gender was established as a risk factor for recurrent disease. This was not entirely unexpected, as female gender has been shown to be a risk factor for CDI in previous reports. Reasons behind this are not well
understood and this study was not designed to investigate this. Yet, results of this study indicated beyond being a risk factor for development of CDI, female gender may further increase the risk of recurrent disease. These results provide more evidence of a need to study any gender-associated predispositions that might be present, both confirmation of their presence and understanding why they may be present. Identification of pathogen- and patient-associated risk factors is an important part of both understanding the root causes of disease and development of successful interventions. These data provide more insight into the risk factors associated with recurrent disease, and susceptible patient populations.

Antimicrobial susceptibility of *C. difficile* isolates from this population of patients was also established. While resistance to antimicrobials is rarely reported in *C. difficile* infections, there is concern about the emergence of metronidazole and vancomycin resistance because of an apparent increase in treatment failures. It was reasonable to suspect that resistance would be more likely in isolates from people with recurrent CDI because of frequent antimicrobial exposure. Resistance to metronidazole and vancomycin was rare among the samples, and would have been non-existent had we used the Clinical and Laboratory Standards Institute (CLSI) susceptibility breakpoint of <8 µg/ml, compared to the EUCAST breakpoints that were used. However, there was a significant difference in MIC of metronidazole in isolates from patients with recurrent disease. This was an interesting finding but the difference between groups was subtle and the clinical relevance of this statistical difference is unclear. While the MICs were different, those differences may not have had any relation to the likelihood of treatment success. A possible explanation is that slight increases in MIC could be related to intestinal efficacy of the antimicrobials, but it could also indicate clinically unrelated
differences among isolates. Variations in comorbid conditions and treatment regimens, as well as prior antimicrobial usage could be responsible for the differences in MIC. The significant differences must not be dismissed, though, because of increasing reports of treatment failures and limited data about the pharmacokinetics of metronidazole and vancomycin in the gut. Antimicrobial exposure data were not available for all patients so the relationship of antimicrobial exposure and MIC was not assessed further. While perhaps of limited or no clinical relevance, the higher MICs in the recurrent disease group raise questions about the mechanisms of recurrent disease and the potential of increasing antimicrobial resistance, and indicate a need for further research.

Fecal microbiota transplantation has attracted much attention in recent years as a tool for the treatment of recurrent CDI. Despite increasing use, little is known about the mechanism of this treatment and factors that influence treatment success. In order to better understand the association between strain and response to FMT, serial stool samples were collected from patients undergoing FMT, and C. difficile was isolated and characterized. Consistent with Chapter 2, NAP1/027 was the predominant ribotype among the recurrent patient population, providing further evidence of the role of NAP/027 in recurrent CDI. Interestingly, the presence of NAP1/027 did not influence the likelihood of response to fecal microbiota transplantation. Clinical cure rate following FMT was high, which was also consistent with previous reports, and the low treatment failure rate might have limited the ability to detect an association between this strain and treatment failure. However, there was no clear evidence that identification of NAP1/027 in a patient was associated with a greater likelihood for FMT failure.

While FMT appears to be a highly effective treatment for recurrent CDI, the true mechanism is not well understood. Specifically, it is unknown whether FMT helps the
body eliminate *C. difficile* or whether it facilitates re-establishment of native intestinal microbiota, therefore “outcompeting” *C. difficile*. Results of this work demonstrate that a microbiological cure is rare, and for the most part *C. difficile* remains in the intestinal tract even after resolution of gastrointestinal symptoms. This is a potential indicator of both the pathophysiology of recurrent CDI and the mechanism of FMT. Presence of *C. difficile* after clinical cure, coupled with a high cure rate, indicates the clinical success of FMT is potentially based on the suppression of *C. difficile* through replenishment of natural intestinal microbiota. This also provides a possible explanation for the lack of success with antimicrobials, as the antimicrobials do not act to restore microbiota and may, in fact, cause further disruption to the gastrointestinal microbiota of individuals with recurrent CDI. The results of this thesis provide insight into the mechanism involved in treatment of recurrent CDI with FMT, and offers information to medical professionals about the benefits of this treatment.

Although interesting findings were noted throughout the studies in this thesis, it is important to note that a small sample size was used, there was a lack of structured and consistent fecal sample submission, and finally, all samples were collected from patients at a single institution. A standardized sampling regimen collected from a larger study population would be useful in substantiating the conclusions drawn from this research. Additionally, samples from hospitals distributed across larger geographic areas would be practical in determining whether risk for recurrent disease is associated with location/hospital/care facility, or with any other number of demographic factors.

Future research should also address the concept of strain-specific characteristics and their relationship to recurrent CDI. Although NAP1/027 was associated with recurrent CDI in this study, other strains were also involved, and more study of pathogen
and host factors that predispose to recurrence is required. Also, study of other potentially hypervirulent strains such ribotype 078 is indicated. Understanding of pathogen or patient factors that predispose to recurrence could be used to identify individuals at risk for recurrent disease, allowing health-care professions to take preventative measure. Additionally, a better understanding of the mechanisms of recurrent disease could enable the identification of future emergent strains to cause recurrent disease.

Studies should incorporate antimicrobial exposure data in order to evaluate the effect of different treatment regimens on the risk of recurrent disease. In addition, it would be interesting to evaluate potential effects of various antimicrobial treatments on the response of individuals to FMT treatments. Knowing whether certain regimens could predispose patients to FMT failure/success would be useful for health care providers and inform future antimicrobial research.

Finally, future research should evaluate post-FMT re-infection rates and patient response to follow-up antimicrobials. If individuals are re-infected at a point in time after recurrent disease has been resolved with FMT, it would be interesting to examine whether a history of recurrent disease and FMT aids or inhibits antimicrobial success. In addition to this, antimicrobial susceptibility data could provide information useful in selecting antimicrobial treatment for recurrent disease.

In conclusion, this thesis provides information on the association between NAP1/027 and recurrent disease, on possible risk factors associated with recurrent disease, on the incidence of FMT clinical cure rates in relation to CDI strain, on the association between NAP1/027 and response to FMT treatment, and on the presence of C. difficile in stool samples after clinical cure. Although sample limitations were encountered, the sample population was based on patients in a clinical setting, and
therefore the results of this research are valuable in a clinical context. More knowledge is needed to better understand the mechanisms of recurrent disease, however the results provided are consistent with clinical observations documenting the virulence of NAP1/027 and the success of FMT treatment. This research has contributed to a better understanding of recurrent CDI, and may be used to develop treatment approaches and minimize risk of recurrent disease. Future research should explore NAP1/027 and recurrent disease in larger, more standardized studies to further evaluate the role of NAP1/027 in recurrent disease, as well as other potential risk factors.


Baron, E., & Tenover, F. (2010). The emerging threat of *Clostridium difficile* infection: new insights into diagnosis and disease management. *CEPHEID*.


Scandinavian Journal of Infectious Diseases, 42(11), 857–861.


difficile isolates using capillary gel electrophoresis-based PCR ribotyping. Journal of Medical Microbiology, 57(11), 1377–1382. doi:10.1099/jmm.0.47714-0


via retention enema. European Journal of Clinical Microbiology and Infectious Diseases. doi:10.1007/s10096-014-2088-9

Lee, C., Petukhova, T., & Kim, P. (2013). Clostridium difficile infection and health-related quality of life at baseline. Unpublished manuscript.


doi:10.1056/NEJMoa051590


doi:10.1007/s10350-006-0511-8


*Clostridium difficile* infection in Canada: patient age and infecting strain type are highly predictive of severe outcome and mortality. *Clinical Infectious Diseases, 50*(2).
doi:10.1086/649213

doi:10.1136/gut.2007.128157

doi:10.1099/jmm.0.056614-0


doi:10.1186/1471-2334-9-103


*Clostridium difficile* is heterogeneous. *Journal of Clinical Microbiology, 46*(9), 3028–3032.


polymerase chain reaction for the diagnosis of C. difficile-associated diarrhea. *Clinical Infectious Diseases, 45*(9), 1152–1160. doi:10.1086/522185


toxin testing for detection of toxigenic Clostridium difficile. Journal of Clinical Microbiology, 45(11), 3601–3605. doi:10.1128/JCM.01305-07


Clostridium difficile–associated diarrhea: an emerging threat to pregnant women.

American Journal of Obstetrics and Gynecology, 198(6), 635–635.


Tannock, G., Munro, K., Taylor, C., Lawley, B., Young, W., Byrne, B., Emery, J., & Louie, T. (2010). A new macrocyclic antibiotic, fidaxomicin (OPT-80), causes less alteration to the bowel microbiota of *Clostridium difficile*-infected patients than does vancomycin. *Microbiology*, 156(11), 3354–3359. doi:10.1099/mic.0.042010-0


