Correlation between phenotypes or genotypes of *Clostridium difficile* and clinical outcome from *Clostridium difficile* infections at Health Sciences North

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science (MSc) in Biology

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THESIS DEFENCE COMMITTEE/COMITÉ DE SOUTENANCE DE THÈSE
Laurentian Université/Université Laurentienne
Faculty of Graduate Studies/Faculté des études supérieures

Title of Thesis Titre de la thèse	Correlation between phenotypes and genotypes of Clostridium difficile and clinical outcome from Clostridium difficile infections at Health Sciences North
Name of Candidate Nom du candidat	Lefebvre, Sebastien Roger
Degree Diplôme	Master of Science
Department/Program Département/Programme	Biology
Date of Defence Date de la soutenance	January 26, 2016

APPROVED/APPROUVÉ

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Abstract

Clostridium difficile, a Gram-positive, anaerobic, spore producing bacillus is the leading cause of nosocomial diarrhea in health-care facilities. Its effect on patients can be detrimental to their recovery, sometimes leading to death; the catalyst to *Clostridium difficile* infection (CDI) is treatment by antibiotics. Focusing research on some of *C. difficile*'s genotypes and phenotypes, we hoped to be able to predict the severity and outcome of the infections based on the strain. *C. difficile* strains isolated from patients were characterized for the presence of the toxin genes and classified by ribotype. They were also tested for antibiotic susceptibility (E-test), toxin production levels, and microbial spore load in stools. The patients symptoms were evaluated to determine if there were any correlations between the genotypes/phenotypes and the infection outcomes. No significant correlation was found between the severity of disease and phenotypic/genotypic attributes of the *C. difficile* strain involved. Thus, other defining factors appear to affect patient prognosis such as the severity of the patients' disease and the presence of co-morbidities. A key finding however was that 30% of the population was at risk of developing CDI due to carriage of toxigenic *C. difficile*.

Keywords

Antibiotic resistance, antibiotic susceptibility, *Clostridium difficile*, genotype phenotype, ribotype, spores, TcdA, TcdB, toxin

Acknowledgments

First and foremost I would like to thank Dr. Francisco Diaz-Mitoma for giving me the opportunity to join his team at the HSNRI. I am grateful for every opportunity I have had as part of the team, and certainly delighted that he agreed to have me as his student. I wish him well in all his future endeavours, and hopefully our paths will cross again.

Secondly, I would like to thank my supervisors, Dr. Amadeo Parissenti and Dr. Reza Nokhbeh. I am extremely grateful and appreciate all the advice they both have provided me in order to finish. I would also like to thank Justo Perez, Yanal Murad and Gustavo Ybazeta for all the guidance they have provided in the lab. Their technical skills, knowledge, and patience was tested and appreciated on more than one occasion.

Finally, I would like to thank all my family and friends for having put up with my craziness for the last 2 years. Thank you Jason for the patience and support you have given me. I don't think I could have made it through without it. Thanks to my Mom and Dad who always support all my ventures and keep reminding me how important education is. Thanks to my brother and sister who put up with me constantly, and all my friends both in and out of the lab for being there when I needed someone to vent.

J'aimerais dédier cette thèse à mon grand-père, Roger Lefebvre. Tu me manques énormément, et j'aurais aimé que tu sois ici pour voir tous le succès que j'ai accomplis.

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ADP	Adenosine diphosphate
B. subtilis	*Bacillus subtilis*
BBA	Brucella blood agar
BHI	Brain heart infusion
BHI-YT	Brain heart infusion, yeast extract, taurocholate
BSB	Brucella supplemented broth
C. difficile	*Clostridium difficile*
CAD	Canadian Dollar
Cat	Catalogue number
CB	Columbia broth
CDI	*Clostridium difficile* infection
CDSA	*C. difficile* selective agar
CDT	Binary toxin
cdt	Binary toxin gene
CDTa	Binary toxin A
CDTb	Binary toxin B
CdtLoc	Binary toxin locus
CLSI	Clinical and Laboratory Standards Institute
CROPs	Combined repetitive oligopeptides
ddH_2O	Double distilled water
EDTA	Ethylenediaminetetraacetic acid

EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
EUCAST	European Society of Clinical Microbiology and Infectious Diseases
GTPase	Hydrolase enzymes that bind and hydrolyze guanosine triphosphate (GTP)
HA-CDI	Healthcare acquired *C. difficile* infection
HSN	Health Sciences North
HSNRI	Health Sciences North Research Institute
LCT	Large clostridial toxins
M	Molar
MD	Maryland
MO	Missouri
MOHLTC	Ministry of Health and Long-Term Care
NAATs	Nucleic acid amplification tests
NaOH	Sodium hydroxyde
NAP1/BI/027	Hypervirulant strain North American pulse field gel electrophoresis 1, restriction endonucleases analysis BI, ribotype 027
No.	Number
ON	Ontario
PaLoc	Pathogenicity locus
PCC	Probe check control
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
PHAC	Public Health Agency of Canada

QC	Quebec
REA	Restriction endonucleases analysis
REB	Research Ethics Board
rRNA	ribosomal ribonucleic acid
SHEA-IDSA	Society for Healthcare Epidemiology of America/Infectious Diseases of America
SPC	Sample processing control
TBE	Tris/borate/EDTA
TcdA	Toxin A
tcdA	Toxin A gene
TcdB	Toxin B
tcdB	Toxin B gene
tcdC	Negative regulator of toxin A and toxin B
TcdE	Putative holoenzyme
tcdE	Putative holoenzyme gene
TcdR	RNA polymerase σ factor
tcdR	Positive regulator of toxin A and toxin B
Thr82Ile	threonine at position 82 changed to an isoleucine
USA	United States of America
VA	Virginia

1.0 Introduction

Clostridium difficile (*C. difficile*) is a Gram-positive, obligate anaerobe, spore forming bacillus that has developed resistance to certain commonly used antibiotics [1], [2]. Its effect on the elderly and patients receiving antibiotic treatment can be detrimental to their recovery, sometimes leading to death [1], [3]. It is widely believed that the catalyst to the infection is treatment by antibiotics; antibiotics damage the gut's microflora allowing for *C. difficile* colonization and release of its toxins [2]. This results in colonal epithelial cell death and an immune response that ultimately leads to colitis and diarrhea. *C. difficile* is the leading cause of nosocomial (health-care acquired) diarrhea and pseudomembranous colitis in the developed world [4]. Furthermore, the primary reservoirs for the organism are carriers who visit the health-care facilities either for treatment, visit, or work [5]. Since the early 2000s, an increase in incidence of *C. difficile* infection (CDI) within hospitals has surged, the cause of which remains unknown [6]. Upon colonization in the colon, *C. difficile* excretes two enterotoxins, TcdA and TcdB, which enter intestinal epithelial cells and disrupt the cytoskeleton, resulting in cell lysis [7]. The activation of cell death and inflammatory pathways then result in the production of intestinal ulcers and diarrhea.

1.1 *C. difficile* history

C. difficile was first described by Hall and O'Toole in 1935 when they conducted a study to characterize the intestinal microflora of newborn children [8]. *C. difficile* has since been found to be the leading cause of health-care acquired (nosocomial) infection in the developed world [9]. The infection has a wide range; however, the specific list of symptoms depends upon the

severity of the illness, and may include watery (sometimes bloody) stool, abdominal cramps, tenderness or distention, hypoalbuminemia, and the possible development of pseudomembranous colitis. The infection can sometimes lead to death [9].

Although the organism was first described in 1935, and pseudomembranous colitis first described by Finney in 1893, *C. difficile* infections did not get much recognition until the 1950s, when clindamycin became the preferred antibiotic of choice for use in treatment of anaerobic infections of the intestinal tract [10]. In 1973, Tedesco et al. conducted a study with 200 patients that were treated with clindamycin at St. Louis' Barnes Hospital, of which 42 patients (21%) developed diarrhea, and 20 patients (10%) were discovered to have pseudomembranous colitis when endoscopy was performed [11]. Eight stool specimens from the study were stored and used in tests 5 years later; 4 of the stool samples were positive for *C. difficile* by culture and also contained toxins in the stool when tested with a cytotoxin assay. This study is considered to be one of the first documented cases of a *C. difficile* nosocomial outbreak [10]. The study of *C. difficile* has focused mostly in three areas in the period between Tedesco's study in 1974 and the 1990s: rodent models and their development of antibiotic-associated typhlitis, the anatomical study of pseudomembranous colitis, and the study of the organism itself. In 1978, *C. difficile* was recognized as a causative agent for pseudomembranous colitis, when two separate research groups isolated the organism from patients with pseudomembranous colitis. [12], [13]

By the 1990s, it was understood that *C. difficile* infections arose in patients when treated by antibiotics, and various tests could be used to diagnose the infection. It was also known that treatment by metronidazole and vancomycin, the latter being the more effective yet more

expensive treatment, was effective in treating the infection [3]. However, a dramatic turn of events occurred in 2005, when over 2,000 fatalities were reported in Quebec in the largest *C. difficile* epidemic recorded. This was attributed to two factors. One factor was the appearance of a newly discovered hypervirulent *C. difficile* strain. The hypervirulent strain was identified by the restriction endonuclease analysis (REA) group BI, North American pulse-field electrophoresis type 1 (NAP1), and polymerase chain reaction (PCR) ribotype 027 (NAP1/BI/027). The second factor was the overuse of antibiotics as a means of treatment [14]. After these events, efforts in the study of *C. difficile* were renewed and focused on understanding the workings of the organism, including methods of infection, colonization, toxin delivery, and sporulation (to name a few).

1.2 Classification

To better understand the changing epidemiology of *C. difficile*, researchers have used various types of molecular classification or "typing". This helps monitor outbreaks, observe regional and global changes of the organism [15]. In the 1980s, phenotyping was much more common and the preferred technique was serotyping, and 15 serogroups were characterized. However, as technology became less expensive and more accessible, genotyping became the detection method of choice. Several typing methods emerged, namely pulse-field gel electrophoresis (PFGE), restriction endonuclease analysis (REA), and PCR ribotyping. Each technique offers its advantages, and each technique has its own nomenclature and classification scheme. PFGE for the purpose of *C. difficile* classification is named North American pulse-field, or NAP. This nomenclature is used in what is probably the most widely recognized *C. difficile* strain, the hypervirulent endemic strain NAP1/BI/027. NAP1 represents that PFGE classification, BI

represents the REA classification, and 027 is the ribotype classification. While any of these techniques can be used for in lab classification, PCR ribotyping has become a gold standard due to its high reproducibility, high sensitivity, high specificity, rapidity and ease of use [16].

PCR ribotying is a fast, reliable, relatively inexpensive and highly reproducible technique that is commonly used in labs throughout the world. The 11 intergenic spacer regions (ISR) between the 16S and the 23S rRNA operons are highly polymorphic, and when amplified by PCR will produce bands of different sizes. Primers which contain a partial, exact sequence for the 16S and 23S rRNA genes were designed by Bidet et al., at the end of the 1990s and are still in use today for ribotyping [16]. A forward primer anneals to bases 1482-1501 of the 16S rRNA gene, while the reverse primer anneals at bases 1-24 of the 23S rRNA to amplify the ISR found between these two genes. Resolving the bands by electrophoresis produce a "fingerprint" and it is this fingerprint that is used to group the *C. difficile* strains together. The bands range from 225bp to 700bp, and there are as many bands as there are rRNA operons in the strains' chromosomal DNA, usually between 7 and 10 bands [16]. As stated, this technique is highly reproducible, meaning that every time the test is repeated, the same fingerprint is generated making this technique a preferred one in the classification of *C. difficile* [16]. More than 300 ribotypes have been identified so far [17]. Sequence-based methods such as whole genome single nucleotide polymorphism typing are slowly starting to replace genotyping methods as they are able to discriminate between very closely related strains [15]. These methods are still quite expensive, but their pricing has dropped significantly over the last few years and will surely continue to do so; it appears as though they will become the new standard of testing in the future.

1.3 Colonization

C. difficile is ubiquitous, found as a spore on many different surfaces, including foods, and in the intestinal tract of humans and animals [18]. While the spores are found on the surfaces, the most common mode of transmission is the fecal-oral route: people ingest the spores by touching contaminated surfaces and foods [9]. The infection that occurs due to this transmission can be divided into one of two categories: endogenous infections and exogenous infections. Endogenous infections originate from the carrier themselves, having the microorganism in their microflora, while exogenous infections occur via transmission from another source, such as a health-care worker or a contaminated individuals [19]. As mentioned earlier, the primary reservoir of the organism is the carriers who come to the health-care facilities for treatment. It is there that the spores gets transferred to the hands of the health-care works, who transport it from patient to patient [5]. However, not everyone who is infected with the spores will develop an infection; various factors will influence the likelihood an individual may have to develop an infection, these include older age (>65 years old), antibiotic usage, reduced immune response and other comorbidities [20].

The spores are able to endure the harsh acid environment of the stomach, and make their way towards the anaerobic colon where they will be able to germinate [21]. Several studies have shown that bile salts such as sodium taurocholate help in the germination of *C. difficile* spores while in the bowel [22]. Furthermore, it was found that glycine can act as a co-factor in germination [22]. Spores germinate best at temperatures of 37^{o}C, and a pH range between 6.5 to 7.5 [23]. The spores germinate in the small intestine after exposure to bile salts and make their way with the aid of flagella and peristaltic intestinal movement to the colon where they will

colonize. *C. difficile* multiplies in the colon; the gut mucosa facilitates the adherence to the epithelial cells [5]. Once *C. difficile* has colonized in the colon, it is then that it will start delivering the toxins that will cause the disease.

1.4 Toxins

Toxigenic *C. difficile* contains three toxins: toxin A (*tcdA*) and toxin B (*tcdB*), which are both found on the same locus, and a binary toxin (*cdt*). TcdA and TcdB have long been known to be the agents that cause pseudomembranous colitis, however in recent years research on CDT has revealed that it may also play a role in the infection [24].

1.4.1 TcdA and TcdB

TcdA and TcdB are part of the large clostridial toxin (LCT) family, and share some homology to other toxins found in other *Clostridia* species, such as the *Clostridium sordellii* lethal toxin and the *Clostridium novyi* α-toxin [25]. TcdA and TcdB are both glucosyltransferases, and are some of the largest bacterial toxins with molecular weights of 308 kDa and 270 kDa, respectively [1], [2], [7], [26]. TcdA and TcdB are produced by *C. difficile* during the late exponential and stationary phases of bacterial growth [7]. The genes for both toxins are found on the *C. difficile* pathogenicity locus (PaLoc), a 19.6 kb region that is present, stable and conserved in toxigenic strains, while absent in the non-toxigenic strains [1], [7], [27].

1.4.2 Pathogenicity Locus

The PaLoc contains several different elements that are essential to the production and delivery of both toxins into the target cells; the locus is also very well described and understood [7]. The genes for Toxin A (*tcdA*) and Toxin B (*tcdB)* have large open reading frames (8133 nucleotides and 7098 nucleotides, respectively) [7]. The G+C content of both toxins is relatively low (28%), which is fairly similar to the rest of the *C. difficile* genome (29%). Furthermore, the two genes are highly homologous (66%), and taking into account the functional homology of the encoded proteins, it has been suggested that the presence of the two toxins is the result of an evolutionary gene duplication event [28].

There are also 3 other open reading frames that are found on PaLoc: *tcdC*, *tcdR* and *tcdE*. *tcdR*, the positive regulator of the PaLoc found upstream of *tcdB*, is upregulated in coordination with *tcdA* and *tcdB* [7]. TcdR is used to activate gene transcription via an alternate RNA polymerase σ factor [29]. *tcdC* is found downstream in opposite direction to *tcdA*, and is highly expressed during the exponential growth phase [7]. Initially, it was observed that the expression of *tcdC* was upregulated while the expression of *tcdA* and *tcdB* were downregulated and vice-versa. It was found that *tcdC* is the negative regulator for the production of toxins on the PaLoc. *tcdC* codes for an anti-sigma factor that destabilizes the TcdR holoenzyme, which prevents the transcription of *tcdA* and *tcdB* [1]. A frameshift mutation is found at position 117 in NAP1/BI/027 hypervirulent strains and was thought to increase the virulence of the strain as it would be ineffective at downregulating toxin production during the growth phase of the organism [30]. There is, however, a conflicting study reporting that the overproduction of toxin occurs despite the mutation in the *tcdC* gene [31]. Finally, *tcdE* is found between *tcdA* and *tcdB*

genes and encodes for a holing-like protein that forms pores in the cell membrane of *C. difficile* to allow for the toxins to exit the microorganism [7]. The sequence of the *tcdE* gene and the structural conformation of TcdE protein suggest that TcdE is related to class I holins, also found in λ phages. These proteins are able to form pores in the cell membrane that would allow the large clostridial proteins through without causing cell death due to depolarization of the cell [32]. It is thought that TcdE has three transmembrane domains, including charged residues in the C terminus as well as a hydrophilic area in the N terminus that may help in avoiding depolarization of the cell while allowing for the toxins to exit the bacterial cell [33]. Figure 1a depicts the pathogenicity locus.

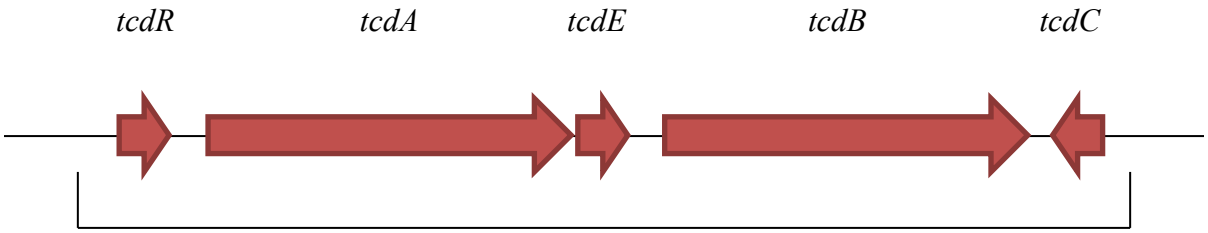
A common practice when working with *C. difficile* is to amplify the *tcdA* and *tcdB* genes by PCR to confirm whether the strain contains both genes. This confirms whether the strain is *tcdA*+ and *tcdB*+, *tcdA*- and *tcdB*- (non-toxigenic), *tcdA*- and *tcdB*+ or *tcdA*+ and *tcdB*-. Furthermore, the primers for *tcdA* are also able to anneal to the smallest of the 3 deletions in the 3' region of the gene, should it be present.

1.4.3 Protein structure

Like other members of the LCT family, the TcdA and TcdB proteins have 4 regions that are conserved among them: an N-terminus that has a biologically active glucosyltransferase domain, followed by a cysteine protease domain, a central region that is hydrophobic and thought to be implicated in the translocation of the protein into the cytosol, and finally a C-terminal domain containing combined repetitive oligopeptides (CROPs) involved in the highly specific cell-surface receptor binding for entry into the epithelial cells [7], [25], [26]. The active toxin

function is located within the N-terminal domain; the first 500 amino acids (approximately) are cleaved off from the rest of the toxin protein by auto-proteolysis via the cysteine protease domain in the endosome and the truncated protein is translocated into the cytosol with the help of the hydrophobic domain of the toxin (Figure 1b), [26].

a.



Pathogenicity locus (19.6 kb)

b.

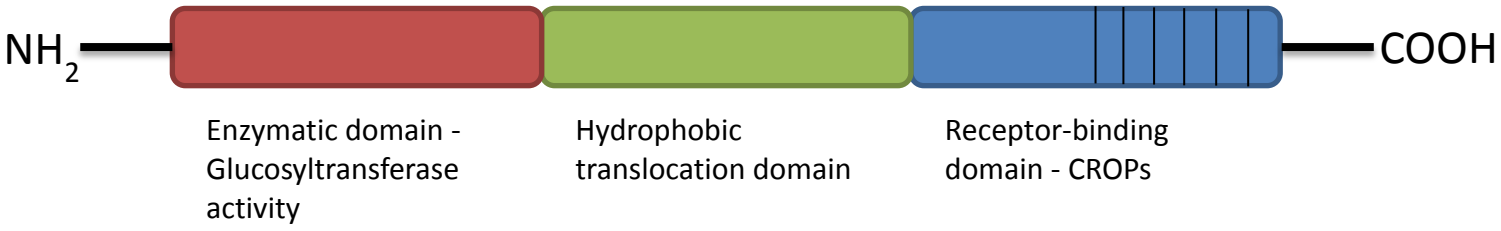


Figure 1 - *C. difficile* pathogenicity locus and TcdB protein structure

(**a.**) The pathogenicity locus is a 19.6 kb region that codes for the toxins TcdA, TcdB and three other open reading frames. *tcdR* is the positive regulator for the toxins, while *tcdC* is the negative regulator. *tcdE* is a putative holin protein that helps perforate *C. difficile*'s cell membrane to allow the toxin to exit. Adapted from Voth et al. (2005) [7] (**b.**) A simplified depiction of the three domain structure of the LCT as described by Voth et al. (2005). Represented are the enzymatic domain, hydrophobic translation domain and the receptor binding domain. Not shown is the cysteine protease domain, found between the enzymatic and hydrophobic translocation domain [7].

1.4.4 Mechanism of action

TcdA and TcdB work by glycosylating the Ras superfamily of GTPases. The Ras GTPases are small monomeric guanine nucleotide-binding proteins that act as molecular switches for various cellular signaling events. The process of glucosylating proteins is irreversible, and inactivates the regulatory proteins, leading to disruption of vital signaling pathways that occur in the epithelial cells of the intestinal tract; this causes the breakdown of the cellular cytoskeleton [7], [34]. Before this process can happen, the toxins must first enter the epithelial cells; this occurs via receptor-mediated endocytosis [1]. The toxins also require the acidic endosome to activate a structural change and translocation into the cytosol of the epithelial cells [7]. The low pH (5.2-6.0) of the endocytic vesicle is essential for the structural change that occurs to the toxin: the N-terminal enzymatic domain of the toxin is cleaved from the rest of the toxin and the exposed hydrophobic portion of the protein forms a pore in the endocytic vesicle through which the activated portion of the toxin translocates into the cytosol [7], [35]. Once in the cytosol, both TcdA and TcdB are able to inactivate Rho, Rac and Cdc42, all part of the GTPase family in the intestinal epithelial cells. The functions of Rho include stress fiber formation, motility, and focal adhesion, while the functions of Rac and Cdc42 are related to the formation of lamellipodium and filopodium [7]. The toxins glycosylate the Rho protein by transferring a sugar moiety to Thr-37 using UDP-glucose as a co-substrate.

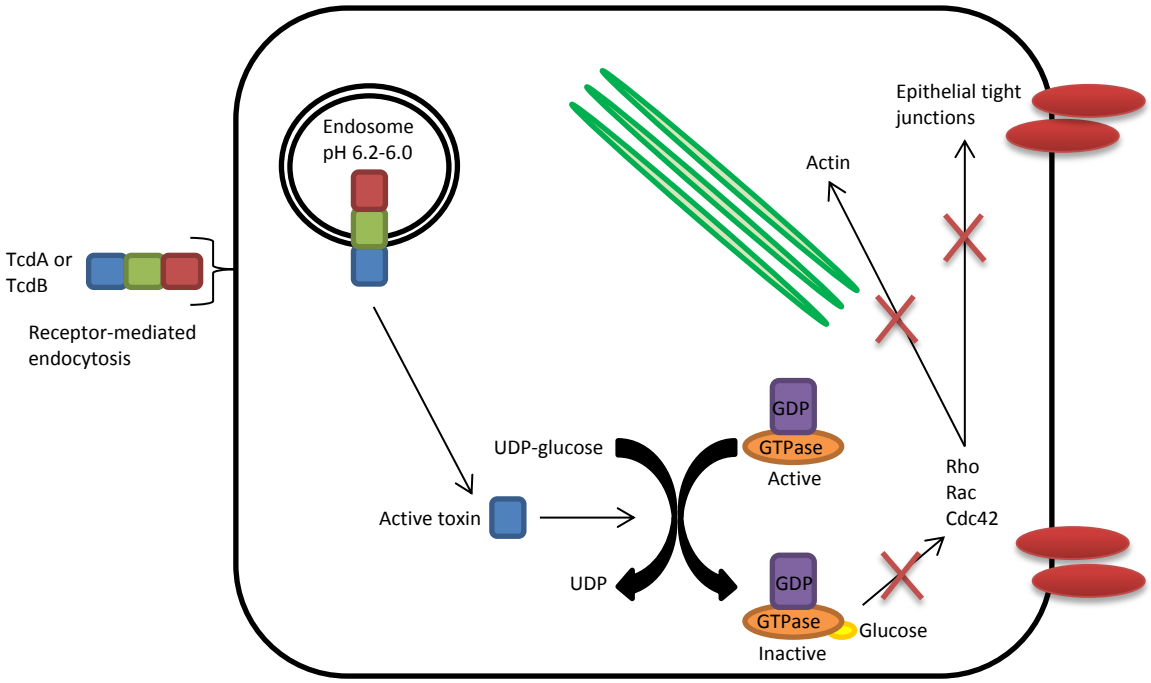


Figure 2 - TcdA and TcdB mechanism of action in colon epithelial cells

Figure 2 shows the mechanism of action of both TcdA and TcdB in colon epithelial cells. First, the toxins must enter the cells through receptor-mediated endocytosis. In the low pH endocytic vesicle, the enzymatic domain of the toxin is cleaved from the protein structure, and the hydrophobic translocation domain of the toxin allows the enzymatic domain to go through the membrane of the vesicle into the cytoplasm of the cell. There, the active toxin glucosylates GTPase, which causes an inactivation of Rho, Rac and Cdc42. Adapted from Voth et al. (2005) [7].

1.4.5 Impact on cell morphology

Cells that have been treated with both TcdA and TcdB have shown a distinct change in the organization of their actin cytoskeleton. This is now seen as the hallmark of cellular intoxication by TcdA and TcdB. Rho (isoforms A, B and C) is a ubiquitous primary regulator of the actin skeleton in eukaryotic cells. Inactivation of the Ras GTPases by the toxins causes a loss of structural integrity; this is brought upon by the decline of F-actin levels, which are regulated by the Ras GTPases. Upon the loss of structural integrity, actin condensation occurs that leads to rounding of the cells, membrane blebbing, and eventually apoptosis [7]. This can occur quickly; it was shown by Qa'Dan et al. that TcdB could elicit cell rounding within 2 hours of treatment with TcdB, and that cell death occurs about 24 hours after treatment [36]. Furthermore, the permeability of the epithelial cells of the colon is increased due to TcdA inactivation of RhoA through protein kinase C; this may lead to the increase in inflammation observed in pseudomembranous colitis. The increase in polymorphonuclear cells found in the colonic epithelial layer may activate the expression of various chemokines, which also may serve as a mechanism that increases the inflammatory response observed in the colon [7]. Physiological events that occur in the affected area include inflammation due to increased epithelial permeability, neutrophilic infiltration, production of chemokines and cytokines, mast cell activation, and damage to the intestinal mucosa, all of which have a direct contribution to the development of pseudomembranous colitis [7].

1.4.6 Binary toxin (CDT)

The binary toxin belongs to a family of binary ADP-ribosylating toxins, which include the *Clostridium botulinum* C2 toxin, the *Clostridium perfringens* iota toxin, and the *Clostridium*

spiroforme toxin. CDT is composed of two separate toxin components, hence the name binary: CDTa and CDTb. While the roles of TcdA and TcdB are well defined in CDI, CDT was overlooked until the early 2000s when the hypervirulent strain NAP1/BI/027 came into prevalence and it was observed that the CDT was present in that strain [2]. Since then, more focus has been given into the study of CDT to understand its role in CDI. The genes coding for CDT are found within the CDT locus (CdtLoc), a 6.2kbp region that encodes for *cdtA*, *cdtB*, and a regulatory protein *cdtR*. CDTLoc isn't found in all *C. difficile* strains; however, when it is, it is either found whole or as a single truncated version. In *C. difficile* strains that do not contain the CdtLoc, a unique 68bp sequence is found in its place [2], [56]. CDTa is a 463 amino acid protein that contains 2 domains: an N-terminus with cell wall receptor binding properties, and a C-terminus that is the enzymatic portion of the toxin with its ADP-ribosyltransferase activity. The second subunit of the toxin, CDTb, is 876 amino acids long, with 4 domains: domain I in the N-terminus is an activation domain, domain II is responsible for membrane insertion and formation of pores, domain III is involved in oligomerization, and the C-terminal domain IV is involved in receptor binding [56]. CDTb is cleaved and activated to form heptamers while binding to the lipolysis stimulated lipoprotein receptor (LSR). The CDTa is recruited to the (CDTb)7-receptor complex. The assembled CDTb and CDTa enter cells via endocytic vesicles, and later, upon acidification of the endosomal vesicles, a pore is formed on the endosomal membrane by the CDTb component, allowing for only CDTa to be translocated into the cytosol. Once in the cytosol, CDTa ADP-ribosylates G-actin resulting in depolymerzation of F-actin [56]. This process of increased depolymerization of the actin skeleton also results in long microtubule-induced membrane protrusions on the surface of the epithelial cells that increases the interaction surface of the cell for adherence of the colonizing *C. difficile* [56]. Knowledge of CDT is

overshadowed by the much more vast knowledge of TcdA and TcdB; however, recent studies suggest that CDT may be just as an important player as TcdA and TcdB in CDI (references).

1.5 Sporulation

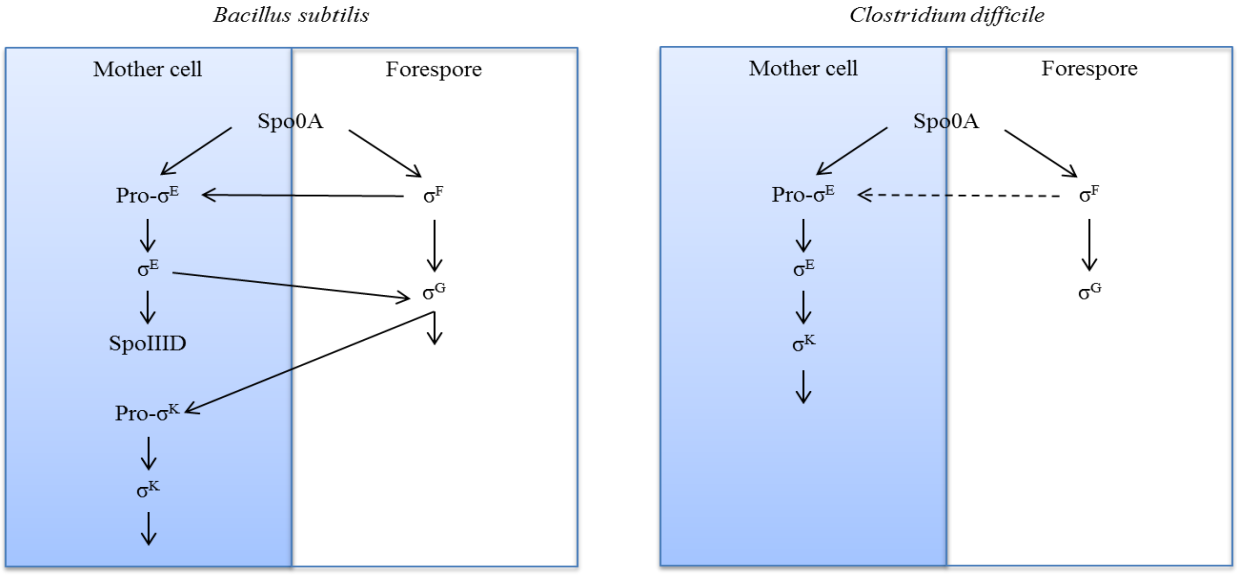
In order for the infection to persist in the colon, and to disseminate to other hosts, *C. difficile* produces spores during the infection of the colon [37]. As mentioned before, the spores are passed along the feces-oral route, and a large amount of spores in the feces is crucial for this transmission to occur [18], [38]. Increased ability to form spores is thought to contribute to the spread and survival of the organism [39]. The sporulation mechanism of *C. difficile* has yet to be fully elucidated; sporulation mechanisms of other spore-forming organisms such as *Bacillus subtilis* and other *Clostridial* species are used as a model to facilitate the study [18], [37], [38], [40], [41]. Spo0A is the transcriptional regulator protein that regulates whether a vegetative cell will initiate sporulation. Factors that may activate the process of sporulation include lack of nutrients, exposure to oxygen, and some recent studies suggest that quorum sensing, a mechanism employed by bacteria using various stimuli (light, chemicals, nutrients) in response to population density to coordinate gene expression and behaviours, may even play a role in sporulation [42]. Several histidine kinases phosphorylate Spo0A that initiate the sporulation cycle [37]. Activation of Spo0A will in turn activate transcription of genes that code for RNA polymerase sigma factors that are specific to spore formation [37], [42].

Prior to sporulation, the DNA is replicated so that there is a copy in the mother cell (the vegetative cell), and another copy in what is going to be the spore, known as the forespore. In *B. subtilis*, Spo0A activates σ^F in the forespore, which then activates pro-σ^E in the mother cell along

with Spo0A. This will then activate σ^E in the mother cell, which together with σ^F in the forespore will activate σ^G in the forespore, followed by pro-σ^K activation to σ^K, both in the mother cell. However, in *C. difficile*, it was noted that the activation of sigma factors in the mother cell and the forespore do not depend on each other, with the exception of pro-σ^E activation in the mother cell by σ^F from the forespore and Spo0A [37]. After the activation of pro-σ^E in the mother cell, this will result in the activation of σ^E followed by σ^K. In the forespore, σ^G is activated by σ^F [37], [42].

The spore is made of several different layers. In the center of the spore lies the spore core that contains the microorganism's genetic material. Surrounding the spore coat is the inner membrane, whose phospholipid composition is very similar to vegetative bacteria and is not very permeable. The germ cell wall surrounding the inner membrane will become the *C. difficile* cell wall upon germination. The next layer is a thick cortex made of proteoglycans followed by an outer membrane that plays a role in spore formation. The spore coat is essential for protection against decontaminating agents and the environment. Finally, the exosporium layer that surrounds the spore is critical in pathogenesis as it helps in transportation of the spore from host to host [37].

a.



b.

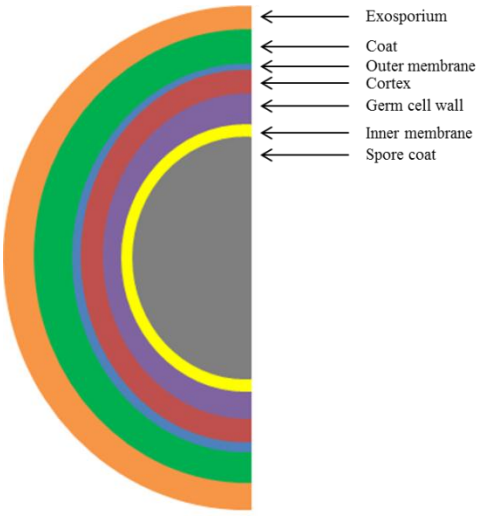


Figure 3 - *C. difficile* sporulation pathway and spore model

(a.) Suggested sporulation pathway as described by Paredes-Sabja et al. (2014), which utilises the well-known *Bacillus subtilis* sporulation pathway as a model. (b.) Layers of a *C. difficile* spore. [37]

1.6 Antibiotic resistance

Clindamycin-associated diarrhea was observed in 1974 and the link between pseudomembrane colitis and *C. difficile* was made in 1978, the prevalence of *C. difficile* infection has been on the rise since the early 2000s [3], [11], [13]. Furthermore, the majority of the observed cases of CDI are in health-care settings such as hospitals and long-term care facilities [6], [43]. One of the reasons for the rise in incidence of CDI is the increased use of antibiotics. The fluoroquinolone-resistant hypervirulent strain NAP1/BI/027 came into prominence in the beginning of the millennium in Quebec, Canada when the use of fluoroquinolones was high in the medical community [44].

Fluoroquinolones are broad-spectrum antibiotics that work by inhibiting bacterial type II topoisomerases that are involved in DNA replication. Because DNA is a large, coiled double helix structure, torsional stress called supercoiling occurs frequently. Also, supercoiling is used to compact DNA. Type II topoisomerases function by cutting both DNA strands and relieving the coiling stress, allowing for replication processes to continue. DNA gyrase subunit A is a type II topoisomerase (GyrA) and is the target of fluoroquinolones. Under normal circumstances, GyrA would bind to the DNA prior to cutting it to form an enzyme-DNA complex. When fluoroquinolones are present, however, they bind to the GyrA enzyme which will then bind to the DNA to form a fluoroquinolone-enzyme-DNA complex. The enzyme can then cut the DNA but the drug interaction disables the enzyme's ability to re-ligate the DNA, which will lead to eventual cell death [45], [46].

Whole-genome sequencing of a collection of *C. difficile* NAP1/BI/027 strains has revealed that resistance to fluoroquinolones was acquired by a single nucleotide mutation in the GyrA (*gyrA*) gene. This causes the threonine at position 82 to be changed to an isoleucine (Thr82Ile). This point mutation does not cause any functional loss in the protein but greatly reduces the binding efficiency of the drug [45], [46]. It was also found that this acquired resistance happened in two separate occasions, and gave rise to two separate lineages that have been named FQR1 and FQR2. These mutational events have happened in the very recent past, FQR1 lineage was thought to have been established in 1993 while the FQR2 lineage was established in 1994. It is thought that it was these mutations that gave rise to acquired resistance to fluoroquinolones which facilitated the outbreak of NAP1/BI/027 strains in the early 2000s [45]. Whole genome sequencing of *C. difficile* strain 630 (epidemic type X), revealed the resistance genes to tetracycline and erythromycin, daunorubicin, bacitracin, nogalmycin, and beta-lactams [47]. It has become evident that *C. difficile* can acquire resistance to antibiotics quite easily, which makes it very problematic to deal with. As mentioned earlier, metronidazole is the first-line of treatment for CDI, and certain strains such as ribotype 001 have started showing lowered susceptibility when tested in 2005 compared to strains of the same ribotype 10 years earlier [48]. This is making the infections harder to treat and is leading to a rise in infection recurrence.

1.7 Epidemiology of *C. difficile*

C. difficile is the cause of the most common nosocomial infection in the developed world, and the incidence of infection has steadily been on the rise [9], [20]. The University of Pittsburgh Medical Centre reported that the number of *C. difficile* infections between 2000 and 2001 had doubled from what had previously been reported between 1990 and 1999 [2]. It was during that

same time that the NAP1/BI/027 strain was first identified as the strain causing outbreaks in the province of Québec in Canada [43]. A study in that province showed an increase in infection rates from 35.63 cases per 100,000 population in 1991 to 156.3 cases per 100,000 population in 2003 [18].

1.7.1 Symptomatic vs. asymptomatic

Patients that are colonized with *C. difficile* can be divided into two groups: symptomatic and asymptomatic. In theory, a symptomatic patient is a patient who is colonized with *C. difficile* and is exhibiting symptoms of the illness, while an asymptomatic patient is a patient that is colonized with *C. difficile* but has no symptoms of the illness. However, this definition is not so obvious in a real life situation, considering that the illness is brought about from the treatment of another illness, in patients who often suffer from a plethora of other co-morbidities. The primary symptom of CDI is the onset of persistent diarrhea, which is defined as having 3 or more unformed stools (stools that take the form of the container) within 24 or less consecutive hours [21], [49]. Recently, physicians are aware of CDI occurring after beginning an antibiotic treatment, and will generally test stool for *C. difficile* upon the onset of diarrhea to confirm the presence of the organism. A symptomatic patient is then defined as a person who has developed diarrhea, and there is confirmation of *C. difficile* by a positive stool test for *C. difficile* toxins, the detection of toxigenic *C. difficile*, or finding evidence of pseudomembranous colitis by endoscopy [21]. It is however harder to define concretely what consists of an asymptomatic patient. There have been many attempts to define the asymptomatic patient. In my thesis, we define a patient as asymptomatic if *C. difficile* is detected via either the Cepheid GeneXpert at HSN's clinical laboratory or by culture, but the patient is not exhibiting any signs of diarrhea.

One must also take into account the patient's normal bowel movements; if a patient normally has looser stools several times a day, it may not be considered to be diarrhea by the physician. This is often seen in cases where patients have loose stool due to other co-morbidities, or current medication for the treatment of these co-morbidities.

1.7.2 Diagnosis

When assessing a patient, at least one of the following criteria must be met to determine whether a patient has a CDI [6]:

- The patient is diagnosed by a physician as having diarrhea (defined as 3 or more loose/unformed stools in 24 consecutive hours or less [49]) , fever, abdominal pain, ileus, or there is confirmation of positive *C. difficile* by enzyme immunoassay (EIA) or by polymerase chain reaction (PCR) tests
- Pseudomembranes are observed by colonoscopy, sigmoidoscopy, histology or pathology
- Toxic megacolon is diagnosed

Furthermore, the CDI is considered to be a health-care associated CDI if:

- The symptoms exhibited by the patients appear 72 hours after admission to the health-care facility
- If symptoms appear within four weeks of discharge from the health-care facility.

These criteria do not apply to patients who are admitted as outpatients or at the emergency department.

1.7.3 Death linked to CDI

Death is sometimes a consequence of the infection. However, not all deaths are attributed to a CDI; a physician must determine whether the death was brought upon by the CDI or if the infection played little to no role in the death of the patient. The Public Health Agency of Canada (PHAC) defined three levels of outcome [6]:

- The death was directly linked to the CDI and the patient had no other condition that could have caused death
- The death was indirectly linked to the CDI; the patient's death was not primarily due to the infection, though it did play a role in the death
- The death was unrelated to CDI.

1.7.4 Rates of CDI

Rates of colonization, infection, morbidity and mortality all vary greatly according to regions; in fact, incidences of infection vary greatly, including Canada. In a report looking at incidences and costs of CDI in Canada, Levy et al. reported that in 2012 there were 9,962 *C. difficile* infections in Ontario, while its neighbouring province of Québec had 16,562 confirmed cases [43]. For the purpose of this study, we will focus on CDI cases at Health Sciences North, in Sudbury, Ontario Canada.

Since 2009, *C. difficile* infections are now "nationally notifiable diseases", and must be reported to the provinces Ministry of Health (in Ontario, this is the Ministry of Health and Long Term Care [MOHLTC]). CDIs are generally reported per 10,000 patient days:

$$\frac{number\ of\ CDI\ cases}{total\ number\ of\ patient\ days}\ per\ year\ \times 10{,}000$$

where patient days is defined as the total number of days all patients have stayed at a health-care facility for the surveillance year [6]. Furthermore, the attributable mortality rate (deaths directly or indirectly caused by CDI) is defined as:

$$\frac{number\ of\ deaths\ directly\ or\ indirectly\ caused\ by\ CDI}{total\ number\ of\ CDI\ cases}\ per\ year\ \times 100$$

The Public Health Agency of Canada (PHAC) released a surveillance report that spanned from January 1st, 2007 to December 31st, 2012. The data was collected from hospitals throughout Canada with the exception of the territories, which were not required to submit rates. The report showed that despite a small decrease in incidences in 2009, the number of health-care associated CDI (HA-CDI) cases have stabilized, despite increased efforts by health care facilities to avoid spread to decrease these numbers. Between 2007 and 2012, the average rate per 10,000 patient days was 6.48, with a low in 2009 with 5.81 per 10,000 patient days and a high in 2008 at 7.47 per 10,000 patient days. The average patient age was 70 years old. From the 18,871 reported cases between 2007 and 2012, 454 people lost their lives, and 152 of those deaths were directly or indirectly linked to the CDI [6]. (Table 1)

About 30% to 45% of all CDI cases are thought to be recurrent [43], [50], [51]. Recurrent *C. difficile* infections are cases in which the symptoms re-appear 28 ± 2 days after the end of the initial treatment for CDI. Recurrent cases can be divided into two categories: relapse in which the same strain is seen to infect the patient account for about 82% of recurrent cases, and reinfection from another strain accounts for 18% of recurrent cases [50]. 10,885 recurrent CDI cases were recorded in Canada in 2012 [43].

Table 1 - Reported *C. difficile* cases in Canada between 2007 and 2012

	2007	2008	2009	2010	2011	2012
No. of HA-CDI cases	3,271	3,192	2,483	3,026	3,417	3,482
No. of total patient days	4,832,597	4,274,020	4,270,868	4,945,856	5,141,450	5,766,774
Rate per 10,000 patient days	6.77	7.47	5.81	6.12	6.65	6.04
Average age	70.5	70.7	69.6	70.4	69.4	69.6
No. of deaths (direct or indirect)	30	23	13	26	36	24

The data was adapted from the surveillance report released by the Public Health Agency of Canada in 2012 entitled "Healthcare-Associated *Clostridium difficile* Infections in Canadian Acute-Care Hospitals". The number of hospital-associated CDI cases, number of patient days, rate per 10,000 patient days, average age, number of deaths (direct or indirect) and attributable mortality rate were drawn from the report and tabulated for clarity [6].

MOHLTC has been keeping their records tabulated and accessible to the public since 2008 (http://www.hqontario.ca/System-Performance/Hospital-Care-Sector-Performance). Between 2009 and 2015, the Ontario average rate of HA-CDI was 3.05 per 10,000 patient days. The Ontario average rate of HA-CDI per 10,000 patient days between 2009 and 2012 has always been lower than the national average rate by about half (3.05, 3.06, 3.41 and 3.39 in Ontario vs. 5.92, 6.21, 6.72 and 6.12 nationally for 2009, 2010, 2011 and 2012 respectively). Compared to the average from all the other hospitals in Ontario, Health Sciences North has been above the provincial average rates except in 2009 and in 2011. It must be noted that since the data includes all Ontario hospitals, there is a bias as the small community hospitals tend to have rates of 0.0 per 10,000 patient days. This is due to the fact that any patient with complications is sent to the bigger acute hospitals for treatment. Hence, it is more accurate to compare the CDI rates of HSN to other acute teaching hospitals as they are classified. In the database provided by the MOHLTC, there are 27 of these hospitals in Ontario. While HSN is closer to those CDI rates, it still is above the acute teaching hospital HA-CDI rates other than 2009 and 2011. When looking at the CDI rates for the years that coincide with the years of samples collected for this project (2013-2015), the annual HA-CDI average rate at HSN was 4.92, 4.18 and 3.57 per 10,000 patient days while the acute teaching hospital averages were 3.00, 2.75 and 2.68 per 10,000 patient days for 2013, 2014 and 2015 respectively [52] (Table 2).

Table 2 - CDI rates per 10,000 patient days at Health Sciences North, compared to Ontario, Ontario acute teaching hospitals and Canada wide rates

	2009	2010	2011	2012	2013	2014	2015
Health Sciences North	1.43	4.73	1.90	4.38	4.92	4.18	3.57
Ontario	3.05	3.06	3.41	3.39	3.00	2.75	2.68
Ontario acute teaching hospitals	3.65	4.04	4.40	4.17	3.97	3.59	3.48
Canada	5.92	6.21	6.72	6.12			

The rates of *C. difficile* infections per 10,000 patient days at Health Sciences North, when compared to the province of Ontario and Canada average CDI rates. Health Sciences North is considered an acute teaching hospital, according to the MOHLTC, and as such the rates for other acute teaching hospitals in the province of Ontario were included to provide a more realistic comparison. This is mostly due to smaller community hospitals having CDI rates of 0.0 per 10,000 days since any complicated patient case is sent to the nearest acute hospital for treatment. Adapted from the MOHLTC website http://www.hqontario.ca/System-Performance/Hospital-Care-Sector-Performance (2016) [52].

1.7.5 Cost of CDI

While most studies focus on the cost of *C. difficile* infections on human lives, we must not forget that the infection also has a high socioeconomic cost. The Center for Disease Control (CDC) in the United States of America (USA) estimates the cost of CDI to be above US $3.2 billion per year [9], [43]. Here in Canada, the total cost of CDI is much lower than the reported cost of CDI in the USA, which is attributed to the fact that we represent only about 10% of the USA population. It is also worthwhile to remember that in the USA, the cost of hospitalization falls on the patient, but in Canada, the health-care bill is paid for by the publicly funded health-care system. Levy et al. set out to find an estimate of the cost of CDI to the Canadian health-care system. According to the data extrapolated by Levy et al., there were an estimated 37,932 identified episodes of *C. difficile* infections in 2012. This number did not only include the HA-CDI, but all reported infections which include community-acquired infections, outpatients, emergency department visits, long-term care facilities, and recurrence cases. A total of 20,002 of the 37,932 CDI cases were treated in hospital, 16,326 were treated in the community, and 1,604 were treated in long-term care facilities. Furthermore, out of the 37,900 cases of CDI, 10,900 cases were recurrent, and of the recurrent cases 7,980 (21%) were relapsed cases (Table 3).

There were many factors to take into consideration when evaluating the cost of CDI. First, all CDI cases had to be divided by severity to determine the type of care that was provided to the patient. The classification used was that of the Society for Healthcare Epidemiology of America-Infectious Diseases Society of America (SHEA-IDSA): mild-to-moderate, severe, and fulminant. Secondly, all attributable costs were taken into account, and this includes: laboratory tests,

hospitalizations (which include hospitalization that were not related to CDI, but extended due to CDI), medication, surgical procedures, and cost of physicians.

After all these considerations, Levy et al., estimated that the cost to the Canadian health-care system was CAD $281 million. Approximately 90% (CAD $260 million) of the cost was due to the cost of hospitalization, CAD $12 million was the price paid to patients of the community, and another CAD $10 million was accrued in lost productivity from patients who could not work due to the infection. The Canadian health-care budget for 2012 was CAD $207 billion, meaning that the cost of treating CDI represents about 0.1% of the health-care budget. It should also be noted that as stated before, the Canadian population represents about 10% of the American population, the CAD $281 million spent on CDI in 2012 represents about 9% of the American reported US $3.2 billion [43]. Cost per capita for treatment of CDI in Canada is CAD 8.08$ (US 6.06$ at the time of redaction) per person while in the United States of America the cost per capita is US 10.23$ per person.

Table 3 - Incidence, type, and severity of CDI in hospitals, communities, and long-term care facilities

Location	Characteristic of infection	Incidence	Percentage
Hospital		20,002	53
	Type of infection		
	New infection	14,593	73
	First relapse	3,134	16
	Subsequent relapse	1,020	5
	Reinfection	1254	6
	Disease severity		
	Mild-to-moderate	12,155	61
	Severe	7,435	37
	Fulminant	412	2
Community		16,326	43
	Disease severity		
	Mild-to-moderate	13,061	80
	Severe	3,265	20
	Fulminant	0	0
Long-term care facilities		1,604.00	4.00

Adapted from Levy et al. (2015) [43]

1.7.6 Risk factors

There are several factors that increase the risk of developing *C. difficile* infection. First and foremost is exposure to antibiotics, especially fluoroquinolones [1]. As stated before, clindamycin was found to be a catalyst to developing CDI back in 1973 when it was the preferred antibiotic to treat intestinal tract infections [3], [10], [11]. With exposure to antibiotics on the rise, *C. difficile* is slowly developing resistance to some of the antibiotics used to treat it, such as metronidazole [3].

Another risk factor is the patient's age. Most recent reports are showing a trend where there is an increased incidence of CDI cases in patients above the age of 65; people over the age of 65 years old are 10 times more likely to acquire a *C. difficile* infection than people younger than 20 years old [1], [2], [18]. It is thought that the sharp increase in CDI cases after the age of 65 is due to immunosenescence: the progressive deterioration of the components of the innate and adaptive immune systems [1]. There have been many studies that show that an effective humoral response to TcdA and TcdB can help prevent a *C. difficile* infection or decrease its effects leading to the immunosenescence theory [1], [53], [54].

Finally, another important risk factor is the environment; in particular health-care facilities. Health-care facilities provide an area of high-risk cross-contamination, especially between health-care providers and patients. Furthermore, susceptible patients may come in contact with objects that previously infected patients may have also been in contact with, namely toilets, hand-rails and bed-rails [1], [55]–[57]. This risk has been of high concern since the early 2000s, and cleaning reforms and more intensive isolation measures throughout health-care facilities

have been put in place to avoid cross-contamination [58], [59]. However, increased lengths of stay in health-care facilities is still a great risk factor to susceptible patients [18].

1.8 Clinical diagnosis and treatment

The first indication of a *C. difficile* infection is the presence of diarrhea. Physicians pay close attention to patients over the age of 65 in health-care facilities who are given a course of antibiotics. Guidelines have been set by the Infectious Disease Society of America (IDSA) and the Society for Healthcare Epidemiology of America (SHEA) with strong recommendations for a best course of action when dealing with a possible CDI [1], [9], [20], [49]. Should a physician suspect that there might be a CDI case (i.e. the presence of diarrhea), a stool sample is collected from the patient where one of several clinical tests can be performed. In previous years, enzyme immunoassay (EIA) was the test of choice for the detection of toxin in feces; however, with greater range in test sensitivity (31-99%) and the drop in cost of nucleic acid amplification tests (NAATs), NAATS such as PCR became the new standard of testing [1], [9]. Due to the higher sensitivity of tests such as PCR, the incidences of CDI may rise due to a potential over diagnosing [60]. However, the potential of over diagnosing is still lower than the false-positive results given by EIA testing [61].

At HSN, the real time-PCR Cepheid GeneXpert has replaced the EIA that was previously being used [61]. The GeneXpert platform is fitted for the company's "all-in-one" cartridges, which contain all the reagents and quality controls needed for the test. The entire test can be completed in less than 1 hour, including the forty-five minute runtime of the instrument, using the patient's stool sample. The cartridges contain 3 sets of primers to help in the detection of toxigenic *C.*

difficile: a set of primer for the *tcdB* gene, a set of primers for the *tcdC* gene that contains a deletion at nucleotide 117, and a set of primers for the *cdt* binary toxin gene. Specifically, should the strain be positive for all three of those genes, it is said to be presumptive for the hypervirulent strain NAP1/BI/027, which is critical knowledge for the health-care facility's infection control department in dealing with appropriate measures of isolation and treatment [62], [63].

In the event that a patient's test is positive for *C. difficile*, the physicians must assess their patients to initiate the best course of treatment. The physicians must first determine the severity of the patient's signs and symptoms which will determine what treatment will be given. IDSA/SHEA both have a three point classification system for the severity of the symptoms of a *C. difficile* infection with a recommended course of treatment for each severity level [9], [20], [49].

1. Mild-to-moderate – Patients with mild-to-moderate CDI basically only have diarrhea as a symptom. Some patients may complain of mild abdominal tenderness and discomfort due to the high frequency of bowel movements, which exceed 3 per 24 consecutive hours. Treatment for mild-to-moderate CDI is a course of metronidazole, 500mg ingested orally three times daily for 10 days. Should the patient be unable to take metronidazole, or if there is no improvement in the patient's health after 5 to 7 days, 125mg of vancomycin may be taken orally 4 times daily for 10 days.
2. Severe – In severe cases, patients will have considerable diarrhea and serum albumin levels smaller than 3g/dl. They will often have an elevated white blood cell count of over 15,000 cells/mm^3 and abdominal tenderness. Treatment for severe disease is a course of vancomycin, 125mg ingested orally 4 times daily for 10 days.

3. Severe and complicated – In a severe and complicated *C. difficile* infection, patients are usually admitted to the intensive care unit due to the severity of the illness. Their symptoms also include fever above 38.5^{o}C, hypotension, significant abdominal distention or ileus, a white blood cell count greater than 35,000 cells/mm^{3} or lower than 2,000 cells/mm^{3}, and serum lactate levels lower than 2.2 mmol/L. Antibiotic treatment at this point is very aggressive, with a recommended 500mg of vancomycin taken orally 4 times daily, 500mg IV of metronidazole every 8 hours, and 500mg in 500mL saline administered rectally (as an enema) 4 times daily. Should antibiotic treatment fail, other more drastic alternative treatments may be used, such as fecal transplant therapy and surgery to remove the affected colon depending on the severity of the case.

Furthermore, patient who present with recurrent cases of CDI are often moved from metronidazole treatment to a vancomycin treatment. After more than 3 recurrences, alternative therapies such as fecal transplant are also explored [9].

1.9 Project rationale

While *C. difficile* research is more common in bigger centres in Canada and around the world, the impact and genetic diversity of *C. difficile* in Northern Ontario is relatively unknown. Northern Ontario is a vast area, and is home to many secluded communities. Health Sciences North provides a health-care hub for all these communities, and patients often travel large distances to get specialized care in Sudbury.

Stool samples that were used in this project were procured from two sources. The first source of samples was from patients admitted to the two wards with the most reported cases of CDI: the

respiratory unit and the oncology and palliative care unit. These patients were asked to participate in our study, and gave consent for us to collect their stool during the first 48 hours of their stay at HSN. This was done according to the HSN's Research Ethics Board (REB) guidelines. The second source of samples was from HSN's clinical lab. As per HSN REB, medical waste samples may be used for research purposes. These stool samples were left over samples from patient who had get tested for CDI during their stay at HSN. These samples come from all wards at HSN.

We have established a library of *C. difficile* samples from patients admitted to HSN in order to examine the genetic diversity of *C. difficile* in Northern Ontario. These samples were then used to assess whether there is a link strain diversity (phenotypic variance) and virulence. Furthermore, using the patients' medical and treatment histories, we then evaluated whether specific bacterial genotypes or phenotypes accurately predicted prognosis and/or treatment outcome.

Bacterial genotypes and phenotypes that were evaluated were antibiotic susceptibility, toxin production, and microbial spore load. We chose these three factors as antibiotics lead to the onset of the infection, toxins cause the symptoms, and spores propagate the organism to its next host.

1.10 Research objectives

1. To build a well annotated cryorepository of *C. difficile* samples from patients at HSN treated for a CDI.

- Isolate, identify and store clinical strains
- Develop a database to record strain genotypes and phenotypes

2. To perform assays on the collected *C. difficile* strains including:
    - Ribotype
    - Toxin production
    - Microbial spore load
    - Antibiotic susceptibility
3. Investigate the correlation between phenotypes and genotypes of *C. difficile* and clinical outcome from CDI at HSN.

2.0 Media and Reagents

The recipes for all media and reagents used in this study are described below:

2.1 Columbia broth (CB)

35g of CB base (BD, Sparks, MD, USA, Cat: 294420) in 1000mL ddH_2O brought to a boil. Media sterilized by autoclaving, stored at room temperature in a 1L Corning bottle.

2.2 Brain heart infusion broth (BHI)

37g of BHI base (Oxoid, Basingstoke, Hampshire, England, Cat: CM1135) in 1000mL ddH_2O brought to a boil. Media sterilized by autoclaving, stored at room temperature in a 1L Corning bottle.

2.3 Brucella supplemented broth (BSB)

28g of Brucella agar base (BD, Sparks, MD, USA, Cat: 294420) in 1000mL ddH_2O brought to a boil. Media sterilized by autoclaving. 10mL vitamin K/hemin solution (QueLab, Montreal, QC, Canada, Cat: 8751) was filtered into the broth with a 2micron syringe filter. The medium was stored at room temperature in a 1L Corning bottle.

2.4 Brain heart infusion yeast extract taurocholate agar (BHI-YT)

37g BHI base (Oxoid, Basingstoke, Hampshire, England, Cat: CM1135), 5g yeast extract (BD, Sparks, MD, USA, Cat: 212750), 1g L-cysteine hydrochloride monohydrate (Sigma-Aldrich, St.

Louis, MO, USA, Cat: C7880), 1g taurocholate (Sigma-Aldrich, St. Louis, MO, USA, Cat: T4009), 15g bacteriological agar (QueLab, Montreal, QC, Canada, Cat: QB-39-0221) were dissolved in 1000mL ddH_2O and brought to a boil. The medium was sterilized by autoclaving and distributed (20mL) into 100mm petri dishes.

2.5 *Clostridium difficile* selective agar (CDSA)

69g of *C. difficile* agar base (Oxoid, Bakingstoke, Hampshire, England, Cat: CM0601), 1g taurocholate (Sigma-Aldrich, St. Louis, MO, USA, Cat: T4009) was dissolved in 920mL of ddH_2O, brought to a boil and sterilized by autoclaving, and then cooled to 60^oC. Medium was supplemented with 2.5mL of 2.4mg/mL Norfloxacin, 2.5mL of 12.8mg/mL Moxalactam, and 70mL defibrinated horse blood (Nutri-Bact, Terrebonne, QC, Canada, Cat:4281). The medium was distributed (20mL) into 100mm petri dishes and stored at 4^oC until use.

2.6 Norfloxacin stock

In a 50mL centrifuge tube, 240mg of Norfloxacin (Sigma-Aldrich, St. Louis, MO, USA, Cat: N9890) and 0.2mL of concentrated hydrochloric acid, 36%-38% (Sigma-Aldrich, St. Louis, MO, USA, Cat: H1758) were added to 50mL ddH_2O. The content of the tube was mixed gently until dissolved. Solution was dispensed into a 100mL volumetric flask and topped up to 100mL with ddH_2O. Solution was syringe filtered (0.2micron) into 15mL centrifuge tubes (10mL/tube), and placed in -80^oC freezer for storage.

2.7 Moxalactam stock

In a 50mL centrifuge tube, 1280mg of Moxalactam (Sigma-Aldrich, St. Louis, MO, USA, Cat: M8158) and 10g of L-cysteine Hydrochloride monohydrate (Sigma-Aldrich, St. Louis, MO, USA, Cat: C7880) were added and topped up to 50mL with ddH_2O. Centrifuge tube was gently mixed until dissolved. Solution was dispensed into a 100mL volumetric flask and topped up to 100mL with ddH_2O. Solution was syringe filtered (0.2micron) into 15mL centrifuge tubes (10mL/tube), and placed in -80^{o}C freezer for storage.

2.8 Brucella blood agar (BBA)

43g of Brucella agar base (QueLab, Montreal, QC, Canada, QB-39-0606) and 15g of bacterial agar (QueLab, Montreal, QC, Canada, Cat: QB-39-0221) were added to 1000mL ddH2O and brought to a boil. The medium was sterilized in the autoclave for 20 minutes on liquid cycle, 120kPa, 121ºC. The medium was cooled to 60ºC in a water bath, and supplemented with 50mL laked sheep blood (Nutri-Bact, Terrebonne, QC, Canada, Cat: 4147) and 5mL vitamin K/hemin solution (QueLab, Montreal, QC, Canada, Cat: 8751). The medium was dispensed in either 150mm petri dishes (70mL) or 100mm petri dishes (20mL).

2.9 Sodium hydroxide stock (1N)

40g of of NaOH (Sigma-Aldrich, St. Louis, MO, USA, Cat: S5881) was dissolved in 1L of ddH_2O.

2.10 Ethylenediaminetetraacetic acid (EDTA)

3.72g of EDTA (Sigma-Aldrich, St. Louis, MO, Cat: E5134) was added to 200mL ddH_2O (0.5 M) and dissolved by adjusting the pH to 8.0 with 1N NaOH.

2.11 10X tris/borate/EDTA (TBE) buffer

In 400mL of ddH_2O, 53g of trizma base (Sigma-Aldrich, St. Louis, MO, USA, Cat: T1503) and 27.5g of boric acid (Sigma-Aldrich, St. Louis, MO, USA, Cat: B7901) were dissolved along with 20mL of 0.5M EDTA. pH was adjusted to 8.0 with 1N NaOH. The volume was topped up to 500mL with ddH_2O.

3.0 Methods

3.1 Sample processing

Stool samples for the project were received from 2 different sources at HSN between December 3rd, 2013 and June 17th, 2015; either by consenting patients to participate in our study (patient recruitment) or from medical waste from a physician ordered *C. difficile* test (clinical samples). All stool samples were tested for *C. difficile* using two methods: by the Cepheid GeneXpert, and by bacterial culture of stool samples on selective media.

3.1.1 Patient recruitment

Patients that were admitted to the Oncology Ward (Med4 North) and the Respiratory Ward (Med6 South) were visited by one of our clinical research co-ordinators (Barbara Rickaby, Pamela Leduc, Carolyn Truskoski) and asked to participate in our study in the 24 hours following their admission to the ward. The two wards in which patients were consented were chosen based on their reported rates of CDI, as well as the frequency of outbreaks. The consenting of patients was approved by HSN's Research Ethics Board (REB). Should the patient consent to participate in our study, they were asked to donate a stool sample that was to be collected by the nursing staff within 72 hours of admission in a specimen cup. The sample was then sent to HSN's clinical laboratory where it was processed for storage and tested on the Cepheid's GeneXpert platform. The samples received from consented patients were given the designation "CDST", followed by the next available number in the database (i.e. CDST0001, CDST0002).

3.1.2 Clinical samples

Clinical stool samples were given to us after use at HSN's clinical laboratory as medical waste. Patients in this case did not need to be consented as medical waste may be used for research purposes. These stool samples were from patients admitted to any ward at HSN that exhibited CDI symptoms from which a physician ordered a test to confirm CDI. The samples were collected in a specimen cup by the nursing staff, and sent to HSN's clinical laboratory. Stool samples were kept in a refrigerator at 4^{o}C until testing by Cepheid's GeneXpert was complete, at which time they were processed for storage. Only stool samples that were positive for *C. difficile* were processed and picked up from the clinical laboratory. Upon receiving the samples, they were numbered arbitrarily using a letter and number system; samples from the clinical lab were given a designation of "CD" followed by the next number available in the database (i.e. CD0001, CD0002, etc.). This was done in accordance with the research ethics board (REB) guidelines, which states that the samples must be given a number that is untraceable to the patient.

3.1.3 Patient profiles

All patient information needed for the research was gathered from Meditech, the medical database that is used at HSN. This was done in accordance with the REB guidelines. This information includes: hospital ward the patient was admitted to, year of birth and age, sex, primary diagnosis, and date of admission and date of discharge or death. As per REB, any documents linking data back to the patient were kept in a locked room or encrypted to prevent unintended disclosures.

3.1.4 Cepheid's GeneXpert diagnostic platform

Cepheid's GeneXpert is real-time PCR instrument that HSN's clinical lab utilises to confirm CDI cases. The test was carried out according to manufacturer's protocol. Each kit contains a sterile swab, transfer pipette, sample reagent, and Xpert *C. difficile* cartridge. The sterile swab was immersed in the stool sample as to get uniform stool coverage around the swab. The swab was then dipped in the sample reagent bottle, and the stem of the swab was snapped off along the dotted line. The sample reagent and swab were vortexed for 10 seconds at high speed. Using the transfer pipette, the entirety of the sample reagent bottle was transferred to the "S" chamber of the Xpert *C. difficile* cartridge. At this point, the cartridge was loaded in the Cepheid GeneXpert instrument and the test was launched. The test detects the presence of the *tcdB* gene, as well as the binary toxin *cdt* and the *tcdC* in the pathogenicity locus with a deletion at nucleotide 117. Included in the cartridge are two quality controls: a Sample Processing Control (SPC) and a Probe Check Control (PCC). *Bacillus globigii* spores are used as SPC to ensure that the stool samples were processed correctly while the PCC ensures proper functioning of the cartridge itself.

3.1.5 Sample preparation and storage

One gram of each stool sample provided was placed in a 15mL conical tube. 4mL of 10% glycerol was then added to the conical tube, and the sample was homogenized by vortexing thoroughly. Samples were brought back to the Health Sciences North Research Institute (HSNRI) laboratory where they were aliquoted into 2mL cryovial tubes. The cryovial tubes were stored in a -80^{o}C freezer until further processing was to take place.

3.1.6 Isolation of *C. difficile*

C. difficile was isolated from the stool samples that were prepared for storage in HSN's clinical laboratory and frozen at -80^{o}C. Once thawed, ethanol shock was performed by taking 0.1mL of stool sample and aliquoting it to cryovials containing 0.1mL of 100% ethanol (anhydrous) (Commercial Alcohols, Brampton, ON, Canada, Cat: P016EAAN). Samples were left to incubate at room temperature for 1 hour. After the ethanol shock was completed, 0.1mL of shocked sample was transferred to a reduced CDSA plate and spread using a T-shaped sterile spreader. Plates were incubated under anaerobic conditions in a Plas-Lab controlled atmosphere chamber containing a gas mixture of 15% CO_2, 75% N_2, <1% H_2 at 37^{o}C for a period of 5 to 7 days. Incubation time was dictated by how well the organism had grown after the 5th day. Were the colonies still too small (smaller than 2mm in diameter), they were allowed to incubate for 2 days more.

After the initial incubation period, the CDSA plates were removed from the anaerobic chamber, and a single colony from each plate was lifted using disposable 1μL inoculating loops and transferred to a 15mL conical tube containing 5mL of pre-reduced BHI broth. The lids of the conical tubes were loosely capped, and the tubes were placed back under anaerobic conditions at 37^{o}C for a 5 day incubation period.

Upon completion of the 5 days incubation period, purity of culture was confirmed by morphological characteristics of colonies (beige to pink colonies, between 2 and 15 mm in diameter depending on incubation period, circular with lobate margins, and a flat and umbonate elevation), and by microscopy. Should the culture be found to be impure, a 1μL sterile

disposable inoculating loop was used to streak a sample of culture on a CDSA plate, which was incubated under anaerobic conditions at 37^{o}C for an extra 5 days. This cycle was repeated until the culture was found to be pure. The pure culture was used for *C. difficile* spore production for storage as well as DNA extraction.

3.1.7 *C. difficile* spore storage

From the pure *C. difficile* isolates cultivated in BHI, a 1µL inoculating loop was charged and streaked liberally over a BHI-YT plate. BHI-YT plates were placed under anaerobic conditions at 37^{o}C for a 7 day incubation period. Resulting colonies were scraped off the plates by pipetting 2mL of sterile ddH_2O directly onto the plate and using a T-shaped spreader to dislodge the colonies. Resulting mixture was transferred to a 2mL cryovial and vortexed thoroughly to homogenize the colonies. Cryovials containing spores are kept in a cryovial box at 4^{o}C indefinitely. To recover the spores, a 1µL inoculating loop can be charged with spores and streaked on a CDSA plate and incubated under anaerobic conditions for 5-7 days at 37^{o}C.

3.1.8 DNA extraction

1.5mL of pure *C. difficile* isolates cultivated in BHI was transferred to a 2mL cryovial. Cryovials were centrifuged at 13,200 RPM for 3 minutes. Supernatant was discarded. Pallet was washed with sterile ddH_2O and vortexed thoroughly. The isolates were centrifuged again (13,200 RPM, 3 minutes) and the supernatant was discarded. Wash and centrifugation were repeated, and supernatant was discarded. 0.5mL of sterile 5% Chelex (Bio-Rad, Hercules, CA, USA, Cat: 1422822) was added to each pallet. Isolates were placed on a hot block at 105^{o}C for 20 minutes,

and then cooled in the refrigerator at 4^{o}C. Isolates were centrifuged once more (13 200RPM, 3 minutes) and the supernatant was transferred to a new cryovial. Supernatant was then frozen at -20^{o}C.

3.2 Characterizations of *C. difficile* strains

3.2.1 Toxinotyping

The genetic method for toxinotyping was adapted from a multiplex PCR protocol described by Lemee et al. (2004) using three pairs of primers: two primers for triose phosphate isomerase (tpi-F [5'-AAAGAAGCTAAGGGTACAA-3'] and *tpi*-R [5'-CATAATATTGGGTCTATTCCTAC-3']) (Sigma-Aldrich, St. Louis, MO, USA, Cat: 99343-003 & 99343-004) which generate a 230bp amplicon and is a housekeeping gene that is conserved in all *C. difficile* strains with a unique sequence to the organism. This is used as a control which determines whether the organism is in fact *C. difficile*. Two primers for *tcdA* are used (*tcdA*-F [5'-AGATTCCTATATTTACATGACAATAT-3'] and *tcdA*-R [5'-GTATCAGGCATAAAGTAATATACTTT-3']) (Sigma-Aldrich, St. Louis, MO, USA, Cat: 99343-007 & 99343-008), which generates a 369bp amplicon, or an 110bp amplicon in strains that have a truncation in the *tcdA* gene. Another pair of primers specific for *tcdB* (*tcdB*-F [5'-GGAAAAGAGAATGGTTTTATTAA-3'] and *tcdB*-R [5'-ATCTTTAGTTATAACTTTGACATCTTT-3']) (Sigma-Aldrich, St. Louis, MO, USA, Cat: 99343-005 & 99343-006) generates a 160bp amplicon. Primers were prepared by first making a 20X stock solution (5µL primers in 95µL RNAse and DNase free ddH_2O) for each primer. Two working stock solutions of primers where then prepared with the following ratios: primer

solution 1 contained 17.9% *tpi*-F, 15.4% *tpi*-R, 35.9% *tcdB*-F and 30.8% *tcdB*-R while primer solution 2 contained 50.7% *tcdA*-F and 49.3% *tcdA*-R.

Qiagen Multiplex PCR Plus kits (Qiagen, Hilden, Germany, Cat: 206152) were used for PCR, with a MasterMix solution made from the kit reagents in the following ratios: 100µL of MasterMix, 20µL of coral dye, 20µL of RNAse free H_2O, 40µL of Q-solution, 10µL of primer solution 1 and 10µL of primer solution 2.

PCR 96-well plate or 8-well strips were loaded with 19µL of MasterMix solution and 1µL of *C. difficile* DNA. DNase/RNAse free ddH_2O was used as blank control, ATCC strain 700057 was used as the *tpi*+/*tcdA*-/*tcdB*- control, ATCC strain 43594 was used as the *tpi*+/*tcdA*+/*tcdB*+ control, and ATCC strain 43598 was used as the *tpi*+/Δ*tcdA*/*tcdB*+ control. Amplification was performed on an Eppendorf MasterCycler Pro 6325 using the following cycles: an initial 5 minutes at 95^{o}C, followed by 30 cycles of: 30 seconds of DNA denaturation at 95^{o}C, 90 seconds of primer annealing at 57^{o}C, and 30 seconds of primer extension at 72^{o}C, and a final step of 10 minutes at 68^{o}C.

PCR amplicons were resolved for 1 hour on a 1% agarose gels (Sigma-Aldrich, St. Louis, MO, USA, Cat: A6877) in a ½ TBE buffer at 100 volts. The gel was stained with 1µg/mL ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA, Cat: E8751) for 15 minutes, and photographed on a UV transilluminator using ProteinSimple FluorChem software.

3.2.2 Ribotyping

PCR-ribotyping was adapted from a method by Bidet & Petit (1999), which itself was adapted from a method by Gürtler (1993), and then O'Neill (1996). The intergenic region in rRNA operons of *C. difficile* were PCR amplified using *C. difficile* genomic DNA and *C. difficile* specific 16S primer [5'-GTGCGGCTGGATCACCTCCT-3'] and 23S primer [5'-CCCTGCACCCTTAATAACTTGACC-3'] (Sigma-Aldrich, St. Louis, MO, USA, Cat: 99343-001 & 99343-002). A 20X primer solution was made by adding 5µL of primer to 95µL of DNase/RNAse free ddH_2O. A Qiagen Multiplex PCR Plus kit (Qiagen, Hilden, Germany, Cat: 206152) was used for PCR, and a MasterMix solution was prepared using the following reagents in these ratios: 100µL of MasterMix, 20µL of coral dye, 20µL of RNAse free H_2O, 40µL of Q-solution, 10µL of 16S primer solution and 10µL of 23S primer solution.

Nineteen µL of the MasterMix solution was added to a 96-well PCR plate or 8-well PCR strip as needed, along with 1µL of *C. difficile* DNA. Plate/strips were placed on an Eppendorf MasterCycler Pro 6325 for the following cycles: an initial 5 minutes at 94^{o}C, followed by 30 cycles of: 60 seconds of DNA denaturation at 95^{o}C, 60 seconds of primer annealing at 57^{o}C, and 60 seconds of primer extension at 72^{o}C, and a final 10 minutes at 68^{o}C after the 30 cycles.

PCR amplicons were resolved by capillary electrophoresis using a DNA-1000 chip for the Agilent Bioanalyzer (Agilent, Waldbronn, Germany, Cat: 5067-1514). The manufacturer's protocol was followed for preparation of the DNA-1000 chip. The chip was placed on the chip priming station, and 9µL of gel-dye matrix was pipetted to the well labelled G and primed for 1 minute with 1mL of air through a syringe. The syringe was then released, and 9µL of gel-dye

was added to the other G well. 5µL of marker was added to wells 1-12, and the ladder well. 1µL of DNA ladder was added to the ladder well, and 1µL of each set of PCR amplicons was pipetted to wells 1-12. The chip was vortexed on the chip vortexer at 2400 RPM for 1 minute. The chip was then placed on the Agilent Bioanalyzer and allowed to run.

Fingerprints generated by the Agilent Bioanalyzer were analyzed by the GelCompar II software (Applied Maths, Austin, Texas, USA) and matched up to our ribotype database.

3.3 Biology of *C. difficile* strains

3.3.1 Microbial spore load

The amount of spores per gram of stool was determined for each of the patient stool samples. This was accomplished by first doing an ethanol shock as described in the *C. difficile* isolation protocol. After the 1 hour shock was complete, a dilution series was prepared for each shocked sample with pre-sterilized ddH_2O to create 1/2, 1/10, 1/100, 1/1000, 1/10000, and 1/100000 dilutions. 0.1mL of each dilution was spread on CDSA plates using a T-shaped spreader and incubated under anaerobic conditions for 48 hours. Colonies were counted after the incubation period to then determine the total spore load for the undiluted sample.

To normalize the data against dry weight of stool samples, the moisture content of each stool was determined in order to have a true representation. 0.5mL of each stool sample was pipetted to cryovials and 0.05mL of 10% bleach was added to each sample and allowed to incubate at room temperature for 1 hour in order to sterilize the stool of microorganisms. The content of the

cryovials was transferred to pre-weighed aluminum tins, and the stool samples were weighed. Stool samples were placed in a drying oven for 1 hour at 120^{o}C until the water had completely evaporated from the control tin. Tin and stool were weighed once more, and the moisture content was calculated as percentage water content.

3.3.2 *In vitro* toxin production

In vitro toxin production of each *C. difficile* isolate was determined using the *C. difficile* Tox A/B II EIA kit from TechLabs (Blacksburg, VA, USA, Cat: T5015). Spores from each sample were recovered on CDSA, and a single colony from each plate was inoculated in 5mL pre-reduced BHI broth in a 15mL conical tube. Tubes were placed under anaerobic conditions at 37^{o}C until culture reached stationary phase (about 48 hours). Each culture was standardized to 0.5 MacFarland standard equivalent by slowly pipetting culture into 5mL of pre-reduced BHI in calibrated glass tubes for the Fisher Scientific Cell Density Meter (model 40). Standardized cultures were placed under anaerobic conditions for 48 ± 2hours. Cultures were transferred to 15mL conical tubes and centrifuged at 4500 RPM for 20 minutes at 4^{o}C. Supernatant was transferred to another 15mL conical tube and stored at 4^{o}C.

Relative toxin levels in each supernatant were measured using the *C. difficile* Tox A/B II EIA kit from TechLab, according to manufacturer's specifications. Provided with the kit is a diluent, a conjugate, a substrate, a positive control, wash buffer, stop solution and microassay plate. The samples were prepared for the test by adding 50µL of culture supernatant to 200µL of diluent in 1.5mL Eppendorf tubes. A series of 6 ten-fold serial dilutions were prepared from this. Then, 50µL of conjugate was added to each needed well with 100µL of prepared diluted samples. 50µL

of the provided positive control was added to one well, and 50µL of diluent as negative control. ATCC 700057 strain was also used as a negative control (non-toxigenic *C. difficile* strain), and ATCC 43594 strain as positive control (toxigenic *C. difficile* strain). An adhesive wrap was placed over the used wells, and the plate was placed at $37^{o}C \pm 2^{o}C$ for 50 minutes. Content of the wells was discarded, and wells were washed with wash buffer 5 times, tapping the inverted plates on paper towels after each wash. 100µL of substrate was added to each well and left to incubate at room temperature for 10 minutes, tapping the plate gently at the start and at 5 minute mark to allow the substrate to mix. 50µL of stop solution was added to each well at the end of the incubation period, and the plate was read on the BioTek Synergy H4 hybrid plate reader at 450/620nm dual wavelength. For the test to pass quality control, the positive control must be ≥0.500, and the negative control <0.080. The test limit for a sample is 0.080, and any sample with a reading lower than this value was considered negligible. Toxin concentrations detectable by the kit are 0.8ng/mL for toxin A, and 2.5ng/mL for toxin B. Upon completion of the test, toxin values were determined, noting the dilution factor for each sample.

3.3.3 Antibiotic susceptibility

Antibiotic susceptibility and the minimal antibiotic inhibitory (MIC) concentration for various *C. difficile* strains was determined using BioMérieux E-Test strips and Oxoid M.I.C.Evaluator strips. Eight different antibiotics were used, based on their frequency of use at HSN's Med4 North and Med6 South clinics. The following antibiotic strips were used: vancomycin (Biomérieux, Marcy-l'Étoile, France, Cat: 412488), metronidazol (Oxoid, Bakingstoke, Hampshire, England, Cat: MA0103F), clindamycin (Oxoid, Bakingstoke, Hampshire, England, Cat: MA0119F), ciprofloxacin (Biomérieux, Marcy-l'Étoile, France, Cat: 412311), amoxicillin

(Biomérieux, Marcy-l'Étoile, France, Cat: 412243), benzyl penicillin (Biomérieux, Marcy-l'Étoile, France, Cat: 412465), cefotaxime (Oxoid, Bakingstoke, Hampshire, England, Cat: MA0112F) and imipenem (Oxoid, Bakingstoke, Hampshire, England, Cat: MA0115F).

Spores were recovered on CDSA plates from the spore stock and placed under anaerobic conditions at 37^{o}C for 5 to 7 days incubation period. Once grown, a single colony from each plate was picked by a 1µL inoculating loop and placed in 5 mL of pre-reduced BSB broth for 48 hour growth under anaerobic conditions at 37^{o}C. After the incubation period, the cultures were used to inoculate 5mL of pre-reduced BSB broth in calibrated glass vials for the Fisher Scientific Cell Density Meter model 40 to standardize the culture to 0.5 MacFarland (OD 0.1 at 600nm). Using a floqswab, a lawn was created on 150mm or 100mm petri dish with BBA agar. On the 150mm plates, 4 E-Test strips were placed at 12, 3, 6 and 9 o'clock positions, while on the 100mm plate, only 1 E-test strip was used. Plates were placed under anaerobic conditions for 48 hours ± 2hours, at which time the plates were taken out, and the MIC observed and recorded. To determine the MIC, the value at which the zone of inhibition ends on the E-Test strip was recorded.

The analysis of the MIC values for the *C. difficile* strains was done by looking at the population's distribution of the MIC values. The population distribution graphs were populated by entering the percentage of samples that were observed at specific MIC values in Microsoft Excel. The population distribution of the MIC values was compared to European Society of Clinical Microbiology and Infectious Diseases (EUCAST). Unfortunately, EUCAST does not have all the

clinical breakpoints of all the antibiotics that were used in this project, so we could not determine the resistance of the strains to certain antibiotics, but trends can be observed in those cases.

3.4 Epidemiology

3.4.1 Classification of patients' symptom severity

Using the guidelines set by the Infectious Disease Society of America (IDSA) and the Society for Healthcare Epidemiology of America (SHEA), the symptoms of the patients were reviewed and given a score between 0 to 3 based on reports by the physicians and clinical lab reports, all available on Meditech.

1) Asymptomatic (score of zero)
    a) no symptoms (formed stool)
2) Mild-to-moderate (score of 1)
    a) diarrhea (unformed stool, in excess of 3 per 24 consecutive hours)
    b) mild abdominal tenderness
3) Severe (score of 2)
    a) considerable diarrhea
    b) serum albumin levels smaller than 3g/dl
    c) elevated white blood cell count of over 15,000 cells/mm^3
    d) abdominal tenderness
4) Severe and complicated (score of 3)
    a) considerable diarrhea
    b) fever above 38.5^oC
    c) hypotension

d) significant abdominal distention or ileus

e) white blood cell count greater than 35,000 cells/mm^3 or lower than 2,000 cells/mm^3

f) serum lactate levels lower than 2.2mmol/L

3.5 Statistical analysis

All statistical analyses (box plots, Welch's t-test, one-way ANOVA) were performed on Prism GraphPad version 5.

4.0 Results

A flowchart representing all the steps from sampling to testing is found in appendix 1, and a tabulated database of all results is found in appendix 2.

4.1 Sample processing and patient demographics

4.1.1 Cohort

A total of 57 *C. difficile* stool samples identified as positive by GeneXpert or by culture in the lab were gathered for the study. The samples were divided into two groups: asymptomatic and symptomatic. 23 of the samples were asymptomatic, 13 of them being males (ages 37-90, mean 73) and 10 females (ages 21-76, mean 60.5), while 34 of the samples were symptomatic, 18 of them being males (ages 55-86, mean 70) and 16 females (ages 44-98, mean 76). Average age of all symptomatic patients was 74.5 years old while the average age for asymptomatic patients was 70. There was no significant difference between the ages of the patients and whether they were asymptomatic or symptomatic (Welch's t-test; p=0.1273) (Figure 4a). The mean of all male patients was 74 years old, and the average for all female patients was 71, and there was no significant difference between the mean ages of patients relative to their sex (Welch's t-test; p=0.3085) (Figure 4b). The mean age of all patients was 70.8 years old. 68.4% of the patients in the study were above the age of 65, and 86% of the patients were above the age of 60.

4.1.2 Patient recruitment samples

1301 patients were visited during the first 24 hours of their stay at Med4 North and Med6 South of HSN. Of the 1301 patients asked to participate in our study, 626 (48%) patients consented to

participate while 675 (52%) patients declined. Of the 626 patients that consented to participate, 220 stool samples were collected, of which 29 (13%) samples were positive for *C. difficile* by culture and were included in this study (Table 4).

4.1.3 Clinical samples

31 samples that had tested positive for *C. difficile* by the GeneXpert from physician ordered tests at HSN's clinical lab were donated to the study. From those 31 samples, 28 samples were positive for *C. difficile* by culture and were included in the study (Table 4).

a.

b.

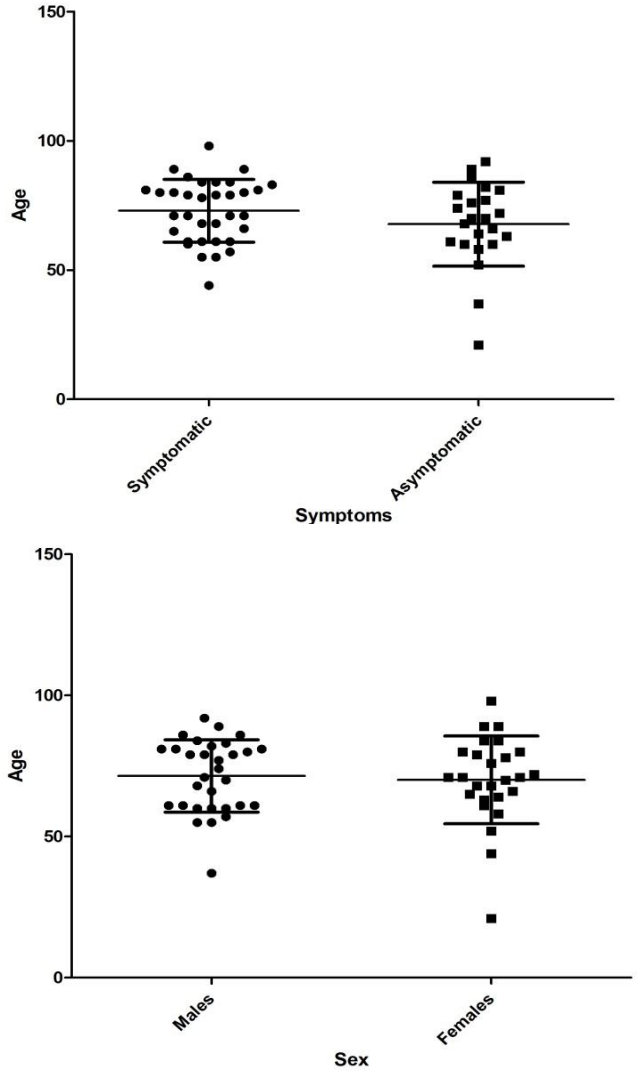


Figure 4 - a. Age of patients vs. patient symptoms b. Age of patients vs. sex of patients

(a.) There was no significant difference between in age between patients that were asymptomatic and the patients that exhibited symptoms in the study. Mean age for symptomatic presentation was 74.5 while asymptomatic patients were on average 70 years old (p=0.1273) (b.) This was also the case when comparing the ages of male and female patients, with a mean age of 74 for males and 71 for females (p=0.3085).

Table 4 - Stool samples received vs. *C. difficile* positive samples from clinical and consented samples

	Samples received	*C. difficile* positive samples
Clinical Samples	31	28
Consented samples	220	29
Total	251	57

Table 4 shows the samples that were received from the clinical lab and from patients that were consented from the Respiratory unit as well as the Oncology and Palliative Care unit. This includes all stool samples, and shows is the number of stool samples that were positive for *C. difficile*.

4.2 Characterization of *C. difficile* strains

4.2.1 Cepheid GeneXpert

All 220 stool samples from consented patients, as well as the 31 stool samples sent to the clinical lab from physician ordered *C. difficile* testing were first tested on the Cepheid GeneXpert. Of the samples that were received from patients that consented to participate in the study, 8 samples were negative for *tcdB, cdt* and *tcdC,* 19 samples were positive for *tcdB*, but negative for *cdt* and *tcdC,* 1 sample was positive for *tcdB* and *cdt*, but negative for *tcdC,* and 1 sample was positive for *tcdB, cdt* and *tcdC*. We did not receive any clinical samples that were negative for *tcdB, cdt* and *tcdC,* 22 samples were positive for *tcdB* but negative for *cdt* and *tcdC,* 5 samples were positive for *tcdB* and *cdt* and negative for *tcdC,* and finally 1 sample was positive for *tcdB, cdt* and *tcdC.* At this point, consented and clinical samples were evaluated separately to note the number of patients that were carriers, which could only be found in consented patients.

Samples that were positive for *tcdB* but negative for *cdt* and *tcdC* make up 72% of the samples. Samples that were negative for *tcdB, cdt* and *tcdC* accounted for 14% of the samples. Samples positive for *tcdB, cdt* and negative for *tcdC* made up 10.5% of the population, while samples positive for all accounted for 3.5% of the population (Table 5). This shows that only a small portion of the sample population is in fact positive for the binary toxin (14% of samples) while the majority of samples would only have the toxins located on the PaLoc.

Table 5 - GeneXpert results for clinical and consented samples

GeneXpert Results	Clinical Samples	Consented Samples	Total	Percentage (%)
tcdB - / *cdt* - / *tcdC*-	0	8	8	14
tcdB + / *cdt* - / *tcdC*-	22	19	41	72
tcdB + / *cdt* + / *tcdC*-	5	1	6	10.5
tcdB + /*cdt*+ / *tcdC*+	1	1	2	3.5
Total	28	29	57	100

The GeneXpert results for both samples collected from clinical lab and from consented patients are shown in table 5. 72% of samples are found to be *tcdB* positive, *cdt* negative and *tcdC* negative.

4.2.2 Toxinotyping

The DNA from all 57 samples that were isolated by culture were characterized for the presence potential genes encoding for toxins (Figure 5). From the consented patients group, 4 samples were *tcdA* and *tcdB* negative, 23 samples were *tcdA* and *tcdB* positive, 1 was *tcdA* positive but *tcdB* negative, and 1 sample contained the *tcdA* gene truncation. From the samples received from the clinical lab, from physician ordered *C. difficile* test, 1 sample was *tcdA* and *tcdB* negative, 22 samples were *tcdA* and *tcdB* positive, 3 samples were *tcdA* positive but *tcdB* negative, and 2 samples contained the *tcdA* gene truncation. No *tcdA* negative and *tcdB* positive samples were found.

tcdA and *tcdB* positive samples accounted for 79% of all samples, while *tcdA* and *tcdB* negative samples accounted for 9% of all samples. *tcdA* positive but *tcdB* negative made up 10.5% of all samples, and the samples containing the truncations in the *tcdA* gene made up 1.5% of all samples (Table 6).

Table 6 - Toxin expression (*tcdA* and *tcdB*) of all stool samples received from patients at HSN

Toxin expression	Clinical Samples	Consented Samples	Total	Percentage (%)
tcdA- / *tcdB*-	1	4	5	9
tcdA + / *tcdB* +	22	23	45	79
tcdA + / *tcdB* -	3	1	4	10.5
tcdA - / *tcdB* +	0	0	0	0
tcdA (del)+ / *tcdB* +	2	1	3	1.5
Total	28	29	57	100

Table 6 demonstrates the toxin expression (*tcdA* and *tcdB*) for samples gathered from the clinical lab and from consented patients. 79% of samples were both *tcdA* positive and *tcdB* positive

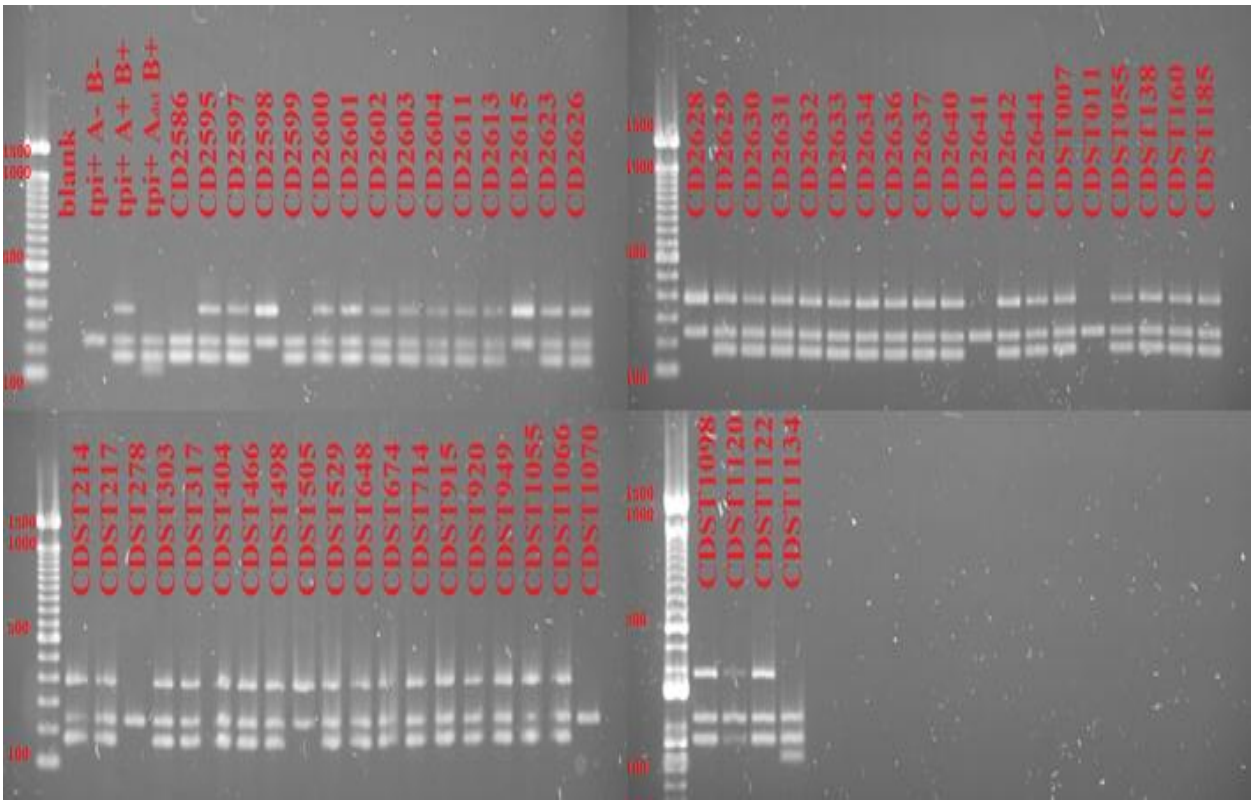


Figure 5 - Characterization of toxin genes (*tcdA* and *tcdB*) among *C. difficile* isolates from patient stool samples at HSN

Figure 5 depicts the results of the separation of PCR-generated amplicons by agarose gel electrophoresis in order to characterize the toxin expression in *C. difficile* for all samples used in the study. The *tpi* gene is a housekeeping gene that is conserved in all *C. difficile* strains with a unique sequence to the organism and generates a 230bp amplicon. *tcdA* generates a 369bp amplicon, or should the truncation of *tcdA* be present, it generates an 110bp amplicon. Finally, the *tcdB* gene generates a 160bp amplicon.

4.2.3 Ribotyping

Following characterization, ribotyping analysis of all 57 *C. difficile* DNA samples was performed. It was found that there were 26 different ribotypes among the 57 samples, 5 of which could not be matched to any other ribotypes in our database (no associated ribotype in database (NARD), and 3 ribotypes were previously found to be unique to Sudbury. Ribotypes of the samples included 001, 002, 012, 017, 046, 056, 075, 077, 106, 126, 137, 174, 251, M, O, and Z. Typical nomenclature of ribotypes are indicated by three numbers, however this is not a standard. The ribotypes that were unique to Sudbury are S03677, S0933 and S2319. The most common ribotypes are 020 and 106 with 9 and 8 samples, respectively (16% and 14%), followed by 001 and 251 with 5 samples each (9%) (Figure 6). There was only one hypervirulent NAP1/BI/027.

We were able to correlate the ribotype groups with the toxin expression of each group. Ribotype 046 and the Sudbury unique strain S0367 were both *tcdA* and *tcdB* negative, while ribotypes 001, 002, 012, 020, 056, 077, 106 137, 174, 251, M, O and Sudbury unique strain S0933 were all *tcdA* and *tcdB* positive. Ribotypes 027, 075, Z and Sudbury unique strain S2319 were *tcdA* positive but *tcdB* negative. Finally, ribotypes 017 and 126 were both strains that contain the truncation in the *tcdA* gene (Table 7).

The ribotype groups were divided between the symptomatic and asymptomatic patients to observe the distributions of the ribotype groups. Ribotype 106 was found to be almost exclusively found among symptomatic patients (88%), as was ribotype 251 (80%), ribotype 001 (80%), and ribotype 056 (75%). Ribotype 020, which is one of the most common ribotypes at

HSN, was found to be distributed almost equally between symptomatic and asymptomatic (45% and 55% respectively). All samples which we could not match up to any other ribotype in our database (NARD) were found to be asymptomatic. The Sudbury unique ribotype strains S0367 and S2319 were only found in asymptomatic individuals, while the Sudbury unique ribotype strain S0933 was found in a symptomatic individual (Figure 7).

The distribution of ribotype groups between sexes was also observed. The ribotype distribution between the two most frequent groups, ribotype 020 and ribotype 106 was fairly close, with 33.3% in females and 66.7% in males for ribotype 020 and 62.5% in females and 37.5% in males for ribotype 106. Ribotype 056 was found predominantly in males (75%), ribotype 001 and ribotype 251 were both found to be closely distributed, with 60% of samples found to come from males and 40% from females. Ribotype 002 was only found in females. Ribotype strains unique to Sudbury S0367 and S0933 were found only in females, while S2319 came from a male (Figure 8).

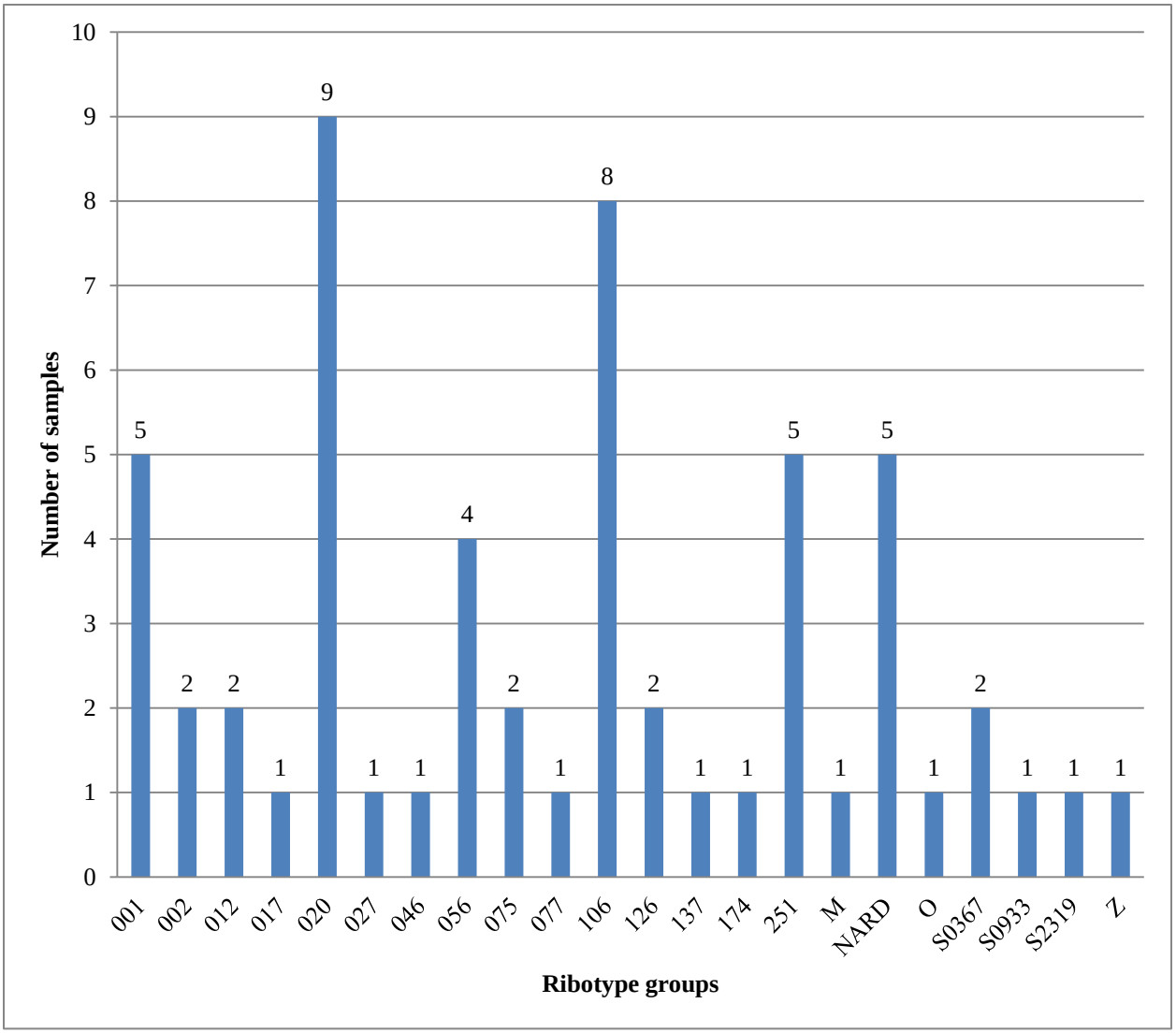


Figure 6 - Ribotypes found among *C. difficile* isolates from patient samples at HSN

Representation of all the ribotypes found in patient samples at HSN. The most common ribotypes found were 020 and 106 with 8 samples per ribotype. There was fairly large diversity of ribotypes found in patients at HSN suggesting that patients are not contracting the organism from the same source.

Table 7 - Association of the different toxinotype profiles with ribotypes from patient samples at HSN

Toxin expression	Ribotypes
tcdA - / *tcdB* -	046, S0367
tcdA + / *tcdB* -	027, 075, S2319, Z
tcdA (del) / *tcdB* +	017, 126
tcdA - / *tcdB* +	
tcdA + / *tcdB* +	001, 002, 012, 020, 056, 077, 106, 137, 174, 251, M, O, S0933

Table 7 demonstrates the toxin expression that was found in all the ribotype groups from samples of HSN patients.

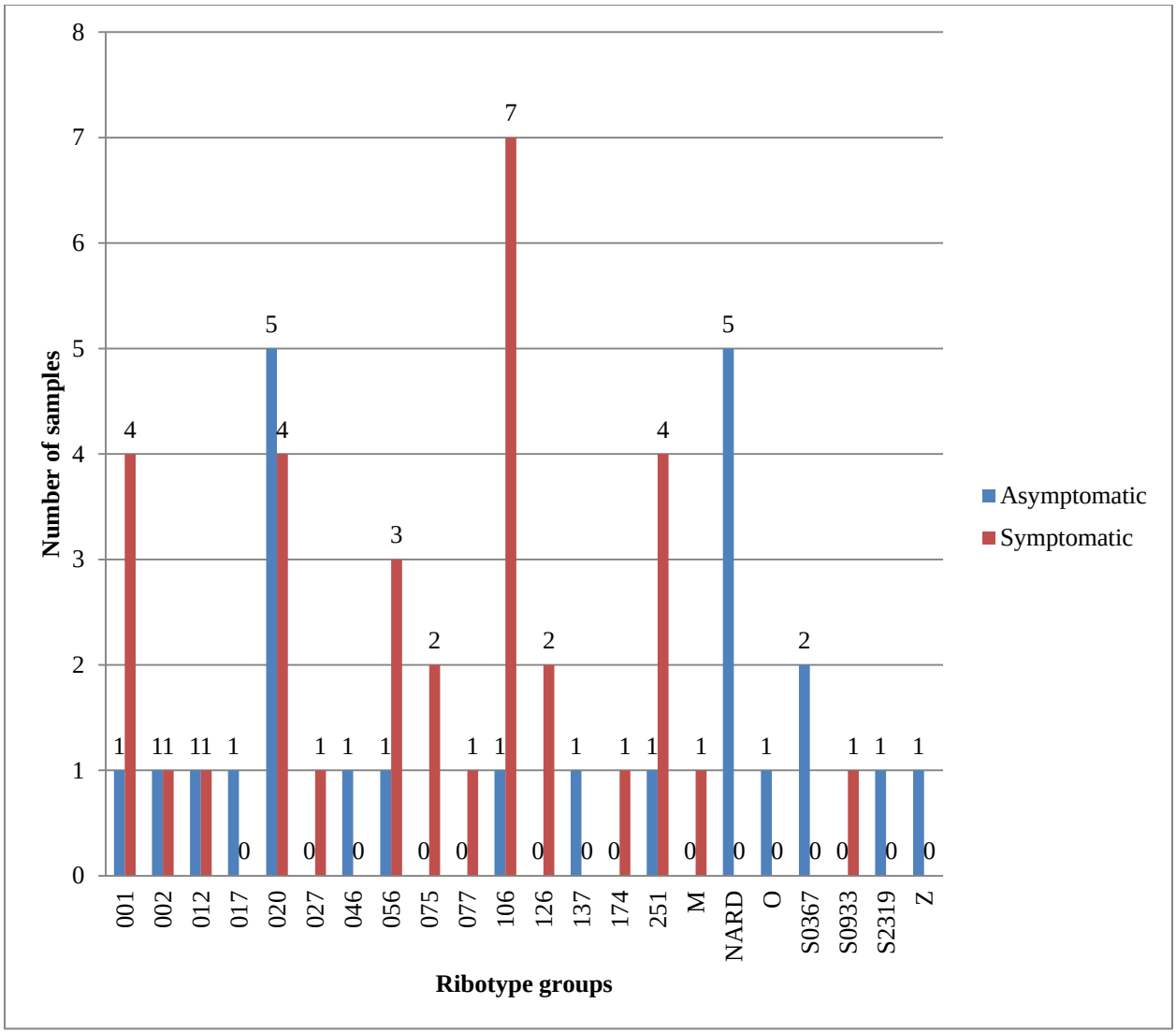


Figure 7 – Distribution of *C. difficile* ribotypes between symptomatic and asymptomatic patients at HSN

The ribotypes from all the samples were grouped by exhibition of symptoms (i.e. asymptomatic or symptomatic) to see if patients were more prone to developing symptoms with certain strains. Of interest was ribotype 020 and 106 as the most commonly found ribotypes in patients at HSN.

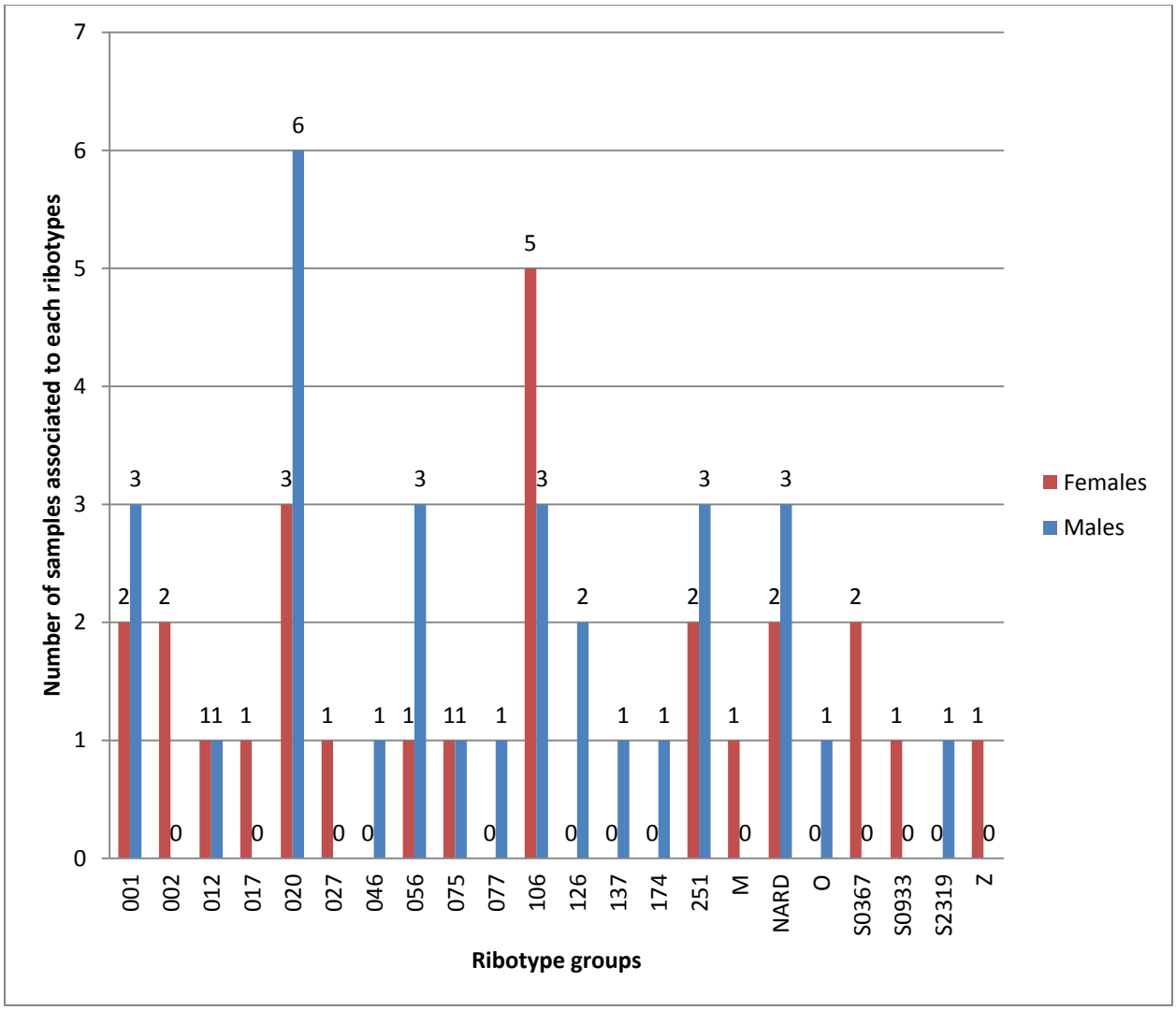


Figure 8 – Distribution of C. *difficile* ribotypes among male and female patients

The ribotypes were divided between the sexes to see if infection rates were different between males and females.

4.3 Biology of *C. difficile* strains

4.3.1 Microbial spore load

The range of the spores that was found in patients stools varied vastly from 196 spores/g of stool to $2.6x10^7$ spores/g of stool. The average quantity of spores was found to be $2.4x10^6$ spores/g of stool with a median of 119,481 spores/g of stool.

The samples were first divided by symptomatic and asymptomatic. There were no significant differences between the groups (p=0.3528). The mean of the symptomatic group was $1.8x10^6$±6.7x105 spores/g of stool while the mean for the asymptomatic group was $3.4x10^6$±$1.6x10^6$ spores/g of stool. Due to the large variance in the spore load in stool, the standard deviation was quite large (Figure 9).

The samples were then grouped by sex. Once again, there were no significant differences between the groups (p=0.0870). The mean for the males was found to be $1.2x10^6$±$6.1x10^5$ spores/g of stool while the mean for the females was $4.0x10^6$±$1.5x10^6$ spores/g of stool. Due to the large variance in spore load between all the samples, the standard deviation was quite large (Figure 10).

Age was taken into account to see if it played a role in the quantity of spores found in the patients' stool. The ages were divided into groups of 10 years (21-30, 31-40, 41-50, 51-60, 61-70, 71-80, 81-90, and 91-100). No significant difference was found between the age groups (p=0.5803); however, the age group of 71-80 years old had a much larger variation in spore load in the stool compared to any other groups (Figure 11).

Comparison of the spore load in stools between all the ribotype groups was impossible as only 5 of the 21 ribotype groups found in patients had 3 samples or more (Figure 12). The 5 ribotype groups with more than 3 samples in each were 001, 020, 056, 106, and 251. A comparison of those groups revealed that there were no significant differences in spore load between any of the groups (p=0.9912) (Figure 13).

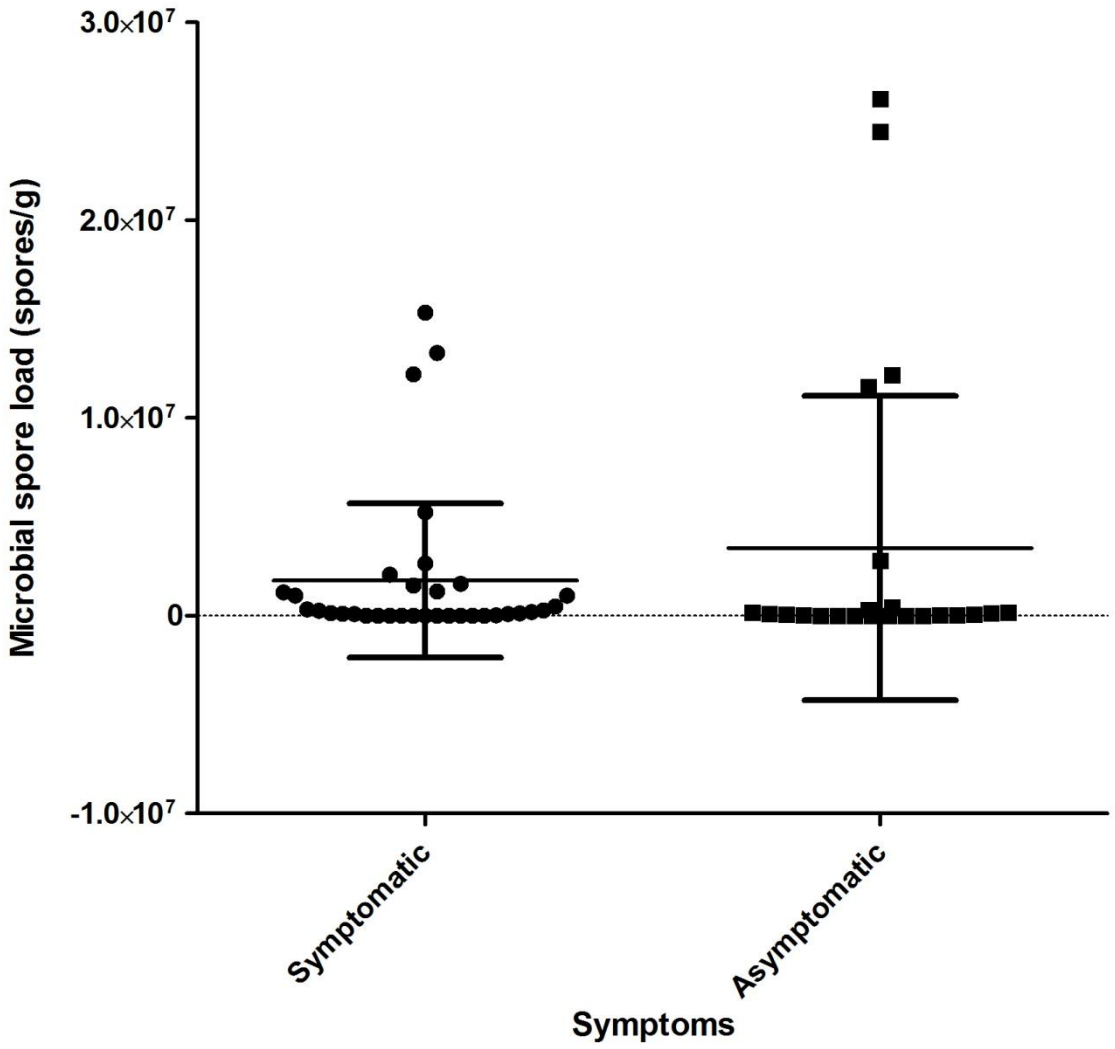


Figure 9 - Microbial spore load (spores/g of stool) found in the stools of symptomatic and asymptomatic patients at HSN

The average number of spores found in symptomatic patient was 1.8x106 spores/g of stool while for asymptomatic it was 3.4x106 spores/g of stool. No significant differences between the groups (p=0.3528). (Welch's t-test)

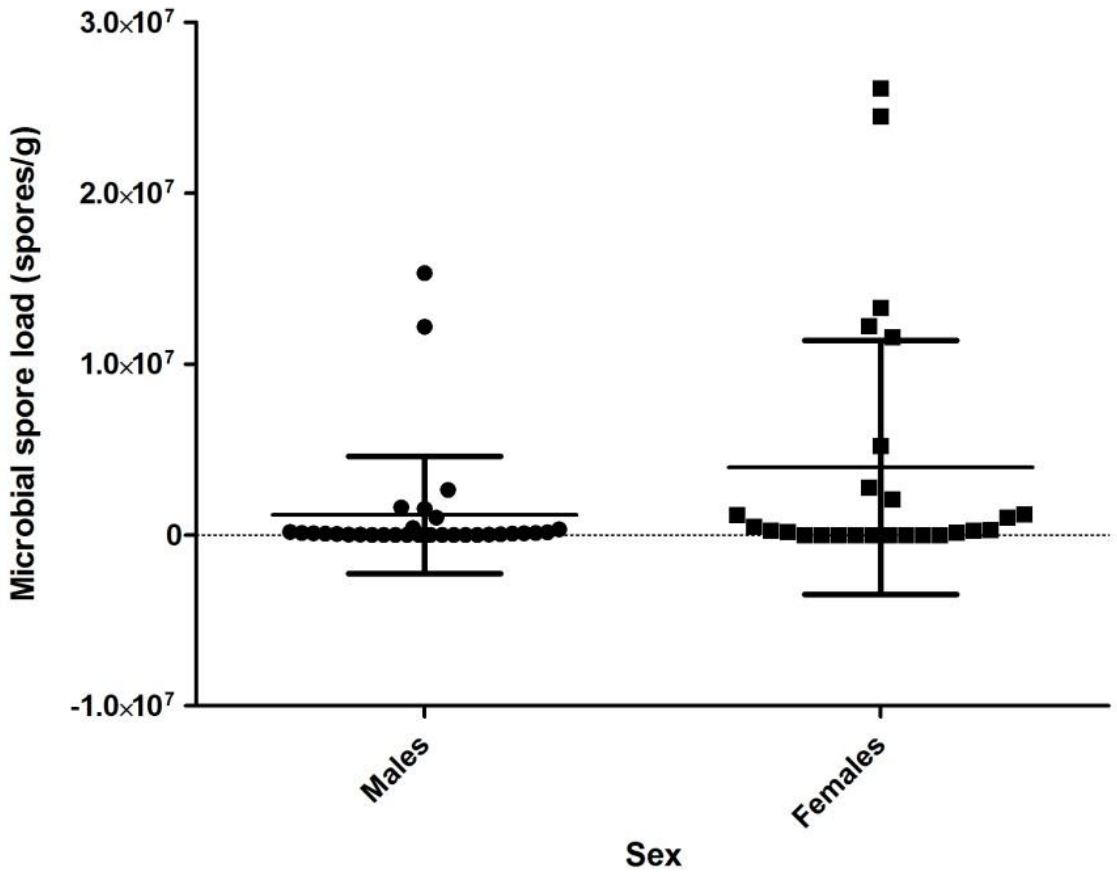


Figure 10 - Microbial spore load (spores/g of stool) found in the stool of male and female patients at HSN

A comparison between the mean of the microbial spore load (spores/g of stool) in male and female patients at HSN revealed that there was no significant differences in mean between the sexes (p=0.0870). (Welch's t-test)

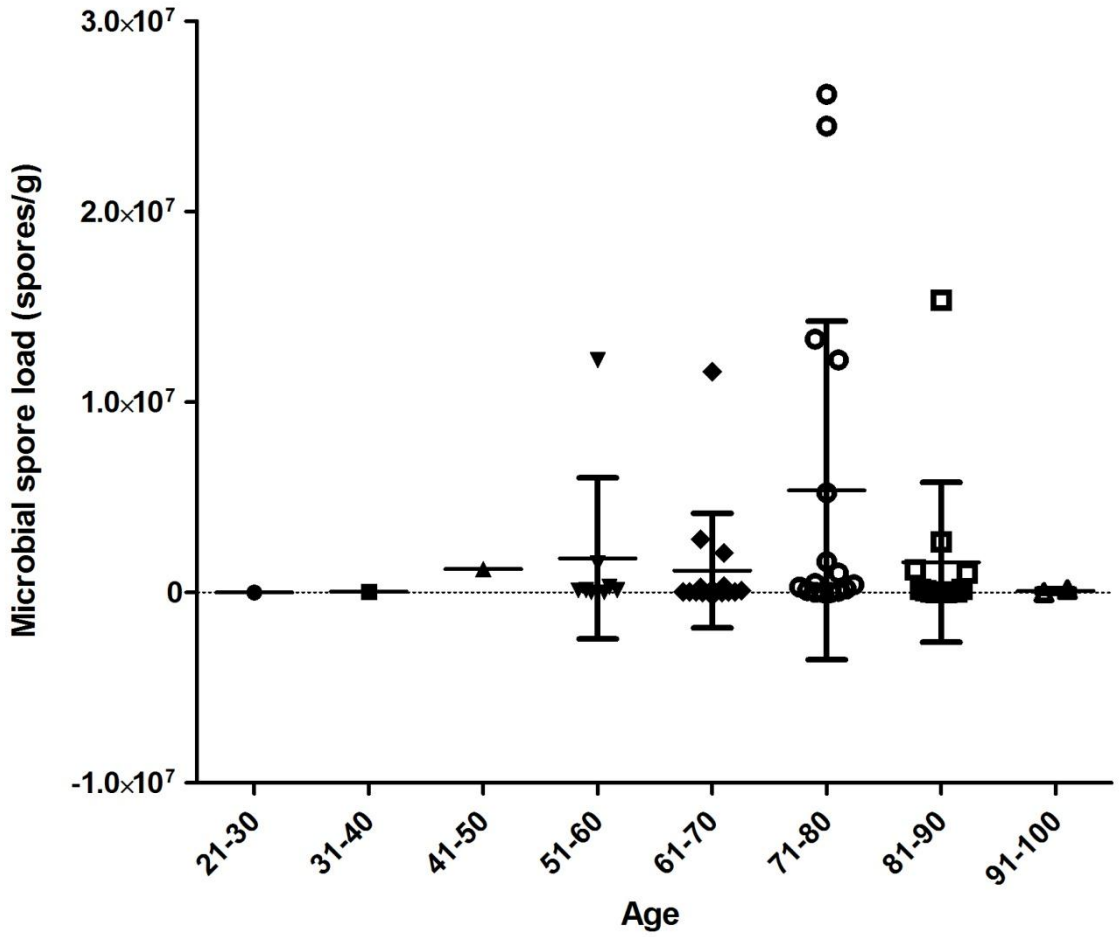


Figure 11 - Distribution of the microbial spore load in stool (spores/g) within 10 year age groups of patients at HSN

The microbial spore load in stool (spores/g) of each patient in the study was placed in their respective age groups. The ages were divided into groups of 10 years (21-30, 31-40, 41-50, 51-60, 61-70, 71-80, 81-90, and 91-100). There were no significant differences between the age groups (p=0.5803), however it is worthy to note that the 71-80 years old group had a much larger variance in quantity of spores in their stool. (One-way ANOVA)

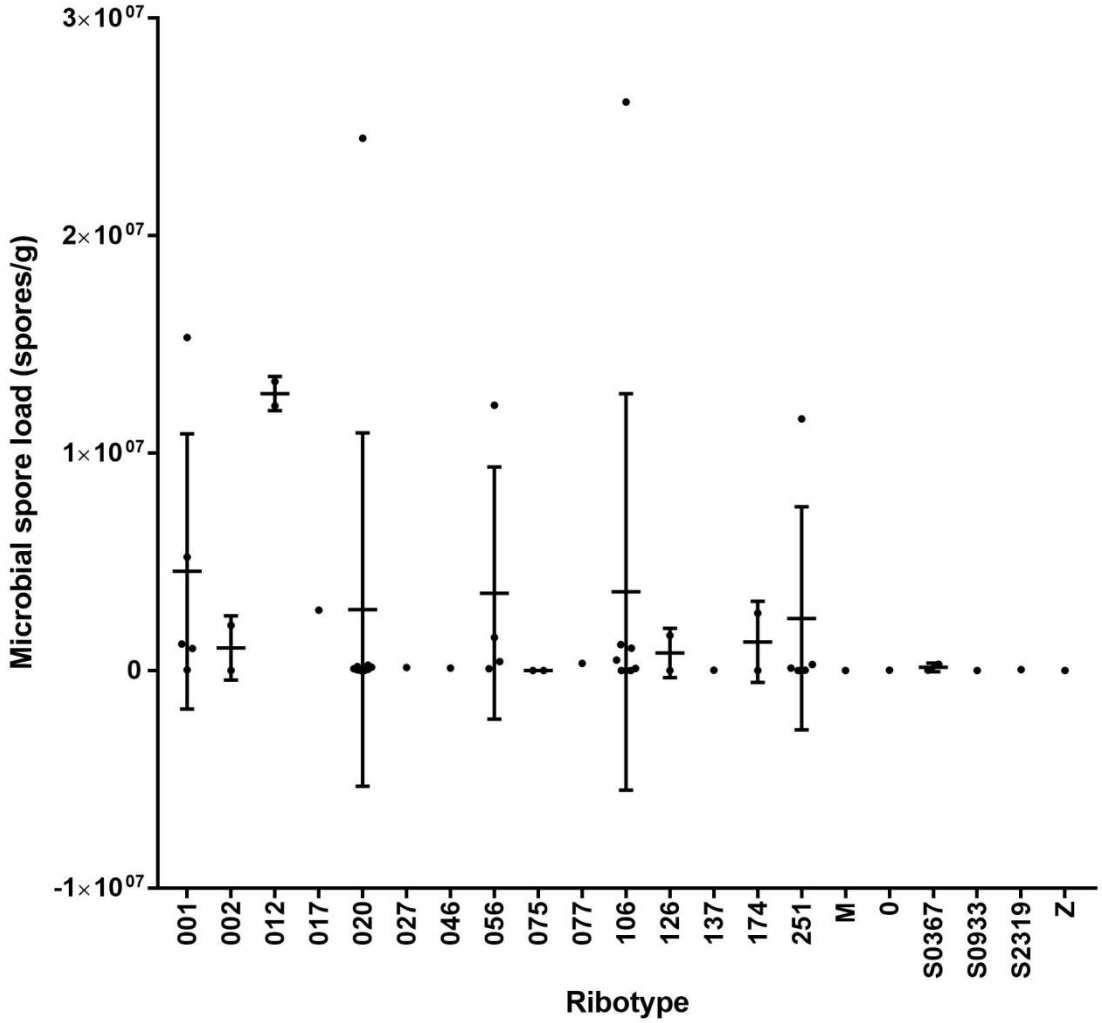


Figure 12 - Distribution of the microbial spore load in stools (spore/g) within ribotype groups found in patients at HSN

Depicted in figure 12 is the distribution of the microbial spore load in stools (spores/g) across all the ribotypes. It was impossible to do any statistical analysis on all the groups as some of the groups had fewer than three samples.

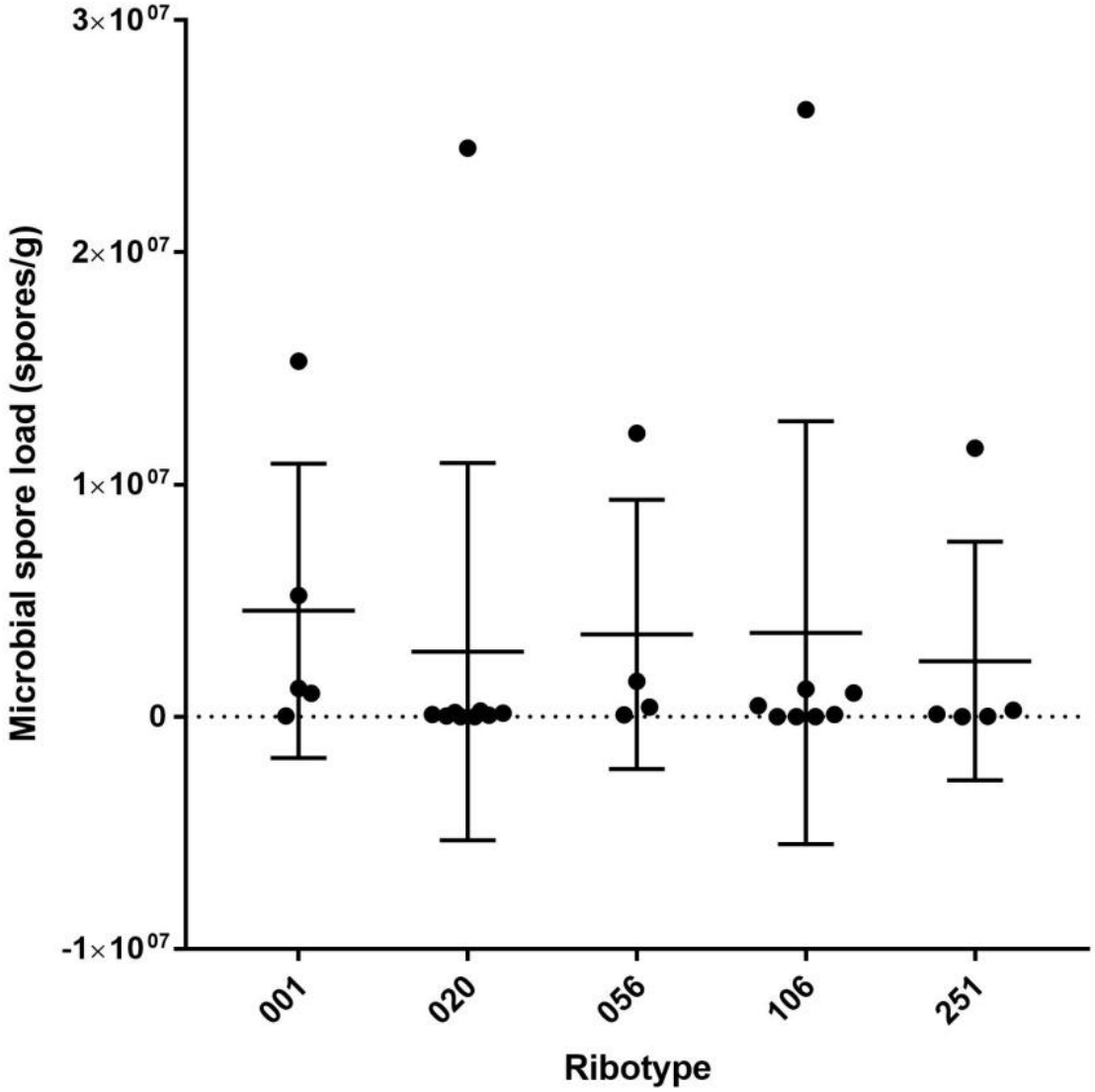


Figure 13 - Distribution of the microbial spore load in stools (spores/g) within the most common ribotype groups found in patients at HSN

The most commonly found ribotypes in patients at HSN (001, 020, 056, 106, and 251) were compared. Due to the great variance in spore load in each of the groups, there were no statistical differences between any of them (p=0.9912). (One-way ANOVA)

4.3.2 *In vitro* toxin production

As was the case with the microbial spore load, some of the ribotype groups were too small to be able to run any statistical analysis. Figure 14 shows the level of toxin produced in each ribotype group, while figure 15 shows the level of toxin produced in the most commonly found ribotype groups among patients at HSN, namely 001, 020, 056, 106, and 251. Obviously, non-toxigenic isolates, namely ribotypes 046 and S0367 produced no toxin. However, certain strains that were in ribotype groups 017, 106, and 251 did not produce any toxins, even though they are known to contain the PaLoc, and other strains from the same group did produce toxin.

All the samples from ribotype groups 001 and 020 produced relatively the same level of toxins, at an average of a dilution factor of 10.37 and 9.30, respectively. Ribotype 020 had some variance, ranging from 9.90 to 10.81 with a mean of 10.46. Ribotypes 106 and 251 showed the greatest range of variance, both having some isolates that did not produce toxin ranging to a dilution factor of 11.46 and 11.19 respectively. There was however no statistical significance between any of the ribotype groups (p=0.4330).

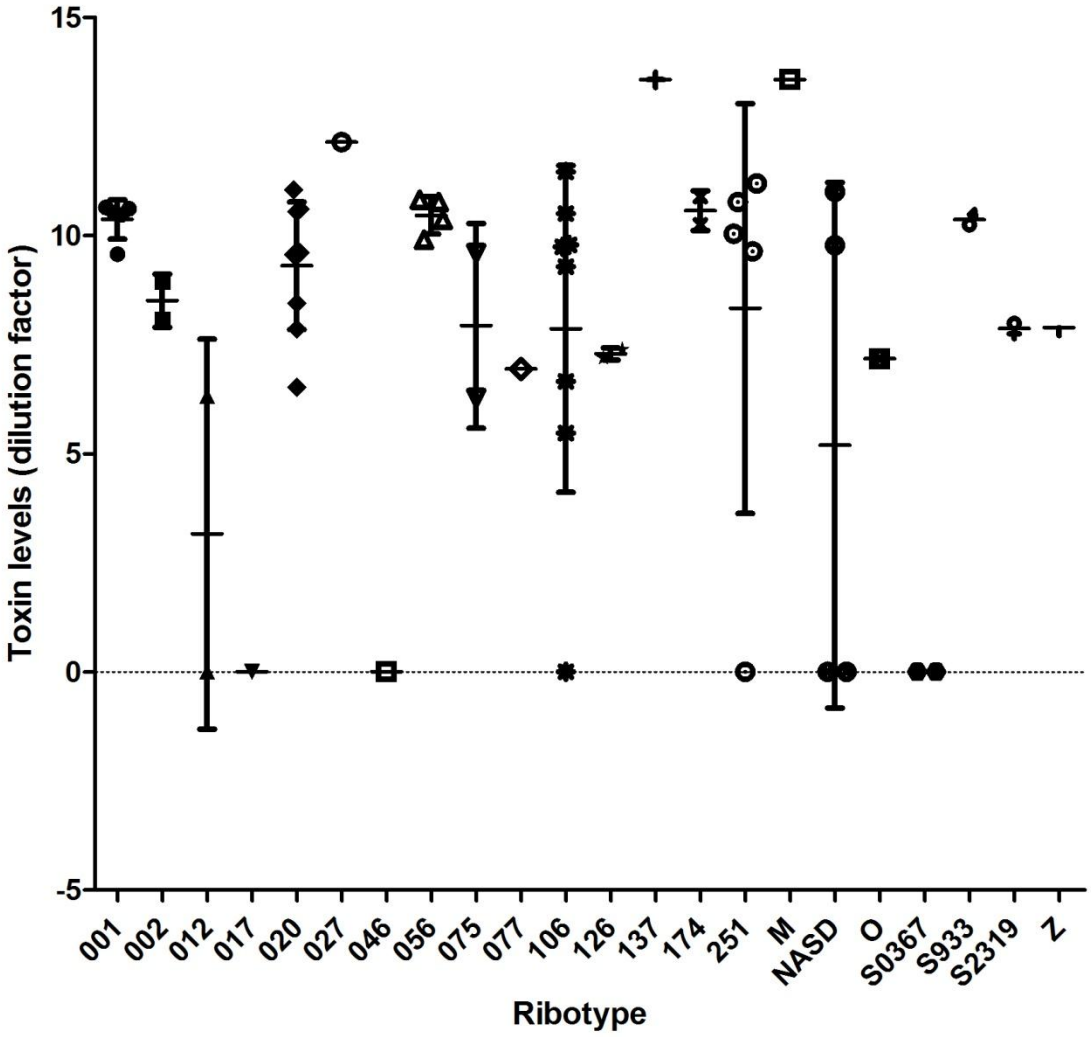


Figure 14 - Toxin levels produced (dilution factor) by *C. difficile* isolates in each ribotype group from patients HSN

Toxin levels of all *C. difficile* strains from patients at HSN were grouped by ribotypes. Because a large number of the ribotype groups contained less than three samples, it was impossible to do any statistical testing. Known non-toxigenic strains from ribotype group 046 and S0367 did not produce any toxins. Conversely, some samples from known toxigenic groups 017, 106 and 251 did not produce any toxins as they should have.

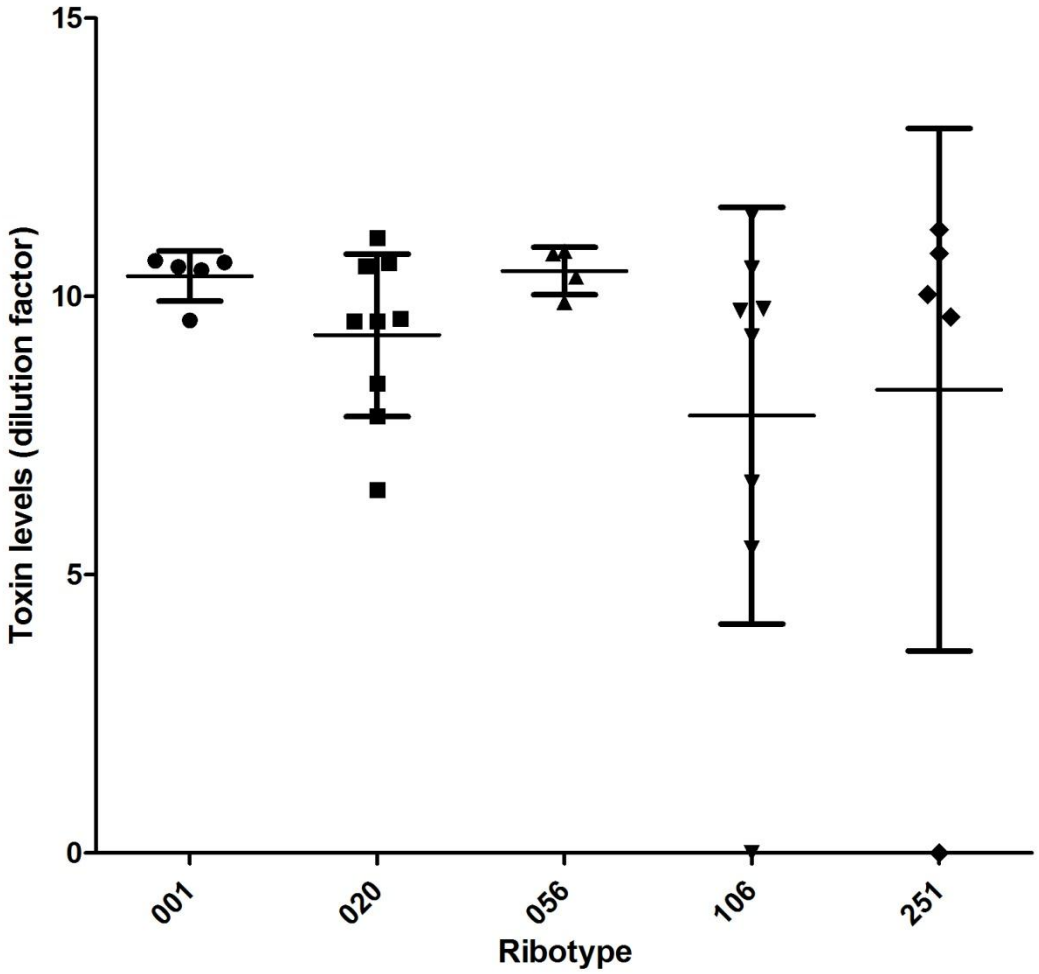


Figure 15 - Toxin level produced (dilution factor) by *C. difficile* strains in each of the most common ribotype groups from patients HSN

Ribotypes 001, 020, 056, 106 and 251 were compared for their toxin expression. All samples from groups 001 and 056 produced toxin with little variance, with an average dilution factor of 10.4 and 10.5, respectively. Ribotype 020 had more variance in production of toxin, with an average dilution factor of 9.3. Ribotypes 106 and 251 had the most variance, and also had strains that did not produce any toxin. Their average toxin levels were 7.9 and 8.3, respectively. There was no significant difference between each group (p=0.4330). (One-way ANOVA)

4.3.3 Classification of patients severity of symptoms

When the 57 cases in the study were reviewed for the severity of each patient's symptoms, 23 (40.3%) of the cases were deemed asymptomatic and as such were given a score of 0. From the remaining 34 cases, 22 (38.6%) were mild-to-moderate (1), 11 (19.3%) were severe (2), and only 1 case (1.8%) was severe and complicated (3).

The severity of the patients' symptoms was evenly distributed between both males and females; with a mean of 0.81±0.14 for the males and 0.85±0.16 for the females. There was no significant difference in severity between males and females (p=0.8554) (Figure 16). From the 26 females in the study, 10 were asymptomatic (0), 11 were mild-to-moderate (1), 4 were severe (2) and 1 was severe and complicated (3). From the 31 males in the study, 13 were asymptomatic (0), 11 were mild-to-moderate (1) and 7 were severe (2).

The severity of the patients' symptoms was analyzed by correlating against the ribotype of the isolates. Again, due to the large number of groups and small number of samples within each ribotype group, no statistical analysis could be done between all groups. However, the most common ribotypes (001, 020, 056, 106, and 251) were analyzed by means of a one-way ANOVA. While ribotype 020 did not have any severity cases above mild-to-moderate 1 (and the only ribotype group not to have any severity classified above mild-to-moderate), there was no significant difference between the ribotypes and the severity of symptoms they cause (p=0.2209) (Figure 17).

Microbial spore load was examined in contrast of the severity of symptoms exhibited by the patients. Asymptomatic patients had the greatest variance in spore count, ranging from 2169 spores/g of stool to $2.6x10^7$ spores/g of stool. While this was interesting to note, there were no significant differences between the severity of symptoms and the microbial spore load (p=0.7802) (Figure 18).

Finally, the level of toxin that each strain is able to produce was analyzed against the severity of symptoms shown in patients. In this case, there were also no significant differences between each severity group, including the asymptomatic group (p=0.4230). It was found that while patients may not have exhibited any symptoms, asymptomatic patients often have stains that produce equal levels of toxins as patients who are showing symptoms, anywhere from mild-to-moderate to severe and complicated. The average mean of toxin produced was 7.0 in asymptomatic, 8.9 in mild-to-moderate, 8.4 in severe, and 8.9 in severe and complicated (Figure 19).

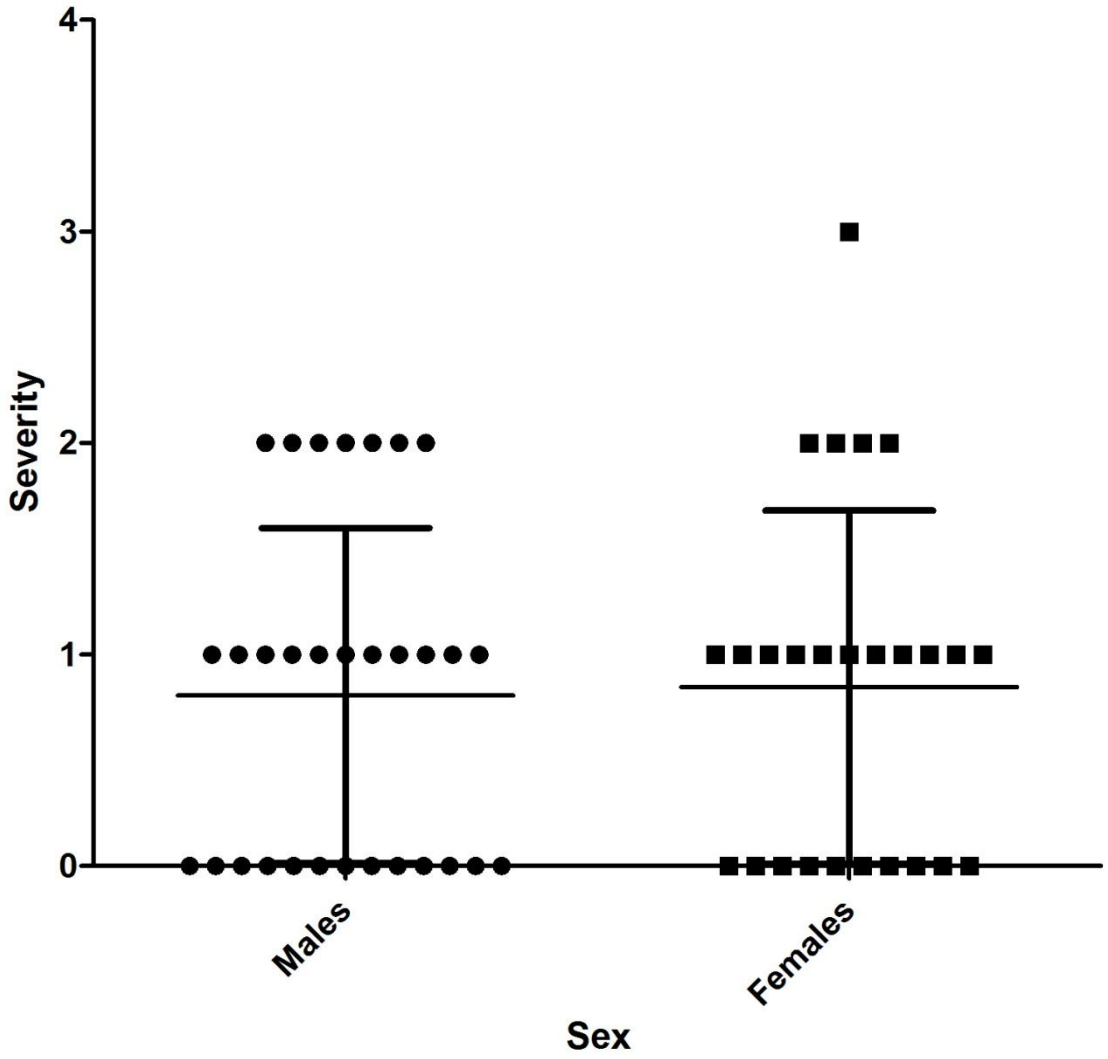


Figure 16 - Severity of patient symptoms distributed between sexes

Of the 31 males in the study, 13 were asymptomatic (0), 11 were mild-to-moderate (1) and 7 were severe (2). Of the 26 females in the study, 10 were asymptomatic (0), 11 were mild-to-moderate (1), 4 were severe (2) and 1 was severe and complicated (3). There was no significant difference between the two groups (p=0.8554). (Welch's t-test)

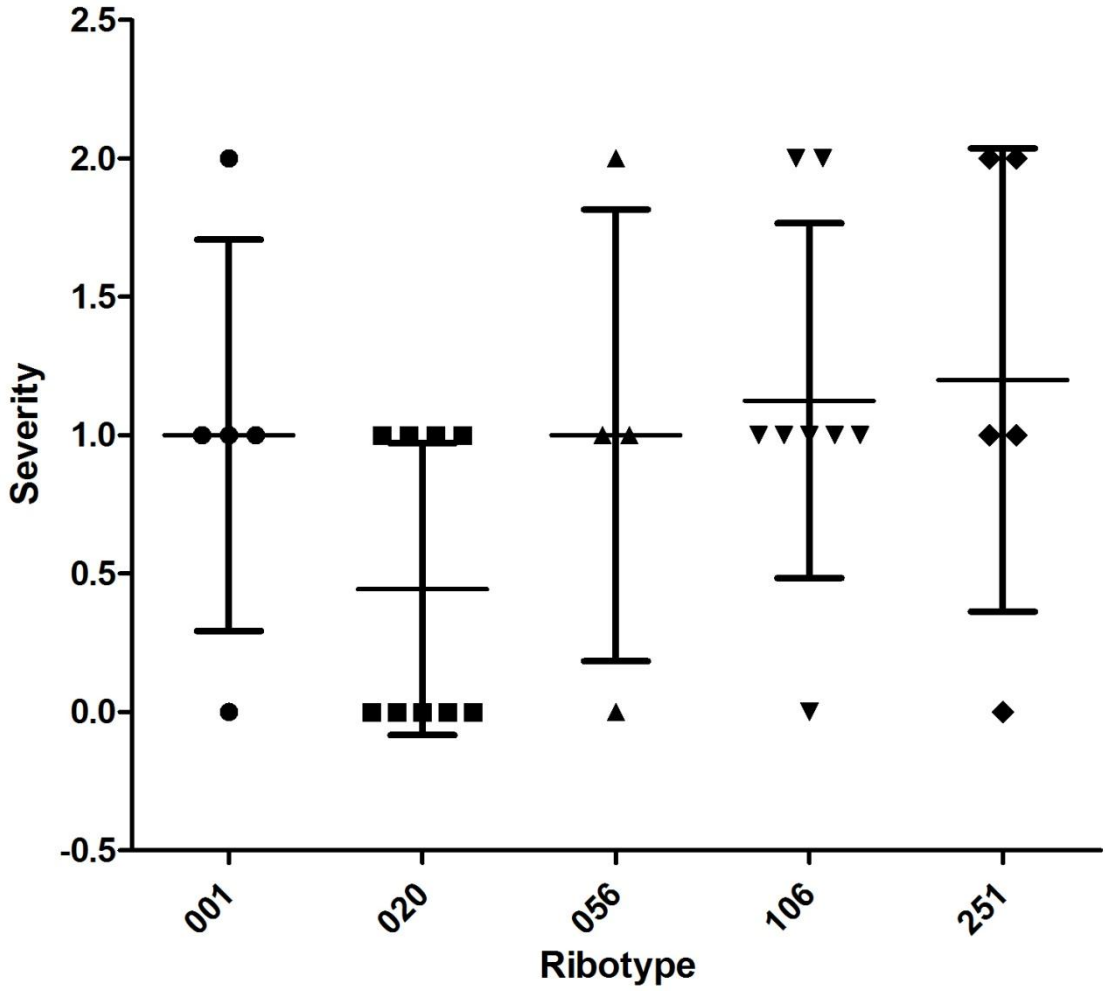


Figure 17 - Severity of the symptoms exhibited by patients do not correlate with the ribotype strain infecting them.

To see if isolates of certain ribotypes affect the severity of the infection, we assessed for association between the severity of symptoms and the most commonly found ribotypes at HSN. Each of the ribotype groups were also found among asymptomatic patients. Ribotype 020 did not cause more than mild-to-moderate symptoms in patients (1), while the other ribotype groups were isolated from patients experiencing severe symptoms (2), and none was involved in severe and complicated cases (3). No significant difference was found between ribotype groups (p=0.2209). (One-way ANOVA)

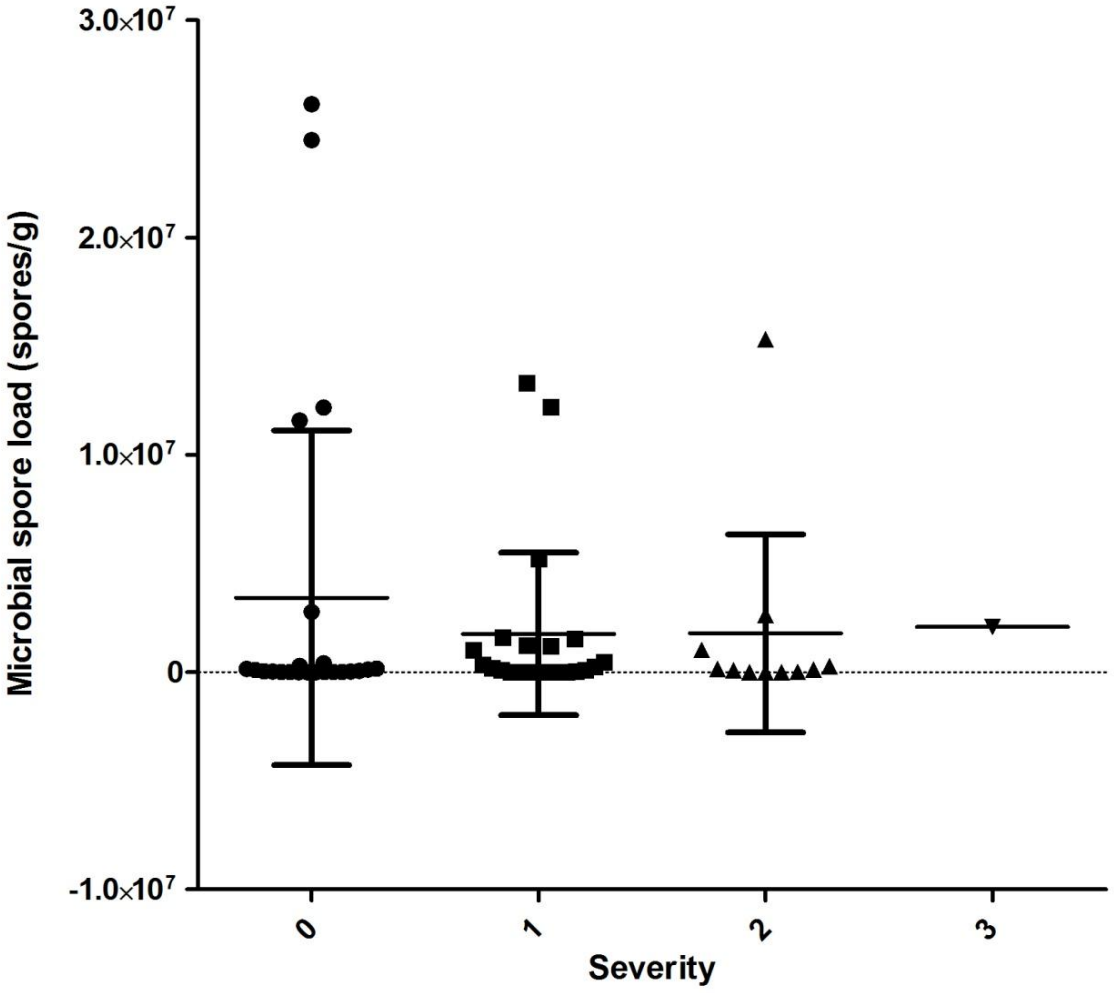


Figure 18 - Microbial spore load (spores/g of stool) found in each category of severity of symptoms exhibited by patients

The greatest variance in microbial spore load was found in asymptomatic patients, where the spore load ranged from 2169 spores/g of stool to $2.6x10^7$ spores/g of stool. However, there was no significant difference between the severity of the patients' symptoms and the microbial spore load found in their stools (p=0.7802). (One-way ANOVA)

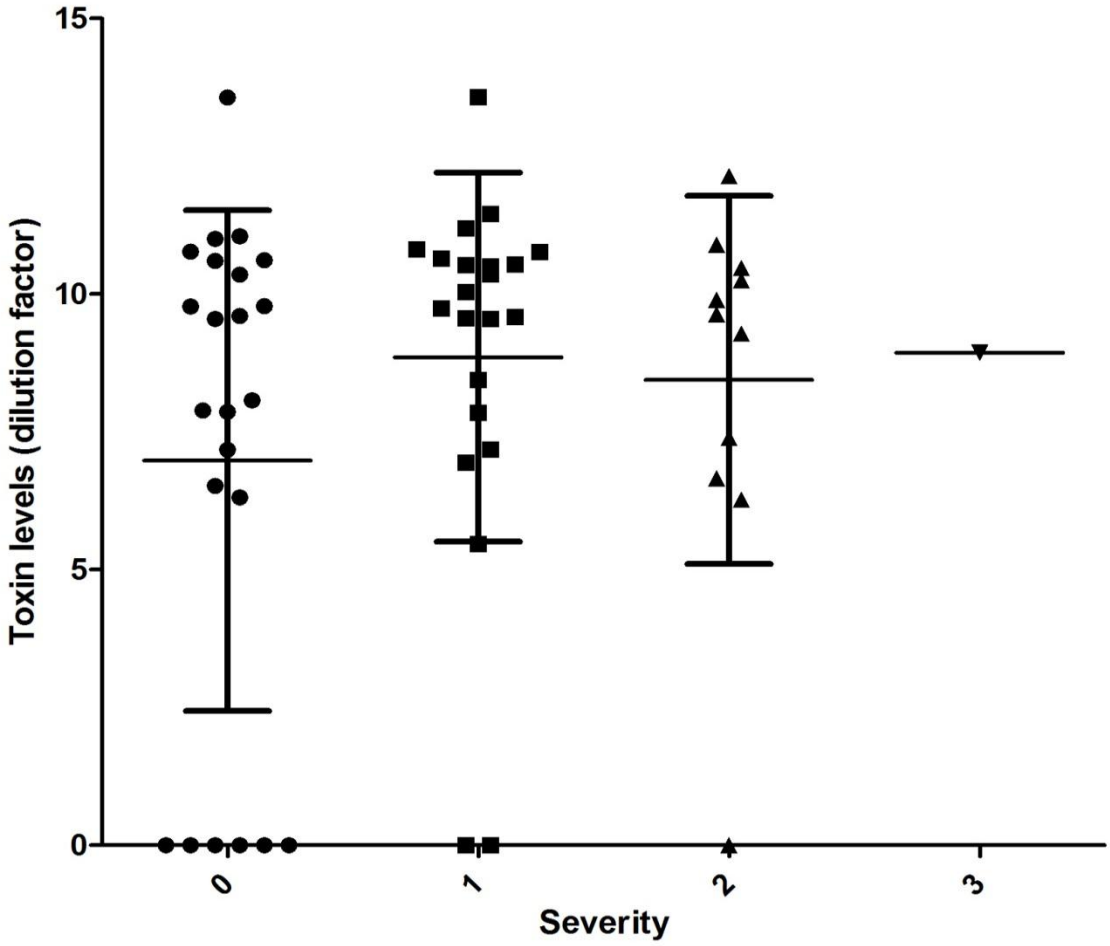


Figure 19 - Toxin levels (dilution factor) produced by *C. difficile* isolates among patients exhibiting different levels of severity of CDI associated symptoms.

Toxigenic C. difficile strains are often found in asymptomatic patients, and as can be seen in figure 19, the asymptomatic patients in the study were colonized with toxigenic strains in many cases. While the average toxin levels in asymptomatic patients are lower (7.0) than the symptomatic patients (8.9 in mild-to-moderate (1), 8.4 in severe (2), and 8.9 in severe and complicated (3) there was no significant difference in the ability of isolates between severity groups to produce toxin (p=0.4230). (One-way ANOVA)

4.3.4 Antibiotic susceptibility

4.3.4.1 Vancomycin

The population distribution of vancomycin resistance closely resembles that of EUCAST. The MIC50 was found to be 0.5mg/L, and the MIC90 was 2mg/L. The epidemiological cut-off (ECOFF) of vancomycin resistance is 2mg/L, and no strain was found to be over this cut-off point, meaning that all strains were found to be susceptible to vancomycin. Population distribution of vancomycin was comparable to that of EUCAST, with EUCAST MIC50 being 0.5mg/L, and MIC90 being 1mg/L (Figure 20).

4.3.4.2 Metronidazole

Metronidazole antibiogram profile showed a bimodal distribution. The MIC50 was found to be 2mg/L and the MIC90 was >256mg/L, while the EUCAST MIC50is 0.25mg/L and MIC90 is 1mg/L. 34 (61%) isolates were susceptible to metronidazole, while 22 (39%) were resistant (ECOFF 2mg/L). The EUCAST distribution was of lower MIC values, with only about 1% of the reported strains being resistant to metronidazole (ECOFF 2mg/L). 8 of the resistant strains were unaffected by metronidazole, and a zone of inhibition was completely absent in these strains (Figure 21).

4.3.4.3 Clindamycin

Clindamycin data did not show a typical normal distribution, and the data was fairly scattered. MIC50 was found to be 0.25mg/L, while MIC90 was found to be 64mg/L compared to EUCAST's MIC50 being 4mg/L and MIC90 at 256mg/L. The ECOFF was set to 16mg/L by

EUCAST, meaning that 50 out of the 57 strains tested (87.5%) were susceptible to clindamycin and 7 (12.5%) were resistant. Five strains (9%) were completely resistant to the E-test strip with a maximum MIC value of 256mg/L and did not show any zone of inhibition (Figure 22).

4.3.4.4 Ciprofloxacin

All 57 strains tested against ciprofloxacin were found to be resistant to the antibiotic. The MIC50 and MIC 90 were both >32mg/L, with the EUCAST MIC50 and MIC90 at 64mg/L (Figure 23). It was expected that all the strains would be resistant to ciprofloxacin as Norfloxacin was used in the CDSA media, and therefore any organism that grew on the media would have to be resistant to quinolones. This also shows the robustness of the C. difficile isolation method we used as no norfloxacin-sensitive colonies were found.

4.3.4.5 Amoxicillin

The MIC50 of HSN strains was found to be 0.125mg/L while the MIC90 was 0.5mg/L, which is lower than the EUCAST MIC50 (1mg/L) and MIC90 (2mg/L) suggesting that the strains found at HSN are slightly more susceptible than that in other regions. The MIC distribution for the HSN strains was skewed slightly to the right, while the EUCAST data skewed more towards the left. Unfortunately, EUCAST did not have an ECOFF value for amoxicillin; therefore, it is impossible to identify a concentration above which the cells exhibit resistance to amoxicillin (Figure 24).

4.3.4.6 Benzyl Penicillin

Similar to amoxicillin, the susceptibility to benzyl penicillin is slightly greater in the strains collected at HSN than they are in the data collected by EUCAST. The MIC50 for the HSN strains was 1mg/L and the MIC90 was 2mg/L, while in the EUCAST data the MIC50 was 1mg/L and the MIC90 was 4mg/L. EUCAST did not provide an ECOFF value, therefore we cannot determine the rate of the resistant isolates (Figure 25).

4.3.4.7 Cefotaxime

There was no data gathered by EUCAST for cefotaxime; therefore, there is no population data from which trends can be observed. Since no ECOFF was determined, we cannot conclude which isolates are indeed susceptible or resistant. However, we did determine that the MIC50 and the MIC90 of the HSN strains to cefotaxime to be >256mg/L, with 30 of the strains (54%) having no zone of inhibition, even at the maximum dosage for the E-test strips (Figure 26).

4.3.4.8 Imipenem

As was the case with cefotaxime, EUCAST has no data regarding antibiotic susceptibility of *C. difficile* to imipenem. The MIC 50 was found to be 4mg/L and the MIC90 16mg/L. 5 strains (9%) had no zone of inhibition at the maximum concentration of the E-test strip (32mg/L) (Figure 27).

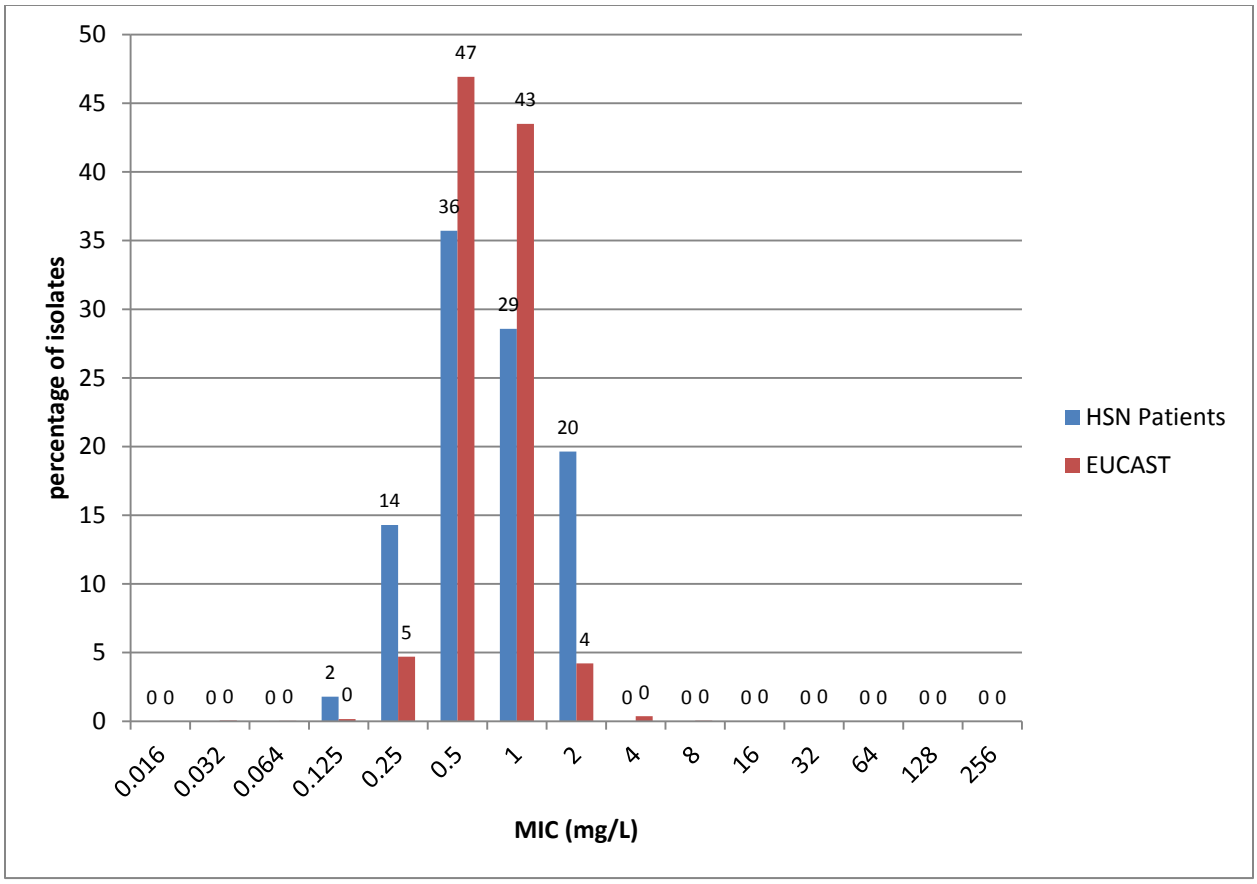


Figure 20 - MIC distribution for vancomycin susceptibility among *C. difficile* isolates found in patients at HSN

ECOFF for vancomycin as described by EUCAST is 2mg/L. All strains collected at HSN were susceptible to vancomycin, with a MIC50 of 0.5mg/L and a MIC90 of 2mg/L. The population distribution at HSN closely resembles the global population collected by EUCAST.

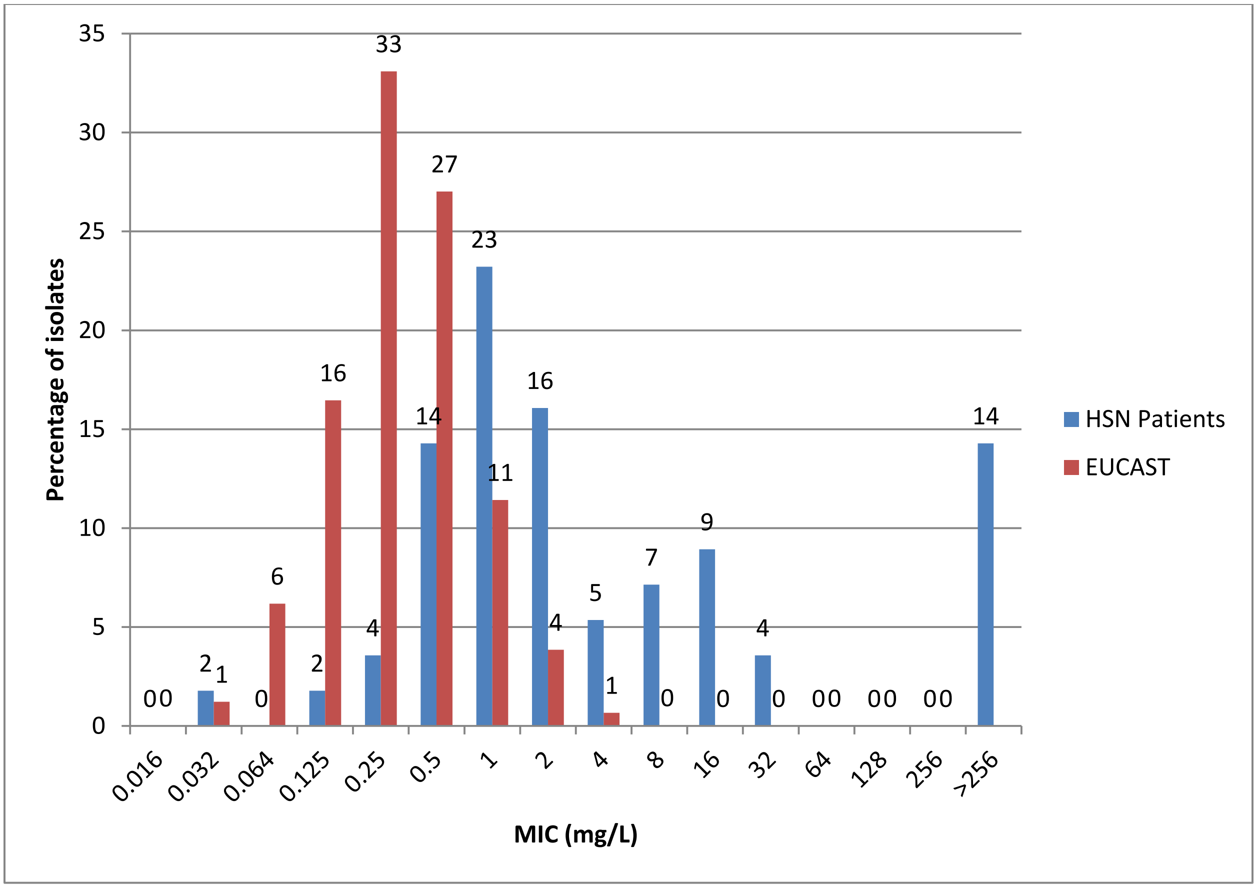


Figure 21 - MIC distribution for metronidazole susceptibility among *C. difficile* isolates found in patients at HSN

ECOFF for metronidazole as described by EUCAST is 2mg/L. The MIC value distribution for metronidazole susceptibility of C. difficile isolates at HSN shows trimodal with susceptible isolates below the 2mg/L, and resistant isolates above the 2mg/L line. 22 isolates were found to be resistant to metronidazole, with 14% being unaffected by highest concentration of metronidazole on the E-test strips.

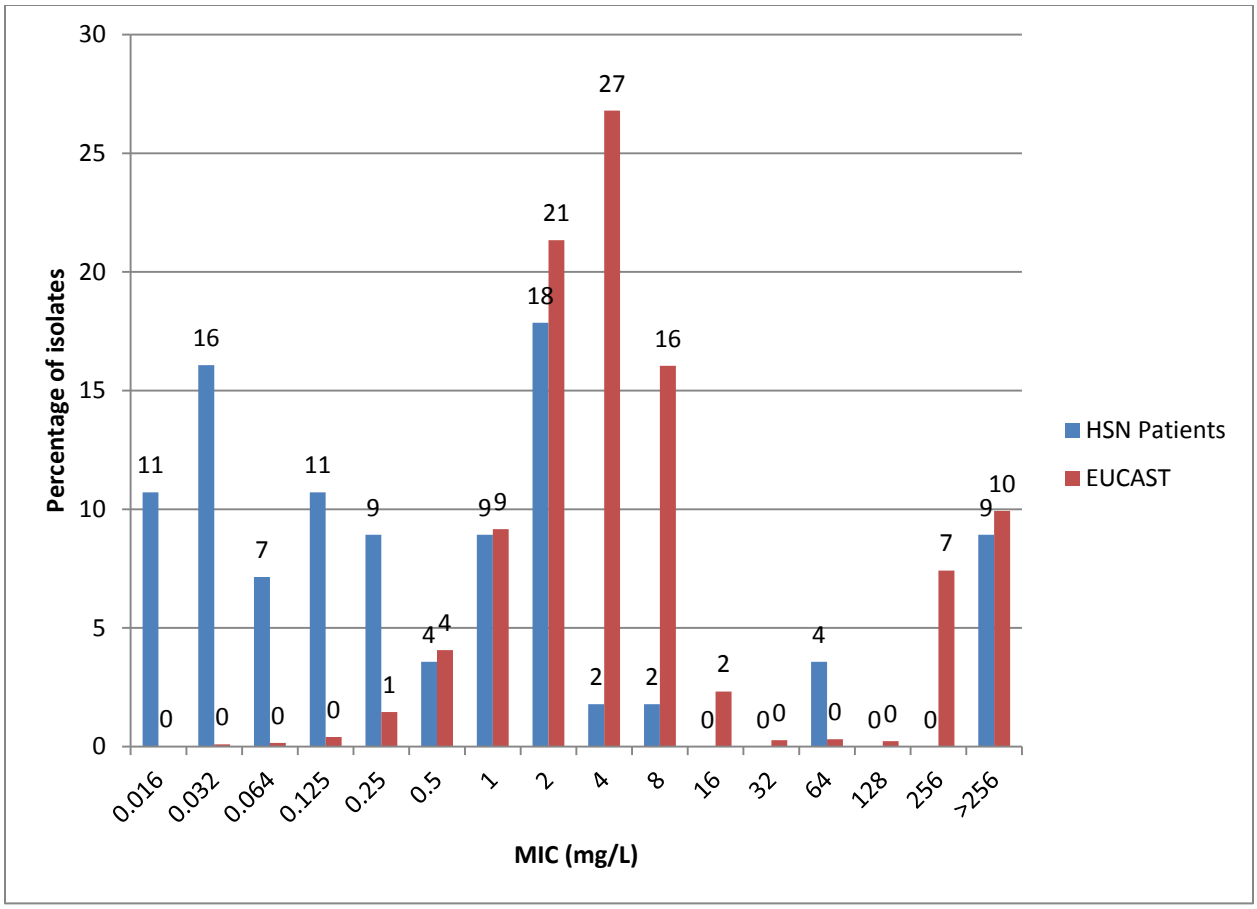


Figure 22 - MIC distribution for clindamycin susceptibility among *C. difficile* isolates found in patients at HSN

ECOFF for clindamycin as described by EUCAST is 16mg/L. The C. difficile isolates all reacted very differently to clindamycin, with 50 out of the 57 strains tested being susceptible and 7 strains being resistant.

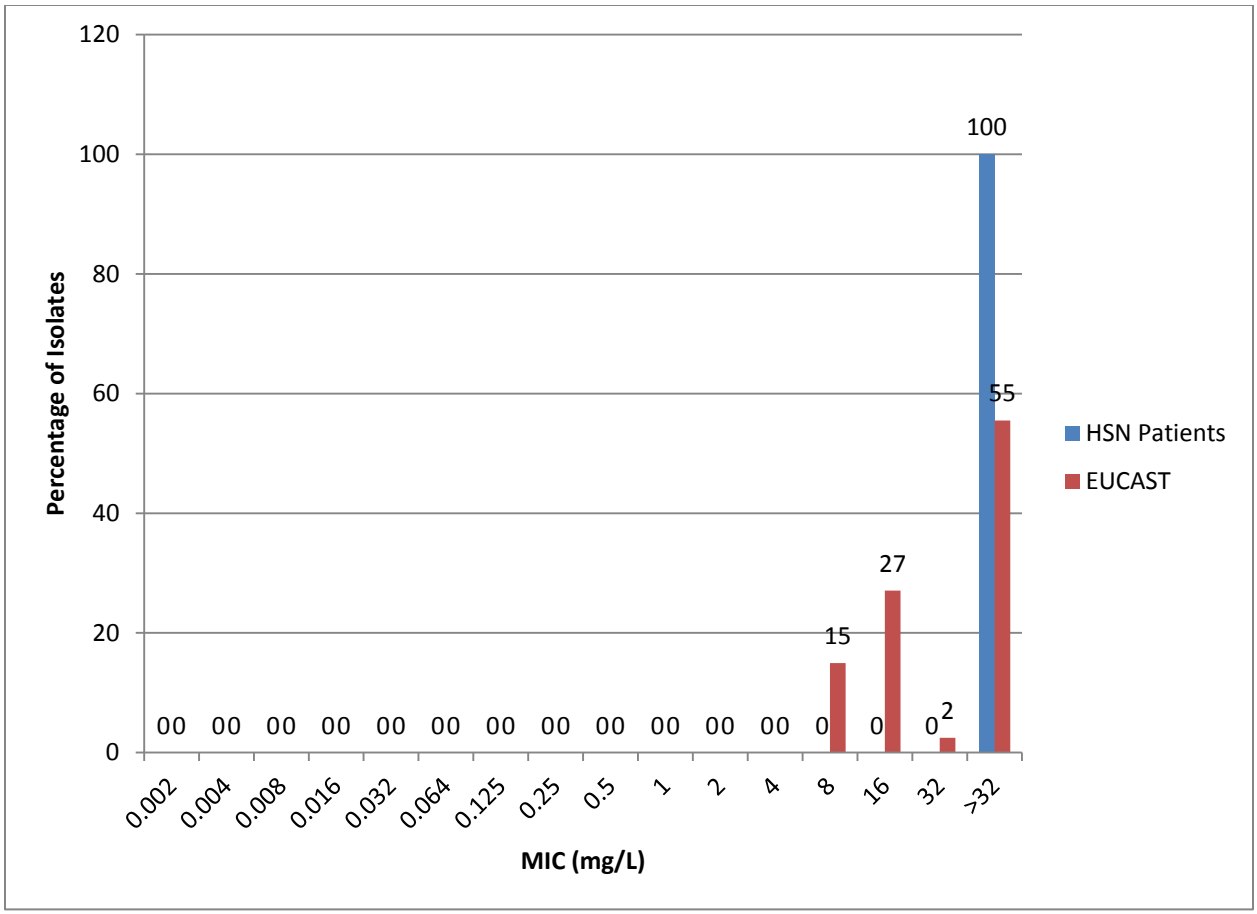


Figure 23 - MIC distribution for ciprofloxacin susceptibility among *C. difficile* isolates found in patients at HSN

No ECOFF was described for ciprofloxacin by EUCAST. However, none of the strains were affected by the E-test strips, and no zones of inhibition were recorded at 32mg/L.

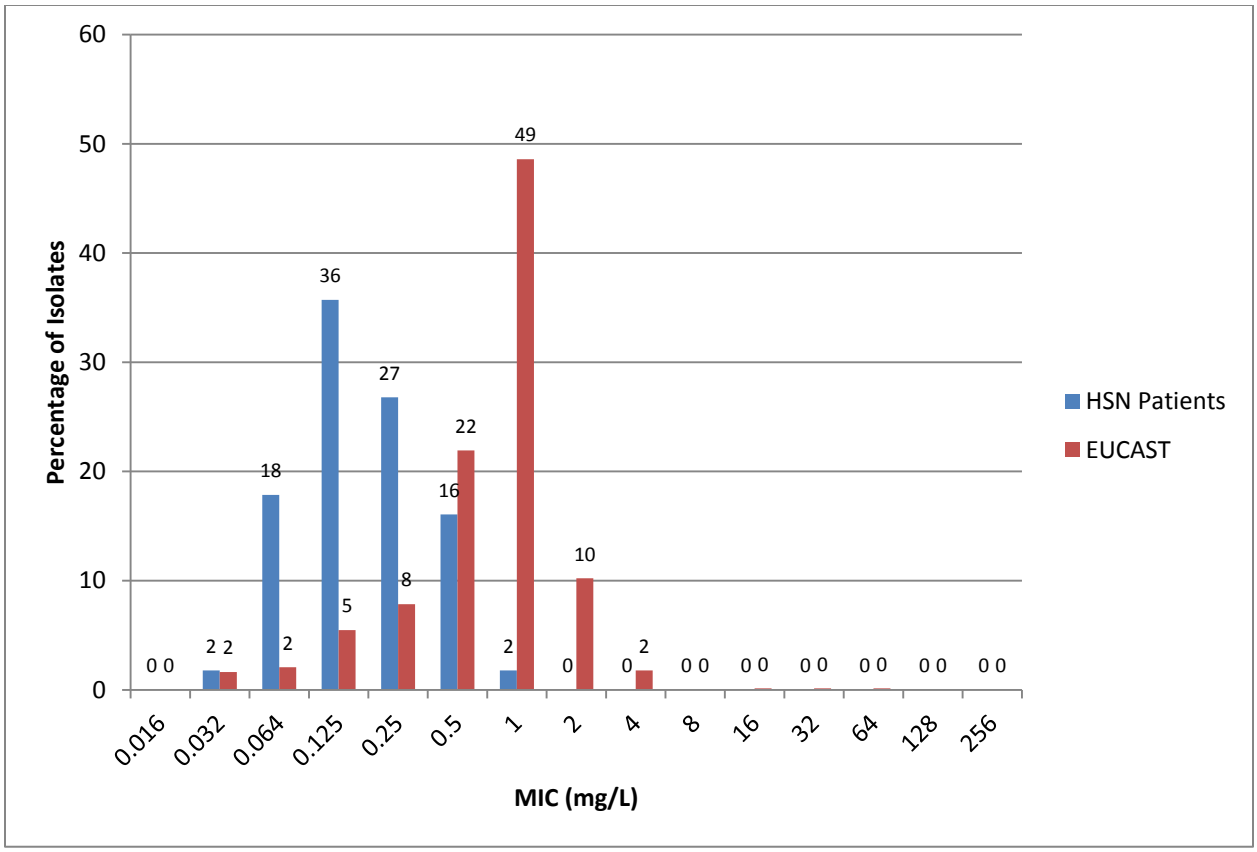


Figure 24 - MIC distribution for amoxicillin susceptibility among *C. difficile* isolates found in patients at HSN

No ECOFF was described for amoxicillin by EUCAST. The MIC50 (0.125mg/L) and MIC90 (0.5mg/L) were both found to be lower than the global population as collected by EUCAST (1mg/L and 2mg/L respectively).

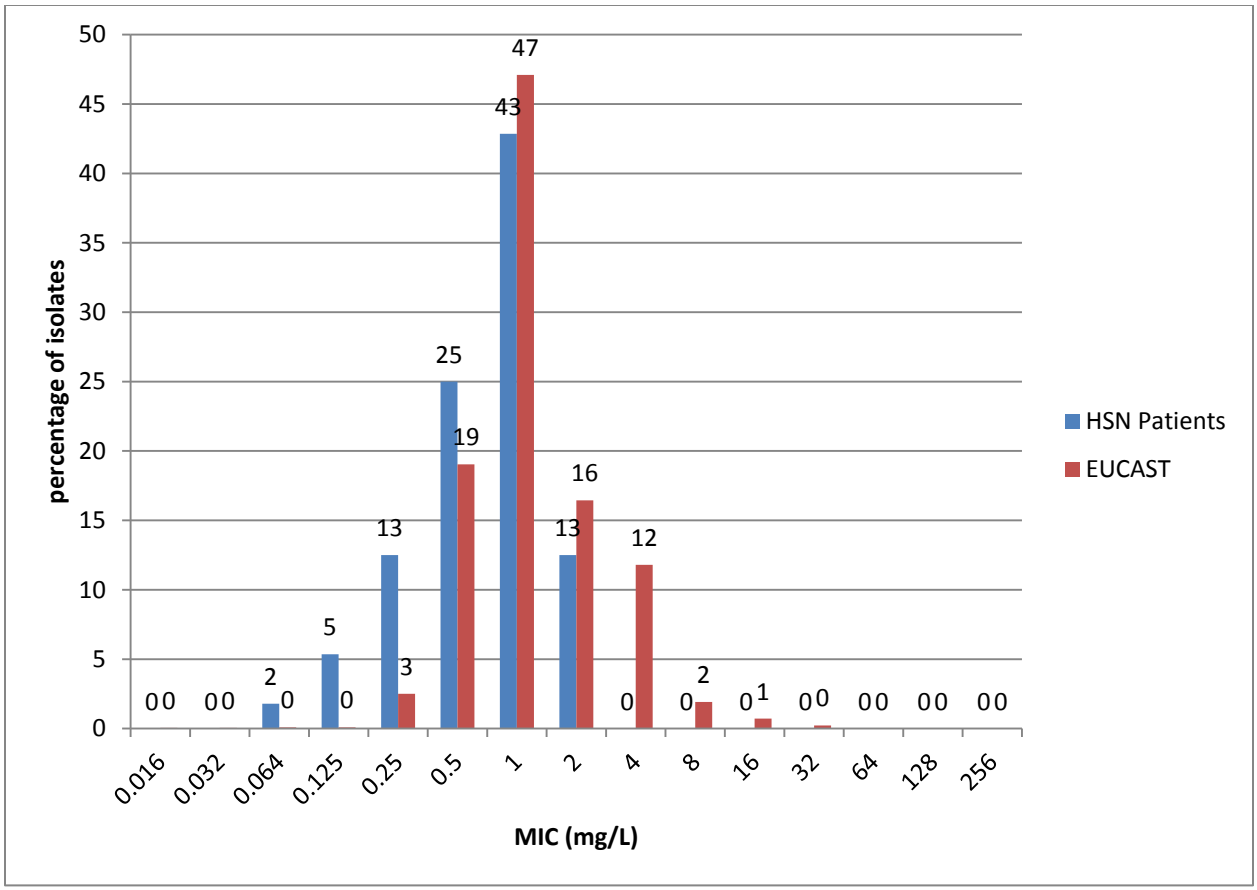


Figure 25 - MIC distribution for benzyl penicillin susceptibility among *C. difficile* isolates found in patients at HSN

No ECOFF was described for benzyl penicillin by EUCAST. The population distribution of susceptibility to benzyl penicillin is slightly greater at HSN than it is globally; the MIC50 was 1mg/L and MIC90 was 2mg/L at HSN compared to 1mg/L and 4mg/L respectively.

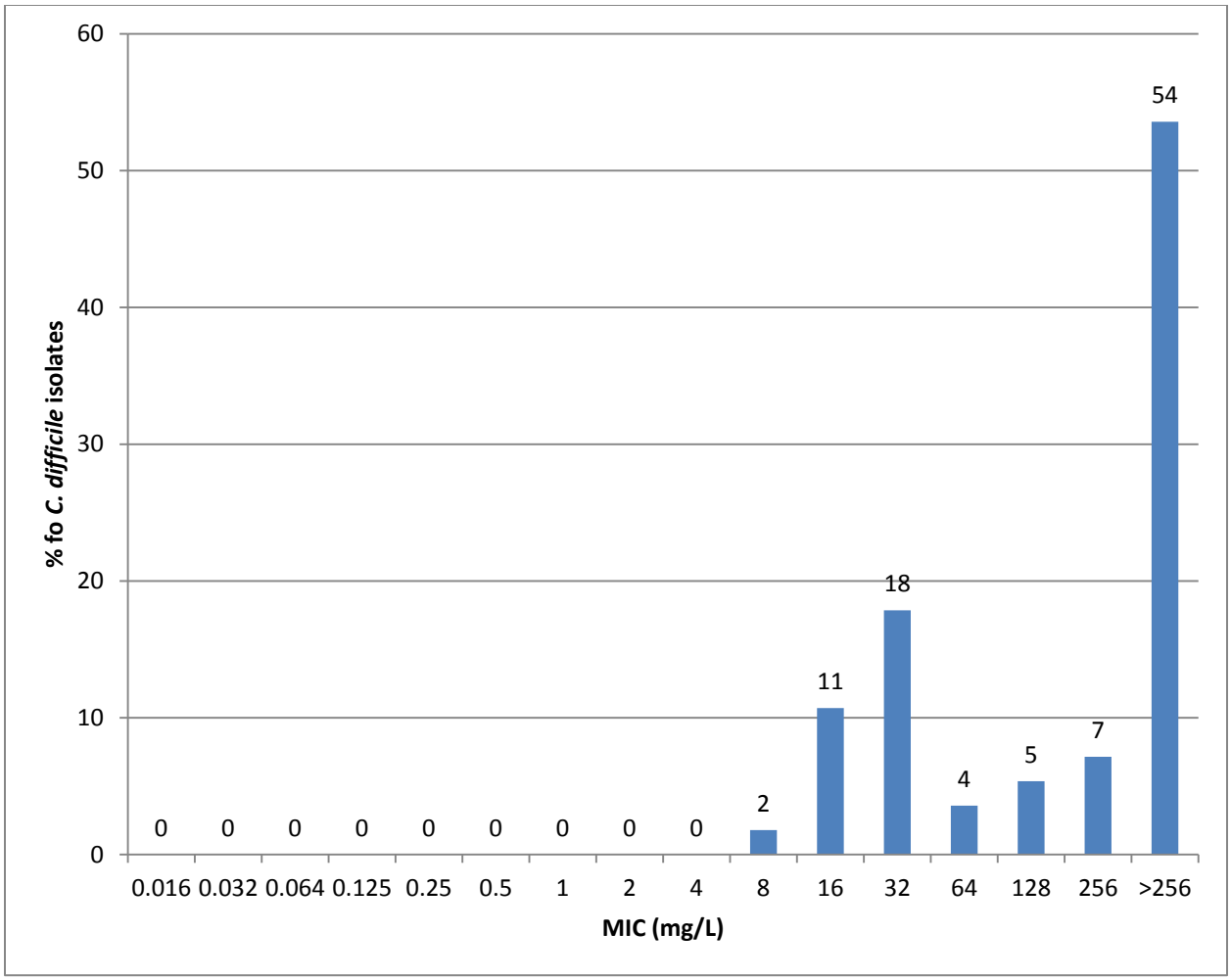


Figure 26 - MIC distribution for cefotaxime susceptibility among *C. difficile* isolates found in patients at HSN

No ECOFF was described for cefotaxime by EUCAST, nor was there data gathered. The majority of strains were unaffected by the E-test strip at a dosage of 256mg/L, suggesting that the strains are resistant to the antibiotic.

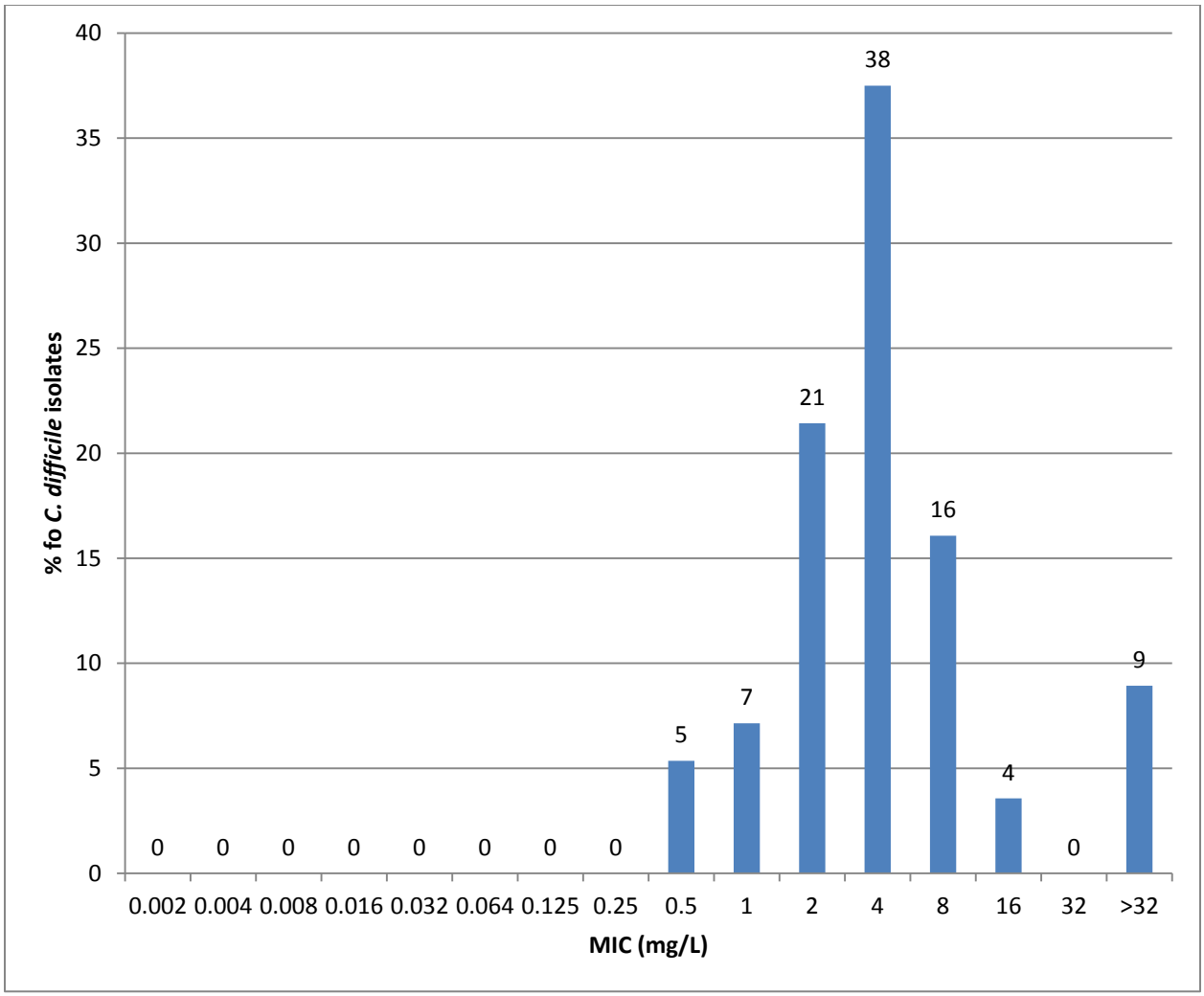


Figure 27 - MIC distribution for imipenem susceptibility among *C. difficile* isolates found in patients at HSN

No ECOFF was described for imipenem by EUCAST, nor was there data gathered. 9% of the strains were unaffected by the E-test strips at a dosage of 32mg/L. The MIC50 was found to be 4mg/L, and the MIC90 was 16mg/L.

4.3.4.9 Summary of antibiotic susceptibility comparison between HSN isolates and EUCAST

Following the comparisons between the isolates from HSN patients and EUCAST data, we can see that the HSN strains are less sensitive to metronidazole than the data given by EUCAST, equally sensitive to benzyl penicillin, vancomycin and ciprofloxacin, and more sensitive to clindamycin and amoxicillin.

5.0 Discussion

5.1 Sample processing and patient demographics

While being in an acute teaching hospital is a prime location for acquiring patient samples for *C. difficile*, we decided to only consent patients from two units at the hospital: the respiratory unit and the oncology and palliative care unit. These two wards were picked based on the number CDI reports which had occurred in recent months, as well as their high rate of hospitalization[67]. This, however, significantly lowered the number of opportunities to meet people who may be asymptomatic carriers. Furthermore, 1301 patients were approached to consent to participate in the study, and 626 (48%) of them agreed to participate. This already lowered the odds by more than 50%. Only about a third of those patients were able to provide a stool sample before being discharged (220 stool samples), and of those only 29 samples (13%) were positive for *C. difficile*. This is not a surprising rate, as it has been reported by several different studies that the carriage rate of *C. difficile* in healthy individuals in anywhere between 5 and 15% [9]. Unfortunately, one of the drawbacks to the rate of carriage in the study is that it involves only people who are admitted to the hospital. The two targeted wards (the respiratory disease and oncology wards) tend to have an older population; indeed, it was very rare that we met patients that were aged 50 or younger (three cases only). This left us quite in the dark in terms of rate of carriage in a population younger than 50 years old.

Medical waste samples received from HSN's clinical lab which were used in the study were procured during the period of patient consenting (November 2013 to July 2015), reducing the number of samples that we could use as we had received over 300 samples since the beginning

of the *C. difficile* projects in 2012. Future plans to restart accrual of patients to include all wards of the hospital over a greater period of time are in the works to overcome some of the shortcomings of this project.

The average age of all patients that participated in the study was 70.8 years old, and the average age of patients with symptomatic CDI was 74.5 years old. Between 2007 and 2012, the Public Health Agency of Canada reported the average age of patients with CDI for every year of the report, which ranged from 69.4 years old to 70.7 years old (Table 1) [6]. The patients who were colonized with *C. difficile* at HSN are close to that age range, albeit a bit older. As mentioned, one of the major risk factors to developing CDI is age, with a sharp increase in incidence for people over the age of 65 [1], [2], [18]. Indeed, 68.4% of the patients in the study were above the age of 65, and 86% of the patients were above the age of 60.

There were no significant differences found in age between the symptomatic patients and the asymptomatic patients (Figure 4a). While one would suspect that the age of the symptomatic patients would be greater than that of the asymptomatic patients, this may have been due to the fact that the majority of hospitalized patients are of older ages. It is a fair assumption that should members of the community in all age groups be included in a similar study, we might see a lowered age mean [68]. There were also no significant differences between the average age of patients between males and females. There have been some research focused on sex bias of *C. difficile* infections [69]. It is known that females exhibit stronger humoral and cell-mediate immune responses, however, this tends to change after menopause leading researchers to believe that this phenomenon is due to the variation in sex hormones between males and females [70].

Women tend to have a more robust immune response and this is partially attributed to estrogen. Estradiol hormones are considered to be more enhancing towards the immune system, while testosterone is found to be more immunosuppressive [70], [71]. During menopause, women's levels of estrogen drops considerably and no longer have an enhancing effect on the immune system [71]. Considering that most CDI cases are post-menopause, it is therefore not surprising that there is no sex discrimination in CDI.

5.2 Characterization of *C. difficile* strains

At HSN, the Cepheid GeneXpert platform is used for laboratory diagnostic testing of stool samples for *C. difficile* and other diseases. The GeneXpert, a real-time PCR-based instrument comes with ready-to-run cartridges that only need the stool sample to be homogenized in a bottle of pre-packaged reagents before being loaded into the cartridge [63]. This method of testing for *C. difficile* infection replaced conventional EIA in 2012 as the primary way of analyzing stool samples. The real-time PCR method detected higher rates of CDI in patients than the EIA method [61], and has the advantage of determining whether an isolate may be the hypervirulent strain NAP1/BI/027 by being able to detect the presence of *cdt* and a deletion at nucleotide 117 of the *tcdC* gene. 72% of the stool samples (41 samples) were only *tcdB* positive, with 14% of the samples (8 samples) being positive for *cdt*.

Most of the research has focused on *tcdA* and *tcdB,* and *cdt* was certainly overlooked. It only came into prominence when it was discovered as an additional virulence factor present in the hypervirulent strain NAP1/BI/027 in the early 2000s [24]. Even in this study, other than knowing whether it was present due to the GeneXpert results, no other work was performed in relation to

cdt. This I believe to be a gap in *C. difficile* research, one that will be filled as more and more researchers are becoming aware of the damage it can potentially do to its host [24]. It is known that the expression of binary toxin (*cdt*) contributes to the increased morbidity resulting in prolonged length of hospital stay [24].

Fourteen percent of stool samples (8 samples) were negative for all three criteria on the GeneXpert, but were found to be positive by culture. This means that the GeneXpert was unable to detect all patients colonized with *C. difficile*. The GeneXpert has certain disadvantages; while the rapidity of the test helps in quickly determining if a patient is *C. difficile*-positive to change the course of his or her treatment, certain strains are missed altogether, giving false negative results. This could have detrimental effects on such patients. A big factor in why 14 stool samples were negative by GeneXpert is most likely because all asymptomatic patients would not have been tested due to not meeting the criteria for CDI. Another possible reason for this is that the low levels of *C. difficile* may have fallen under the detection limit of the instrument [72]. Furthermore, some strains may have not met the criteria of the test; 4 of the strains in the study are *tcdA* positive but *tcdB* negative. Should those strains be *cdt* negative and not contain the deletion at nucleotide 117 of the *tcdC*, they would be missed altogether by the instrument. The false negative rate of the GeneXpert over a larger number of samples and longer period of study was found to be 2.2%, which is still considered a relatively low rate of failure [72]. Using primers for a gene such as *tpi* could help identify all cases of colonized *C. difficile* patients.

Characterizing *C. difficile* strains for toxin expression is important, especially for determining the presence of the PaLoc [64]. The absence of PaLoc accounts for many of the asymptomatic cases

of *C. difficile* colonization, but not all. Eighteen out of the 23 asymptomatic patients were colonized with toxigenic *C. difficile* putting them at risk of developing CDI should a course of broad-spectrum antibiotics be started. With this knowledge in hand, a physician could decide on a course of antibiotics that is also detrimental to *C. difficile*, such as metronidazole or vancomycin. However, toxin expression is not a standard clinical laboratory test; furthermore, it is conducted using the chromosomal DNA of cultured *C. difficile* cells, which takes 5 to 7 days to grow. This could be too late for use in patient management.

Seventy nine percent of the *C. difficile* isolates in the study were *tcdA* and *tcdB* positive. 9% were *tcdA* and *tcdB* negative, and 10.5% were *tcdA* positive and *tcdB* negative. A very small portion of the population (1.5%) contained the truncation in the *tcdA* gene. With 91% of the isolates known to be toxigenic, and only 60% of the study population showing symptoms of CDI, it is clear as to why knowing the toxin expression could help in prevention of the infection. To avoid this problem, the best course of action would be to test each patient that is a potential candidate for antibiotic therapy for *C. difficile* colonization (using conventional testing means i.e. GeneXpert at HSN), giving the physicians a more complete picture when choosing which antibiotic to treat their patients with.

Ribotyping has become a gold standard in epidemiological studies and in the classification of *C. difficile*. This method is fast, reliable, inexpensive and highly reproducible. The most frequent ribotypes at HSN found in this study were 020 and 106, which is consistent with previous results on the epidemiology of *C. difficile* at HSN [67]. Ribotype 020, with the highest frequency (9 cases, 15.8%), was distributed fairly evenly between symptomatic and asymptomatic patients. A

recent study has found that ribotype 020, a frequent strain in humans, is also found in different animals (such as cattle) and in water [17]. This could explain why it is a very common strain affecting humans worldwide [17]. As for ribotype 106, its presence was predominantly in symptomatic patients. This ribotype is also found in hospitals in England, and is one of the most predominant strains in the UK. It was described as being the second most frequently found strain at the District General Hospital in South East England after ribotype 027. It was found to cause severe CDI in 78% of CDI cases, and a mortality rate of 10.8% [73]. It is also important to note that prior work done at HSNRI had found a significantly high frequency of hypervirulent NAP1/BI/027 strain, which was the third most prominent strain infecting patients at HSN [67]. In this study, only one case of NAP1/BI/027 was found.

From our previous *C. difficile* study at HSNRI, we also discovered several new ribotypes that were unique to HSN. Ribotypes S0367, S0933 and S2319 were present in this study as well. The only Sudbury-specific isolate that was found in a symptomatic patient was S0933, which is also toxigenic. The fact that there are *C. difficile* isolates that are unique to Sudbury is very interesting, as it shows the existence of a large diversity among *C. difficile*. Furthermore, there are another 5 isolates that we could not match up to any other ribotype in our database (no associated ribotype in database, NARD). However, further testing on those isolates at another facility with a much larger database would corroborate whether they are indeed previously unidentified strains.

As part of our study, we wanted to see if there was a bias for ribotypes between sexes brought on by the sexual dimorphism in immune system between males and females as mentioned before in

the discussion. Unfortunately, the biggest problem with using ribotypes as a form of classification is the fact that there are over 300 different ribotypes described thus far [17]. This is quite problematic considering the rather small sample size that this study has compared the number of ribotype groups available. Many isolates found were isolated only once in a ribotype group, resulting in group sizes being too small for any statistical analyses. As mentioned, sequence-based methods are now becoming more accessible and much cheaper, and many researchers are slowly transitioning to that method of classification [15]. A classification system that is coming to prominence uses sequence-based classification to classify the *C. difficile* population into 6 phylogenetic clades (1, 2, 3, 4, 5 and C-I). This much smaller grouping system would benefit research greatly as the isolates are grouped in similarity of their genetic sequence, which would associate like with like as opposed to arbitrary grouping based on intergenic spacer regions.

5.3 Biology of *C. difficile* strains

5.3.1 Microbial spore load

Sporulation is an intricate microbial process that *C. difficile* employs to survive in harsh environments such as an aerobic environment or nutritional stress. Additionally, the spores are thought to be crucial for transmission from host-to-host [18], [37]–[39], [42]. It is for these reasons that we thought that we would find more spores in asymptomatic patients; since *C. difficile* isn't thriving in the colon, it would make sense it would sporulate more to propagate to other hosts. While we did see an elevated level of spore count among asymptomatic patients, there were no significant differences between the symptomatic and asymptomatic patients stool

samples (Figure 9). We also compared stool spore counts between males and females and between symptomatic and asymptomatic patients. There was a larger range in the spore count in females than there was in males, but still there was no significant difference between the two groups (Figure 10). When the patients were divided up in age groups of 10 years apart, the age range that had the greatest variance in spore load was the 71-80 year group (Figure 11). There was also no significant difference between the spore counts in the stools of patients of different age groups, which may be due to the small sample size.

The association of spore count with different ribotypes was investigated. Because of the large number of ribotypes, it was hard to discern if there was any one ribotype that produced more spores than any other. Because so many of the ribotype groups had lesser than three samples in each group, no statistical analyses could be performed (Figure 12). We thus decided to restrict our examination to ribotype groups that had more than three samples. Only 5 of the groups had 3 or more samples (ribotypes 001, 020, 056, 106, and 251). Each of the 5 groups had a very large variation in spore counts in the stool. Due to these large variances, we could not find any significant differences between the groups in spore count (Figure 13).

Spore count in stool may not be a relevant way of testing how effective a strain is at propagating itself. Firstly, the location and the size of the infection in the colon would both affect the number of spores in the stool. A smaller infection would logically have fewer spores as there are fewer vegetative cells. Secondly, the stage of progression of the infection would also affect the number of spores in the stool. If an infection has been persisting for a few days, it is more likely to have more spores in the stool than a new infection from newly colonized *C. difficile*. The effects of the

human immune system are also not being taken into consideration when doing a raw spore count. To have a better idea of how well the microorganism is at propagating itself, the spore count would need to be normalized against a vegetative cell count in stool. This can prove quite problematic as the cells die off quite quickly once exposed to oxygen; depending on the environment (dry vs moist). Vegetative cells usually die off within 15 minutes but can survive up to 9 hours [55], [74]. Furthermore, considering the number of different types of bacteria found in the human digestive tract and therefore in the stool, a selective and differential media would need to be used on stool that is relatively fresh.

5.3.2 *In vitro* toxin production

Unfortunately, due to the nature of the sampling and the time that elapsed before any test in this study was run, we could not test the toxin content of the stool samples. When stool samples are frozen, the toxin titres drop down significantly by day five [75]. When we tried to test the stools for toxin, we could not get a reading on the EIA test (data not shown). We still wanted to know how much toxin each strain could potentially produce, and to see if certain ribotypes could produce more or less toxin. It is already known that the NAP1/BI/027 produces 16 to 23 times more toxin than other PFGE types [73]. Again, due to the small number of samples in certain ribotypes, we could not perform any meaningful statistical analyses related to toxin production and ribotype.

In the most frequent ribotypes (001, 020, 056, 106 and 251), there were no significant difference in the level of toxin produced between different ribotype groups (Figure 15). There was very little difference between ribotypes 001 and 056. Ribotypes 106 and 251 however had a large

degree of variance, with both of these ribotypes having a sample that did not produce any toxin at all. Both ribotypes however were positive for PaLoc. Furthermore, each one of the strains was cultured on 3 separate occasions and the samples still did not yield any toxin. While this may be attributed to environmental and growing conditions, all other strains positive for the PaLoc grew and produced toxin. A whole-genome sequencing analysis may reveal if there is a mutation in the PaLoc that interferes with toxin production.

5.4 Classification of patients' symptom severity

Following the guidelines set by SHEA and IDSA, the severity of the symptoms from each patient in the study were identified. From the 57 samples of *C. difficile* that were in this study, only one patient was in the severity index 3 – severe and complicated. One of the key features of a severity index 3 is that patients are often in the intensive care unit as their symptoms are so severe. Since a large portion of the clinical samples and all of the consented patient samples came from the respiratory and the oncology wards, it is surprising that we did not have more severity index 3 patients. Linking the severity of the symptoms with the biology of *C. difficile* was key to this project. While describing characteristics of the bacteria is useful information, seeing how the biology affects the patient would lead to better CDI patient management in the future.

It did not appear that the sex of the patient affected the severity of the symptoms; symptom severity was distributed fairly evenly in both males and females (Figure 16). There is little evidence in the literature that sex plays a major role in the severity and the outcome of CDI [1], [69]. There was no evidence to suggest that strains from a specific ribotype group affected the

severity of symptoms in one type of patient than another (Figure 17). While it was quite interesting to note that no strain from ribotype 020 caused symptoms more severe than mild-to-moderate, there is nothing to suggest that it could not. Moreover, each of the other ribotypes (i.e. 001, 056, 106 and 251) were found in asymptomatic patients, as well as in patients with mild-to-moderate (1) CDI and severe (2) CDI. Other than non-toxigenic strains, it is impossible to predict the severity of the symptoms based on ribotype [76]. As was the case with asymptomatic and symptomatic patients, there was no significant difference between microbial spore load and severity of infection (Figure 18). Lastly, the potential levels of toxin that each isolate could produce did not affect the severity of the symptoms in patients. Stains that were high toxin producers were found in asymptomatic patients, and low toxin producing strains were found in patients with severe (2) CDI. For further studies, it would be useful to compare the level of toxin in patient stool to the severity of infection in patients to confirm this lack of relationship between ribotype and both toxin load and severity of infection.

5.5 Antibiotic susceptibility

The data available for susceptibility breakpoints for *C. difficile* is surprisingly low. Only 3 of the antibiotics used for this part of the project (vancomycin, metronidazole and clindamycin) had defined epidemiological cut-offs (ECOFF). These breakpoints are determined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or the Clinical and Laboratory Standards Institute (CLSI). There are two types of breakpoints defined: wild-type breakpoints and clinical breakpoints.

Wild-type breakpoints are determined from bacteria that have acquired resistance to a certain antibiotic. In the case of *C. difficile*, fluoroquinolone breakpoints would be considered wild-type breakpoints as *C. difficile* has acquired the gene for resistance to that type of antibiotics. Those cases are fairly easy to identify as there is little to no effect from the antibiotic during the test, leaving little to no zones of inhibition. Nowadays with whole-genome sequencing, it is easy to tell if indeed the organism has acquired an antibiotic resistance gene or genes, though it is not always clear if resistance is due to one gene or from a polygenic phenotype. Clinical breakpoints are harder to determine and require a large set of data to be able to derive the breakpoint. That is because the clinical breakpoints separate the strains based on their likelihood of killing by antibiotic treatment. Therefore in order to determine these breakpoints, data not only needs to include the MIC, but also the success or failure rate of antibiotic therapy [77]. For this reason, without breakpoints from such agencies as EUCAST and CLSI, we cannot say whether or not *C. difficile* is susceptible or resistant to a certain antibiotic; we can only report the trends observed.

EUCAST had no data compiled for either cefotaxime or imipenem. 54% of the strains tested against cefotaxime had no zone of inhibition at the maximal dosage of 256mg/L, suggesting that there might be an antibiotic resistance that was acquired in certain strains. This was also the case for imipenem, 9% of the strains had no zone of inhibition at the maximal dosage of 32mg/L.

Amoxicillin and benzyl penicillin did not have any ECOFF defined by EUCAST; they are however compiling data for these two antibiotics. In both cases, the HSN population distribution appeared to be more susceptible than the global population gathered by EUCAST. The MIC50 and MIC90 were both lower than that reported by EUCAST.

Ciprofloxacin does not have an ECOFF defined by EUCAST. Ciprofloxacin is a fluoroquinolone, and *C. difficile* has an antibiotic resistance mechanism to fluoroquinolones, so it was not surprising that 100% of the strains had no zones of inhibition at the maximal dosage of 32mg/L [47]. Conversely, all strains were susceptible to vancomycin, which is the antibiotic used to treat CDI when metronidazole fails [1].

The results for clindamycin were the most erratic from all the antibiotics tested; there were no discernable trends observed, and 7 of the 57 (13%) isolates were resistant to the antibiotic.

The levels of susceptibility and resistance in *C. difficile* seen might be due to the frequency of use of the antibiotics at HSN versus elsewhere in the world. Metronidazole, under the name of Flagyl, was found to be given quite frequently to patients visiting the Respiratory unit and Oncology and Palliative care unit. Clindamycin and amoxicillin were found to be of lower use on those floors.

The most concerning results from the antibiotic susceptibility testing involved treatment with metronidazole. 22 isolates out of the 57 (38.5%) tested were found to be resistant to metronidazole. Moreover, 8 of those isolates were unaffected by the metronidazole and had no zone of inhibition, again suggesting that they may have acquired a gene for resistance to metronidazole. This is very concerning considering that metronidazole is the first line of treatment for CDI [9]. Based on these numbers, almost 40% of metronidazole treatments for CDI at HSN are likely to fail, resulting in persistence of disease. Knowing which isolates are resistant

to metronidazole ahead of treatment would benefit the patient in that they could be treated with vancomycin immediately and avoid the prolonged illness. In order to do this, antibiotic susceptibility testing in the means of E-test would need to be done to every patient strain.

6.0 Concluding remarks

Based on the research conducted in this study, we cannot find any evidence that we can predict the severity of symptoms that will be exhibited in patients based solely on certain genotypic and phenotypic characteristics of *C. difficile* such as toxin and spore production. The severity of the symptoms is likely due to a combination of bacterial characteristics and the patients' capability at mounting an immune response, and on the co-morbidities that may have an effect on the course of their illnesses.

It appears that ribotyping, while being quite a convenient and easy method of classifying strains of *C. difficile*, is not a very practical way of classifying the organism for the severity of infection. There are far too many groups, with often too little variance between each group. Ribotyping is, nevertheless, a well-established method of strain classification in epidemiological studies.

Finally, results for antibiotic susceptibility were especially concerning for metronidazole. 38.5% of isolates were found to be resistant to this first line of treatment antibiotic, likely jeopardizing the recovery of patients and prolonging their illness.

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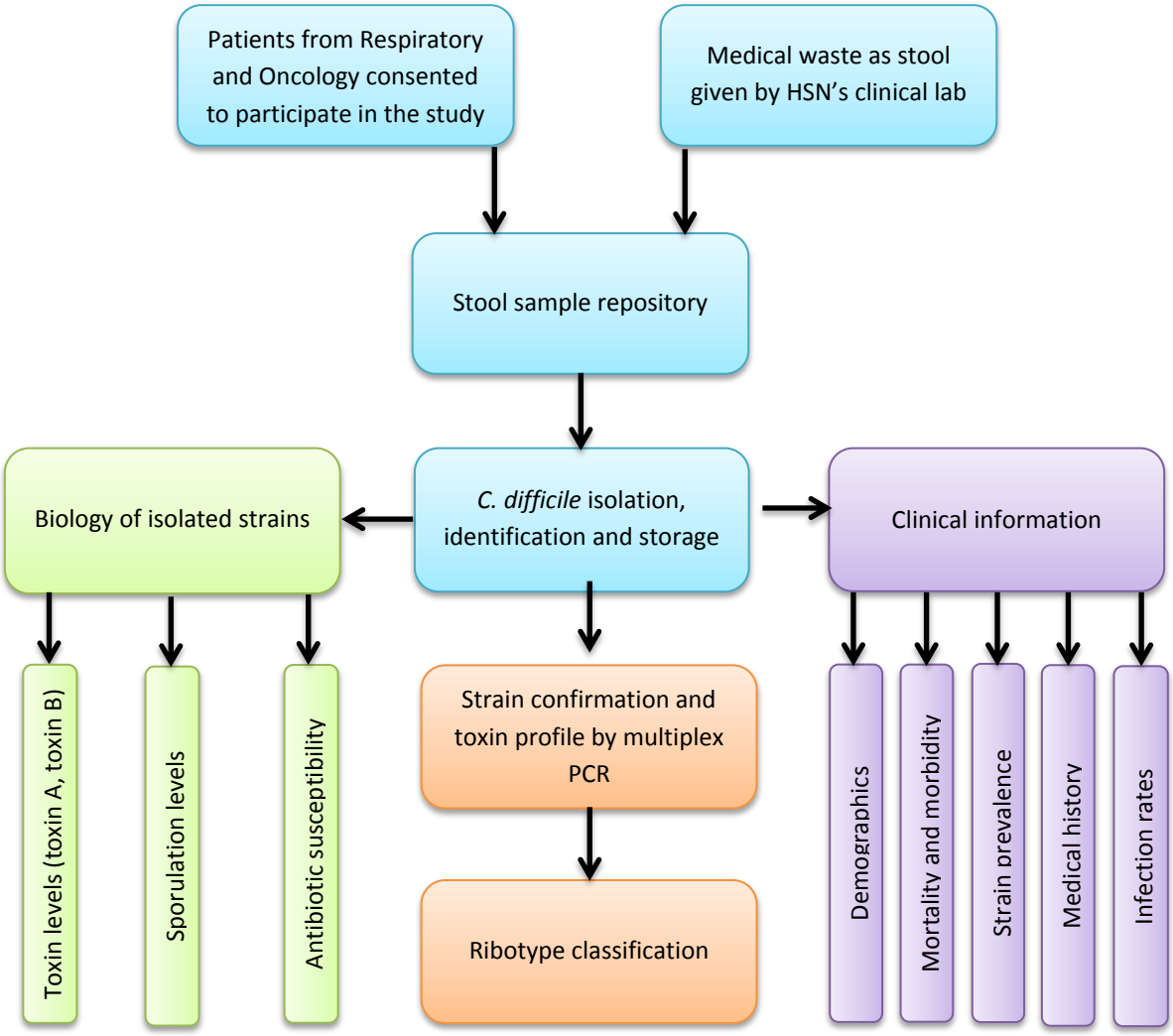
Appendix I: Flow chart of Project



Appendix II: Database results

Sample ID	Age	Sex	Symptoms	Severity index	GeneXpert			Characterization 1				Rybotype	Spore count in stool (spore/g)	Toxin production	Antibiotic Susceptibility			
					Toxin B	Binary toxin	TcdC	tpi	Tox A	Tox B	Tox A^{del}				CM	VA	MZ	CI
CD2586	79	M	S	1	+	+	-	+	-	+	+	126	1613861	7.189824559	R	S	R	R
CD2595	61	M	S	1	+	-	-	+	+	+	-	106	8219	11.19401808	R	S	R	R
CD2597	68	M	S	2	+	-	-	+	+	+	-	056	90698	9.897845456	R	S	R	R
CD2598	81	M	S	2	+	+	-	+	+	-	-	075	581	6.270528942	R	S	R	R
CD2599	79	M	S	2	+	+	-	+	-	+	+	126	11304	7.388878339	S	S	R	R
CD2600	55	M	S	2	+	-	-	+	+	+	-	251	119481	9.632995197	R	S	R	R
CD2601	71	F	S	2	+	-	-	+	+	+	-	251	281405	3.201633861	R	S	R	R
CD2602	55	M	S	1	+	-	-	+	+	+	-	106	103448	10.50263294	R	S	R	R
CD2603	61	M	S	1	+	-	-	+	+	+	-	077	330973	4.655351829	R	S	R	R
CD2604	80	F	S	1	+	-	-	+	+	+	-	001	5232143	10.64421682	R	S	R	R
CD2611	60	M	S	1	+	-	-	+	+	+	-	106	2734	5.472487771	R	S	R	R
CD2613	68	F	S	1	+	-	-	+	+	+	-	M	304	13.57478272	R	S	R	R
CD2615	84	F	S	2	+	+	+	+	+	-	-	027	147305	12.13955135	R	S	R	R
CD2623	71	F	S	1	+	-	-	+	+	+	-	106	480088	11.46066066	R	S	R	R
CD2626	80	M	S	1	+	-	-	+	+	+	-	020	196	7.852997588	R	S	R	R
CD2628	98	F	S	1	+	+	-	+	+	-	-	075	512	9.590961241	R	S	R	R
CD2629	84	F	S	2	+	-	-	+	+	+	-	106	1026764	6.655351829	R	S	R	R
CD2630	89	F	S	1	+	-	-	+	+	+	-	106	1192771	10.36632221	R	S	R	R
CD2631	57	M	S	1	+	-	-	+	+	+	-	056	1525745	10.81634371	R	S	R	R

Appendix II: Database results (Continued)

Sample ID	Age	Sex	Symptoms	Severity index	GeneXpert			Characterization 1				Rybotype	Spore count in stool (spore/g)	Toxin production	Antibiotic Susceptibility			
					Toxin B	Binary toxin	TcdC	tpi	Tox A	Tox B	Tox A^{del}				CM	VA	MZ	CI
CD2632	84	M	S	2	+	-	-	+	+	+	-	174	6643	10.89299809	R	S	R	R
CD2633	61	M	S	1	+	-	-	+	+	+	-	251	25000	6.944858446	R	S	R	R
CD2634	61	M	S	1	+	-	-	+	+	+	-	251	2368	10.04001568	R	S	R	R
CD2636	81	M	S	2	+	-	-	+	+	+	-	001	15323944	10.47613863	R	S	R	R
CD2637	80	F	S	1	+	-	-	+	+	+	-	020	187540	9.559185866	R	S	R	R
CD2640	65	F	S	3	+	-	-	+	+	+	-	002	2085271	8.938991439	R	S	R	R
CD2641	79	F	S	1	+	+	-	+	-	-	-	056	12211302	10.76752239	R	S	R	R
CD2642	44	F	S	1	+	-	-	+	+	+	-	001	1227848	9.569096095	R	S	R	R
CD2644	71	F	S	2	+	-	-	+	+	+	-	106	6000	9.285402219	R	S	R	R
CDST0007	89	M	A	0	+	-	-	+	+	+	-	020	12147	9.553053253	R	S	R	R
CDST0011	63	F	A	0	-	-	-	+	-	-	-	S0367	13943	0	R	S	R	R
CDST0055	89	F	S	1	-	-	-	+	+	+	-	S0933	2083	9.742140985	R	S	R	R
CDST0138	61	F	A	0	+	+	+	+	+	+	-	251	11582090	10.77082905	R	S	R	R
CDST0160	86	M	S	1	+	-	-	+	+	+	-	020	93277	10.54457812	R	S	R	R
CDST0185	82	M	A	0	+	-	-	+	+	+	-	137	14880	13.56985561	R	S	R	R
CDST0214	58	F	A	0	+	-	-	+	+	+	-	NASD	5696	9.777419716	R	S	R	R
CDST0217	64	F	A	0	+	-	-	+	+	+	-	NASD	17039	11.00450139	R	S	R	R
CDST0278	81	M	A	0	-	-	-	+	-	-	-	NASD	175000	0	R	S	R	R
CDST0303	78	F	S	1	+	-	-	+	+	+	-	012	13297491	4.378511623	R	S	R	R

Appendix II: Database results (Continued)

Sample ID	Age	Sex	Symptoms	Severity index	GeneXpert			Characterization 1				Rybotype	Spore count in stool (spore/g)	Toxin production	Antibiotic Susceptibility			
					Toxin B	Binary toxin	TcdC	tpi	Tox A	Tox B	Tox A^{del}				CM	VA	MZ	CI
CDST0317	66	F	S	1	+	-	-	+	+	+	-	020	258667	8.441284272	R	S	R	R
CDST0404	74	M	A	0	+	-	-	+	+	+	-	056	417234	10.35270557	R	S	R	R
CDST0466	21	F	A	0	+	-	-	+	+	+	-	002	2169	8.0725346	R	S	R	R
CDST0498	76	F	A	0	+	-	-	+	+	+	-	106	26150833	9.780703794	R	S	R	R
CDST0505	37	M	A	0	+	+	-	+	+	-	-	S2319	53249	7.867896464	R	S	R	R
CDST0529	72	F	A	0	+	-	-	+	+	+	-	020	24492754	9.6043679	R	S	R	R
CDST0648	71	M	S	1	+	-	-	+	+	+	-	001	1015873	10.5341083	R	S	R	R
CDST0674	60	M	A	0	+	-	-	+	+	+	-	NASD	126073	0	R	S	R	R
CDST0714	60	M	A	0	-	-	-	+	+	+	-	012	12185185	6.314696526	R	S	R	R
CDST0915	86	M	A	0	+	-	-	+	+	+	-	001	37202	10.61581353	R	S	R	R
CDST0920	92	M	A	0	+	-	-	+	+	+	-	020	159504	10.60214209	R	S	R	R
CDST0949	68	F	A	0	+	-	-	+	+	+	-	Z	5870	7.8899602	R	S	R	R
CDST1055	66	M	A	0	-	-	-	+	+	+	-	O	13878	7.177917792	R	S	R	R
CDST1066	70	M	A	0	-	-	-	+	+	+	-	020	32203	6.523561956	R	S	R	R
CDST1070	79	M	A	0	-	-	-	+	-	-	-	046	110145	0	R	S	R	R
CDST1098	83	M	S	2	+	-	-	+	+	+	-	174	2642857	10.24555271	R	S	R	R
CDST1120	52	F	A	0	-	-	-	+	-	-	-	S0367	287599	0	R	S	R	R
CDST1122	77	M	A	0	+	-	-	+	+	+	-	020	63924	11.04957632	R	S	R	R
CDST1134	70	F	A	0	+	-	-	+	-	+	+	017	2775362	0	R	S	R	R

Legend

M: Male F: Female A: Asymptomatic S: Symptomatic

CM: Clindamycin VA: Vancomycin MZ: Metronidazole CI: Ciprofloxacin