Clostridioides difficile Molecular Epidemiology in Alberta, Canada

by

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Abstract

Clostridioides difficile is a major cause of infectious diarrhea in the hospital setting resulting in significant morbidity among elderly patients with recent antibiotic and healthcare exposure. Antibiotics disrupt the resident microbiota allowing for germination of *C. difficile* spores conferring susceptibility to *C. difficile* infection (CDI). In CDI cases, production of *C. difficile* A and B toxin causes diarrheal symptoms which progress in severity to pseudomembranous colitis, toxic megacolon, and death. In addition to the A and B toxins, some strains also contain a "binary" toxin. Non-toxigenic *C. difficile* lacking the A, B, and binary toxin genes do not cause disease. Asymptomatic colonization is also observed, particularly in children and infants, in which colonization rates are high. This population represents a potential reservoir of pathogenic strains in the community. There are currently no reports on the *C. difficile* genotypes colonizing Canadian children. *C. difficile* genotyping and culture are lengthy processes not routinely performed in the clinical microbiology laboratory, which impairs timely investigation of potential *C. difficile* outbreaks. *C. difficile* ribotyping, a genotyping method, requires a *C. difficile* isolate for genetic characterization. This thesis addresses these deficits by: 1) characterizing the *C. difficile* strains present in children and adults from Alberta, Canada, and 2) developing a direct from stool *C. difficile* ribotyping method.

To characterize the *C. difficile* molecular epidemiology in Alberta, 308 *C. difficile* isolates recovered from symptomatic (diarrhea and vomiting) and asymptomatic children recruited — through the Alberta Provincial Pediatric EnTeric Infection TEam (APPETITE) study — were genotyped using PCR ribotyping. Ribotypes identified in APPETITE children were compared to ribotypes identified in 79 adult and 18 pediatric *C. difficile* infection cases. Ribotype 106 was the most prevalent (20.8%) molecular fingerprint identified in the APPETITE children. Similarly,

ribotype 106 was predominant in pediatric CDI cases (27.8%) in contrast to ribotype 027 (44.3%) in adult CDI cases. Isolate ribotypes identified in APPETITE study children were also present in adult and pediatric CDI cases. With respect to toxin genes, isolates from APPETITE study children and pediatric CDI cases contained toxin A and B genes (ranging from 88.1-94.1%), whereas 53.2% of isolates from adult CDI cases contained the binary toxin gene in addition to the toxin A and B genes. Of note, the presence of toxin in stool did not significantly differ ($p=0.22$) between symptomatic and asymptomatic children in the APPETITE study.

Using samples from the APPETITE study and CDI cases with direct PCR ribotyping, a direct from stool *C. difficile* ribotyping technique and algorithm was developed. A total of 187 stools containing toxigenic *C. difficile* were subjected to direct ribotyping. The success rate for direct ribotyping from stool was 66.8%; whereas 33.2% of the remaining samples required broth enrichment to produce a ribotype. Direct ribotyping was observed to correlate with the *C. difficile* bacterial load based on the qPCR cycle threshold (Ct) values targeting the 16S and *tcdB* genes, as the Ct values were significantly lower ($p<0.001$) in directly ribotyped stools as compared to enriched stools. Similarly, toxin positive stools were more likely to be ribotyped directly from the stool compared to toxin negative stools ($p<0.001$). High concordance (94.7%) was observed between direct and isolate ribotypes. Non-matching samples were due to mixed infections with more than one ribotype (4.8%) or inability to recover an isolate (0.5%). Overall, potentially mixed infections were identified in 7.5% of the samples. Additional validation revealed direct ribotypes were highly concordant (87.0%) with isolate ribotypes independently generated at the National Microbiology Laboratory. Additionally, a multiplex qPCR assay detecting triose phosphate isomerase (*tpi*), an enzyme involved in glycolysis present in toxigenic and non-toxigenic *C.*

difficile, and the toxin B gene (*tcdB*) was developed for simultaneous detection of toxigenic and non-toxigenic *C. difficile*.

In conclusion, this thesis identifies the prevailing *C. difficile* ribotypes circulating in children as well as CDI cases in Alberta, Canada, which have not been previously reported, and describes the development of a novel direct from stool PCR ribotyping method for *C. difficile* surveillance.

Preface

This thesis is an original work by Colin Lloyd. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, "Alberta Provincial Pediatric EnTeric Infection TEam (APPETITE): Epidemiology, Emerging Organisms, and Economics", No. Pro00050790, OCTOBER 30, 2014 and also from the University of Calgary Research Ethics Board.

A portion of **Chapter 2** has been published as an abstract: Lloyd C, Parsons B, Du T, Golding GR, Lee B, Chui L, Freedman, S. *Clostridium difficile* Molecular Epidemiology in a Prospective Cohort of Canadian Children Compared with Cases of *C. difficile* Infection. Open Forum Infectious Diseases. 2017 Oct 1;4(suppl_1, Fall 2017):S678. I was responsible for analyzing all of the data generated and the experimental work performed after the ribotyping validation (section 2.2.8) as well as writing the first draft and revising the abstract. Dr. Brendon Parsons performed testing on all of the APPETITE samples using the Luminex® xTAG® Gastrointestinal Pathogen Panel (section 2.2.3). I also assisted Dr. Parsons in carrying out the testing, and Dr. Parsons also reviewed and contributed to writing the abstract. Tim Du and Dr. George Golding performed molecular characterization of the isolates included in the PCR ribotyping validation (section 2.2.8) and contributed to writing the abstract. Dr. Bonita Lee aided in study conceptualization. Dr. Linda Chui aided in study conceptualization, provided oversight of the project, and reviewed and contributed to writing the abstract. Dr. Stephen Freedman reviewed and contributed to writing the abstract.

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10.1016/j.diagmicrobio.2020.115259. Online ahead of print. Volume 99, issue 3, March 2021. I was responsible for the experimental work, data collection and analysis, writing the first draft, and revising the paper. Dr. Brendon Parsons aided with study conceptualization, reviewed and contributed to writing the paper. Dr. Binal Shah-Gandhi aided with DNA extractions (section 3.2.3) and reviewed the paper. Sarah Morin aided with identifying mixed *C. difficile* infections (section 3.2.2) and reviewed the paper. Tim Du provided ribotype consultation (section 3.2.5), performed isolate ribotyping at the NML for comparison to direct ribotypes (section 3.2.7), and provided feedback on the paper. Dr. George Golding reviewed the paper. Dr. Linda Chui aided in developing the methodology and in study conceptualization, provided oversight and direction, and reviewed and contributed to writing the paper.

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List of Abbreviations

AC	Adult *C. difficile* infection
AGE	Acute gastroenteritis
APL-ProvLab	Alberta Precision Laboratories - ProvLab
APPETITE	Alberta Provincial Pediatric EnTeric Infection Team
BB	Banana Broth™
BHI	Brain heart infusion
CA-CDI	Community-Acquired *C. difficile* infection
CC	*C. difficile* culture
CCNA	Cell cytotoxicity neutralization assay
CDC	Centers for Disease Control and Prevention
CDI	*C. difficile* infection
CDQC	*C. DIFF QUIK CHEK COMPLETE*®
CDT	*C. difficile* transferase
CdtLoc	Cdt Locus
CLS	Calgary Laboratory Services
CNISP	Canadian Nosocomial Infection Surveillance Program
CPD	Cysteine protease domain
CPE	Cytopathic effect
CROP	Combined repetitive oligopeptide
Ct	Crossing threshold
DFS	Direct from stool
DNA	Deoxyribonucleic acid
ED	Emergency Department
EIA	Enzyme immunoassay
EIP	Emerging Infections Program
ELISA	Enzyme linked immunosorbent assay
FMT	Fecal microbiota transplantation
GDH	Glutamate dehydrogenase
GI	Gastrointestinal

GPP	Gastrointestinal Pathogen Panel
GTD	Glucosyltransferase domain
GTPase	Guanosine triphosphatase
HA-CDI	Hospital-Acquired *C. difficile* infection
HAI	Hospital acquired infection
HC	Healthy children
HCF	Healthcare facility
HeLa	Henrietta Lacks
IBD	Inflammatory bowel disease
IDSA	Infectious Diseases Society of America
IgA	Immunoglobulin A
IgG	Immunoglobulin G
ISR	Intergenic spacer region
LB	Lysis buffer
LOS	Length of stay
LTCF	Long-term care facilities
NAAT	Nucleic acid amplification test
NAP	North American Pulsotype
NPV	Negative predictive value
NTCD	Non-toxigenic *C. difficile*
PaLoc	Pathogenicity Locus
PBS	Phosphate Buffered Saline
PC	Pediatric *C. difficile* infection
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PHAC-NML	Public Health Agency of Canada – National Microbiology Laboratory
PMC	Pseudomembraneous colitis
PPI	Proton pump inhibitor
PPV	Positive predictive value
qPCR	Quantitative polymerase chain reaction
REA	Restriction Endonuclease Analysis

RT	Ribotype
ROC	Receiver Operator Characteristic
SC	Symptomatic children
SHEA	Society for Healthcare Epidemiology of America
SNP	Single nucleotide polymorphism
SIMOA	Single molecule array
UAH	University of Alberta Hospital
UV	Ultraviolet
WGS	Whole Genome Sequencing

Chapter 1 Introduction

1.1 Identification of *Clostridioides difficile* and *C. difficile* infection

Clostridioides difficile was first isolated from an infant by Hall & O'Toole in 1935 and was initially named *Bacillus difficilis* reflective of its difficulty to culture (1). It was described as a motile, Gram positive, anaerobic bacilli producing subterminal spores (1,2). *B. difficilis* also produced a heat labile toxin pathogenic to animals; however, this characteristic was not shared by all strains (2). In 1938 *Bacillus difficilis* was renamed *Clostridium difficile* (3) and later reclassified to the genus *Clostridioides* in 2016 (4) but the name *Clostridium difficile* is still widely accepted.

The connection between *C. difficile* and human disease was made in the 1970s (5). The association between pseudomembranous colitis (PMC) and antibiotic use was an initiator to the discovery of *C. difficile* infection (CDI). Tedesco *et al.* (6) reported that among patients receiving clindamycin, nearly 21% had diarrhea and 10% developed PMC synonymously referred to at the time as "clindamycin colitis". A similar disease was demonstrated using animal models, and Green (7) postulated that penicillin associated gastrointestinal illness in guinea pigs might be due to the activation of a latent virus. Further study by Bartlett *et al.* (8) found that clindamycin induced lethal enterocolitis in hamsters, but hamsters were protected with vancomycin treatment. This suggested that a bacterium may be responsible for the antibiotic associated gastrointestinal illness as vancomycin targets mostly Gram-positive bacteria (9). Using this same hamster model, Bartlett *et al.* (9) identified a *Clostridium* strain from a clindamycin-treated hamster which caused enterocolitis when pure cultures were injected into the cecum of healthy hamsters. Interestingly, cell-free supernatants of the *Clostridium* cultures also caused enterocolitis when inoculated into healthy hamsters, but enterocolitis was absent when the cecal contents of diseased hamsters were

first incubated with commercial gas gangrene antitoxin (derived from the toxins of five *Clostridium* species associated with gas-gangrene) prior to inoculation into healthy hamsters suggesting the illness was likely due to a toxin-producing *Clostridium* (9).

In 1977 & '78 several reports were published linking *C. difficile* and its toxins to PMC in humans. Two separate groups had reported that stools of PMC patients contained a toxin that caused cytopathic effect (CPE) in cell cultures and both gastrointestinal illness and mortality in animal models. This toxin could be neutralized using *C. sordellii* antitoxin (10,11). Bartlett *et al.* confirmed the link between *C. difficile*, its toxins, and human disease in 1978 by isolating an organism matching the characteristics of *C. difficile* from the stool of PMC patients. These isolates demonstrated a consistent pathology in both hamsters and cell cultures as had been previously observed and was neutralized by both gas-gangrene and *C. sordellii* antitoxin (5). Several studies further confirmed this observation (12,13). Consequently, Chang *et al.* (14) developed the first *C. difficile* diagnostic assay called the cell cytotoxicity neutralization assay (CCNA) and *C. difficile* toxin was detected in 96% of patients with antibiotic associated PMC.

1.2 *C. difficile* Toxins

1.2.1 Pathogenicity Locus

The genetic element responsible for *C. difficile* toxin production is a 19.6 kb deoxyribonucleic acid (DNA) segment referred to as the pathogenicity locus (PaLoc) (**Figure 1.1**). Strains of *C. difficile* are classified as toxigenic or non-toxigenic based on the presence of the PaLoc. In non-toxigenic *C. difficile* strains, which are non-pathogenic, the PaLoc is replaced with a 115 bp non-

coding sequence (15). Considerable diversity in the PaLoc exists between *C. difficile* strains. The PaLoc encodes five genes being *tcdR, tcdB, tcdE, tcdA,* and *tcdC* (**Figure 1.1**).

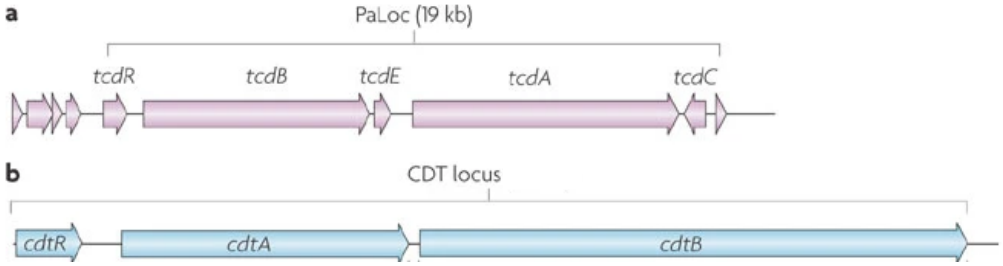

Figure 1.1. Genetic organization of the Pathogenicity Locus (PaLoc) and the CDT locus (CdtLoc). (a) The 19kb PaLoc encodes the two *C. difficile* toxin genes *tcdA* and *tcdB,* two regulatory genes *tcdR* and *tcdC*, and the toxin exporter *tcdE.* (b) Additionally, some strains contain the 6.2 kb CdtLoc encoding *cdtA* and *cdtB* which express subunits of the binary toxin and a regulatory gene *cdtR*. Adapted by permission from (15) Springer Nature: Nature Reviews Microbiology © 2009.

1.2.2 *tcdA* & *tcdB*

tcdA and *tcdB* encode for the two toxins TcdA & TcdB (commonly referred to as toxin A and toxin B), which are the primary mediators of *C. difficile* infection. TcdA and TcdB are highly homologous and belong to the large clostridial toxin family including toxins from *C. sordellii*, *C. novyi, & C. perfringens* (16). Transcription of *tcdA* and *tcdB* occurs primarily during stationary growth phase (17). TcdA & TcdB are similar in size (308 and 270 kDa, respectively) and consist of four domains; the N-terminal glucosyltransferase (GTD) domain, the cysteine protease (CPD) domain (also known as the auto-protease domain), the pore-forming domain, and the combined repetitive oligopeptide (CROP) domain at the C-terminus (16,18). The GTD is the main enzymatic component, the CROP domain plays a role in receptor binding, and the CPD and pore-forming domain are involved in toxin maturation and cleavage of GTD into the cytosol (16). The proposed mechanism for toxin entry into host cells is that the CROP domain first binds to host cell

carbohydrates and receptors present on the cell's surface (16). The toxin then enters the cell by endocytosis, and upon acidification of the endosome the pore forming domain associates with the endosomal membrane (16,19). The CPD and GTD translocate into the cytosol through an endosomal pore, and association with inositol hexakisphosphate causes auto-proteolysis releasing the GTD into the cytosol where it glucosylates Rho guanosine triphosphatases (GTPase) (16,19). Rho GTPase glucosylation interferes with the actin cytoskeleton which in turn disrupts tight junction stability and gut epithelial tissue integrity, causing disorder and permeability of the intestinal epithelium (18,19). Action of the toxins results in both apoptotic and necrotic cell death (19). Both toxins contribute to virulence; however, there is debate as to which toxin is more potent. Clinical isolates without the TcdA toxin are frequently identified in CDI cases suggesting TcdA might not be essential for the pathogenicity of these strains (20,21). Clinical isolates expressing TcdA but not TcdB have been identified (22) but are rare, which may be a consequence of *tcdB* focused diagnostic testing (21).

1.2.3 *tcdC*, *tcdR* & *tcdE*

The remaining genes of the PaLoc play a role in positive & negative regulation of toxin expression and toxin export. TcdR is a positive regulator of both *tcdA* and *tcdB* and is similar to positive regulators of *C. tetani* and *C. botulinum* toxin as well as bacteriocin in *C. perfringens* (23). TcdR acts as a sigma factor and increases transcription of the toxin genes by associating with RNA polymerases (23). TcdR is primarily transcribed in the stationary growth phase (17) and its production is modulated by environmental factors such as glucose, amino acid, and carbon source availability (23). TcdC is a negative regulator of toxin production and interacts with either TcdR, the RNA polymerase, or both to inhibit toxin gene transcription (24). In contrast with the other PaLoc genes, *tcdC* is primarily transcribed during the exponential growth phase (17). TcdE is a

holin-like transporter associated with the cytoplasmic membrane and exports *C. difficile* toxin outside of the bacterium while retaining the cytoplasm (25). Homologs of TcdE are found in *C. sordellii* and *C. perfringens* (25). Similar to *tcdA, tcdB,* and *tcdR, tcdE* is transcribed during the stationary growth phase (17).

1.2.4 CDT Locus

In addition to the two large clostridial toxins TcdA & TcdB, a third toxin is present in some *C. difficile* strains. Most commonly referred to as the binary toxin, *C. difficile* transferase (CDT) has an actin ribosylating action which is similar to the iota toxin of *C. perfringens* (26). The genes encoding CDT are located on the 6.2 kb Cdt locus (CdtLoc) which is composed of the two toxin subunit genes *cdtA* and *cdtB* as well as a regulatory gene *cdtR* (**Figure 1.1**) (26). CDTa is the enzymatic adenosine diphopshate-ribosyltransferase subunit and CDTb plays a role in cell entry and release of CDTa (27). The proposed mechanism of CDT is that CDTb binds to host cell receptors and forms CDTb oligomers that are bound to both CDTa and the host cell receptor (27). The CDTab complex is endocytosed and acidification of the endosome results in endosomal pore formation by CDTb and the release of nascent CDTa into the cytosol (27). Host chaperones then fold CDTa into its active conformation where it targets host actin molecules (27). CDT causes several changes in the host cell such as destabilization of the actin cytoskeleton, release of fibronectin, and formation of microtubule protrusions enhancing *C. difficile* adherence to host cells (28).

1.3 Pathophysiology, Treatment, and Clinical Presentation

1.3.1 Pathophysiology

CDI can be conceptualized as a disorder of the microbiome. The CDI pathophysiology process begins with the ingestion of *C. difficile* spores. Spores are ingested orally through the fecal-oral route via contact with the contaminated environment. Spores localize to the lower gastrointestinal (GI) tract where they reside dormant in cases of asymptomatic colonization; when environmental conditions become favorable through the loss of colonization resistance mediated by the host microbiome, these spores will germinate into vegetative cells (**Figure 1.2**). The normal, non-CDI, host microbiome provides resistance to *C. difficile* colonization by preventing *C. difficile* germination (**Figure 1.2**). Colonization resistance from the normal host microbiota is potentially conferred by several mechanisms including bile acid and nutrient metabolism as well as antimicrobial activity due to bacteriocins, bacteriophages, and host-defense molecules (**Figure 1.2**) (29). Dysbiosis of the healthy, protective, gut microbiome, commonly due to antibiotics or other factors, confers susceptibility to CDI (**Figure 1.2**). When spores enter a susceptible host, the spores germinate, colonize the lower GI tract, and produce toxins. The toxins act on intestinal epithelial cells destabilizing gut epithelial tissue integrity (**Figure 1.2**). This in turn activates innate inflammatory responses, activation of monocytes and macrophages, release of proinflammatory cytokines, and invasion of neutrophils (30,31).

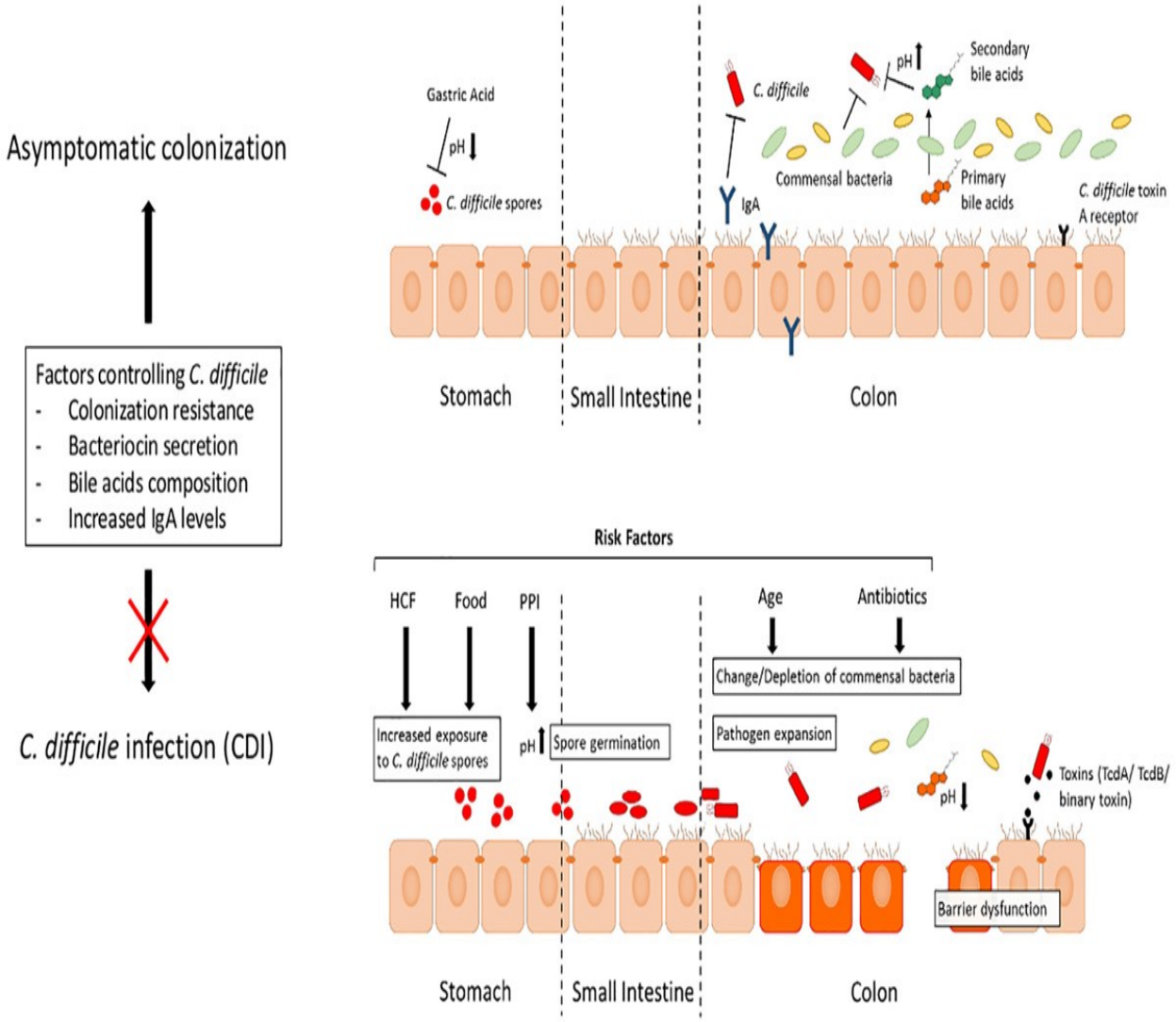

Figure 1.2. Pathophysiology and risk factors of *C. difficile* infection. *C. difficile* can persist asymptomatically (top) or cause infection (bottom) depending on several factors. In asymptomatic carriage (top) several factors prevent *C. difficile* infection such as: gastric acid pH, host defenses such as immunoglobulin A (IgA), inhibitory secondary bile acids produced through primary bile acid metabolism by the microbiome, and nutrient competition and bacteriocin production by commensal bacteria. However, in the susceptible host (bottom) protective factors are absent or disrupted by several factors such as: increased exposure to spores through healthcare facilities (HCF), contaminated food, and increased gastric pH from proton pump inhibitors (PPI), as well as dysbiosis of the microbiome through antibiotic use and increased age leading to increased primary bile acid availability and reduced inhibition from secondary bile acids leading to overgrowth, toxin production, and *C. difficile* infection. Adapted from (32) © 2018 Schäffler and Breitrück with permission under Creative Commons CC BY 4.0 license.

1.3.2 Treatment

Antibiotic treatment is the standard therapy for CDI. Based on recent Infectious Diseases Society of America/Society for Healthcare Epidemiology of America (IDSA/SHEA) guidelines (33), vancomycin and fidaxomicin are recommended for adult CDI while metronidazole is no longer recommended but can be used in pediatric cases. Antibiotic therapy works in principle by eliminating or reducing the *C. difficile* burden from the GI tract such that the normal microbiome and colonization resistance will reestablish over time. However, recurrent CDI occurs in 20-42% of CDI cases with the potential for several sequential recurrences over time (34). In addition to recurrence, reinfection is possible in which a second strain of *C. difficile* germinates and causes disease. Reinfection and recurrence are indistinguishable clinically and require genotypic characterization of *C. difficile* isolates. Another emerging treatment for CDI is fecal microbiota transplantation (FMT). FMT attempts to rapidly reconstitute a healthy microbiome by infusing the GI tract with a microbiome derived from a donor's stool (29). With successful treatment and resolution of symptoms, *C. difficile* may either persist asymptomatically or is cleared from the patient.

Host immune response also plays a role in CDI pathophysiology as anti-toxin A IgG concentrations are higher in asymptomatic carriers and lower in patients with recurrent disease (35,36). Patients receiving monoclonal antibodies against TcdA and TcdB have demonstrated lower CDI recurrence (37). Bezlotoxumab, a monoclonal toxin B antibody, is a biotherapeutic which has been shown to be superior at reducing CDI recurrence compared to actoxumab, which is a monoclonal toxin A antibody (38). In addition, *C. difficile* vaccines are in development (39)

which likely prevent CDI in a similar way by enhancing the host immune response to *C. difficile* toxins in vaccinated individuals when challenged with an active infection.

1.3.3 Clinical Presentation

CDI is often referred to as *C. difficile* associated disease as *C. difficile* produces a spectrum of illness. The most common presentation in mild to moderate CDI cases is diarrheal illness, generally considered to be ≥3 stools per 24 hours, associated with recent antibiotic exposure (40). Bloody diarrhea is a rare presentation for CDI (41). Colitis is common and presents with abdominal pain, nausea, and anorexia in addition to diarrheal illness (40). Fever, dehydration, and signs of infection such as leukocytosis and inflammation (40,41) may also be present. In severe CDI, PMC occurs with the formation of yellow-white raised pseudomembranes composed of neutrophils, destroyed intestinal cells, and inflammatory elements which are visible on the colorectal mucosa (**Figure 1.3**) (42,43). Pseudomembrane formation is highly indicative but not specific to CDI (43). Severe cases are also characterized by increasing abdominal pain, profuse diarrhea, leukocytosis and hypoalbuminemia (40). Although rare, in 3-8% of CDI cases, severe fulminant colitis occurs, associated with ileus, toxic megacolon, colonic perforation, sepsis/septic shock and death (40,43). Surgical intervention such as colectomy may be required to prevent mortality in fulminant CDI cases. In some serious CDI cases with paralytic ileus, atypical presentation can occur with decreased frequency of diarrhea (40,41).

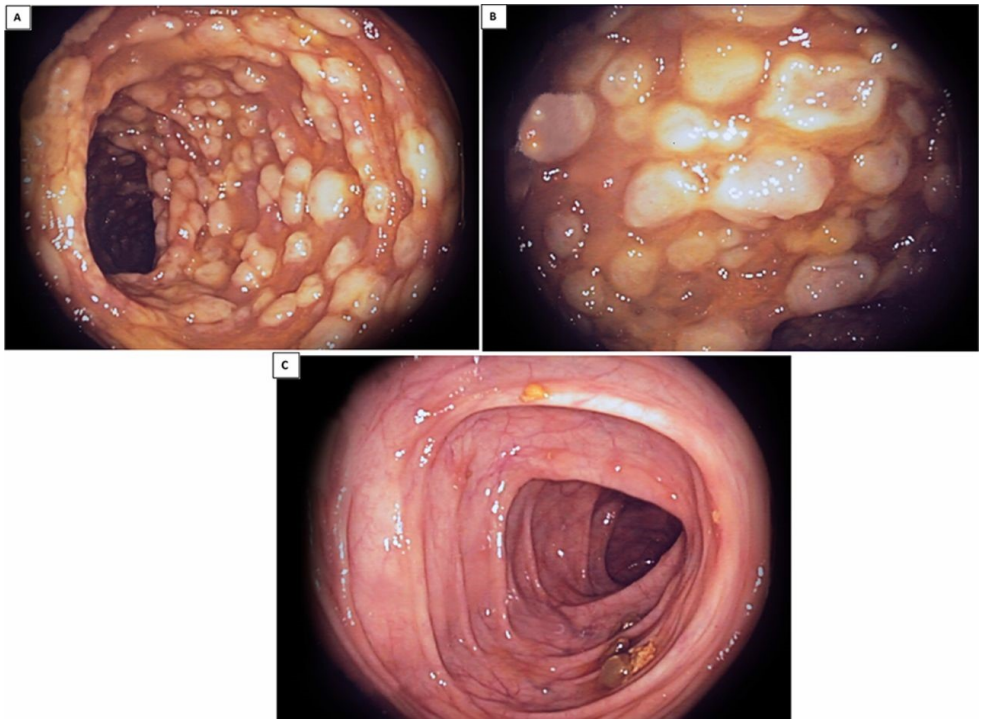

Figure 1.3. Pseudomembranous colitis in *C. difficile* infection. (A,B) Hallmark raised, white-yellow pseudomembranous plaques on the colon of a CDI patient. (C) Absence of pseudomembranes following vancomycin treatment (44). Adapted with permission from BMJ Publishing Group Limited.

Asymptomatic colonization with *C. difficile* can also occur in healthy individuals without exhibiting the symptoms as described above. Colonization can be both transient, as particular strains are acquired and lost over time, as well as persistent carriage with the same strain. Additionally, colonization is a major confounder for diagnosis as it must be determined for each symptomatic patient whether the current symptoms are attributable to *C. difficile* or if there is an alternative cause of diarrhea, which requires both diagnostic testing together with clinical assessment (45). The European Society of Clinical Microbiology and Infectious Diseases recommends that alternative diarrheal etiologies should be excluded in potential CDI diagnoses (46) as to avoid over diagnosis and unnecessary treatment of colonized patients.

1.4 *C. difficile* Diagnostic Assays

Optimal diagnostic testing is a source of significant debate in the *C. difficile* literature as many assays lack either specificity or sensitivity, and cannot distinguish colonization from infection. In addition, hospital acquired diarrhea is not specific to CDI (47,48) and *C. difficile* is implicated in only 15-25% of antibiotic associated diarrhea cases (41,47), which can make CDI difficult to diagnose accurately.

1.4.1 Cell Culture Cytotoxicity Assay (CCNA)

The CCNA is one of the earliest *C. difficile* assays and detects primarily toxin B (46,49). A stool filtrate is inoculated onto a cell culture monolayer and observed for the development of CPE demonstrated by rounding of the cells in 24 to 48 hours. If CPE occurs, the assay is repeated by adding *C. sordellii* or *C. difficile* antitoxin prior to inoculation of the stool filtrate. The antitoxin will neutralize the toxin thus preventing CPE. CCNA is regarded as the reference method for *C. difficile* toxin detection and can detect picogram levels of toxin (41,50). A wide variety of cell lines have been validated for use with CCNA including human fibroblast, Vero, and HeLa cells (14,46,49). Pre-analytical handling can affect CCNA sensitivity as improper sample storage or transportation can cause degradation of the heat labile toxins; variations in assay performance can differ between cell lines and filtrate preparation methods (49). CCNA is generally considered to have high specificity for CDI but is only 75-85% sensitive (51). While this assay is currently the best method for toxin detection, its turnaround time is 1-2 days and there is a requirement of technical expertise and maintenance of cell lines, thus making it impractical for routine CDI diagnoses.

1.4.2 *C. difficile* Culture (CC)

C. difficile isolation is performed by inoculating the stool sample on specific media with further confirmation of isolates as toxigenic by enzyme immunoassay or nucleic acid amplification assay as described in the sections below. Frequently used media for *C. difficile* isolation are the cycloserine-cefoxitin, fructose agar and cycloserine-cefoxitin, egg yolk with lysozyme agars (52). Common additives to *C. difficile* specific media include cycloserine and cefoxitin to limit growth of competing organisms as well as taurocholate and lysozyme to promote spore germination (53,54). Ethanol shock or heat treatment of samples prior to inoculation can be used to reduce competing organisms and to select for *C. difficile* spores (55,56). Using pre-reduced (anaerobic) media for *C. difficile* culture is advised (49). Commercially available chromogenic media include CHROMID® *C. difficile* agar (bioMérieux, Marcy-l'Étiole, Lyon, France) on which presumptive *C. difficile* colonies appear black and CHROMagar™ *C. difficile* (CHROMagar Microbiology, Paris, France) which identifies *C. difficile* by UV fluorescence. White *C. difficile* colonies have been observed on CHROMID® plates due to the inability of some strains to hydrolyze esculin (57). *C. difficile* is identifiable on sheep blood agar by its characteristic "horse barn" odor, chartreuse fluorescence under UV light (52), and is confirmed by standard microbiological methods (colony morphology, Gram stain, latex agglutination, and biochemical testing) (49). Sensitivity of CC can be enhanced by enrichment in broth prior to inoculation onto agar (55). Commercial *C. difficile* specific broths include cycloserine-cefoxitin mannitol broth with taurocholate and lysozyme (Anaerobe Systems, Morgan Hill, CA, USA) and *C. difficile* Banana Broth™ (Hardy Diagnostics, Santa Maria, CA, USA), which contains thioglycolic acid and L-cysteine for aerobic incubation based on the formulation of Cadnum et al. (58). A major limitation of current *C. difficile* media is the inability to differentiate colonies based on toxigenicity. However, Darkoh *et al.* (59) have

described a differential medium on which toxin producing colonies appear blue, but it is not widely used and is not produced commercially. Similar to CCNA, CC is tedious, labor intensive, and has a lengthy turnaround reporting time which makes this method unrealistic for routine diagnostics. However, CC remains essential for genotypic characterization and antimicrobial susceptibility testing.

1.4.3 Enzyme Immunoassay (EIA)

Following the development of the CCNA, several enzyme immunoassay (EIA) platforms have been developed including rapid lateral flow EIAs, such as the *C. DIFF QUIK CHEK COMPLETE*® (Techlab, Blacksburg, Virginia, US). The principle of these tests uses immobilized antibodies that bind to their target antigen and a reporter molecule indicates the presence of target antigen visually by color change or fluorescence. *C. difficile* EIAs offer identification of several targets including glutamate dehydrogenase (GDH), TcdA, and TcdB. GDH is a metabolic enzyme and is secreted in high levels (60) from both toxigenic and non-toxigenic *C. difficile* strains. GDH EIAs have been shown to be comparably sensitive to CC with ~96% sensitivity and specificity (46); however, GDH EIAs lack specificity because the assay cannot differentiate between toxigenic and non-toxigenic strains. Consequently, GDH EIAs are recommended as a rapid *C. difficile* screening test in combination with a toxin EIA (61). Toxin EIAs are widely used for *C. difficile* diagnosis due to its faster turnaround time as compared to CCNA. However, it has been demonstrated that many toxin EIAs lack adequate sensitivity. Reported toxin EIA sensitivities and specificities range from 40-100% depending on the assay (49), and studies using CC as a reference method generally report lower sensitivities than those using CCNA (49). Similarly, systematic review and meta-analysis (46) of commercial toxin EIA assays indicated only 57% sensitivity as compared to CC and 83% sensitivity compared to CCNA but toxin EIAs are highly specific (99%) compared to either assay.

1.4.4 Nucleic Acid Amplification Test (NAAT)

Nucleic acid amplification tests (NAAT) identify toxigenic *C. difficile* by amplification of the toxin genes. NAAT is similar to CC as it detects the presence of toxigenic *C. difficile*. One widely used assay, the GeneXpert® *C. difficile* (Cepheid, Sunnyvale, California, US) targets *tcdB* as to not miss potential cases due to strains containing *tcdA* deletions. Another variant of this assay is the GeneXpert® *C. difficile Epi* assay that also detects *cdtA* and a Δ117 deletion in *tcdC*, which presumptively identifies the epidemic strain ribotype 027; however, other strains can also give positive results which necessitates genotyping for confirmation (62–66). There are also reports of clinically relevant CDI cases due to toxin A and B negative, binary toxin positive *C. difficile* strains, in particular ribotype 033, that have been identified by atypical results from the GeneXpert® *C. difficile Epi* assay ($tcdB^{-}/cdtA^{+}$), which is currently the only frequently used commercial assay that detects the binary toxin (64–66). Assays that do not include binary toxin detection will not identify these cases (64–66). Additionally, GeneXpert® reports cycle threshold (Ct) values which allows for comparison of the relative *C. difficile* burden between samples. Systematic review and meta-analysis of commercial NAAT assays determined the sensitivity and specificity to be >94% regardless of whether CC or CCNA was the reference method (46). Sensitivity and specificity of the GeneXpert® *C. difficile* assay ranges from 94.4-100% and 93.0-99.2% respectively with positive (PPV) and negative predictive values (NPV) of 78.9-94.7% and 99.3-100% respectively (49), which is similar to other commercial NAAT assays. NAAT and toxin EIAs have a comparable turnaround time of approximately 1 hour, which makes them ideal as screening assays for detecting toxigenic *C. difficile*.

1.4.5 NAAT vs. EIA and multistep algorithms

There are no *C. difficile* diagnostic assays currently available that perform well as standalone tests. All *C. difficile* diagnostic tests are limited in that identification of the organism (NAAT, GDH EIA, and CC) or its toxins (CCNA & toxin EIA) does not definitively differentiate between CDI patients and *C. difficile* carriers, which is a distinction that must be made based on clinical judgement (45). CC, GDH EIA, and NAAT are highly sensitive methods and detect both CDI cases and colonized patients. Laboratories switching from toxin EIA based detection to NAAT have seen a rise in CDI rates (67) ranging from 43 to 67% (68,69) due to the increased sensitivity of NAAT assays. This has raised concerns regarding over diagnosis by misclassification of colonized patients (i.e. diarrhea due to etiologies other than *C. difficile,* or NAAT/CC positive with low or minimal toxin production (70)) as CDI cases. There are however several advantages to using higher sensitivity NAAT assays. Better identification of *C. difficile* carriers, which pose a potential transmission risk, can allow for improved CDI prevention leading to decreases in the CDI rate over time (61). CCNA and toxin EIA are highly specific for CDI; however, the lower sensitivity of EIA assays has created concern regarding false negatives and missing cases. Several studies have shown that toxin tests provide greater clinical utility compared to NAAT assays (69,71,72) because toxin positive patients have significantly higher mortality compared to patients in which the organism but not the toxins are detected (71). In Polage *et al.'s* (72) study comparing outcomes between $NAAT^{+}/toxin^{+}$ patients (*tcdA* or *tcdB* positive and toxin A or B positive) and $NAAT^{+}/toxin^{-}$ patients, $NAAT^{+}/toxin^{+}$ patients had higher *C. difficile* organism burden, toxin burden (as determined by a toxin quantification assay), hypervirulent ribotype 027 and 078 prevalence, duration of diarrhea, fecal lactoferrin (indicative of intestinal inflammation), and CDI related mortality and complications compared to $NAAT^{+}/toxin^{-}$ patients. Additionally,

NAAT$^+$/toxin$^-$ patients had similar outcomes compared to patients in which *C. difficile* was not detected by either NAAT or toxin EIA (72).

The inadequacies among individual *C. difficile* assays has led to the use of multistep testing algorithms for the detection of *C. difficile*. Generally, these algorithms will start with a sensitive screening test with high NPV and rapid turnaround time such as toxin NAAT or GDH EIA (i.e. "first test") (61) allowing for exclusion of *C. difficile* negative patients. Patients positive by the first test are confirmed to have *C. difficile* and are followed up by a highly specific test such as toxin EIA (i.e. "second test") to identify the presence of *C. difficile* toxin (61). The difficulty with multi-step algorithms is in classifying patients for which *C. difficile* is detected (positive by the first test) in the absence of detectable toxin (negative by the second test) which then requires clinical evaluation to determine the relevance of *C. difficile* detection (46). In all cases confirmation of toxigenic *C. difficile* is required; for example, if a patient is positive by GDH EIA but negative by toxin EIA then NAAT targeting the toxin gene(s) is used as an arbiter to determine if toxigenic *C. difficile* is present. Patients can be stratified using the multi-step algorithm as: *C. difficile* negative (negative by the first test, i.e. GDH$^-$ or NAAT$^-$), potentially *C. difficile* colonized (positive by the first test but negative by the second test, i.e. GDH$^+$ or NAAT$^+$ but toxin$^-$), and CDI cases (positive by the first and second test, i.e. GDH$^+$ or NAAT$^+$ and toxin$^+$) (46,73). Patients classified as *C. difficile* colonized by the algorithm require further clinical evaluation to determine whether treatment is required (46) as some toxin EIA negative patients can have severe CDI (73). Additionally, some laboratories may forego multi-step *C. difficile* testing algorithms in favor of NAAT only testing as this method can rapidly screen samples for *C. difficile* toxin genes and report positive results without further delays incurred by the inclusion of a toxin EIA assay to confirm toxin production. With respect to infection control, patients

positive for toxigenic *C. difficile*, which includes *C. difficile* colonized as well as CDI cases, require isolation and contact precautions (73).

One approach to using NAATs as a standalone *C. difficile* assay has been to stratify patients based on the bacterial load as determined by quantitative polymerase chain reaction (qPCR) assays. Studies assessing the bacterial load, using quantitative culture and qPCR (both in-house and quantitative commercial NAAT assays such as the GeneXpert® *C. difficile*), demonstrated that higher bacterial loads were associated with toxin positive samples (74–76). However, other studies have shown no association between clinically significant diarrhea (≥3 diarrheal stools per 24 hours) and the bacterial load or toxin concentration (77). As there is a correlation between high loads of toxigenic *C. difficile* and toxin positivity, Ct values, which correspond to the bacterial load, reported by qPCR based assays can potentially be used to discriminate potential *C. difficile* carriers (likely to test toxin negative) and CDI cases (likely to test toxin positive) as indicated by various publications (74,76,78). In practice, using Ct value thresholds of between 25 and 27 (thresholds vary between studies) to predict toxin positivity have reported sensitivities, specificities, PPVs, and NPVs of 96.0%, 65.9% (increased to 78.0% including CCNA as a reference), 57.4%, and 97.1% respectively in one study (78) and 29-82%, 73-97%, ~70%, ~80% respectively in another study (76). Using a Ct <25 cut off, one study found low Ct was significantly associated with ribotype 027, toxin positivity, and mortality (79). However, the ability of Ct values to accurately categorize all high risk patients (presumably toxin positive) from low risk or asymptomatic carriers (presumably toxin negative) is insufficient for clinical use (79,80). Thus using standalone NAATs to classify patients based on Ct thresholds is not recommended but could be used as a supplement to routine diagnostics (79,80).

1.4.6 Multiplex syndromic panels

A relatively new development in diagnostic microbiology are syndromic multiplex panels that simultaneously detect multiple pathogens using polymerase chain reaction (PCR), and these types of testing platforms can eliminate the utilization of various growth media for upfront bacterial culture and technologist time for other conventional detection methods (81). Several gastrointestinal syndromic panel assays include *C. difficile* in their targets, such as the Luminex® xTAG® GI Pathogen Panel (GPP) (Luminex® Corporation, Austin, Texas, US) and BIOFIRE® FILMARRAY® GI Panel (BIOFIRE® Diagnostics, Salt Lake City, Utah, US). These multi-analyte assays are both sensitive and specific for *C. difficile* (82–85). With respect to *C. difficile,* these assays share the same advantages and disadvantages as standalone NAAT assays for *C. difficile*, such as the GeneXpert®, with the added benefit and complexity of identifying coinfections. *C. difficile* is one of the most frequently identified pathogens using these assays and is identified in ~30% of coinfections (82,84,85). *C. difficile* coinfection confounds the interpretation of multiplex panels as it is unclear whether *C. difficile* is present as a colonizer or actively contributes to disease (82,85–87). One study (88) found no difference in severity, recurrence or length of stay (LOS) in patients when *C. difficile* was identified alongside another pathogen.

1.5 *C. difficile* Genotyping

Several strain typing methods have been developed for *C. difficile* with early methods focusing on phenotypic characterization (i.e. antimicrobial susceptibility, serotyping, phage-typing) and genotypic methods have developed more recently which are currently used for characterizing *C. difficile* strains (49). Genotypic methods differ with respect to the region(s) of the *C. difficile* genome that are analyzed as well as discriminatory power, technical expertise, and turnaround

time. Most genotyping methods use genomic restriction sites, repetitive regions, or housekeeping genes, but toxinotyping is unique as it is the only method that characterizes *C. difficile* based on variations in the PaLoc and is the only method that reflects *tcdA* and *tcdB* sequence diversity between strains (89).

There is no consensus on a standard genotyping method, thus making comparison between studies difficult. This has also resulted in the adoption of confusing multi method genotype designations for certain *C. difficile* strains; for example: ribotype (RT) 027, North American Pulsotype (NAP) 1 by pulsed-field gel electrophoresis (PFGE), restriction endonuclease analysis (REA) type BI, and toxinotype III by toxinotyping. Additionally, there is loose correlation between different genotyping methods thus inferring the results of one genotyping method based on another might result in inaccuracies (i.e., not all NAP1 strains are RT 027) (90).

1.5.1 Pulsed-field Gel Electrophoresis (PFGE)

Pulsed-field gel electrophoresis for *C. difficile* is fairly technical, lengthy, and has issues with inter-laboratory interpretation (91), but PFGE has been the standard *C. difficile* typing method in North America and provides high discriminatory power (92). This method discriminates *C. difficile* isolates by restriction digestion of genomic DNA using *Sma*I, which infrequently cuts the *C. difficile* genome resulting in large DNA fragments (49). Consequently, the large DNA fragments require an alternating, pulsed electric field during agarose gel electrophoresis to provide sufficient resolution for comparison (49). PFGE has had several drawbacks due to issues with DNA degradation, in particular with the REA type J strain (92), and difficulty with spore lysis. However, gel additives such as thiourea and harvesting log phase cultures (6-8 hours growth (92,93)) has resolved these issues (91,92). PFGE categorizes strains into NAP types (NAP 1-12)

which are groups of related *C. difficile* strains having PFGE patterns (pulsotypes) with ≥80% similarity (90,92).

1.5.2 PCR Ribotyping

PCR ribotyping is an easy to use, robust, and simple *C. difficile* genotyping method and has become the standard *C. difficile* typing tool in both Europe and Australia (91), but is less discriminatory than PFGE (92). Early PCR ribotyping began with the discovery of a variable length region of DNA located between the 16S and 23S rRNA genes of *rrn* operons that could be used to differentiate *C. difficile* strains (94). This region referred to as the intergenic spacer region (ISR) which is composed of (from 16S to 23S): an ISRstart sequence, a 172 bp region, several 9 bp repeat sequences and spacer sequences of 0, 33, or 53 bp in length, and an ISRend sequence (**Figure 1.4**) (95). The ISRstart and ISRend are consistent between *rrn* operons; however, the 172 bp region can be absent and the length of the spacer sequences is variable between *rrn* operons (**Figure 1.4**) (95). Between 9 and 12 *rrn* operons can be present in the genome depending on the strain (96). Thus, when ISRs are amplified by PCR the variable sizes for each unique ISR within the *rrn* operons generates amplified fragments ranging in length from 200-700 bp (49). Amplicons separated by electrophoresis generate a characteristic banding pattern which can differentiate *C. difficile* strains.

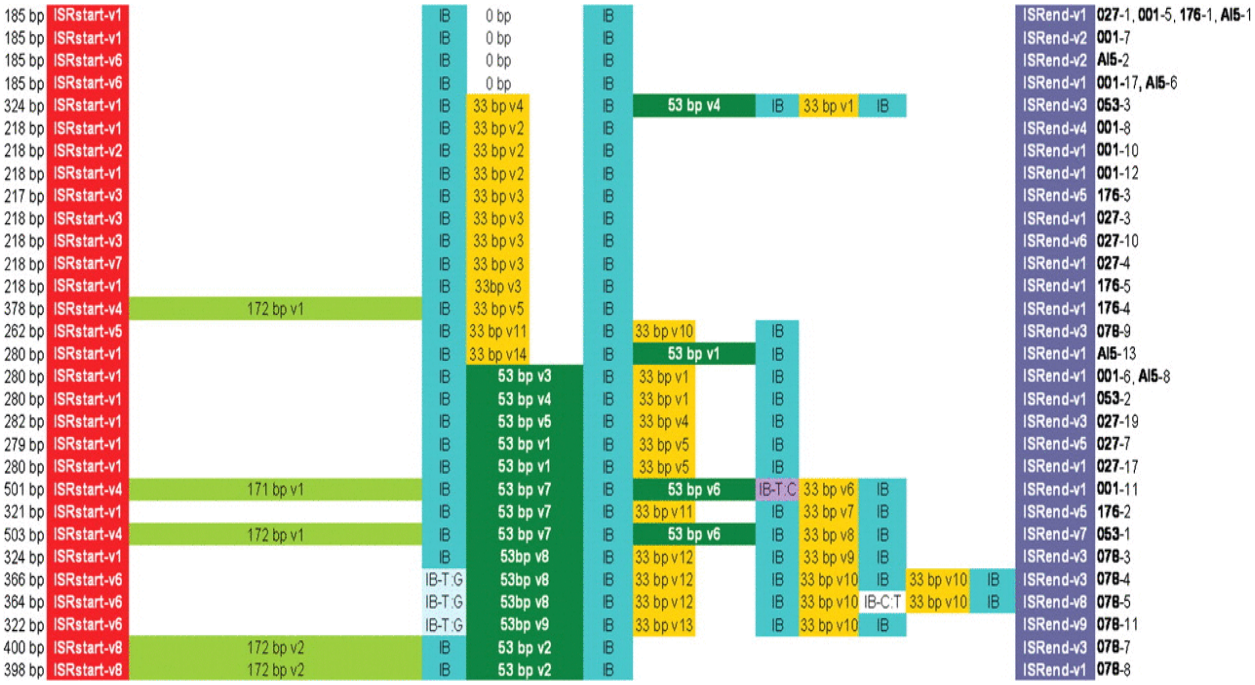

Figure 1.4. The intergenic spacer region (ISR) between the *C. difficile* 16S and 23S rRNA genes. The ISR is composed of a start and end sequence, a 172 bp region which differs between strains, 9 bp repeats (IB) and spacer sequences of varying length (0, 33, 53 bp). Respective ribotypes for each *rrn* operon and ISR are shown at right in bold. Reproduced with permission of Microbiology Society, from reference (95); permission conveyed through Copyright Clearance Center, Inc.

One of the first PCR primer sets developed for ribotyping was described by O'Neill *et al.* (97) and provided similar discriminatory power compared to *C. difficile* serotyping. Stubbs *et al.* (98) identified 116 ribotypes from a collection of 2,030 *C. difficile* isolates using this method and developed the nomenclature used internationally for ribotype designations (e.g. 001, 027, 106, etc.). The O'Neill (97) 23S reverse primer was developed based on *C. botulinum* sequences. This led Bidet *et al.* (99) to develop a new set of PCR primers based on 16S and 23S sequences from *C. difficile*. Compared to the O'Neill primers, the Bidet 16S primer binds closer to the 3' end of the 16S gene, towards the ISR (**Figure 1.5**), generating comparatively smaller PCR amplicons which provide better resolution with agarose electrophoresis (99). Agarose gel electrophoresis was the primary method used to resolve ribotyping amplicons, but the study by Indra *et al.* (100)

demonstrated that capillary electrophoresis produced similar results with better resolution for closely related ribotypes and required less hands-on time. Additionally, ribotypes generated by capillary electrophoresis as compared to agarose gel analysis are more easily exchanged electronically using shared software between laboratories (100). One limitation of PCR ribotyping is the availability of reference collections & databases which has led many institutions to develop internal nomenclature making comparison between studies difficult (101). However, Indra *et al.* (100) developed an online ribotype database (http://webribo.ages.at) onto which users can upload ribotype chromatograms generated from capillary electrophoresis and ribotypes are automatically assigned to the samples. To further facilitate comparability of ribotypes between laboratories, Fawley and colleagues (101) performed an international isolate ribotyping method validation among 4 *C. difficile* surveillance laboratories using the Bidet *et al.* (99) primers and capillary electrophoresis to develop the international consensus PCR ribotyping method. Another ribotyping primer pair was developed by Janezic *et al.* (102) and binds closer to the ISR, partially spanning into the ISR, relative to the O'Neill and Bidet primers (**Figure 1.5**). Janezic *et al.* (102) demonstrated direct PCR ribotyping from stool, using agarose electrophoresis, without the need for *C. difficile* culture and isolation.

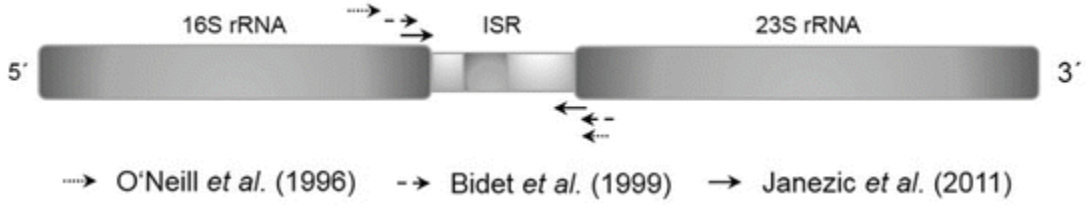

Figure 1.5. 16S-23S PCR ribotyping primer binding regions. Graphical representation of PCR ribotyping primer binding sites (solid arrow, Janezic; broken arrow, Bidet; dotted arrow, O'Neill) along the *rrn* operon that differ in proximity to the ISR. Adapted from (103) by permission from Springer Nature: Springer Methods Molecular Biology © 2016.

1.6 Epidemiology

1.6.1 *C. difficile* Burden

C. difficile is one of the most prominent causes of hospital acquired infectious diarrhea. Centers for Disease Control and Prevention (CDC) Emerging Infections Program (EIP) point prevalence surveys of hospital acquired infections (HAI) identified gastrointestinal illness as the 3rd most and 2nd most prevalent HAI in 2011 and 2015 respectively and *C. difficile* was identified in >70% of the gastrointestinal HAIs (104,105). In 2011, an estimated 453,000 infections, 83,000 first recurrences, and 29,300 deaths were associated with CDI in the United States (106). CDI causes considerable economic burden with an estimated cost of $1 billion to >6.3 billion USD (107,108). In Canada, there were 20,623 adult healthcare-associated CDIs (HA-CDI) between 2009 and 2015 reported under the Canadian Nosocomial Infection Surveillance Program (CNISP) (109). During the period of 2011-2015, HA-CDI incidence rates decreased by 35.8% from 6.7 to 4.3/10,000 patient days (109). The majority of HA-CDIs occurred in older patients (65.8%, >65 years old; 34.2%, 18-64 years old); however, there was a significant increase in the proportion of HA-CDIs in younger patients over the surveillance period (109). An estimated 37,932 CDIs occurred in Canada in 2012 resulting in an associated cost of $280 million (110). Across Alberta there are typically 1000 cases of CDI annually, and the Edmonton and Calgary zones have the highest prevalence per 10,000 patient days (111). The 2019 HA-CDI rate in Alberta is currently consistent with past rates at 2.8/10,000 patient days (112).

1.6.2 Changes in Epidemiology

One of the most significant changes in the epidemiology of CDI is the emergence of the epidemic strain designated 027/NAP1/BI. In the early 2000s, significant increases in CDI

morbidity and mortality were observed in Quebec with 30 hospitals reporting a fivefold rise in incidence compared to previous years (113). One hospital in particular, the Centre Hospitalier Universitaire de Sherbrooke, reported morality rates of 13.8% in 2003 which is nearly triple the rate observed in previous years (4.7% in 1991-92) (113). Similar observations were made in the United States with increasing CDI prevalence and severity in the early 2000s (114). Infection with this strain has been associated with increased mortality and severity in elderly patients compared to non-NAP1 strains (93). Similarly, Walker *et al.* observed greater mortality with clade 2 (ribotype 027) and clade 5 (ribotype 078) strains compared to other *C. difficile* genotypes (115).

Ribotype 027 contains several virulence factors that likely contributed to its emergence as a virulent epidemic strain. These include increased production of TcdA and TcdB (113), greater sporulation (116), presence of the binary toxin genes (113), an 18 bp and Δ117 *tcdC* deletion (117), and resistance to fluoroquinolones (114,118). It has been proposed that *tcdC* deletions may interfere with negative repression of toxin expression (113) and deletions of 18 bp, 36 bp, 39 bp in *tcdC* as well as truncating mutations, such as Δ117 in ribotype 027, have been described (117). Some studies show that ribotype 027 strains carrying *tcdC* deletions produce relatively more toxin compared to other strains (113); however, studies replicating these *tcdC* deletions show no increased toxin production relative to wild type *tcdC* (119,120). Furthermore, ribotype 027 strains contain mutations in the DNA gyrase *gyrA*/*gyrB* genes which confer fluoroquinolone resistance (121).

Historically, CDI was considered primarily a HAI; however, there is increasing recognition of CDI cases arising in the community. Identification of CDI cases in previously low risk populations, typically lacking hallmark risk factors for CDI, such as young individuals without antibiotic exposure and pregnant women (122–124) has led to an increased awareness of

community-acquired CDI (CA-CDI). One study observed 41% of CDI cases were CA-CDI; however, this also included cases with indeterminate acquisition, which corresponds to the timeframe in between hospital and community-acquired infection (124). Studies have observed a *C. difficile* prevalence of 1.5-2.1% in stools submitted from community patients (122,125), which in one study was comparable to *Salmonella spp.* (125). The prevalence of CDI in the community may be underestimated as CDI is rarely considered in this population and therefore directed *C. difficile* testing is rarely performed (125). One study of Canadian provincial CDI surveillance data observed increases in the CA-CDI incidence rates in five Canadian provinces between 2011 and 2016/2017 (126). Ribotype 078 is another virulent strain and is associated with CA-CDI in an age group of <80 years causing similar severity of illness as ribotype 027 (127). Similar to ribotype 027, ribotype 078 strains typically contain the binary toxin genes, a 39 bp *tcdC* deletion, and a C184T point mutation in *tcdC* resulting in a premature stop codon (127).

1.6.3 Risk Factors and Special Populations

CDI risk factors are primarily related to the host, microbiome, and increased risk of *C. difficile* exposure (34), and CDI patients often have multiple interrelated risk factors (**Figure 1.2**). With respect to the host, this includes age (generally considered ≥65 years), sex (women may be at increased risk for CDI (106) but varies between studies), and comorbidities such as: inflammatory bowel disease (IBD), solid organ and hematopoetic stem cell transplant, chronic kidney disease, human immunodeficiency virus, and cancer (33,34,128). Antibiotics are the most crucial risk factor for CDI due to their disruption to the microbiota. Many antibiotics have been linked to CDI and particular high risk classes include cephalosporins, fluoroquinolones, carbapenems, and clindamycin (33). In addition to antibiotics, other treatments such as gastric acid suppressants (H_2 receptor antagonist & proton-pump inhibitors (PPI)), GI surgery or interventions (such as feeding

tubes), immunosuppression, and chemotherapy are CDI risk factors (33,128). The role of PPIs and H_2 receptor antagonist as a CDI risk factor is contested (129). Increased exposure of susceptible patients to *C. difficile* spores such as prior hospitalization and greater LOS also increases CDI risk (128). Interestingly, asymptomatic colonization with *C. difficile* (toxigenic and non-toxigenic) is associated with lower risk of CDI (130).

CDI is a prominent issue in long-term care facilities (LTCF) with 36% of the estimated US CDI cases in 2011 having disease onset in these facilities (106). CDC EIP surveillance sites reported 12,821 cases associated with LTCFs between 2011 and 2015 and LTCF-CDI incidence significantly decreased over this period from 311.19 to 138.88/100,000 persons (131). LTCF residents are at particular risk for CDI as many are elderly with frequent hospital exposure, potential antibiotic exposure, several comorbidities, and, in some cases, a history of CDI (33). Additionally, LTCF residents are often asymptomatically colonized with *C. difficile* and spores have been recovered from residents' skin and environment, which may play a role in CDI transmission in these facilities (33,132).

The majority of CA-CDI patients share many of the risk factors stated above; however, these risk factors are often missing in a minority of CA-CDI patients. As stated above in section 1.5.2, peripartum women are at risk for CDI and antibiotic use is often encountered in this population prophylactically for preventing neonatal group B *Streptococcus* infection and in caesarean section (133,134). In addition to antibiotic use and prior hospitalization, caesarean section and comorbidities (including underlying illness, chorioamnionitis and postpartum endometritis) were identified as CDI risk factors in peripartum women (134).

Children are also at risk for *C. difficile* infection. Compared to adult CDI, pediatric CDI is generally less severe and rarely results in recurrence (~10-20%), colectomy, intensive care unit

admission, or death (135–138). Pediatric CDI is predominantly community acquired (51-75%), which is generally less severe than pediatric HA-CDI (136–138). Studies suggest that pediatric CDI is increasing and incidence generally follows a U-shape trend with highest incidence in early (around 1-5 years) and late childhood (around 13-17 years) (136,138–140). Risk factors for pediatric CDI are similar to adult CDI but emphasis is on children with comorbidities such as Hirschsprung's disease, IBD, and cancer (33,34,138).

1.6.4 Reservoirs, Transmission, and Prevention

C. difficile, being a spore forming pathogen, is identified in several reservoirs both in hospital and community environments. In the hospital the primary sources of *C. difficile* are symptomatic CDI patients, asymptomatic *C. difficile* carriers, and environmental contamination. While *C. difficile* was predominantly believed to be nosocomially acquired, the study by Eyre *et al.* (141) provided insight pertaining to the role of symptomatic patients in *C. difficile* transmission. In their study; only 35% of CDI cases had genetic similarity suggestive of transmission from prior CDI cases; whereas, 45% of CDI cases were unlikely to result from similar transmission during the study period. While asymptomatic carriers do not require treatment, unless they progress to infection, they remain a potential transmission risk for pathogenic *C. difficile*. One study linked 29% of CDI cases to asymptomatic carriers, and similarly 30% of cases were linked to other CDI patients (142). Isolation of asymptomatic carriers is costly and is not recommended (143); however one study reported significant decreases in CDI incidence with screening and isolation of asymptomatic carriers (144). *C. difficile* carriers and CDI cases in the LTCF setting also transmit *C. difficile* to other LTCF residents and can potentially transmit *C. difficile* to susceptible hospitalized patients when transferred into the hospital from the LTCF (145).

CDI prevention is focused primarily on isolation (including patients and medical materials), contact precautions (use of gloves and gowns), and disinfection (patient rooms and shared materials) (143). The hospital environment can be a source of *C. difficile* as shedding of spores can contaminate the bed, table, telephone, and call bell of patient rooms (132,142). Patient's skin may be colonized with *C. difficile,* even in *C. difficile* negative patients*,* (132) which can transfer to the hands of healthcare workers and to medical materials (143). Use of gloves and hand washing with soap and water is recommended when interacting with CDI patients as alcohol is ineffective against spores; however, alcohol hand wash is useful for other HAI pathogens such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci* (143). Sporicidal disinfectant such as sodium hypochlorite (bleach) is recommended for cleaning *C. difficile* patient rooms (143). Implementation of infection prevention measures may differ depending on setting i.e: during an outbreak as compared to endemic spread (143).

One of the most effective approaches to CDI prevention is antibiotic stewardship. One study (121) showed that restriction of primarily cephalosporin and fluoroquinolone prescription resulted in dramatic decreases in CDI incidence driven by significant decreases in fluoroquinolone resistant *C. difficile* (ribotype 027 and resistant isolates of ribotypes 106, 001, and 017). This study suggested that antimicrobial stewardship was more effective at reducing CDI incidence compared to other infection prevention measures (121).

There is a much broader range of reservoirs present in the community as *C. difficile* has been identified in animals (both farm and companion) (146–149), food (meat and vegetables) (146,150,151), the environment (water and soil) (146,151), and colonized healthy individuals (31,146). A multitude of animals have been reported to harbor *C. difficile*. In farm and companion animals *C. difficile* has been reported in pigs, cows, horses, dogs, cats, and hamsters (149). Among

more exotic animals, this includes elephants, bears, non-human primates, poultry and ostriches (149). Although genotypes of *C. difficile* found in animals overlap with those identified in CDI patients; there have been no confirmed zoonoses (147–149) but there is compelling evidence for zoonotic transmission. Isolates of ribotype 078 and other ribotypes belonging to sequence type 11 were found to be highly related (in some cases >99%) between animal and human sources by whole genome sequencing suggesting inter and intra species transmission (152,153). Wide-spread dissemination of these genotypes was observed with related isolates identified on the local, national, and international level, and multiple antibiotic resistance markers were identified in the genomes of these isolates (152,153). These findings further stress the importance of *C. difficile* as a One Health pathogen and emphasize the role of antimicrobial stewardship both inside and outside of the hospital.

Asymptomatic carriage occurs at high rates during early life decreasing with age to approximately 4-15% in healthy adults, which is similar to the 3-21% rate of asymptomatic colonization in hospitalized patients (31). Infants and children have been widely reported to be asymptomatically *C. difficile* colonized with prevalence ranging from 4-71% varying between studies (31,154). The highest prevalence of colonization occurs during early infancy (within the first year of life) and decreases throughout childhood eventually mirroring adult rates of asymptomatic colonization after 2 years of age (31). Several factors are associated with increased infant *C. difficile* colonization including: feeding method (increase in formula fed compared to breastfed) (155), method of delivery (caesarean section vs vaginal birth), food diversification, hospitalization, age, pre-term birth, and studies differ as to whether antibiotic use and companion animals (dogs and cats) increase *C. difficile* colonization (156–158). *C. difficile* acquisition in infants is hypothesized to be primarily environmental; however, in some cases there is evidence

suggesting acquisition can be originated from the mother (159,160). Both toxigenic and non-toxigenic strains can colonize infants and in some cases both types of strains may be identified concurrently (157,158,161,162). Asymptomatic colonization in infants is hypothesized to occur by three potential mechanisms being: passive immunity from antibodies present in the mother's milk, absence of *C. difficile* toxin receptors in the maturing gut, and the lack of a *C. difficile* occluding microbiome during early infancy (161,162). One study demonstrated that infants recently colonized with toxigenic *C. difficile* produce an immune response against the *C. difficile* toxins (162). As *C. difficile* is often identified in both symptomatic and asymptomatic children (154) distinguishing pediatric CDI from asymptomatic carriage can be incredibly difficult particularly in younger children. Therefore, it is recommended that in symptomatic infants, other diarrheal etiologies should be ruled out as well for CDI diagnosis (33,46).

1.7 Rationale for Research

Studies investigating the *C. difficile* genotypes colonizing children have been performed in cohorts from the United Kingdom (157), France (158), Belgium (163), Sweden (161), China (164), and the United States (162). Genotypes identified in children colonized with *C. difficile* are similar to those identified in CDI cases (157,158). Contact with infants has previously been identified as a potential risk factor for CA-CDI (122). Together, these findings suggest that infants may play a role as a reservoir of pathogenic *C. difficile* strains in the community setting (158). As there is a lack of data reporting the *C. difficile* genotypes colonizing children in Canada and the potential for toxigenic strains present in children to transfer to susceptible adults, I sought to investigate the *C. difficile* genotypes from these two populations and to determine their genetic relatedness.

Additionally, *C. difficile* culture and genotyping is seldom performed in the clinical microbiology laboratory due to the lengthy turnaround time of culture, but direct from stool ribotyping has the potential to generate a *C. difficile* ribotype without the need for culture. Similar to the development of the isolate ribotyping method, the next evolution of direct from stool ribotyping is its transition from agarose to capillary electrophoresis. There is currently no direct from stool *C. difficile* PCR ribotyping method developed using capillary electrophoresis. Therefore, I also aimed to develop a *C. difficile* direct from stool ribotyping method for implementation in the clinical microbiology laboratory.

1.8 Hypothesis

The *C. difficile* genotypes identified in colonized children are genetically related to those from adult and pediatric cases of infection in Alberta, Canada, and children colonized with *C. difficile* are a community reservoir of pathogenic *C. difficile* strains that can be potentially transmitted to susceptible individuals.

1.9 Objectives

The aim of my study was to characterize the molecular epidemiology of *C. difficile* from Alberta, Canada through the following objectives:

1. To compare the genetic relatedness between *C. difficile* isolates, with respect to the toxin genes (*tcdA, tcdB,* and *cdtB*) and ribotypes, from colonized children and isolates from adult and pediatric CDI cases in Alberta, Canada (**Chapter 2**).

2. To develop and validate a direct from stool PCR ribotyping method using capillary electrophoresis, and to establish a direct ribotyping algorithm for future implementation in the clinical microbiology laboratory for *C. difficile* surveillance (**Chapter 3**).

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Chapter 2 Genetic relatedness of *C. difficile* from colonized children and *C. difficile* infection cases*

2.1 Introduction

Clostridioides difficile is a prevalent cause of hospital acquired diarrheal illness in adults, but this opportunistic pathogen's role in children has been less characterized. *C. difficile* infection (CDI) affects both adults and children; however, infants (≤2 years old) are frequently observed to be asymptomatically colonized. Colonization with toxigenic and non-toxigenic *C. difficile* is common in infants, but strains containing the binary toxin, such as the epidemic ribotype (RT) 027, are rarely observed in colonized children (1,2). Prevalence of *C. difficile* carriage in infants varies between studies, but is generally highest between birth and the first year of life decreasing with age (3). *C. difficile* testing is not recommended for infants because of the high asymptomatic colonization rates in this population with preference given to pursuing alternative etiologies (4).

The majority of pediatric CDI cases occur in early childhood, overlapping with the highest prevalence of colonization, and are primarily acquired from the community (CA-CDI) with studies suggesting a potential rise in pediatric CDI rates (5–7). Additionally, infants colonized with infectious strains represent a large *C. difficile* reservoir residing in the community (1). Previous studies have suggested that contact with infants could be a potential risk factor for developing CDI (8) and similar genotypes have been identified between adults and colonized children in France (1) and the U.K. (2). Studies investigating *C. difficile* genotypes in the pediatric population have

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often focused on pediatric CDI (9,10), and there are few studies investigating genotypes present in colonized children (1,2,11,12) in particular children from North America (13).

The etiological agents of pediatric acute gastroenteritis (AGE) are largely unknown as not all children who experience diarrhea and/or vomiting submit a stool sample for microbiological testing, and moreover fewer children will have a pathogen identified (14). The Alberta Provincial Pediatric EnTeric Infection TEam (APPETITE) is a pediatric case-control study that aims to identify the causes of pediatric AGE in Alberta. APPETITE includes pediatric patients (<18 years old) both symptomatic with AGE and asymptomatic, healthy pediatric controls (14). In order to characterize the etiological agents associated with AGE, rectal swabs along with stools were collected from pediatric patients and controls for molecular testing using the multi-analyte Luminex® xTAG® GPP (14). *C. difficile* was frequently identified in the children from the APPETITE cohort (15,16), which provides an excellent opportunity to study the *C. difficile* genotypes circulating in the pediatric community in Alberta. Additionally, *C. difficile* genotypes in the APPETITE children can be compared to those from adult and pediatric CDI cases to determine genetic relatedness between the cohorts. In this chapter, the genotypes of *C. difficile* isolates from symptomatic and asymptomatic children recruited through the APPETITE study will be compared to isolates from adult and pediatric CDI cases.

2.2 Methods and Materials

2.2.1 Study Subject Recruitment

2.2.1.1 Symptomatic Children and Asymptomatic Children – APPETITE Study

Symptomatic children (n=188) were recruited through the APPETITE study (15). Children symptomatic with AGE (≥3 diarrheal and/or vomiting episodes in the preceding 24 hours and a total duration of symptoms <7 days) were recruited from emergency departments (ED) of the Stollery Children's Hospital (Edmonton, Alberta) and the Alberta Children's Hospital (Calgary, Alberta) (14). In addition, non-hospital based recruitment of symptomatic children was performed through Health Link Alberta, which is a provincial service providing healthcare advice (17). Children recruited through Health Link Alberta were deemed well enough for caregivers to provide ongoing supportive care at home without a physician's assessment (17). A summary of the ages of the symptomatic children are included in **Table 2.1**.

Healthy children (n=120), without AGE, were included as part of the APPETITE study control group. These children were recruited from the EDs, seeking care for non-gastrointestinal related events; other healthy children were from the Calgary Thornhill Community Health Centre, a public health clinic for early childhood vaccinations (15). Ethics approval for the APPETITE study was granted by the University of Calgary and University of Alberta Research Ethics Boards; parental consent along with informed assent were obtained as appropriate (15). Ages of the healthy children are included in **Table 2.1.**

2.2.1.2 CDI Cases

Adult (n=79) and pediatric CDI (n=18) cases that received testing at the University of Alberta Hospital (UAH) were also included for comparison. Ages of the pediatric and adult CDI cohorts are shown in **Table 2.1**.

Table 2.1. Summary of participant ages included in the study.

	Symptomatic Children (n=188)	Healthy Children (n=120)	Adult CDI (n=79)	Pediatric CDI (n=18)
Mean (years)	1.16	0.85	67.47	7.48
Median (y)	0.82	0.58	73.00	6.55
Interquartile Range (y)	(0.63-1.11)	(0.34-1.01)	(58.0-82.0)	(2.34-12.30)
Min. (y)	0.06	0.17	21.00	1.11
Max. (y)	9.98	5.19	97.00	16.49

2.2.2 Specimen Collection

Stools (n=293) and dry flocked rectal swabs (n=15) from symptomatic and healthy APPETITE study participants were collected through the APPETITE study (18). Rectal swabs were collected if the study participant was unable to provide a stool sample. Briefly, samples were collected while participants were present in the EDs or the vaccination clinic (15). If a specimen was not obtained while present in these locations, as well as for all of the participants recruited through Health Link, then a specimen collection kit was given to the caregiver and was retrieved by courier (15). APPETITE samples were kept refrigerated approximately 1-6 days until received in the laboratory where they were frozen at -80°C before undergoing further testing. CDI patients'

stools tested positive for *C. difficile* at the University of Alberta were refrigerated approximately 1-2 weeks until retrieval for further testing.

2.2.3 APPETITE *C. difficile* Screening[†]

APPETITE samples were screened for the *C. difficile* toxin genes *tcdA* and *tcdB* using the GPP (Luminex® Corporation, Toronto, ON, Canada) as described previously (18). The GPP assay is a multi-analyte polymerase chain reaction (PCR) panel assay which includes testing for the following bacteria, viruses, and parasites: Adenovirus genotypes 40/41, *Campylobacter spp., Clostridioides difficile* (*tcdA* & *tcdB*)*, Cryptosporidium, Entamoeba histolytica,* pathogenic *Escherichia coli* (including the shiga toxins (stx_1 & stx_2), serotype O157, and enterotoxigenic *E. coli* (ETEC))*, Giardia,* Norovirus genogroups GI/GII, Rotavirus group A, *Salmonella spp., Shigella spp., Vibrio cholerae,* and *Yersinia spp.* (19). For stool samples, ~100 mg stool was suspended in 1 mL NucleiSENS® Lysis Buffer (LB) (bioMérieux, Montreal, QC, Canada). For rectal swabs, 500 µL 1X phosphate buffered saline (PBS) (ThermoFisher Scientific, Waltham, MA, USA) was used to rinse the rectal swab and, after brief vortexing, 300 µL of the 1X PBS rinse was suspended in 700 µL LB (bioMérieux). Suspensions were prepared in Bertin Corp SK38 soil grinding tubes (ESBE Scientific, Saint-Laurent, QC, Canada) spiked with 10 µL of the internal control xTAG® MS2 bacteriophage (Luminex® Corporation). Tubes were mixed for 10 minutes using a Fisherbrand™ analog vortex mixer (ThermoFisher Scientific) at high speed (setting 8-10), incubated in ambient conditions for 15 minutes, and centrifuged for 5 minutes at 15,871 X *g* using an Eppendorf™ Centrifuge 5425 (ThermoFisher Scientific). Two-hundred microliters of suspension was added to the easyMAG® cassette and subjected to a pre-lysis step on the

[†] Dr. Brendon Parsons performed testing on all of the APPETITE samples using the Luminex® xTAG® Gastrointestinal Pathogen Panel at APL-ProvLab, Edmonton, Alberta.

NucleiSENS® easyMAG® instrument (bioMérieux). After pre-lysis, the sample was mixed with 125 µL of NucleiSENS® easyMAG® MagSIL beads (bioMérieux) diluted 1:1 with UltraPure Distilled water (Alberta Precision Laboratories-ProvLab (APL-ProvLab), Edmonton, AB, Canada) then the nucleic acid was extracted using the Specific A 1.0.2 program on the NucleiSENS® easyMAG® DNA extractor (bioMérieux). A 70 µL eluate was used for the GPP assay performed according to the manufacturer's instructions (18). For the GPP assay, PCR was performed using 10 µL of the extracted nucleic acid with reagents from the proprietary Luminex® xTAG® GPP test kit (Luminex® Corporation): 2.5 µL xTAG® RNAse-free Water, 7.5 µL xTAG® One Step Buffer 5X, 2.5 µL xTAG® GPP Primer Mix, 0.5 µL xTAG® BSA, and 2.0 µL OneStep Enzyme Mix with the following PCR cycling conditions: 1 cycle of 53°C for 20 min, 1 cycle of 95°C for 15 min, 38 cycles of 95°C, 58°C, and 72°C for 30 seconds each, and 1 cycle of 72°C for 2 min using an Eppendorf® MasterCycler® Pro S thermal cycler (Luminex® Corporation). Five microliters of PCR product was mixed with 20 µL xTAG® GPP Bead Mix (Luminex® Corporation) in a 96-well plate, and 1 µL of xTAG® 0.22 SAPE (Luminex® Corporation) reporter dye and 74 µL xTAG® Reporter Buffer (Luminex® Corporation) was added to the wells. This mixture was hybridized at 60°C for 3 min and then 45°C for 45 min in the thermal cycler (Luminex® Corporation). Samples were then analyzed using the MAGPIX® instrument and Luminex® xPONENT® software (Luminex® Corporation). Mean fluorescent intensity recordings for each enteric pathogen target (listed above), generated by the MAGPIX®, were analyzed in Luminex® TDAS GPP v1.11 software (Luminex® Corporation) and the pathogens identified were reported for each sample. Samples positive for toxigenic *C. difficile* between February 2015 and August 2017 were included.

2.2.4 CDI *C. difficile* Testing

CDI patient stools were tested at the UAH using a two-step algorithm. This included the *C. DIFF QUIK CHEK COMPLETE*® (CDQC) (Techlab, Blacksburg, VA, USA) and the GeneXpert® *C. difficile* (Cepheid, Sunnyvale, CA, USA). The CDQC detects glutamate dehydrogenase (GDH) and toxin A & B, and all stools testing toxin negative by CDQC were further tested for *tcdB* by GeneXpert®. Adult CDI cases from April 2015 to September 2015 and pediatric CDI cases from between April 2015 and February 2016 were included.

2.2.5 *C. difficile* Culture, DNA Extraction, and Storage

C. difficile was isolated from stool specimens by direct inoculation onto CHROMID® *C. difficile* agar (bioMérieux), and incubated in anaerobic conditions at 37°C for 48 hours. Anaerobic conditions were generated using the Advanced® Anoxomat™ instrument (Advanced Instruments, Norwood, MA, USA) with an atmospheric composition of 0.2% oxygen, 9.9% carbon dioxide, 9.9% hydrogen, and 80.2% nitrogen in a sealed Anoxomat™ anaerobic jar containing an anaerobic indicator (ThermoFisher Scientific) and catalyst (Advanced Instruments). Alternatively, rectal swabs collected from the APPETITE study, that were suspended in 500 µL 1X PBS during Luminex® xTAG® GPP pathogen screening, were used for *C. difficile* isolation by directly inoculating 20-50 µL of the 1X PBS suspension onto CHROMID® agar with incubation using the same conditions as described above. Black colonies, presumptive of *C. difficile,* on CHROMID® plates were subcultured, selecting one colony per sample, to pre-reduced brain heart infusion (BHI) agar (APL-ProvLab) and incubated anaerobically at 37°C for 36 to 48 hours. *C. difficile* growth was identified on BHI plates based on green-yellow colony color with a typical “horse barn” odor and chartreuse fluorescence under exposure to long wave 365 nm ultraviolet light. A sweep of

clonal colonies from the BHI plate were subcultured into pre-reduced BHI broth (APL-ProvLab) and incubated anaerobically for 5 to 6 hours. DNA was extracted by centrifuging 400 µL of BHI broth cultures at 14,674 X *g*; the supernatant was removed, and the pellet was resuspended in 250 µL InstaGene™ matrix (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). The cell suspension was boiled at 90°C for 30 min using a VWR Standard Heatblock (VWR Scientific, Mississauga, ON, Canada) and 200 µL supernatant was retained for PCR ribotyping. In addition, a 250 µL aliquot of BHI broth was added to 2 mL skim milk medium (APL-ProvLab) and kept frozen at -70°C for storage.

2.2.6 PCR Ribotyping and Capillary Electrophoresis

DNA extracted from *C. difficile* cultures was quantified using a Nanodrop™ 2000 spectrophotometer (ThemoFisher Scientific) and then standardized to 100 ng/µL by diluting in UltraPure™ DNAse, RNAse-Free Distilled Water (ThemoFisher Scientific). PCR ribotyping was performed using 200 ng of DNA with a 100 nM carboxyfluorescein (FAM)-labelled 16S forward (ThermoFisher Scientific) and unlabelled 23S reverse (Integrated DNA Technologies, Coralville, IA, USA) Bidet *et al.* ribotyping primers (20) (**Table 2.2**), 200 µM dNTPs (ThermoFisher Scientific), 1X PCR buffer (Qiagen, Mississauga, ON, Canada), and 0.025 U/µL HotStarTaq® Polymerase (Qiagen). Fifty microliter reactions were subjected to PCR cycles as shown in **Table 2.2** using the Eppendorf® MasterCycler® Pro S thermal cycler. PCR ribotyping amplicons were analyzed using capillary gel electrophoresis on the Applied Biosystems® 3130 genetic analyzer as previously described (21). In brief, 1 µL amplicon was diluted 1:10 with 8.5 µL of Hi-Di™ formamide (ThermoFisher Scientific) and 0.5 µL GeneScan™ 1200 LIZ™ sizing standard (ThermoFisher Scientific). Amplicons were then subject to 95°C for 5 min using an Applied

Biosystems® GeneAmp™ 2720 thermal cycler (ThermoFisher Scientific) and were snap-chilled using a -20°C cold-block (Fisher Scientific, Nepean, Ontario, Canada). Amplified fragments were then separated using POP-7™ polymer in a 36-cm capillary array (ThermoFisher Scientific) with the following conditions: 5kV injection voltage for 5 seconds, and 6.5 kV run voltage for 103 minutes to separate amplicons. Complete capillary electrophoresis parameters are shown in **Appendix A.**

Table 2.2. PCR ribotyping primer sequences with corresponding PCR cycling conditions.

Target	Primers (5'→3')	Cycling Conditions	Reference
16S-23S ISR	F: FAM-GTGCGGCTGGATCACCTCCT R: CCCTGCACCCTTAATAACTTGACC	1 cycle (95°C x 15 min) 24 cycles (95°C x 1min, 57°C x 1min, 72°C x 1min) 1 cycle (72°C x 10 min)	(20)

2.2.7 Ribotype Assignment

Electropherograms produced from capillary gel electrophoresis were analyzed using GeneMapper® v4.0 software (ThermoFisher Scientific) and a molecular weight sizing table was generated for amplified fragments (peaks) between 200-700 base pairs. The sizing table was imported into BioNumerics software v6.01 for visualization as synthetic bands (Applied Maths, Austin, TX, USA). A threshold of 10% of the highest peak's height was applied to synthetic band profiles to assign bands within BioNumerics; however, ribotypes were visually compared and determined. Ribotypes were assigned by comparing to a reference database of ribotypes developed through national *C. difficile* surveillance at the Public Health Agency of Canada - National Microbiology Laboratory (PHAC-NML, Winnipeg, MB, Canada). Ribotypes not matching a ribotype pattern using standard nomenclature were designated as "ns" (non-standard ribotype) and

given a number (ex: ns123). Ribotypes with only "ns" indicate that no assignment has been given by the PHAC-NML.

2.2.8 PCR Ribotyping Validation, Pulsed-field Gel Electrophoresis and Toxin Gene Amplification.‡

As part of a *C. difficile* ribotyping validation to develop PCR ribotyping in our laboratory, *C. difficile* isolates from 76 APPETITE children and all of the 97 CDI cases (n=173) were sent to the PHAC-NML where molecular characterization of the isolates was performed using PCR ribotyping, pulsed-field gel electrophoresis (PFGE), and toxin gene amplification of *tcdA, tcdB,* and *cdtB* by endpoint PCR (**Figure 2.1**). In addition to the 76 APPETITE *C. difficile* isolates included in the PCR ribotyping validation, a further 232 *C. difficile* isolates were recovered from APPETITE children and had PCR ribotyping performed only (**Figure 2.1**). Using this data, the relatedness between *C. difficile* isolates from children and adults was compared by identifying differences in the toxin genes and ribotypes present in *C. difficile* isolates from both populations.

‡ Dr. Nancy Price performed the *C. difficile* culture for all of the CDI cases, 62 APPETITE stools, and 15 APPETITE rectal swab samples at APL-ProvLab, Edmonton, Alberta. Tim Du and Dr. George Golding performed molecular characterization of the isolates included in the PCR ribotyping validation at the Public Health Agency of Canada – National Microbiology Laboratory in Winnipeg, Manitoba.

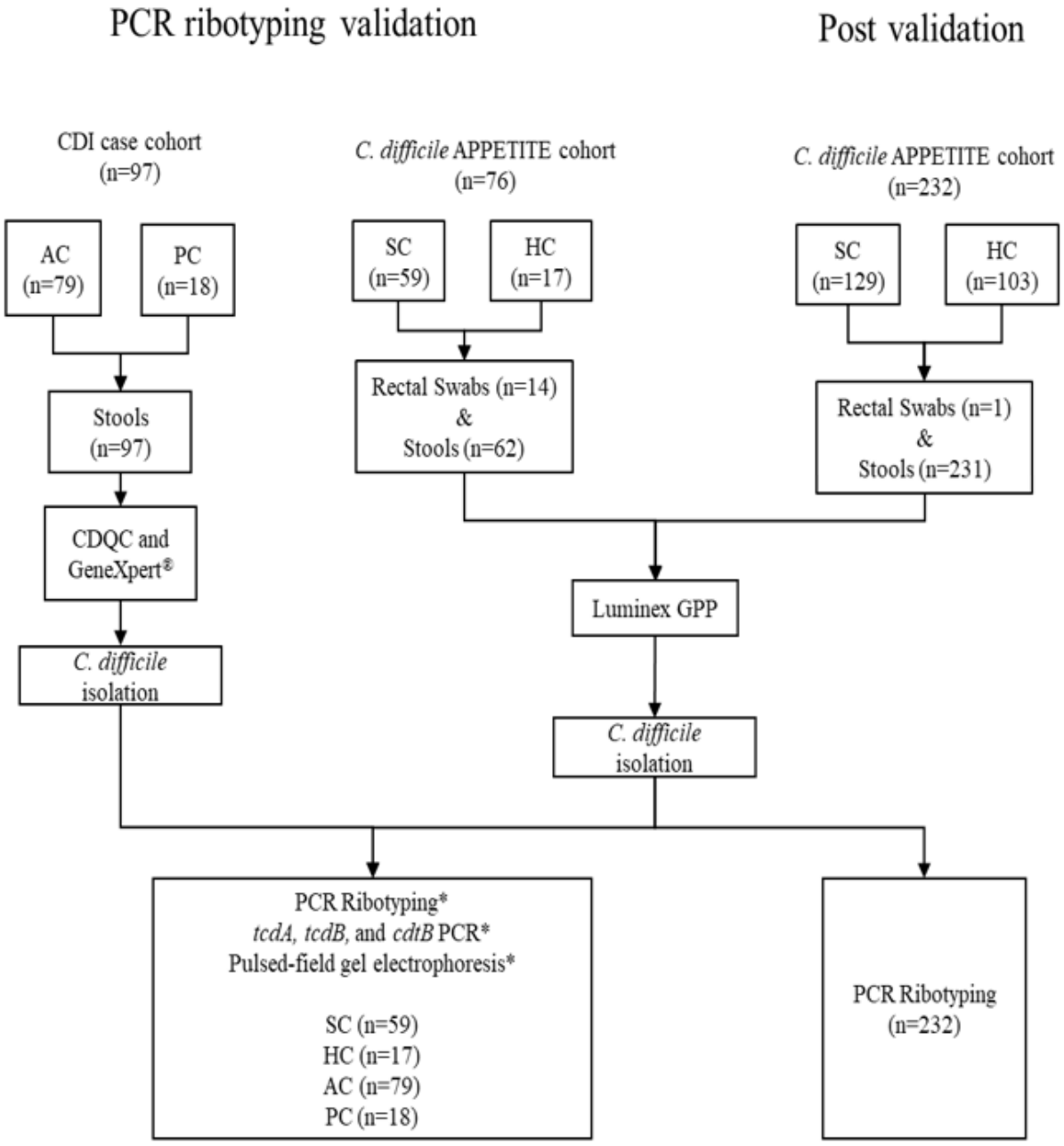

Figure 2.1. Samples, screening methods, and genotyping assays performed. Samples were collected from the four cohorts being symptomatic children (SC), healthy children (HC), adult CDI cases (AC), and pediatric CDI cases (PC). All samples were screened for toxigenic *C. difficile* by either *C. DIFF QUIK CHEK COMPLETE*® (CDQC) and/or GeneXpert® for CDI cases (AC & PC) or by Luminex® xTAG® GPP for the APPETITE cohort (SC & HC). All samples included had *C. difficile* recovered and genotypic characterization performed. As part of a PCR ribotyping validation, isolates from the AC, PC, SC, and HC cohorts were sent to the Public Health Agency of Canada – National Microbiology Laboratory and had molecular characterization (*) performed. After the PCR ribotyping validation, additional isolates from the APPETITE cohort were characterized by PCR ribotyping.

2.2.9 Statistical Analyses

Simpson's index of diversity, a statistic reflecting the abundance and distribution of a population, was calculated using Microsoft Excel (Microsoft, Redmond, WA, USA). The calculation for Simpson's index of diversity is shown in **Appendix B**. Populations with higher index scores reflect greater diversity compared to those with lower index scores. Fisher's exact test was used for all analyses comparing proportions of isolates or samples with respect to toxin gene profiles, ribotypes, and toxin positivity. Statistical analyses were performed using GraphPad Software v8.4.3 (GraphPad Software Inc., San Diego, CA, USA) with $p<0.01$ considered significant.

2.3 Results

2.3.1 *C. difficile* toxin genes t*cdA*, *tcdB*, and *cdtB*

In order to determine if *C. difficile* isolates from the APPETITE children and CDI cases were similar with respect to the toxin genes, *tcdA, tcdB,* and *cdtB* genes were amplified and their profiles were identified and compared between isolates from each cohort as shown in **Table 2.3**. The toxin profiles were classified into the following categories for each cohort: non-toxigenic (*tcdA, tcdB* and *cdtB* negative), toxigenic (*tcdA & tcdB* positive but *cdtB* negative), and binary toxigenic (*tcdA, tcdB,* and *cdtB* positive). None of the healthy children carried non-toxigenic strains and no binary toxigenic strains were identified from the pediatric CDI patients. The toxin gene profiles in isolates recovered from the APPETITE children (SC and HC) and pediatric CDI patients (PC) were similar as they were primarily toxigenic strains (88.1-94.1%) with few binary

toxigenic (0-5.9%) and non-toxigenic (0-11.1%) strains identified. This contrasted with the adult CDI (AC) cases as the toxin profiles among these isolates were primarily binary toxigenic strains (53.2%), while the remainder were toxigenic (43.0%) and non-toxigenic strains (3.8%). There were 10 non-toxigenic isolates recovered from samples tested positive by CDQC or PCR (GeneXpert® or GPP), which indicates the presence of a toxigenic strain. It is possible that a toxigenic strain, in addition to the non-toxigenic strain identified, was not characterized for these cases as only a single isolate was characterized for each sample (**Table 2.3**). The proportion of isolates with a particular toxin gene profile was compared between cohorts. The proportion of binary toxigenic isolates was significantly higher in adult CDI cases ($p<0.001$) compared to the other cohorts. There were no other significant differences in the proportion of isolates belonging to a particular toxin gene profile between any of the cohorts.

Table 2.3. *C. difficile* toxin gene profile distribution between cohorts

Toxin Profile	Symptomatic Children (n=59)	Healthy Children (n=17)	Adult CDI (n=79)*	Pediatric CDI (n=18)
***tcdA*$^+$*/tcdB*$^+$*/cdtB*$^-$**	52 (88.1%)	16 (94.1%)	34 (43.0%)†	16 (88.9%)
***tcdA*$^+$*/tcdB*$^+$*/cdtB*$^+$**	2 (3.4%)	1 (5.9%)	42 (53.2%)	0 (0.0%)
***tcdA*$^-$*/tcdB*$^-$*/cdtB*$^-$**	5 (8.5%)	0 (0.0%)	3 (3.8%)	2 (11.1%)

†One isolate containing a deleted form of tcdA and intact tcdB was included.
*Proportion of binary toxigenic isolates was significantly higher compared to the other cohorts ($p<0.001$).

2.3.2 *C. difficile* Ribotype Comparison between APPETITE Children and CDI cases

In order to determine if the *C. difficile* genotypes present in symptomatic and healthy children from APPETITE were similar to those from adult and pediatric CDI patients, the ribotypes identified in each cohort were compared (**Table 2.4** and **Table 2.5**). A total of 72 ribotypes were identified from the isolates with RT 106 (n=74, 18.3%), 027 (n=39, 9.6%), 629 (n=33, 8.1%), 020 (n=32, 7.9%), and 014 (n=28, 6.9%) comprising 50.9% (n=206) of the isolates (**Table 2.4** and **Table 2.5**). The remaining 49.1% (n=199) of isolates were distributed among 67 different ribotypes, of which 34 (8.4%) corresponded to only a single isolate. In the APPETITE children, 53 ribotypes were identified from symptomatic children and 38 ribotypes from healthy children with RT 106 being the predominant strain comprising 22.3% (n=42) of isolates from symptomatic children and 18.3% (n=22) of isolates from healthy children (**Table 2.4** and **Table 2.5**). With respect to CDI cases, a total of 29 ribotypes were identified in adult CDI and 10 ribotypes were identified in pediatric CDI cases (**Table 2.4** and **Table 2.5**). The predominant ribotype in adult CDI cases was RT 027 (n=35, 44.3%), whereas, RT 106 (n=5, 27.8%) was most prevalent in pediatric CDI cases, which was similar to the APPETITE children. Ribotype diversity, as measured by the Simpson's index score, was highest in the symptomatic and healthy APPETITE children as well as pediatric CDI cases with scores of 0.92, 0.93, and 0.91 respectively. Diversity was lowest amongst adult CDI cases with an index score of 0.80 (**Appendix B**).

Comparing ribotypes between the APPETITE and CDI cohorts showed that 34 out of the 72 ribotypes identified were shared between cohorts (**Table 2.4**), whereas the remaining 38 ribotypes were unique to their respective cohorts (**Table 2.5**). Several ribotypes were identified in multiple cohorts as 15 ribotypes were shared between only two cohorts, while 14 ribotypes were shared between three cohorts and 5 ribotypes were identified in all cohorts (**Table 2.4**). Comparing

the toxin gene profiles between shared ribotypes, for those isolates which had toxin gene PCR performed (n=173) (**Figure 2.1**), most of the shared ribotypes between the APPETITE *C. difficile* isolates and CDI cases were toxigenic strains with the exception of binary toxigenic (RT 019, 027, & 078) and non-toxigenic (RT 010 and 039) strains that were also shared (**Appendix C**). Interestingly, the mutual RT 009 isolates from both a symptomatic child and adult CDI case (**Appendix C**) differed in toxin gene profile as the adult CDI isolate was non-toxigenic and the symptomatic child isolate was toxigenic.

To determine if there was a significant difference in the proportion of isolates between cohorts for ribotypes that were shared (**Table 2.4**), the proportion of isolates with the same ribotype were compared between the cohorts. Significant differences in the proportion of isolates between cohorts were observed only for RT 027 ($p<0.01$, adult CDI compared to all cohorts) and 106 ($p<0.01$, only for symptomatic children compared to adult CDI) (**Table 2.4**). While not significantly different, the proportion of isolates between specific cohorts approached significance (p-values: 0.0178-0.0878) for RT 106, 629, 020, 014, 039, 056, and 009 (**Table 2.4**). The remaining shared ribotypes did not approach significant differences in any of the comparisons of the proportion of isolates between any of the cohorts, and results of all pairwise Fisher's exact test comparisons are included in **Appendix D**.

Table 2.4. Shared ribotypes generated from APPETITE and CDI cases

		Cohort				Total isolates (n=405)
		SC	HC	AC	PC	No. (%)
Shared Ribotypes	**106**	42	22	5	5	74 (18.3%)*Δ
	027	2	2	35		39 (9.6%)*
	629	16	13	2	2	33 (8.1%)Δ
	020	16	13	2	1	32 (7.9%)Δ
	014	18	6	2	2	28 (6.9%)Δ
	076	7	8	2		17 (4.2%)
	056	4	3	4		11 (2.7%)Δ
	002	7	1	2		10 (2.5%)
	010	5	3	1		9 (2.2%)
	077	6	3			9 (2.2%)
	039	4		1	2	7 (1.7%)Δ
	057	3	2		2	7 (1.7%)
	072	1	2	2	1	6 (1.5%)
	009	1	4	1		6 (1.5%)Δ
	325	4	2			6 (1.5%)
	ns180	3	2		1	6 (1.5%)
	ns70	2	1	2		5 (1.2%)
	137	1	3	1		5 (1.2%)
	296	3	2			5 (1.2%)
	012	2	2	1		5 (1.2%)
	019	3		1		4 (1.0%)
	046	2	1	1		4 (1.0%)
	511	2	1	1		4 (1.0%)
	103	1	3			4 (1.0%)
	328	1	3			4 (1.0%)
	078		1	2		3 (0.7%)
	ns205	2	1			3 (0.7%)
	017		2	1		3 (0.7%)
	075		1	2		3 (0.7%)
	015	1		1		2 (0.5%)
	ns123	1		1		2 (0.5%)
	354	1	1			2 (0.5%)
	207	1	1			2 (0.5%)
	ns103	1	1			2 (0.5%)

The total number of isolates included was 405. Symptomatic children (SC), healthy children (HC), adult CDI cases (AC), and pediatric CDI cases (PC). *Ribotypes significantly differed ($p<0.01$) and Δribotypes approached significant differences (p-values: 0.0178-0.0878).

Table 2.5. **Unique ribotypes identified in symptomatic and healthy children from APPETITE as well as adult and pediatric CDI cases**

	Symptomatic Children (SC)	Healthy Children (HC)	Adult CDI (AC)	Pediatric CDI (PC)
Unshared Ribotypes	005 (2), 023, 085, 153, 293, 351, 530, ns, ns107, ns113 (2), ns145, ns164 (2), ns166, ns165, ns184, ns195, ns202, ns203, ns204, ns281, ns293, ns326	097, 154 (3), ns23, ns138, ns223, ns235, ns267, ns296	043, 126, 157, ns108, ns152, ns167	004, ns178

Brackets indicate the number of isolates. The "ns" ribotype does not have a number designated by the PHAC-NML.

With respect to only those isolates included in the ribotyping validation (n=173) (**Figure 2.1**), 20 ribotypes were shared (n=139) (**Appendix C**) between the different cohorts. PFGE pulsotypes were available for the toxigenic isolates included in the ribotyping validation (n=163/173, as 10 were non-toxigenic (**Table 2.3**) and PFGE was not performed), and PFGE pulsotype was used to further investigate genetic relatedness between isolates that shared ribotypes between cohorts (n=139) as shown in **Figure 2.2.** Of the 20 ribotypes that were shared between cohorts included in the ribotyping validation (n=139), 11 ribotypes had isolates that also shared a PFGE pulsotype with a total of 19 unique ribotype-pulsotype combinations identified in isolates from different cohorts (n=90) (**Figure 2.2**). Sixteen unique ribotypes and pulsotypes were shared between only two cohorts while 3 were shared across all four cohorts being (ribotype-pulsotype): 020-0033, 106-0046, and 106-0612. These results generated from the two molecular typing methods further confirm the clonal relatedness of these strains inferring that these isolates from the different cohorts might be related.

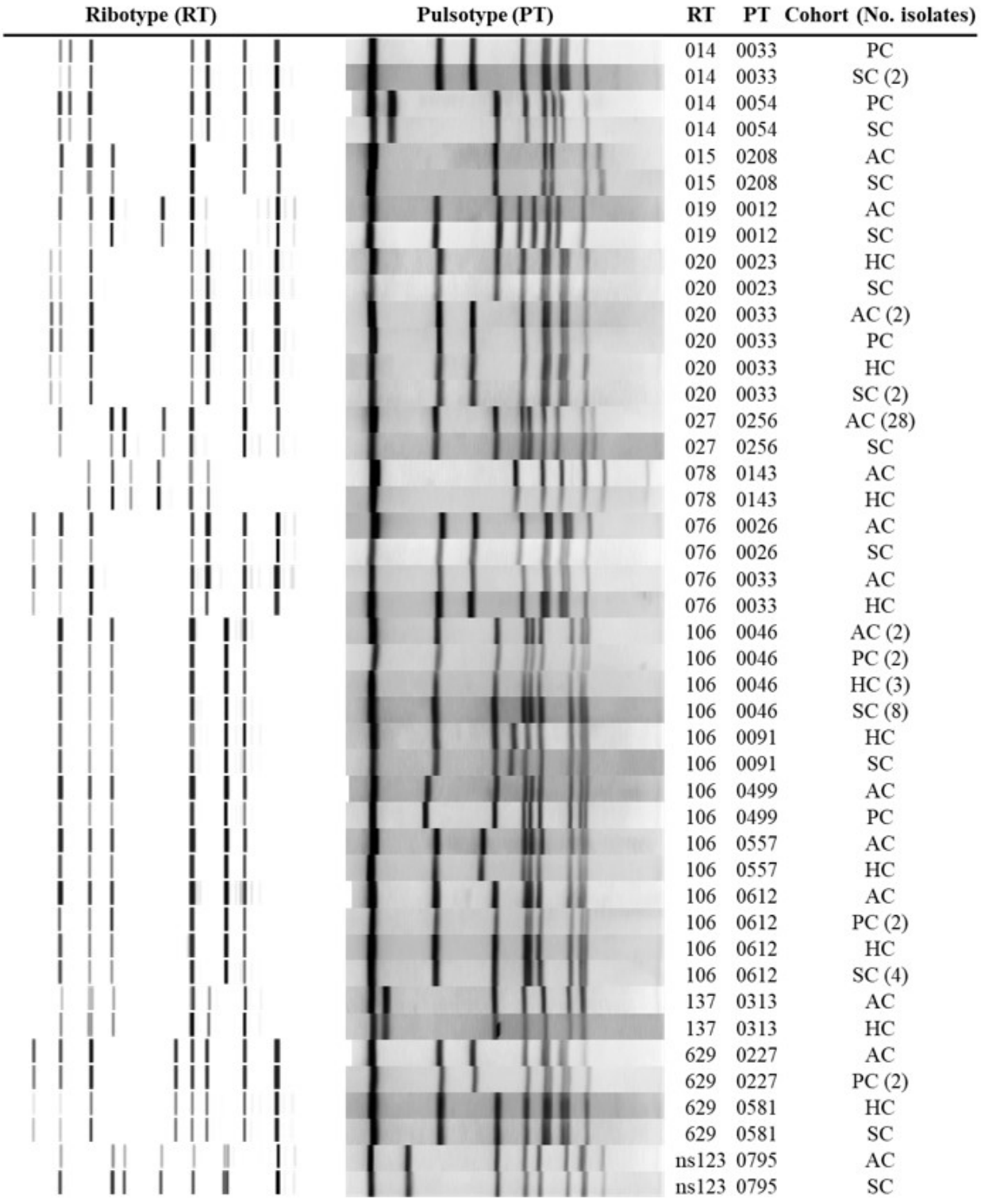

Figure 2.2. Shared *C. difficile* genotypes with matching ribotype and pulsotype identified both in the APPETITE children and CDI cases. Symptomatic children (SC), healthy children (HC), adult CDI (AC), and pediatric CDI (PC). Brackets indicate number of isolates.

2.3.3 *C. difficile* Toxin Production in Symptomatic and Healthy APPETITE Children

As toxigenic *C. difficile* isolates (**Table 2.3**) were identified by PCR in both symptomatic and healthy APPETITE children included in the ribotyping validation; toxin enzyme immunoassay (CDQC) was performed on 59 stool samples from 45 symptomatic and 14 healthy children as 3 stools had insufficient quantity for further testing. *C. difficile* toxin was detected in 28/45 (62.2%) of the symptomatic children and 6/14 (42.9%) of the healthy children. There was no significant difference in toxin positivity between the symptomatic and healthy APPETITE children tested (p=0.22).

2.3.4 *C. difficile* Ribotype Comparison between Symptomatic and Healthy APPETITE Children

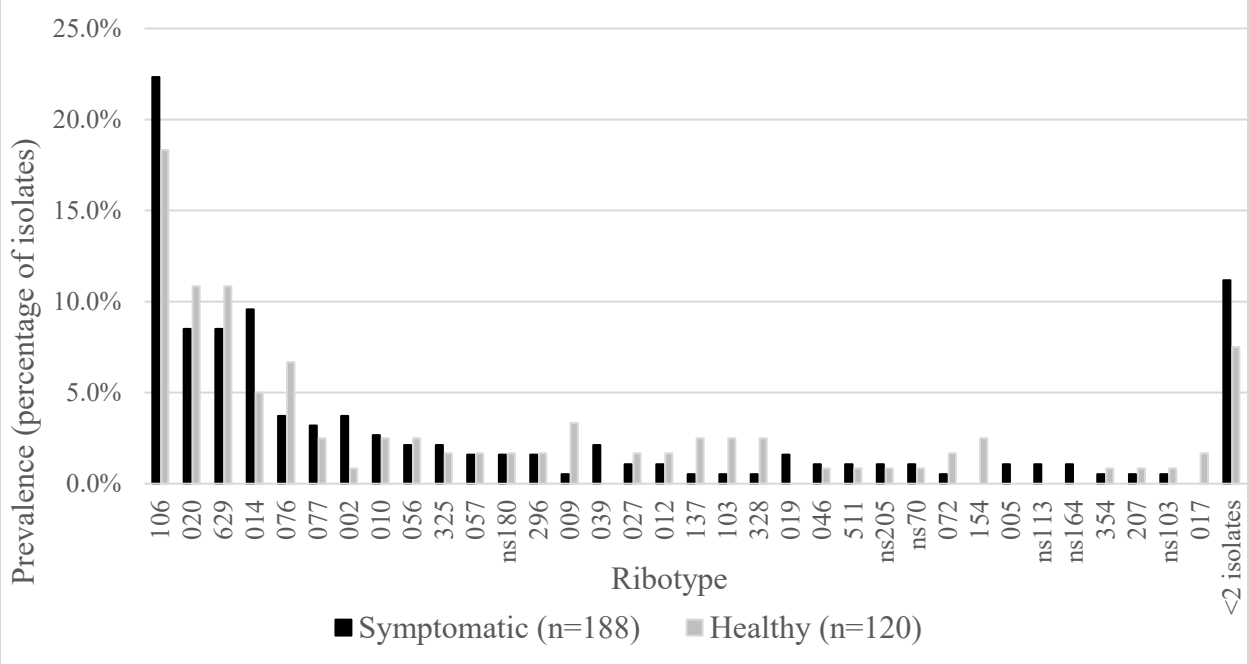

Figure 2.3. *C. difficile* ribotype prevalence in symptomatic and healthy APPETITE children. Thirty unique (i.e. not mutual) ribotypes represented by only single isolates are grouped together as <2 isolates.

The molecular epidemiology of *C. difficile* in the APPETITE children was also analyzed with respect to symptomatic and healthy children. Ribotypes did not show major differences between the symptomatic and asymptomatic children (**Figure 2.3**). The four most prevalent ribotypes in symptomatic children were 106 (n=42, 22.3%), 014 (n=18, 9.6%), 020 (n=16, 8.5%), and 629 (n=16, 8.5%), and in the healthy children these were 106 (n=22, 18.3%), 020 (n=13, 10.8%), 629 (n=13, 10.8%), and 076 (n=8, 6.7%) (**Figure 2.3** and **Table 2.4**). Overall, 27 out of 64 RTs were shared between healthy and symptomatic children (**Figure 2.3** and **Table 2.4**). As with symptoms, ribotype prevalence was also similar between *C. difficile* isolates from APPETITE children recruited from Calgary (n=235) and Edmonton (n=73) which were mostly ribotypes 106, 020, and 629 (**Appendix E**). Twenty-three ribotypes were shared between these groups of children whereas 35 out of 58 ribotypes were unique to Calgary and 6 out of 29 were identified only from Edmonton (**Appendix E**).

2.4 Discussion

There is limited data regarding the *C. difficile* strains colonizing infants and children particularly in North America. The most prevalent toxigenic strains identified among colonized European children were RTs 020, 014, & 077 from French children (1), 020/014, 017 & 005 among children from England (2), and 014, NPR3, & 502 among a cohort of Belgian children (11) and 001, 014, and 020 from Swedish children (22). The most prevalent toxigenic ribotypes in colonized children from China were HB03, 001, & 017 (12). Isolates of RT 002, 005, 012, 014, 015, 017, 020, 046, 072, 077, and 078 were common between this study and those previously published (1,2,11,12). Ribotypes 020 and 014 were widely reported in the literature (1,2,11,22), and were highly prevalent among children in this study (**Table 2.4**). Surprisingly, ribotypes 020 and 014

were not reported in the colonized Chinese children (12). None of the previous studies identified RT 027 in colonized children (1,2,13), whereas in this study 4 RT 027 isolates were identified in children in addition to isolates from other *cdtB* positive strains including RT 019 and 078 (**Table 2.4**). The low prevalence of RT 027 in CDI patients from Paris, France and Oxfordshire, England may be attributed to the absence of this genotype in colonized infants from these areas (1,2), whereas RT 027 is still present in the U.S. (23) and Canada (24). However, non-RT 027 binary toxin positive ribotypes were identified in the previous studies such as RT HB53 and 078 in colonized children from China (12) and England (2) respectively. Binary toxin positive strains such as RT 027 have been associated with worse clinical outcomes in adult CDI (25) and have been identified in pediatric CDIs (10); however, their significance in colonization is unknown. It's likely that carriage of *cdtA/cdtB* positive strains confers no additional pathogenicity in colonized children relative to *tcdA* and *tcdB* positive strains; however, there remains the potential risk of transmitting these virulent strains to susceptible caregivers.

Studies of colonized children differ in the inclusion of symptomatic children with some studies including these children (2,26) while others included only asymptomatic individuals (1,11,12). Previous studies surveyed only children under two years (2,11) or between 0 and 3 years (1,12) which likely reflected *C. difficile* carriage as opposed to infection. In this study, the symptomatic children and healthy children included were primarily <2 years of age (IQR: 0.63-1.11 years for symptomatic children and 0.34-1.01 years for healthy children, **Table 2.1**), which differed compared to the pediatric CDI cohort which were older (IQR: 2.34-12.30 years, **Table 2.1**). As infants are often asymptomatically colonized with toxigenic *C. difficile,* discerning colonization from infection can be particularly challenging, and care should be taken to exclude potential CDI cases when including symptomatic children in studies of *C. difficile* colonization. In

the APPETITE cohort, severe AGE was not associated with *C. difficile* detection in the children from APPETITE regardless of whether *C. difficile* was detected alone or alongside another pathogen (16) suggesting *C. difficile* was most likely present as a colonizer in these children. Additionally, in this study there was no difference in toxin positivity between symptomatic and healthy children, which provides further evidence for *C. difficile* colonization in the APPETITE children.

In this study, ribotypes were shared between children and adult CDI cases which is similar to previously published studies (1,2). However, many of the shared ribotypes consisted of only single isolate pairs from one or both of the cohorts. Genotypes (ribotype-pulsotype) 020-0033, 106-0046, and 106-0612 were shared between adults and children and these ribotypes were quite prevalent in their respective cohorts. It is possible this relationship between isolates from different cohorts arose due to an overall higher prevalence of these ribotypes in their respective cohorts, which suggests perhaps these ribotypes are present in an environment shared between the cohorts. Additionally, significant differences in ribotype distribution between cohorts were observed particularly for RT 106 with the APPETITE cohort and pediatric CDI having a higher proportion of isolates compared to adult CDI cases. Similarly adult CDI cases had a significantly higher proportion of RT 027 isolates compared to APPETITE and pediatric CDI. It's likely that this observation was due to the differences in the ages of the cohorts with ribotype 027 identified more in older individuals, and ribotype 106 in younger individuals.

Interestingly, matching ribotype (and pulsotype) 027 & 078 isolates were identified in both the APPETITE and adult CDI cases (**Figure 2.2**). Also, two symptomatic children both had isolates of ribotype ns164 with pulsotype 0145 (**Table 2.5**) and were presumably siblings, based on recruitment questionnaire responses, suggesting each acquired *C. difficile* from a shared

environment or potentially transmitted this strain between each other. Further investigation using whole genome sequencing (WGS) of these isolates is warranted to better discriminate the relatedness of these isolate pairs. A study from Oxfordshire, U.K. compared genetic relatedness between *C. difficile* isolates from infants and CDI cases using WGS (2). Based on estimates of the *C. difficile* evolutionary rate and within-host diversity, genetic relatedness was assessed as isolates with ≤2 single nucleotide polymorphism (SNP) difference were considered to represent potential transmission or recent acquisition, whereas isolates with ≤10 SNPs difference were considered to have likely arisen from a shared exposure. When comparing CDI cases sampled prior to the infants to determine if infants acquire *C. difficile* from CDI cases, 18% of the infant isolates were potentially transmitted from a CDI case (≤2 SNPs), whereas 50% of infant isolates were consistent with a shared exposure within the past 5 years (≤10 SNPs). This suggests that in most cases infants likely acquire *C. difficile* from their environment, which may be contaminated with *C. difficile* resembling isolates from CDI cases, as opposed to direct acquisition from CDI cases. The investigators also compared isolates from CDI cases and infants in order to assess potential transmission due to infant *C. difficile*. Based on this study from Oxfordshire, only 2% of these CDI isolates were potentially transmitted from an infant to a CDI case (≤2 SNPs), whereas 15% of adult CDI isolates were consistent with a shared exposure between infants and CDI cases (≤10 SNPs) suggesting that, for most CDI cases, acquisition of *C. difficile* from colonized infants is rare and is unlikely to explain the majority of cases. Interestingly, Alam *et al*. (27) recently characterized toxigenic *C. difficile* isolates from community and hospital environments in Houston, Texas and found RT 027 was significantly more prevalent in both clinical isolates and isolates from the hospital environment as compared to the community. As noted previously, the proportion of RT 027 was significantly higher in adult CDI cases ($p<0.001$), which could reflect primarily healthcare

facility related *C. difficile*. Contrarily, the proportion of RT 106 was significantly higher, and approached significant differences, in both APPETITE and pediatric CDI children compared to adult CDI cases, suggesting RT 106 may be circulating primarily in the community. The majority (88.9%) of pediatric CDI isolates in this study overlapped with ribotypes from APPETITE children which may also reflect primarily community-acquired strains (6,28) as pediatric *C. difficile* acquisition is believed to be mediated through environmental contamination (29). Perhaps in pediatric CDI cases, the ribotype identified was present as a colonizing strain prior to the development of CDI and is then subsequently identified.

Ribotype 106 was the most prevalent ribotype identified, regardless of symptoms, in both APPETITE children and pediatric CDI. Interestingly, in a study of *C. difficile* genotypes in pediatric CDI patients from a Chicago children's hospital (30), which used restriction endonuclease analysis (REA) for genotyping, identified REA group DH, which is associated with RT 106, as the most prevalent genotype. Ribotype 106 was also the second most prevalent ribotype identified from CDI cases in England from 2007-2010 (31). However, RT 106 was notably absent in symptomatic and healthy children from Oxfordshire, England and was not particularly prevalent among CDI cases in this same region between 2006 and 2013 (2). The absence of RT 106 was also consistent in the studies from France, Belgian, and Sweden (1,11,22), which contrasts the high RT 106 prevalence observed in this study. The CAN-DIFF study, which aims to characterize the ribotypes and antimicrobial susceptibilities of *C. difficile* identified from CDI cases located across Canada, noted that the most prevalent ribotypes between 2013-2017 to be 027, 106, 020, and 014 (24), which also resembles the overall ribotype prevalence in this study with the exception of ribotype 629. Interestingly, the prevalence of RT 106 significantly increased whereas RT 027 significantly decreased in Canada over the CAN-DIFF study period (24). Taking this into account,

these findings suggest that RT 106 may be an emerging strain in Canada or has recently increased in prevalence due to decreases in CDI attributable to RT 027 (24).

This study is subject to several limitations. First, ribotyping and PFGE lack discriminatory power compared to whole genome sequencing which is optimal for comparing relatedness between *C. difficile* isolates. Thus, isolate relatedness in this study may be overestimated as shared genotypes may differ in loci that were not covered by either ribotyping or PFGE. However, one strength of this study compared to those previously (1) was the inclusion of two genotyping methods to compare relatedness. Only a minor proportion of the isolates recovered from the APPETITE cohort (76/308, 24.7%) had both toxin gene PCR and PFGE performed in addition to PCR ribotyping, which limited the comparisons that could be made between the APPETITE and CDI cohort. As APPETITE was an on-going prospective study, *C. difficile* isolates from APPETITE were available over a much longer timeframe as compared to the CDI cases, which did not allow for longitudinal comparison of *C. difficile* isolates from children and CDI cases. Another limitation was that no supporting epidemiologic data such as contact tracing or exposure setting (hospital versus community acquisition) was available to further explain the observed genetic relatedness between isolates from children and adults. Additionally, the majority of APPETITE children were recruited from the Calgary area (n=235/308) with comparison made to adult and pediatric CDI cases from Edmonton (separated by ~300 km) suggesting, for most cases, isolate relatedness most likely was not due to transmission. As only one isolate was characterized for each sample, mixed *C. difficile* cases were identified for 10 samples indicated by the recovery of a non-toxigenic isolate from a stool containing toxigenic *C. difficile* (**Table 2.3**) and in these cases the toxigenic strain was not characterized for ribotype comparison. Lastly, this study

benefitted, compared to those prior (1,2,13,22), by including a large cohort of both symptomatic and asymptomatic children.

In summary, this chapter reports the *C. difficile* ribotypes identified in children and CDI cases from Alberta, Canada and highlights the involvement of RT 106 in both colonization and pediatric CDI. Additionally, toxigenic *C. difficile* isolates from children were similar to those found in adult CDI cases supporting previously published observations in European children (1,2).

2.5 References

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Chapter 3 Development of a direct from stool *C. difficile* ribotyping method and algorithm for the clinical microbiology laboratory*

3.1 Introduction

C. difficile is a Gram positive, spore-forming, toxin-producing bacterium and is highly prevalent in cases of hospital acquired gastrointestinal infection (1). PCR ribotyping has been widely used to determine the clonal relationship of different strains. This method is based on the variable copy number and size of intergenic spacer regions located between the 16S and 23S rRNA genes to generate a unique ribotype profile. This bacterium has gained notoriety from the early 2000s with the emergence of an epidemic strain, ribotype (RT) 027, causing outbreaks of *C. difficile* infection (CDI) in Canada, the United States, and Europe (2). Several studies have shown a decreasing prevalence of this ribotype but with others increasing such as RT 106, 002 and 056 in Canada and the U.S. (3,4). With the decrease in RT 027, there is a greater heterogeneity of ribotypes identified in CDI cases (4). *C. difficile* molecular epidemiology also differs worldwide with RT 018 and toxin A^-/B^+ RT 017 being prevalent in China, Korea, and Japan (5–7), and RT 001, in addition, to RT 027 and 020/014 in Europe (8). Ribotype 078 is also concerning due to its association with community-CDI cases and disease severity similar to RT 027 (9). CDI manifests as a spectrum of disease progressing in severity from diarrhea to pseudomembranous colitis, toxic megacolon, and in some cases, death. The major risk factors for CDI are antibiotic usage, preceding hospitalization (10), and age, especially for individuals ≥65 years of age (11). Disruption of the gut microbiota (such as with antibiotic treatment) is key for *C. difficile* pathogenesis as this

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allows the organism to colonize, grow, and produce toxins. The primary *C. difficile* virulence factors are the two toxins, TcdA and TcdB, encoded by the *tcdA* and *tcdB* genes (12). A third "binary" toxin *C. difficile* transferase, encoded by *cdtA* and *cdtB,* can also be present. Strains lacking toxin genes are considered to be non-toxigenic and non-pathogenic.

C. difficile genotyping is an important component of infection control as it allows for surveillance of emerging strains, transmission patterns, and cluster detection. Genotyping is also important for distinguishing between CDI recurrence and reinfection. Current genotyping methods require culture and isolation of the organism, which can be lengthy (two to three days) and labor intensive. Consequently, many clinical microbiology laboratories only screen for the presence of toxin or toxin genes and culture is not routinely performed unless genotyping is requested. Current genotyping protocols for *C. difficile* require the selection of a single colony; consequently, a mixed population containing multiple strains of different genotypes will not be detected using this method (13) and the potential outbreak strain might be missed.

There are a variety of commonly used *C. difficile* genotyping methods including PCR ribotyping, which provides adequate discriminatory power (14) and reproducibility. Other methods like multilocus variable-number tandem-repeat analysis and whole genome sequencing (WGS) are also used and are more discriminatory. In recent years, WGS has been developed and adopted as a tool for tracking clusters and transmission in some major laboratories worldwide. However, due to the expense of the equipment required to set up a whole genome sequencing laboratory, specially trained personnel, and a bioinformatics pipeline, it is not practical for implementation in a frontline hospital laboratory. Alternatively, PCR ribotyping is simple and robust, and can be easily performed in frontline microbiology laboratories. The purpose of this

chapter is to develop a testing algorithm for direct *C. difficile* ribotyping from stool using capillary electrophoresis for analysis in comparison to conventional isolate ribotyping.

3.2 Methods and Materials

3.2.1 Samples and Diagnostic Testing

To develop the direct from stool ribotyping method, stool samples positive for toxigenic *C. difficile* and *C. difficile* isolates previously identified in **Chapter 2** from the Alberta Provincial Pediatric EnTeric Infection TEam (APPETITE) study and from one adult CDI case were used. The sample type and number of samples included is described under the relevant methods subheadings. For validation of the direct ribotyping method, toxigenic *C. difficile* positive stools (n=187) were collected from the University of Alberta Hospital (UAH) (n=113) and Calgary Laboratory Services (CLS) (n=74) microbiology laboratories. Both laboratories identify toxigenic *C. difficile* using a two-step algorithm. At the UAH**,** the *C. DIFF QUIK CHEK COMPLETE*® (CDQC) (Techlab, Blacksburg, VA, USA) and the GeneXpert® *C. difficile* (Cepheid, Sunnyvale, CA, USA) are used (as described in **Chapter 2, section 2.2.4**), while CLS employs the LIAISON® *C. difficile* GDH (DiaSorin, Mississauga, ON, Canada) and GeneXpert® *C. difficile/Epi* (Cepheid). Both CDQC and GeneXpert® assay results were collected to correlate with direct ribotyping performance.

3.2.2 *C. difficile* Enrichment, Culture, and Isolation[†]

C. difficile positive stools used in the direct ribotyping validation were enriched (n=187) by using a sterile swab coated in unformed stool or 250 µL liquid stool and inoculated into Brain

[†] Sarah Morin aided with identifying mixed *C. difficile* infections at APL-ProvLab, Edmonton, Alberta.

Heart Infusion (BHI) broth (Alberta Precision Laboratories-ProvLab (APL-ProvLab), Edmonton, AB, Canada) and incubated under anaerobic condition for 24 hours at 35°C. Anaerobic conditions were generated as described in **Chapter 2 section 2.2.5**. If enrichment failed or the sample was unable to produce a direct ribotype using BHI, the sample was alcohol shocked as follows: 250 µL liquid stool or ~100 mg unformed stool was suspended in 250 or 500 µL 1X phosphate buffered saline respectively (ThermoFisher Scientific). Then 500 µL of 95% ethyl alcohol (Commercial Alcohols, Brampton, ON, Canada) was added, vortexed using a Fisherbrand™ analog vortex mixer (ThermoFisher Scientific) at high speed (setting 8-10) to homogenization, incubated for 35 min at ambient temperature, and the stool was pelleted by centrifugation at 1150 X *g* in an Eppendorf™ Centrifuge 5425 (ThermoFisher Scientific). The pellet was added to Hardy Diagnostics *C. diff* Banana Broth™ (BB) (Micronostyx, Ottawa, ON, Canada) using a sterile swab and incubated aerobically for 24 to 72 hours (depending on color change of the broth from red to yellow, which is indicative of growth) at 35°C for enrichment. Alternatively, the pellet was inoculated using a sterile swab onto CHROMagar™ *C. difficile* agar (Micronostyx) and incubated anaerobically for 24 hours at 35°C for *C. difficile* isolation.

For *C. difficile* isolation from BHI and BB, 500 µL of broth was shocked with 95% ethanol and incubated as described above for the stool samples, centrifuged at 13,523 X *g*, and the pellet was inoculated onto CHROMagar™ plates. Similarly, 250 µL BB culture was inoculated directly onto CHROMagar™ plates and incubated as described above. Presumptive *C. difficile* colonies on CHROMagar™ (indicated by opaque, colorless, pale-blue fluorescing colonies under long wave 365 nm ultraviolet (UV) light) were subcultured onto BHI agar (APL-ProvLab) and incubated anaerobically for 48 hours at 35°C. *C. difficile* colonies on BHI plates were confirmed for physical

characteristics such as odour and fluorescence under 365 nm UV light and were confirmed by real-time PCR.

3.2.3 DNA Extraction‡

DNA from stool and broth was extracted using the NucliSENS® easyMAG® instrument (bioMérieux, Montreal, QC, Canada) as described in **Chapter 2, section 2.2.3** with minor adjustments. Stool (250 µL liquid or ~100 mg unformed) or broth (100 µL) was suspended in 1 mL NucliSENS® Lysis Buffer (bioMérieux) using a Bertin Corp SK38 Soil grinding tube (ESBE Scientific, Saint-Laurent, QC, Canada). Tubes were mixed for 10 minutes on an analog vortex mixer with a tube adaptor at high speed, incubated in ambient conditions for 15 minutes, and centrifuged for 5 minutes at 15,871 X *g*. DNA was extracted from 200 µL of lysate using the NucliSENS® easyMAG® extractor (bioMérieux) and a 70 µL DNA eluate was collected from the instrument for use in molecular assays. DNA from C. *difficile* isolates was extracted from a 1 µL loopful of culture swept from the BHI agar plate and suspended in 100 µL InstaGene™ matrix (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada), boiled for 30 min at 95°C using an Eppendorf® MasterCycler® Pro S thermal cycler (Luminex® Corporation, Toronto, ON, Canada), and the supernatant was used as template for qPCR and PCR ribotyping.

3.2.4 Real-Time PCR (qPCR) and Triosephosphate isomerase Primer Design

DNA extracts from isolates, stools, and broths were used as template in a singleplex qPCR panel targeting the 16S, *tcdA*, *tcdB* (15) and *cdtB* (16) genes (Integrated DNA Technologies, Coralville, IA, USA), as shown in **Table 3.1**. Additionally, primers and probes targeting the *C. difficile* specific triosephosphate isomerase (*tpi*) gene, a glycolytic enzyme present in both

‡ Dr. Binal Shah-Gandhi aided with DNA extractions at APL-ProvLab, Edmonton, Alberta.

toxigenic and non-toxigenic *C. difficile* (17), were developed using Beacon Designer software v8.20 (Premier Biosoft, San Francisco, CA, USA) using GENBANK accession LC151869.1 as a reference sequence. Primer and probe specificity was confirmed using a panel of organisms and stool samples submitted and tested for *C. difficile* at the UAH. Non-*C. difficile* organisms chosen for the specificity panel were obtained from the APL-ProvLab Quality Control Department (Edmonton, AB, Canada), and *C. difficile* ribotype 027 DNA was previously isolated in **Chapter 2**. DNA was extracted by suspending non-*C. difficile* organisms in 100 µL rapid lysis buffer (prepared by APL-ProvLab Bacterial Typing Unit, Edmonton, AB, Canada) and heating for 15 minutes at 95°C using a VWR Standard Heatblock (VWR Scientific, Mississauga, ON, Canada). Stools used for the specificity panel were *C. difficile* negative or contained non-toxigenic *C. difficile* obtained from the UAH. Singleplex qPCR assays for 16S, *tcdA, tcdB, cdtB,* and *tpi* were performed using the Applied Biosystems™ 7500 Fast Real-Time PCR instrument with 5 µL template DNA, 222 nM forward and reverse primers, 333 nM probe, and 1X PrimeTime® Gene Expression Master Mix (Integrated DNA Technologies) as outlined in **Table 3.1**. Multiplex qPCR targeting *tpi* and *tcdB* genes were performed using the same conditions. Extracted DNA from 9 isolates (8 from APPETITE and 1 from an adult CDI case), isolated in **Chapter 2**, was used as template for the in-house *tpi* and *tcdB* multiplex qPCR, and compared to the end point PCR with the same targets performed at the Public Health Agency of Canada – National Microbiology Laboratory (PHAC-NML, Winnipeg, MB). Additionally, Ct values of the *tpi* and *tcdB* multiplex qPCR assay and singleplex 16S, *tcdA, tcdB,* and *cdtB* were compared using 4 of the 187 samples which were part of the direct ribotyping validation. Ribotype 027 isolate DNA was used as a positive control in addition to a no-template control for all qPCR assays.

Table 3.1. Real-Time PCR (qPCR) and PCR ribotyping primer and probe sequences with corresponding PCR cycling conditions

Target	Primers and Probes (5'→3')	Cycling Program	Reference
16S	F: GCAAGTTGAGCGATTTACTTCGGT R: GTACTGGCTCACCTTTGATATTYAAGAG P: /56-FAM/TGCCTCTCA/ZEN/AATATATTATCCCGTATTAG/3IABkFQ/	1 cycle (95°C x 20 sec), 40 cycles (95°C x 3 sec, 60°C x 30 sec)	
tcdA	F: CAGTCGGATTGCAAGTAATTGACAAT R: AGTAGTATCTACTACCATTAACAGTCTGC P: /56-FAM/TTGAGATGA/ZEN/TAGCAGTGTCAGGATTG/3IABkFQ/		(15)
tcdB	F: TACAAACAGGTGTATTTAGTACAGAAGATGGA R: CACCTATTTGATTTAGMCCTTTAAAAGC P: /56-FAM/TTTKCCAGT/ZEN/AAAATCAATTGCTTC/3IABkFQ/		
cdtB	F: AAAAGCTTCAGGTTCTTTTGACAAG R: TGATCAGTAGAGGCATGTTCATTTG P: /56-FAM/CAAGAGATC/ZEN/CGTTAGTTGCAGCATATCCAATTGT/3IABkFQ/		(16)
tpi	F: TGGACTATGTTGTAATAGGA R: CAGCTTCTCTTTGTTCTAA P: /5YakYel/TTGGGTCTA/ZEN/TTCCTACTTCTAATGCTT/3IABkFQ/		(This study)
16S-23S ISR (Isolate)	F: FAM-GTGCGGCTGGATCACCTCCT R: CCCTGCACCCTTAATAACTTGACC	1 cycle (95°C x 15 min), 24 cycles (95°C x 1min, 57°C x 1min, 72°C x 1min), 1 cycle (72°C x 10 min)	(18)
16S-23S ISR (Stool & Broth)	F: /56-FAM/GCTGGATCACCTCCTTTCTAAG R: TGACCAGTTAAAAAGGTTTGATAGATT	1 cycle (95°C x 15 min), 35 cycles (95°C x 1min, 57°C x 1min, 72°C x 1min), 1 cycle (72°C x 10 min)	(19)

PCR ribotyping primers differed depending on sample type (shown in brackets)

3.2.5 PCR Ribotyping and Capillary Electrophoresis[§]

Isolate ribotyping was performed as previously described in **Chapter 2, section 2.2.6** using the Bidet *et al.* (18) PCR ribotyping primers and cycling conditions (**Table 3.1**). To simulate potential direct from stool ribotyping results that could arise from the presence of 2 concurrent strains, isolate ribotyping with a combination of 2 unique ribotypes in each reaction (056 & 106, 629 & 076, 020 & 014, 106 & 629, 056 & 076) was performed using 200 ng of DNA extracted from isolates as previously described in **Chapter 2.**

To develop the direct ribotyping method, DNA extracted from 10 APPETITE stool samples described in **Chapter 2,** was used. Direct ribotyping was performed using the Janezic *et al.* (19) 16S-23S ribotyping primers with the PCR cycling protocol as shown in **Table 3.1.** These assays were set up as described for isolates except 5 µL of DNA template was added to a 50 µL PCR mixture, and amplification was performed using the Eppendorf® MasterCycler® Pro S thermal cycler (Luminex® Corporation). To determine reproducibility of the direct ribotyping method, three stool samples from the UAH, also included in the direct ribotyping validation set (n=187), spanning a range of 16S Cts were selected and DNA was extracted from each stool sample two times (two biological replicates) and direct ribotyping was performed on each extract three times (three technical replicates). Stool and broth ribotyping in the direct ribotyping validation (n=187) was performed as described above, however; template DNA, extracted from the NucliSENS® easyMAG®, was first standardized by diluting in UltraPure™ DNAse, RNAse-Free Distilled Water (ThermoFisher Scientific) based on the 16S qPCR cycle threshold (Ct values) according to a "Ct key" established in this study. The "Ct key" was developed by performing direct ribotyping on

[§] Tim Du aided in comparing direct and isolate ribotypes for 40 samples included in the direct ribotyping validation to determine if the ribotypes matched at the Public Health Agency of Canada – National Microbiology Laboratory in Winnipeg, Manitoba.

serial dilutions of DNA extracted from 38 stools from the UAH, which were also part of the direct ribotyping validation (n=187), to establish the correlation of direct ribotyping with the 16S qPCR Ct values. For stools included in the ribotyping validation (n=187), if no ribotype or an unresolved ribotype was produced (i.e., no bands or fewer bands than expected for a known ribotype), a higher concentration of template DNA was used on repeat ribotyping PCR. If the repeated PCR assay failed, the sample was enriched in broth before attempting another ribotyping run. Capillary electrophoresis was performed as previously described (20) and as outlined in **Chapter 2, section 2.2.6**. DNA isolated from ribotypes 106 and 629 were included in each PCR and capillary electrophoresis as positive controls in addition to a no-template control.

3.2.6 Ribotype Assignment and Analysis

Electropherograms were analyzed as described in **Chapter 2, section 2.2.7** with the exception that the range of amplified fragments (peaks) analyzed was adjusted to between 175-700 base pairs to account for differences in peak size generated between the Bidet *et al.* (18) and Janezic *et al.* (19) ribotyping primers. There is a shift in the ribotyping patterns when primers from Bidet *et al.* (18) and Janezic *et al.* (19) were compared due to the difference in binding location of the primers on the *rrn* operon (**Figure 1.5**). For verification, DNA extracted from 9 different *C. difficile* isolates from APPETITE participants included in **Chapter 2** was used as template for isolate ribotyping to compare the peak sizes generated by these two set of primers. Peak sizes were adjusted prior to importing into the BioNumerics v6.01 software (Applied Maths, Austin, TX, USA). Direct ribotypes not matching the isolate, or reference collection, due to the presence of additional bands, were considered a composite ribotype consisting of >1 unique ribotype pattern.

3.2.7 Inter-laboratory Ribotype Comparison[**]

Twenty-three stools were used to compare ribotypes and toxin profiles generated in our laboratory to those identified at the PHAC-NML. Direct ribotyping and singleplex *tcdA, tcdB,* and *cdtB* qPCR were performed for comparison to the isolate ribotype and end point PCR for the *tcdA, tcdB,* and *cdtB* genes generated at the PHAC-NML. Of the 23 samples included, 6 were part of the direct ribotyping method validation (n=187), whereas the other 17 were not part of the validation but were tested at the UAH.

3.2.8 Statistical Analyses

The proportion of toxin positive and toxin negative directly ribotyped stools were compared by Fisher's Exact Test. Differences in Ct values between directly ribotyped stools compared to enriched stools were analyzed by the Mann-Whitney U test. Receiver operator characteristic (ROC) curves were generated to compare the diagnostic capability of different target genes to identify stools for direct ribotyping. Specificity and sensitivity of target gene Ct thresholds were derived from the ROC curve. All statistical analyses were performed using GraphPad Software v8.4.3 (GraphPad Software Inc., San Diego, CA, USA) with $p<0.01$ considered significant.

[**] Tim Du performed isolate ribotyping and toxin gene PCR at the Public Health Agency of Canada – National Microbiology Laboratory in Winnipeg, Manitoba for comparison to direct ribotype.

3.3 Results

3.3.1 Direct Ribotyping Development

3.3.1.1 Ribotyping Primer Comparison

The Janezic *et al.* (19) and Bidet *et al.* (18) ribotyping primers anneal and amplify different regions of the *rrn* operon (refer to **Figure 1.5**); consequently, the ribotype patterns produced by each primer set would not be directly comparable. In order to determine the differences in ribotype patterns produced by each primer set, isolate ribotypes were generated and compared using both sets of ribotyping primers for 9 unique ribotypes. Fragment sizes produced by the Janezic primers were ~25 base pairs smaller than those produced by the Bidet primers (**Figure 3.1**). Therefore the amplicons produced by Janezic's primers were manually increased by 25 base pairs to allow for comparison of amplified products between the two primer sets. Using this translation method, ribotypes generated using the Janezic primers can be directly compared to ribotype patterns produced by the Bidet primers (**Figure 3.1**).

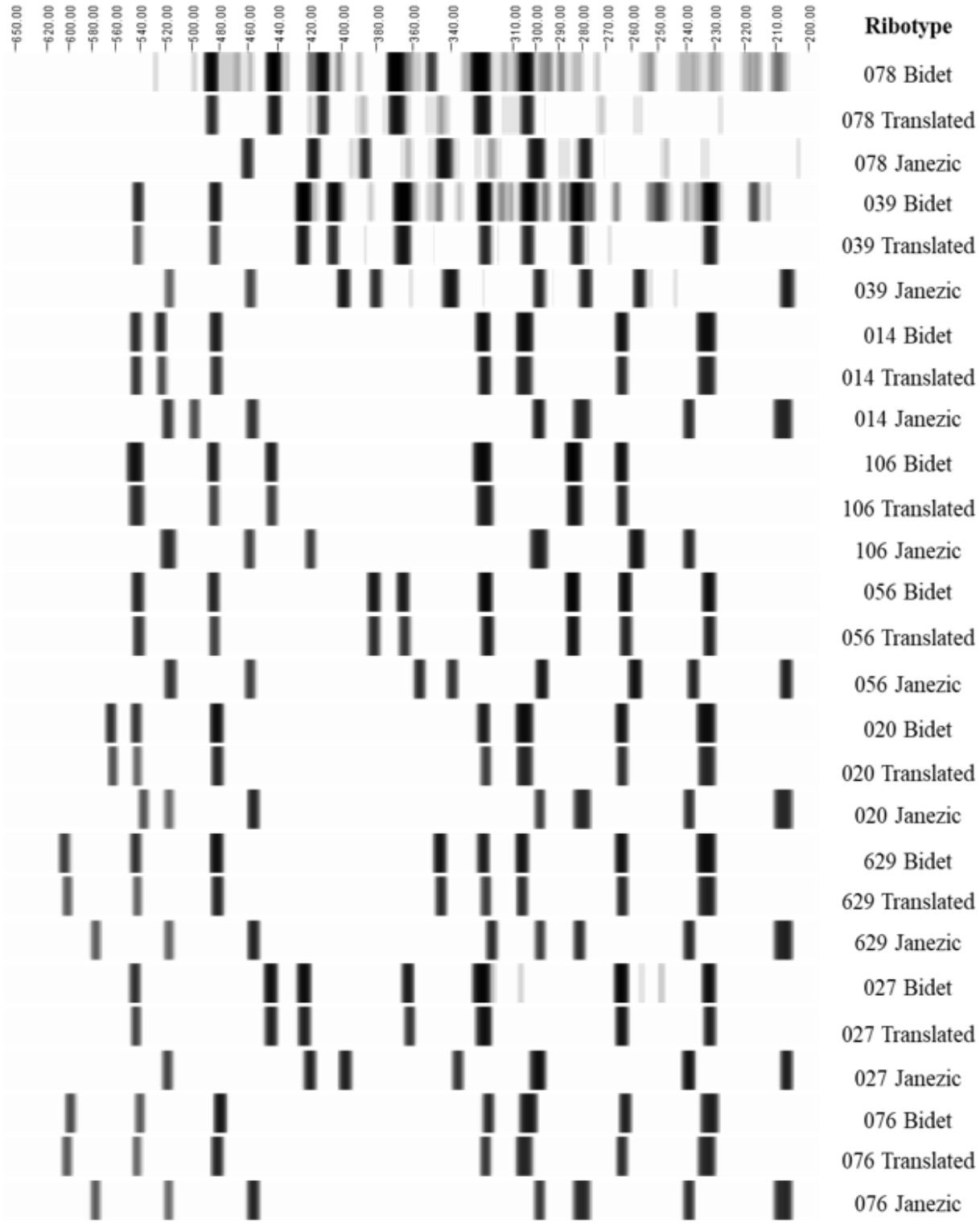

Figure 3.1. Translation between Bidet and Janezic ribotypes by a 25 base pair shift. Ribotype patterns can be translated between the Janezic and Bidet primer sets using a 25 base pair shift as the Translated ribotype has been shifted to match the Bidet isolate ribotype. Molecular weight marker (base pairs) is shown above the ribotype patterns.

3.3.1.2 Simulated Ribotyping with Concurrent *C. difficile* Strains

There is the potential for encountering stools that contain more than 1 strain of *C. difficile* concurrently with direct from stool ribotyping. In order to simulate potential ribotype patterns likely to be produced in stools containing >1 concurrent *C. difficile* strain, isolate ribotyping was performed with equal amounts of isolate DNA corresponding to two unique ribotypes using a selection of six ribotypes: 106, 056, 629, 076, 020, and 014 (**Figure 3.2**). As expected, when DNA from more than one ribotype is present in the PCR assay, a composite ribotype, made up of each unique ribotype, is produced. However, as shown in **Figure 3.2**, the composite ribotype of 629 and 076 (629/076 mix) does not reflect the pattern of >1 strain as ribotype 629 contains all of the fragments present in ribotype 076. This finding suggests that in stools containing >1 ribotype that are highly similar to each other, concurrent strains may not be detectable based on direct ribotyping alone.

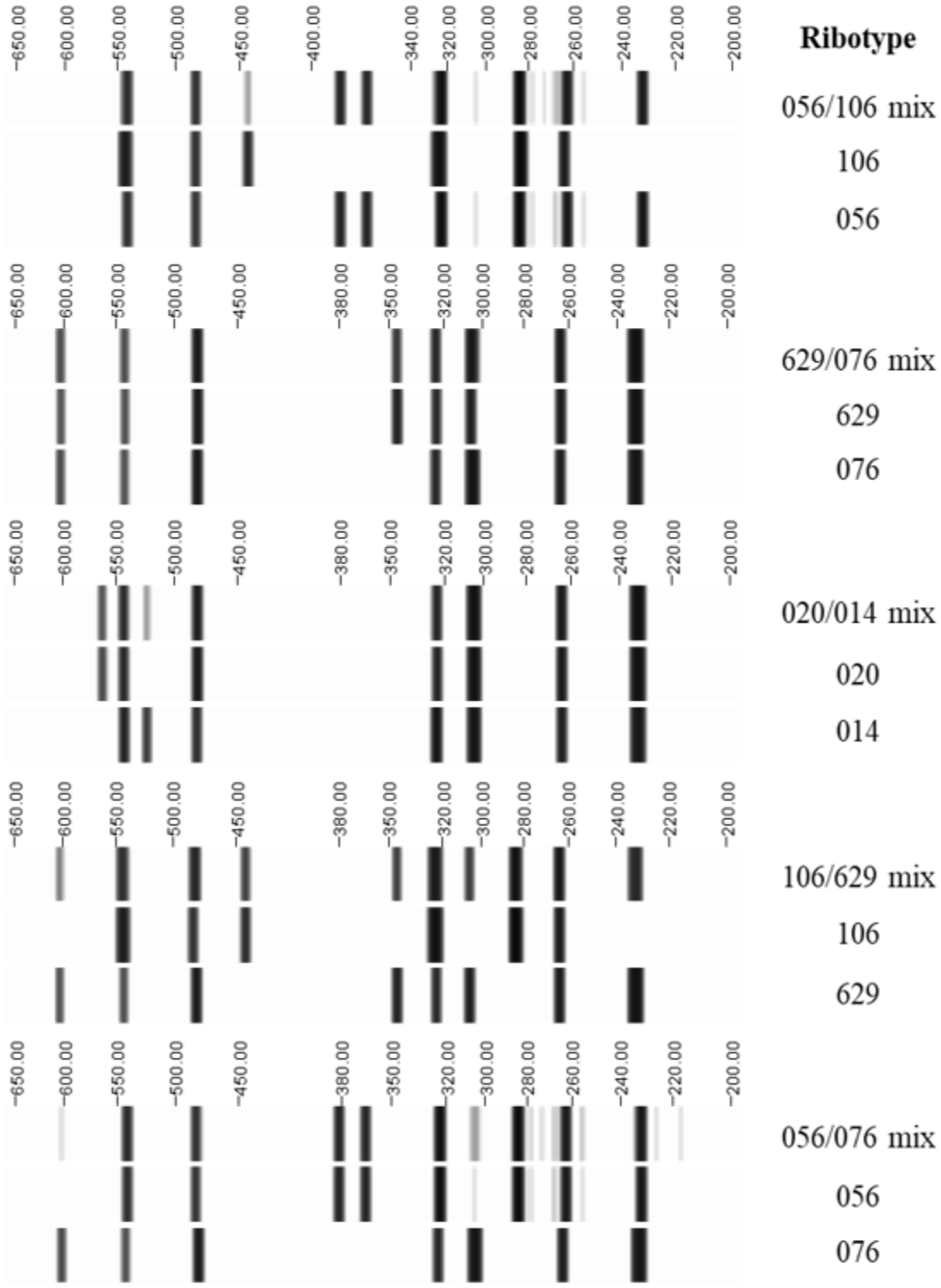

Figure 3.2. Simulated *C. difficile* mixed infection with >1 ribotype. Isolate DNA, recovered in Chapter 2, from two unique ribotypes was subject to PCR using 200 ng of template DNA each to simulate mixed infection with >1 ribotype. Molecular weight marker (base pairs) is shown above the ribotype patterns.

3.3.1.3 Preliminary Direct Stool Ribotyping

To develop the direct from stool ribotyping methodology, a protocol was established by adapting the 24 PCR cycle count used in the isolate ribotyping methodology to the 35 cycles used by Janezic *et al.* (19) (**Table 3.1**) in order to increase the sensitivity for ribotyping with stool samples. Using DNA extracted from 10 *C. difficile* positive stools from the APPETITE study, described in **Chapter 2**, direct ribotyping was performed and, inconsistency was observed as both complete and incomplete ribotypes were generated (**Figure 3.3**). It was suspected that the incomplete ribotypes were due to the presence of a low bacterial load in those samples. Another possibility was insufficient copies of particular ISRs, and thus those ISRs were not sufficiently amplified nor detected by capillary electrophoresis.

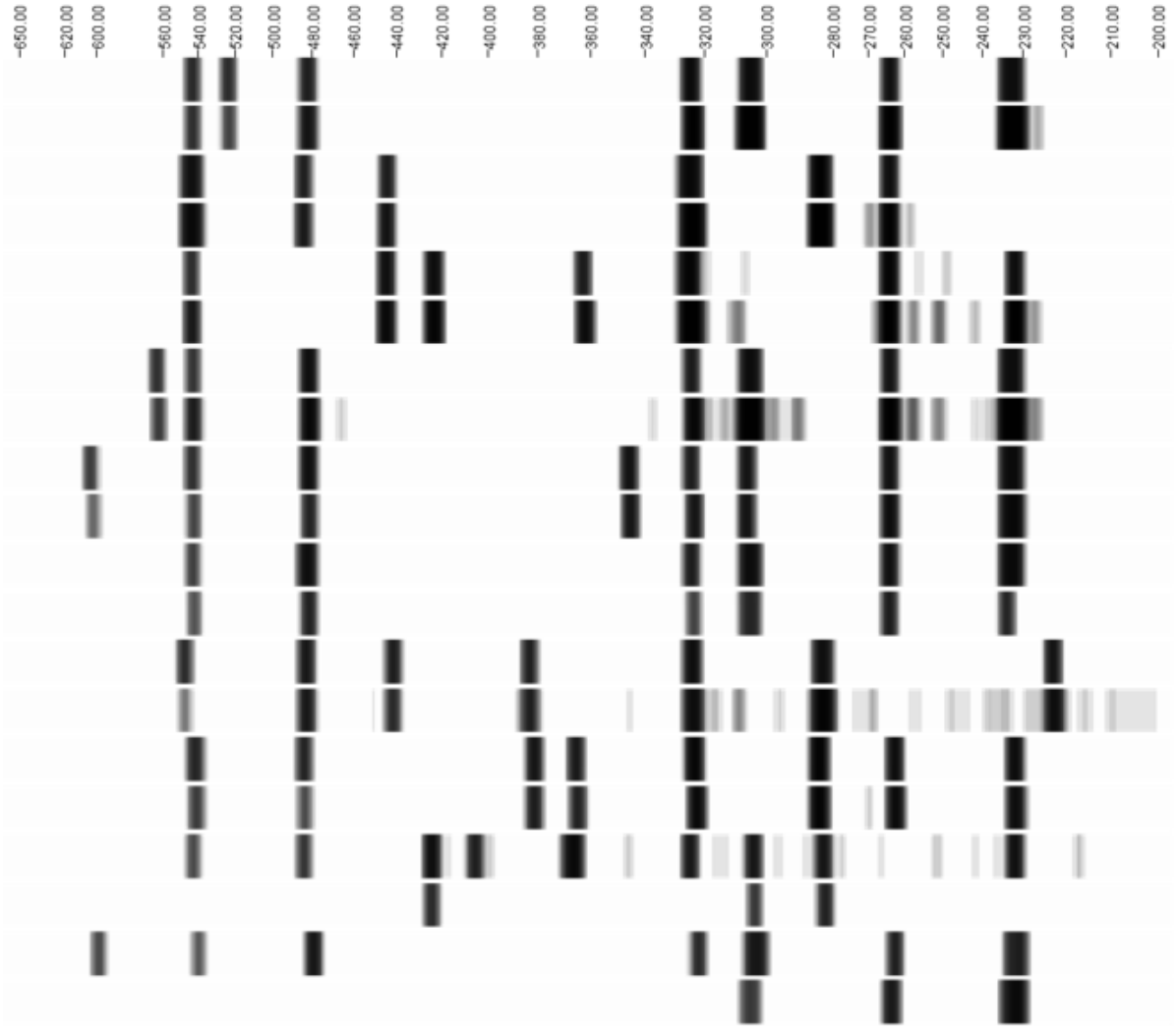

Figure 3.3. Variation in direct from stool ribotyping success between stool samples. Using DNA from 10 APPETITE stools, most samples produced the complete ribotype pattern compared to the isolate, whereas the bottom two samples did not produce the complete pattern as 6 bands (RT 039) and 4 bands (RT 076) were missing in the stool compared to the isolate respectively. Molecular weight marker (base pairs) is shown above the ribotype patterns.

3.3.1.4 Development of Singleplex 16S, *tcdA*, *tcdB*, and *cdtB* and Multiplex *tpi* and *tcdB* qPCR Assays

In order to determine the relative bacterial load and to develop an assay for *C. difficile* toxin gene detection, singleplex qPCR assays targeting *C. difficile* 16S, *tcdA, tcdB, cdtB,* and *tpi* genes were developed. Using previously published primers for 16S, *tcdA, tcdB,* and *cdtB* as well as a newly developed primer set for *C. difficile* specific *tpi* (triosephosphate isomerase), which is an enzyme involved in glycolysis, were included (**Table 3.1**). The specificity of each primer and probe set was assessed using a panel of organisms as well as stools that were *C. difficile* negative or contained non-toxigenic *C. difficile* (**Table 3.2**). As expected, primers and probes for all targets were specific and reacted only with the positive control (*C. difficile* ribotype 027) DNA; stools containing non-toxigenic *C. difficile* strains were positive for only 16S as anticipated (**Table 3.2**).

In addition, results from a multiplex qPCR assay targeting *tpi* and *tcdB* using DNA extracted from isolates and stools are shown in **Table 3.3** and **Table 3.4** respectively. With a *C. difficile* inclusive panel of DNA from 9 isolates obtained from stools in **Chapter 2**, *tpi* and *tcdB* multiplex assays were performed and the results showed concordance between our laboratory and the end point PCR assay performed at the PHAC-NML (**Table 3.3**). In addition, the *tpi* and *tcdB* multiplex PCR was tested alongside a singleplex qPCR panel including 16S, *tcdA, tcdB,* and *cdtB* using DNA from 4 toxigenic *C. difficile* positive stool samples identified at the UAH and results are shown in **Table 3.4**. In general, *tcdB* and *tpi* Ct values of the multiplex qPCR were similar compared to the toxin genes of the singleplex qPCR. Upon validation of the qPCR primer and probe specificity, singleplex qPCR assays were performed as a panel including 16S, *tcdA, tcdB,* and *cdtB* targets for estimation of the bacterial load and detection of the toxin genes in *C. difficile* positive stool samples based on the Ct values produced.

Table 3.2. qPCR specificity panel of organisms and stools.

Organism[a]/Sample Number	**16S**	***tcdA***	***tcdB***	***cdtB***	***tpi***
Bacteroides fragilis, vulgatus	-	-	-	-	-
Campylobacter coli, concisus, fetus, helveticus, hyointestinalis, jejuni, lari, upsaliensis	-	-	-	-	-
Clostridioides difficile (Ribotype 027)	-	-	-	-	-
Clostridium bifermentans, innocuum, perfringens, ramosum, septicum, sordellii, sporogenes, tertium, tetani	+	+	+	+	+
Enterobacter aerogenes, cloacae	-	-	-	-	-
Enterococcus faecalis, faecium	-	-	-	-	-
Escherichia coli (O103:H2, O111:H8, O121:H19, O145: Non-motile, O153:H25, O157:H7, O165:H25, O174:H2, O26:H11, O45:H2, and O8:H14)		-	-	-	-
Klebsiella pneumoniae	-	-	-	-	-
Micrococcus luteus	-	-	-	-	-
Morganella morganii	-	-	-	-	-
Prevotella melaninogenica	-	-	-	-	-
Proteus mirabilis, vulgaris	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-
Salmonella enterica subsp. enterica serovars (Enteritidis, 4,[5],12:i:-, Braenderup, Brandenburg, Hadar, Heidelberg, Infantis, Javiana, and Typhimurium)	-		-	-	-
Serratia marcescens	-	-	-	-	-
Shigella boydii, dysenteriae, sonnei	-	-	-	-	-
Staphylococcus aureus, epidermidis, saprophyticus	-	-	-	-	-
Vibrio parahaemolyticus	-	-	-	-	-
Yersinia enterocolitica	-	-	-	-	-
Non-toxigenic *C. difficile* stools (by diagnostic testing, n=14)	+	-	-	-	**NT**
C. difficile negative stools (by diagnostic testing n=20)	-	-	-	-	**NT**

[a]Species belonging to the same genus are separated by a comma. All qPCR assays were performed in singleplex reactions. **NT**, not tested.

Table 3.3. Inclusivity panel of *C. difficile* isolates for *tpi* and *tcdB* detection by multiplex qPCR.

Sample	Ribotype	Multiplex qPCR	Endpoint PCR	
		tpi/*tcdB*	*tpi*	*tcdB*
1	039	POS/NEG	POS	NEG
2	039	POS/NEG	POS	NEG
3	085	POS/NEG	POS	NEG
4	010	POS/NEG	POS	NEG
5	106	POS/POS	POS	POS
6	019	POS/POS	POS	POS
7	014	POS/POS	POS	POS
8	020	POS/POS	POS	POS
9	017	POS/POS	POS	POS

Nine isolates from APPETITE and an adult CDI case, identified in Chapter 2, were tested by multiplex qPCR and results were compared to the end point PCR results from the PHAC-NML.

Table 3.4. Cycle threshold (Ct) comparison between *tpi* and *tcdB* multiplex qPCR and 16S, *tcdA*, *tcdB*, and *cdtB* singleplex qPCR.

	Multiplex qPCR	Singleplex qPCR			
Sample	*tpi*/*tcdB*	16S	*tcdA*	*tcdB*	*cdtB*
1	26.43/**26.20**	22.21	25.90	**26.12**	NEG
2	27.22/**27.92**	23.67	26.53	**27.50**	26.58
3	29.54/**28.78**	24.14	27.70	**28.51**	NEG
4	36.51/**36.36**	31.45	34.51	**34.56**	34.93

Four stool samples containing toxigenic *C. difficile* were tested by both methods with Ct values shown below. The tcdB Ct values are bolded for each qPCR reaction.

3.3.1.5 Development of the "Ct key" for Direct Ribotyping Standardization

With qPCR assays developed, further investigation into the variability of direct from stool ribotyping performance was done by performing direct ribotyping on serial dilutions of DNA extracted from 38 toxigenic *C. difficile* stool samples obtained at the UAH, spanning a range of *C. difficile* 16S Cts, indicative of varying *C. difficile* loads (**Figure 3.4, A**). Interestingly, direct ribotype patterns could not be produced from undiluted samples across a range of 16S Ct values (**Figure 3.4, A**) but this characteristic varied from sample to sample. For dilutions of DNA with 16S Ct values >30, a ribotype was not produced the majority of the time (**Figure 3.4, A**). By testing diluted DNA based on a range of neat 16S Ct values, it appeared that the optimal dilution for direct ribotyping (**Figure 3.4 A**, white backgrounds, and **Figure 3.4 B**) was inversely correlated with the neat 16S Ct of the stool DNA extract; meaning, the lower the 16S Ct value is in the neat DNA extract, the greater the dilution is required to produce the optimal ribotype pattern (**Figure 3.4 A, Samples 1-8**). In some cases if the DNA was diluted too far, a ribotype would not be generated (**Figure 3.4 A, Samples 2, 7-12, 14, 18, 20-23, 25-26**). For many samples the ribotype pattern was acceptable across a wide range of dilutions (**Figure 3.4 A,** gray backgrounds) suggesting that one particular dilution could be sufficient within a discrete range of neat DNA 16S Ct values allowing for standardization across multiple stool samples containing varying neat DNA 16S Ct values. Based on these findings, a "Ct key" was developed allowing for standardization as shown in **Figure 3.4**, in which neat DNA extracts for direct ribotyping should be diluted according to the 16S Ct as such: 1:500 (Ct ≤19), 1:50 (Ct 19-22), 1:10 (Ct 22-24), and both 1:5 and neat (Ct >24).

A

Sample	Dilution							
	Neat	1:5	1:10	1:50	1:100	1:500	1:1000	1:5000
1	16.6	18.9	19.9	22.2	23.5	26.3	28.4	30.7
2	17.5	19.6	20.5	23.4	24.8	27.0	27.8	NEG
3	17.6	25.1	20.7	23.1	24.2	26.8	27.7	37.5
4	17.9	20.1	21.1	24.2	24.7	27.2	28.1	37.2
5	17.9	20.2	21.1	23.5	24.7	27.6	28.3	33.6
6	18.5	20.7	21.6	24.1	25.0	27.9	29.2	
7	18.5	20.8	22.0	26.4	25.8	31.9	31.0	NEG
8	18.8	21.2	22.2	24.8	25.8	28.7	29.7	34.3
9	19.0	21.4	22.6	25.1	26.1	28.7	30.4	39.0
10	19.2	21.7	22.5	25.4	27.0	29.3	30.4	
11	20.3	22.5	23.5	27.2	27.5	30.3	31.6	NEG
12	20.7	23.0	23.9	26.9	28.1	31.6	33.4	
13	20.9	22.8	23.9	26.1	27.8			
14	20.9	23.2	24.3	26.6	27.8	30.6	31.4	34.0
15	21.1	23.5	24.5	28.0	29.8			
16	21.2	23.6	24.5	26.8	28.2	30.6	31.7	
17	21.3	22.7	23.6	26.5	28.1	30.0		
18	21.3	23.5	24.4	27.2	28.6	31.6	31.9	
19	21.4	23.7	24.6	27.2	28.9			
20	22.2	24.5	25.4	27.9	28.8	32.1	32.9	
21	22.7	25.2	26.0	28.7	29.8	32.6	34.1	
22	23.1	24.5	25.6	28.2	29.4			
23	23.6	25.4	26.3	30.3	30.8			
24	25.2	27.6	28.6	31.2				
25	25.6	27.9	28.9	32.0	32.7			
26	26.0	28.3	29.3	32.7	34.0			
27	26.3	27.7	28.8					
28	28.7	31.3						
29	28.8	30.4	31.2					
30	29.7	32.4	33.5					
31	30.6	32.2						
32	30.7	32.5	33.4					
33	30.7	32.4						
34	31.2	33.4						
35	31.5	34.0						
36	31.6	34.1						
37	34.0	35.4						
38	38.4	37.0						

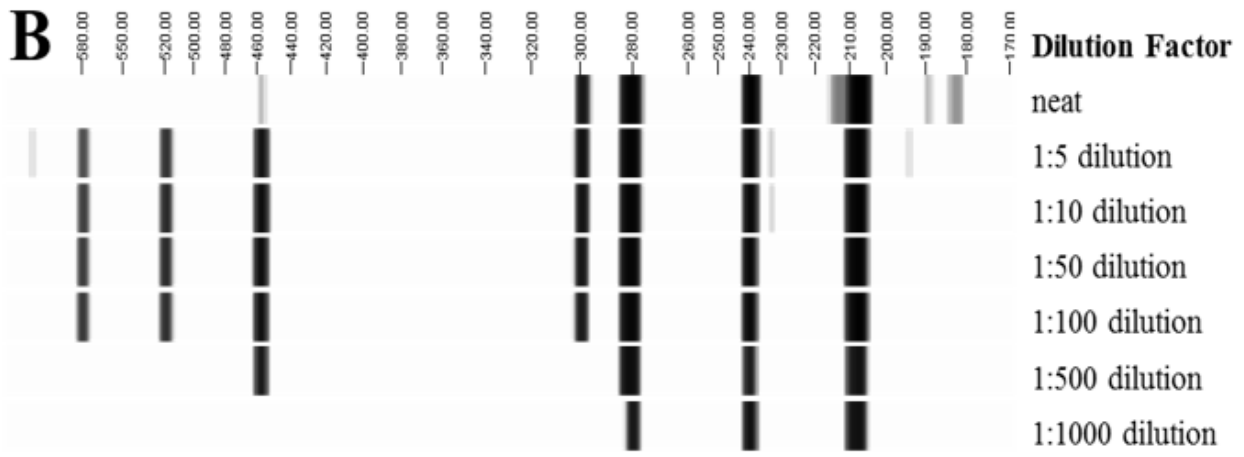

Figure 3.4. Development of the 16S "Ct key" for direct ribotyping. DNA extracts from stools positive for toxigenic *C. difficile* with varying 16S Cts were subject to serial dilution, direct ribotyping, and ribotype assessment (A). Dilutions producing incomplete ribotype patterns or lacking amplification by PCR are shown in black. Dilutions producing the complete ribotype pattern are shown in gray. The lowest dilution producing the best ribotype pattern indicated by the least background (i.e. the optimal pattern) are shown in white. For sample 6 1:50 dilution (dark gray) the LIZ1200 sizing standard did not sufficiently resolve and a ribotype pattern was not accessible. NEG samples produced no 16S Ct value. Ribotype patterns produced by each dilution of Sample 12 (B). Molecular weight marker (base pairs) is shown above the ribotype patterns.

3.3.1.6 Direct Ribotyping Reproducibility

With standardization achieved, the next step was to demonstrate the reproducibility of the direct ribotyping method using three stool samples with varying 16S Ct values (**Table 3.5,** 16S Ct: 18.2, 22.4, and 26.7). Using newly extracted DNA for each sample, the average 16S Ct values of the stools across all three extractions were 18.9, 24.1 and 30.6 with sample 1 and 2 producing consistent ribotyping results compared to the initial direct ribotyping result, whereas sample 3 was unable to generate a ribotype from the new extractions (**Table 3.5**). It was apparent for sample 3 that the increase in 16S Ct in the new DNA extracts was detrimental to direct ribotyping, which further confirmed the previous observation that direct ribotyping is dependent on bacterial load. If the bacterial load is low, there are potential inconsistencies in the results due to sampling error.

Table 3.5. Reproducibility of direct from stool ribotyping.

Sample	Biological replicate No.	Avg. 16S Ct	16S Ct	Ribotyping Replicate 1	Ribotyping Replicate 2	Ribotyping Replicate 3
	initial		22.4	106, 1:10	-	-
1	1	24.1	25.0	106, 1:5	106, 1:5	106, 1:5
	2		24.9	106, 1:5	106, 1:5	106, 1:5
	initial		18.2	015, 1:500	-	-
2	1	18.9	18.7	015, 1:500	015, 1:500	015, 1:500
	2		19.8	015, 1:50	015, 1:50	015, 1:50
	initial		26.7	027, 1:5	-	-
3	1	30.6	31.0	INC.	INC.	INC.
	2		34.2	INC.	INC.	INC.

To demonstrate reproducibility, three stools spanning a range of 16S Ct were subject to direct ribotyping using two biological replicates and three technical replicates. Each biological replicate represents a new DNA extract tested by qPCR and direct ribotyping. The initial result reflects the first time the sample was directly ribotyped and subsequent biological replicates were used to confirm the initial result in order to demonstrate reproducibility. All 16S qPCR assays were performed only once. INC., incomplete as no ribotype pattern was produced. The ribotype and dilution tested are shown for each replicate.

3.3.2 Direct Ribotyping Validation

3.3.2.1 Direct Ribotyping Performance and Correlation with Commercial Diagnostic Assays

With standardization of the direct ribotyping method achieved through the "Ct key" as shown in **Figure 3.4 A**, validation was the next step in assessing the performance of the direct ribotyping method. In order to validate and determine the performance of the direct from stool (DFS) ribotyping method, 187 toxigenic *C. difficile* positive stools from UAH and CLS were subjected to the DFS ribotyping method and ribotyping performance was assessed with respect to *C. difficile* diagnostic assay result (**Figure 3.5**). Of the 187 samples tested, 125 (66.8%) stools produced a DFS ribotype, whereas 62 (33.2%) failed. As shown in **Figure 3.4,** direct ribotyping performance appeared to correspond to the bacterial load, and the 62 stools that were not directly

ribotyped were enriched in broth to increase the *C. difficile* load available for direct ribotyping (**Figure 3.5**). Enrichment in BHI broth resulted in 32 (51.6%) samples producing a ribotype from the extracted DNA (**Figure 3.5**). It was suspected that in the 30 samples that were not directly ribotyped using BHI enrichment that potential competing organisms had limited the growth of *C. difficile*. Using *C. difficile*-specific Banana Broth™ for enrichment the remaining 30 (48.4%) stools were directly ribotyped from DNA extracted from the broth (**Figure 3.5**).

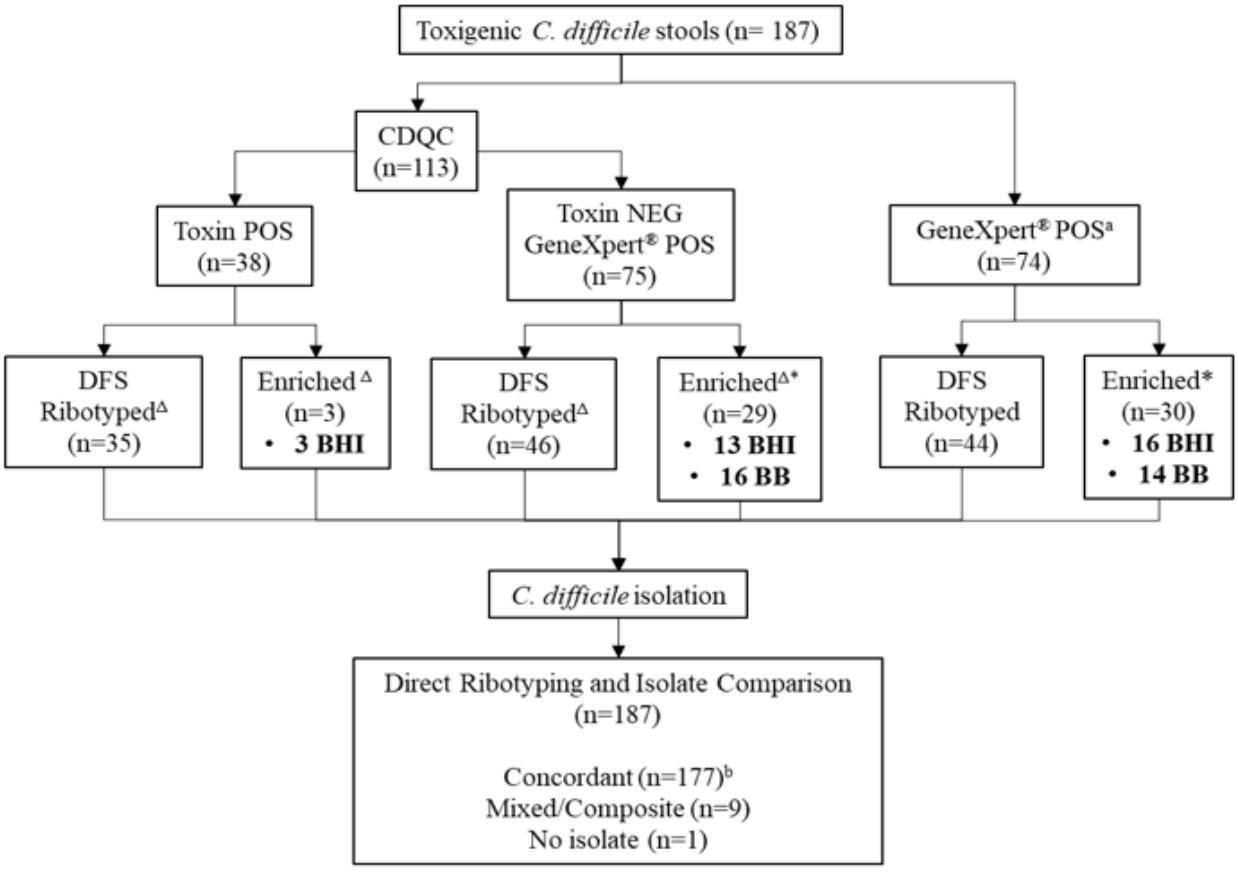

Figure 3.5. Summary of diagnostic testing and direct ribotyping results. [a]Six samples presumptively identified as ribotype 027 by GeneXpert® *C. difficile/Epi.* [Δ]Toxin positive stools were significantly more likely to be directly ribotyped (Fischer's Exact Test, $p<0.001$ (35/38, 92.1% vs. 46/75, 61.3%)). *Eleven stools were negative for *tcdB* by qPCR (eight Toxin NEG/GeneXpert® POS and three GeneXpert® POS of which one was also 16S negative). [b]Includes five mixed samples containing >1 ribotype but the direct ribotype matched one of the isolates. Composite: ribotypes did not match the reference database or isolate(s).

Of the 113 samples tested by CDQC, 38 samples were toxin positive and 75 were toxin negative but contained toxigenic *C. difficile* (*tcdB* positive by GeneXpert®) (**Figure 3.5**). DFS ribotyping was successful for 35 toxin positive samples with 3 requiring enrichment (**Figure 3.5**), whereas 46 toxin negative samples were directly ribotyped and 29 required enrichment (**Figure 3.5**). The remaining 74 samples were not tested by CDQC but were positive for *tcdB* by GeneXpert® with 44 directly ribotyped and 30 of them required enrichment (**Figure 3.5**).

3.3.2.2 Direct from Stool Ribotyping Correlation with the *C. difficile* Bacterial Load

To determine if *C. difficile* toxin enzyme immunoassay (EIA) or DNA detection by singleplex 16S and *tcdB* qPCR and GeneXpert® *tcdB* correlated with DFS ribotyping performance, the *C. difficile* toxin positivity and Ct values of stools that yielded a DFS ribotype versus those that failed were compared. Directly ribotyped stools had both significantly ($p<0.001$) lower 16S and *tcdB* Ct, as determined by qPCR panel, as well as GeneXpert® *tcdB* Ct values compared to stools that required enrichment (**Figure 3.6, A**). Also, 11 GeneXpert® positive stools were negative for *tcdB* (and 16S for one stool) using the qPCR panel, but all were resolved upon repeat testing after broth enrichment (**Figure 3.5, Figure 3.6**).

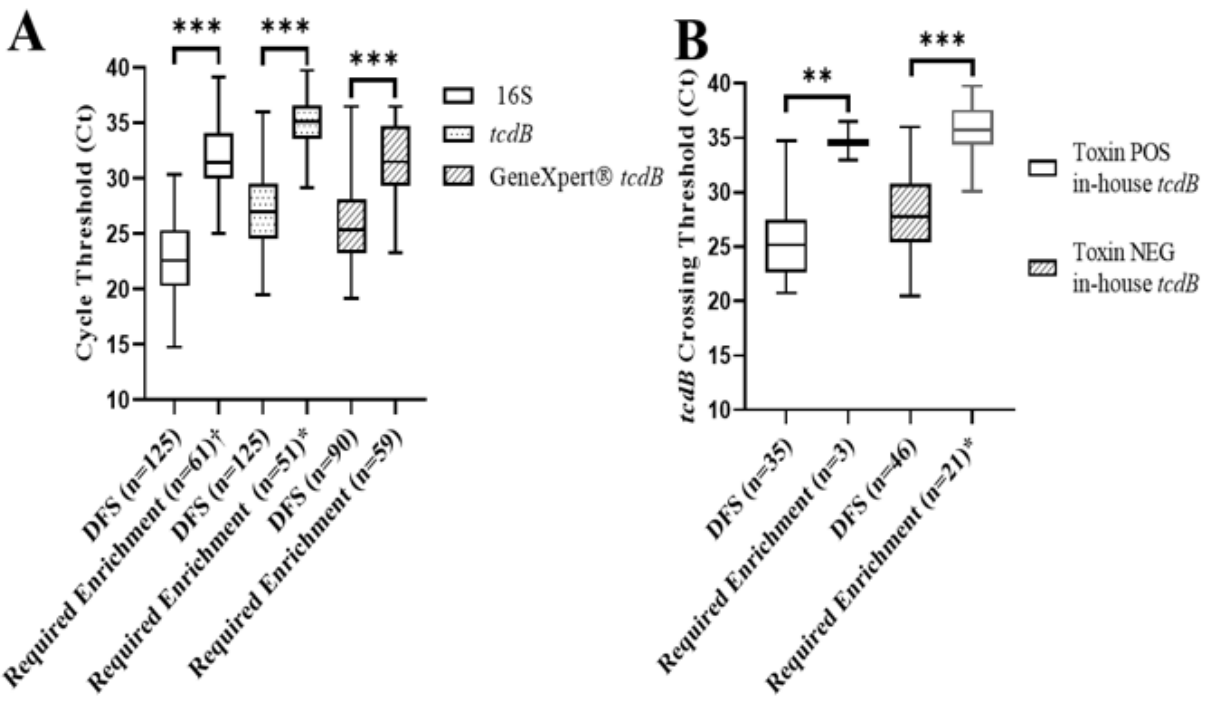

Figure 3.6. Cycle threshold (Ct) comparisons between directly ribotyped stools (DFS) and stools that required enrichment with respect to 16S and *tcdB* qPCR (A), GeneXpert® *tcdB* (A), and *tcdB* qPCR with respect to toxin positivity (B). †One GeneXpert® POS stool was negative for 16S and was omitted.*Excludes 11 *tcdB* negative stools in panel A and 8 stools testing toxin and *tcdB* negative in panel B (Figure 3.5). Mann-Whitney U Test was used to compare Ct between DFS ribotyped and enriched stools in each group with *** and ** representing $p \leq 0.001$ and $p \leq 0.01$ respectively.

Directly ribotyped toxin EIA positive ($p<0.01$) and toxin EIA negative ($p<0.001$) stools had significantly lower *tcdB* Ct values, as determined by qPCR, compared to enriched stools (**Figure 3.6, B**). Also, toxin positive stools were significantly more likely to yield a DFS ribotype ($p<0.001$) compared to toxin negative stools (**Figure 3.5**). To compare Ct values generated by the 16S and *tcdB* qPCR assays, and GeneXpert® *tcdB* assay as predictors of DFS ribotyping, ROC curve analysis was performed. Area under the curve values were highest for 16S (0.97) and *tcdB* (0.96) compared to GeneXpert® *tcdB* (0.85) (**Figure 3.7**). Retaining ≥95% specificity, Ct thresholds derived from the ROC curve of <27.31, <30.2, and <25.05 were 88.8%, 80.0%, and 42.2% sensitive using the 16S, *tcdB,* and GeneXpert® *tcdB* targets, respectively.

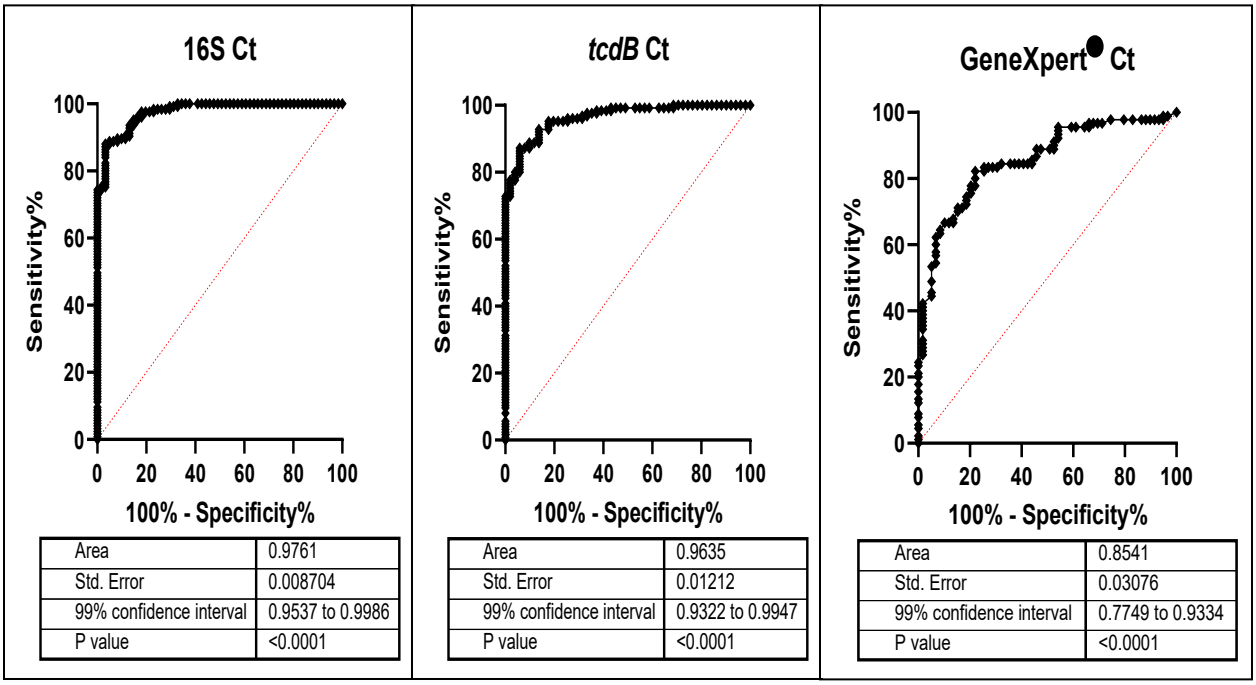

Figure 3.7. Receiver operator characteristic (ROC) curves comparing 16S Ct and *tcdB* Ct by singleplex qPCR panel and GeneXpert® *tcdB* Ct as a predictor for direct ribotyping of stool samples.

3.3.2.3 Comparison of Direct and Isolate Ribotypes

To establish whether the direct ribotype represented ribotypes derived from isolates obtained from the same sample, isolate ribotyping and direct ribotyping were performed and compared. Of the 187 samples included in the validation, 186 samples had ≤5 isolates subjected to ribotyping and toxin gene qPCR, and there were 177 (94.7%) samples that directly correlated between the direct and isolate ribotype patterns (**Figure 3.5**). One (0.5%) sample was ribotyped from Banana Broth™ (RT 076) but failed to obtain an isolate for ribotype comparison (**Figure 3.5,** "No isolate"). Of the 177 samples, 6 samples with concordant direct and isolate ribotypes were presumptively identified as RT 027 by the GeneXpert® *C. difficile/Epi* of which only 4 were RT 027 and 2 were *cdtB* positive RT 011 and ns64 (**Figure 3.5**). Fourteen (7.5%) potentially mixed

C. difficile cases were also identified (**Table 3.6**). When >1 strain was suspected (e.g., toxin gene or ribotype discordance between stool/broth and isolate), additional isolates and broth enrichments were ribotyped and tested by qPCR panel until mixed ribotype status was confirmed if possible. Five (2.7%) mixed ribotype samples had a direct ribotype matching one of the isolates and were included in the 177 matching samples (**Figure 3.5, Table 3.6**, Samples: 1, 4-6 and 8). The other 9 (4.8%) mixed samples had composite direct ribotypes that did not match any of the isolate ribotypes (**Figure 3.5, Table 3.6**, Samples: 2, 3, 7, 9-14).

Table 3.6. Summary of mixed *C. difficile* cases

Sample No.	Direct Ribotype	Isolate Ribotypes		
1	014*	014*	084	
2	043*†	ns366*	024	
3	Composite*	017*	084	
4	**024Δ**	**024***	**075Δ**	
5	**039***	**039**	**106***	**009**
6	**039***	**039**	**103***	
7	Composite Δ	056*	ns290*	
8	**014Δ**	**014***	**019Δ**	
9	Composite Δ	020*	176Δ	
10	Composite*	629*	ns113	
11	Composite*	005*	153*	
12	011*†	103*	001*	
13	Composite*	ns107*		
14	Composite*	057*		

Samples 1-12 were confirmed and samples 13 and 14 were suspected to have >1 ribotype.
Composite: ribotypes did not match the reference database or any particular isolate.
Bolded samples had matching direct and isolate ribotypes but differed in toxin gene profile.
*Toxigenic (*tcdA/tcdB*) & Δtoxigenic + binary (*tcdA/tcdB/cdtB*).
†Samples matched the database but were actually a composite.
NT denotes new type as this ribotype was not present in the reference collection

3.3.2.4 Characterization of *C. difficile* Isolates in Samples with Mixed Ribotypes

Of the 14 samples with mixed ribotypes identified, 12 (6.4%) were confirmed through isolation of more than one ribotype (Samples: 1-12, **Table 3.6** and **Appendix F**), while 2 (1.1%) could not be confirmed because only one ribotype was recovered by culture (Samples 13 and 14, **Table 3.6**). The complete ribotyping and toxin gene qPCR results for each mixed ribotype case is shown in **Appendix F**. With respect to the 14 mixed samples, one particular sample (7.1%) was DFS ribotyped as RT 014 - matching the toxigenic isolate - while a second non-toxigenic strain, RT 084, was randomly identified by isolation from the same sample (Sample 1, **Appendix F** and **Table 3.6**). Two samples (14.3%) had direct ribotypes matching the reference collection, but their isolates revealed that the direct ribotype was actually a composite of two individual ribotypes being: Sample 2, stool RT 043 was a composite of isolate RTs 024 and ns366 and Sample 12 with RT 011 from BHI broth was a composite of isolate ribotypes RT 103 and 001 (**Appendix F** and **Table 3.6**). Seven (50%) mixed ribotype samples had composite direct ribotypes and did not match the reference collection or the isolate shown in Samples 3, 7, 9-11, 13 and 14 (**Appendix F** and **Table 3.6**). There were 4 mixed cases (28.6%) identified by differing toxin gene profiles between stool/broth and isolate ribotype (Samples 4, 5, 6, and 8 **Appendix F** and **Table 3.6**). In these cases, an additional isolate was identified (*cdtB* positive RTs 075 and 019 in Samples 4 and 8; *tcdA/tcdB* positive RTs 106 and 103 in Samples 5 and 6), which accounted for this difference. Interestingly, Sample 7 (**Appendix F** and **Table 3.6**) also fit into this category with a composite direct ribotype and was *cdtB* positive; however, a *cdtB* positive isolate was not recovered. In two cases, Sample 5 and 6, an additional non-toxigenic strain (RT 009) and a toxigenic strain (RT 103) respectively, were recovered after subculture to a new Banana Broth™ (**Appendix F**).

3.3.2.5 Isolate Ribotypes Identified in Direct Ribotyping Validation

Overall 63 different ribotypes were identified from 199 isolates, 172 were from non-mixed samples (**Figure 3.5** "Concordant n=177" subtracting 5 mixed cases that were also concordant being Samples 1, 4, 5, 6, and 8 in **Table 3.6**) and a total of 27 isolates identified from the mixed samples in **Table 3.6**, recovered during the validation of the direct ribotyping method. The prevalence of the different ribotypes identified is shown in **Figure 3.8** with the majority (51.8%) of *C. difficile* isolates corresponding to a diverse range of RTs being: 106 (n=16, 8.0%), 020 (n=16, 8.0%), 027 (n=14, 7.0%), 014 (n=14, 7.0%), 002 (n=14, 7.0%), 056 (n=10, 5.0%), 015 (n=10, 5.0%), and 629 (n=9, 4.5%). Several binary toxin positive ribotypes were identified including RT 011, 019, 023, 027, 075, 078, 153, 176, 328, ns101, ns111, ns164, ns296, ns363, and ns64

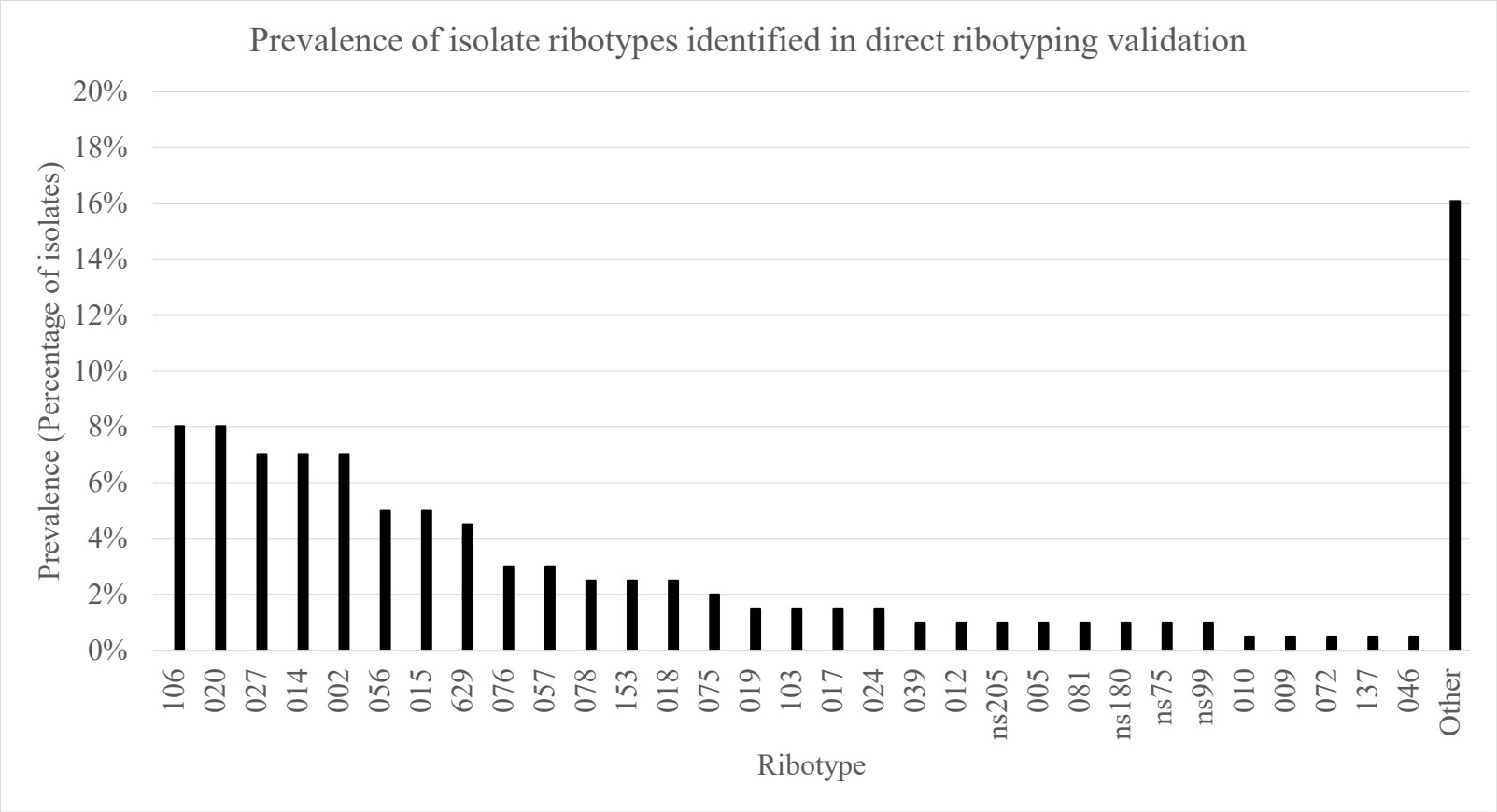

Figure 3.8. ***C. difficile*** **isolate ribotypes identified during the direct from stool ribotyping validation.** Ribotypes included in the Other category consisted of ribotypes with only a single isolate.

3.3.3 Inter-laboratory Direct and Isolate Ribotype Comparison

To further validate the direct ribotyping method, direct ribotypes and toxin gene profiles were compared to isolate ribotypes and toxin gene profiles generated at the PHAC-NML for 23 samples. There was high concordance (20/23, 87.0%) between both laboratories, despite different ribotyping and toxin gene amplification methods used (qPCR vs endpoint PCR) (**Table 3.7**). Ribotypes differed for only three samples (Samples 1, 12 and 15, **Table 3.7**) and the toxin gene profiles were consistent between laboratories. In two cases (Samples 1 & 15), the PHAC-NML isolate ribotype differed from the direct ribotype due to mixed infections (**Table 3.7**). For Sample 12 the direct and isolate ribotypes differed as ns235 and 001 have indistinguishable banding patterns but ns235 contains a doublet (two peaks) in the highest molecular weight band compared to a singlet (one peak) in ribotype 001.

Table 3.7. Inter-laboratory direct and isolate ribotype comparison

Sample	Direct Ribotype	Isolate Ribotype(s)	NML Isolate Ribotype
1	**014***	**084** **014***	**084**
2	ns99*	ns99*	ns99*
3	020*	020*	020*
4	020*	020*	020*
5	027$^{\Delta}$	027$^{\Delta}$	027$^{\Delta}$
6	012*	012*	012*
7	075$^{\Delta}$	-	075$^{\Delta}$
8	629*	-	629*
9	012*	-	012*
10	002*	-	002*
11	012*	-	012*
12	ns235*	-	001*
13	293*	-	293*
14	ns297$^{\Delta}$	-	ns297$^{\Delta}$
15	**ns178†***	**002*** **015***	**002***
16	629*	-	629*
17	103*	-	103*
18	076*	-	076*
19	027$^{\Delta}$	-	027$^{\Delta}$
20	014*	-	014*
21	002*	-	002*
22	014*	-	014*
23	014*	-	014*

Sample toxin profile and direct ribotype refers to the result from either stool or broth. Isolation was performed for only the samples with isolate ribotypes. Mixed *C. difficile* samples are bolded.
*Toxigenic (*tcdA/tcdB*) & $^{\Delta}$toxigenic + binary (*tcdA/tcdB/cdtB*).
†This sample matched the database but was actually a composite

3.4 Discussion

Janezic *et al.* (19) first reported PCR ribotyping directly from stool using agarose gel electrophoresis for analysis. Recently, another direct *C. difficile* ribotyping assay using capillary electrophoresis has been described by Rossen *et al.* (21). Both Janezic *et al.*'s and Rossen *et al.*'s

studies can be compared to the method described in this chapter as there are several differences and interesting comparisons between the three studies. Both Janezic *et al.* and Rossen *et al.* identified samples that potentially contained mixed *C. difficile*, which was indicated by additional bands in the direct ribotype relative to the isolate ribotype. In this study, additional bands in the direct ribotype were contributed by a second strain present in the stool (**Appendix F**). Fourteen mixed *C. difficile* cases were identified in this chapter, whereas none of the other studies confirmed potential mixed *C. difficile* cases. Broth enrichment was a major difference between studies as enrichment was not performed in the other studies, and enrichment likely promoted the growth of strains that might be present in the stool at low numbers, which aided in identifying mixed *C. difficile* cases.

The amount of *C. difficile* DNA in the stool, reflective of *C. difficile* burden, was previously hypothesized as a limitation for direct *C. difficile* ribotyping (19). This study demonstrated lower 16S and *tcdB* qPCR Ct values, indicating higher bacterial loads, correlated with a higher chance of obtaining a ribotype directly from stool (**Figure 3.6**). Amplicons generated by ribotyping PCR (generally those at high molecular weights) are absent in the direct ribotype when the bacterial load is insufficient, resulting in an incomplete ribotyping pattern (**Figure 3.3**), and this is in agreement with Rossen *et al.* (21). For some stools, undiluted DNA extracts did not generate a direct ribotype across a range of stool 16S Cts (**Figure 3.4**). In some cases, this was likely due to the presence of PCR inhibiting compounds present in stool that are less concentrated when the DNA extract is diluted and thus a ribotype can be generated even after minimal 1:5 dilution, which was also observed by Rossen *et al.* (21). Alternatively, direct ribotyping could have been inhibited by addition of too much template DNA in the reaction, which likely occurred for stools with low 16S Ct values (**Figure 3.4**).

In both the Janezic *et al.* (19) and Rossen *et al.* (21) studies, a much higher proportion of stools were directly ribotyped (86.9% and 100% respectively) as compared to this study (66.8%). These discrepancies in direct ribotyping success are likely attributable to differences in methodological approaches. With respect to Janezic *et al.*, this difference in performance was likely due to an amplicon concentration step prior to electrophoresis in which the volume of the amplified PCR products was reduced from 50 µL to 25 µL by heating. This procedure was not considered in this study due to the potential for generating aerosolized amplified products in the laboratory which can be a source of contamination in subsequent PCR reactions. In this study amplicons were directly subjected to electrophoresis and were not concentrated post-PCR which likely accounted for the lower success rate in this study compared to Janezic *et al.* However, it is likely that the increased sensitivity of capillary gel electrophoresis, compared to agarose gel electrophoresis which was used in Janezic *et al.*'s study, contributed to the direct ribotyping success rate in this study. Compared to Rossen *et al.,* there is a significant difference in methodology as Rossen *et al.* developed two new primer sets for direct ribotyping. The Rossen *et al.* primers were designed in order to amplify ISR fragments both below and above 400 base pairs. As noted above, ISR fragments at higher molecular weight are more often missing when the bacterial load is insufficient and specific amplification of these fragments by the Rossen *et al.* primers likely increased the sensitivity of direct ribotyping resulting in a greater direct ribotyping success rate compared to this study. In addition, Rossen *et al.* used 10 µL of template DNA for direct ribotyping compared to the 5 µL used in this study which likely increased direct ribotyping success. As success of direct from stool ribotyping was dependent on the bacterial load, improvements to the direct ribotyping assay could include increasing the amount of template DNA; however, this would require a revised "Ct key" to in order to re-standardize the direct ribotyping

assay. It is important to note that while the direct from stool ribotyping success rate was lower in this study, compared to the other studies, all of the samples were able to be directly ribotyped with the inclusion of broth enrichment.

A 25 base pair shift used in this study to compare ribotypes of Janezic *et al.* and Bidet *et al.* (18) is consistent with Janezic's (22) observation of a 24 base pair shift. Lastly, Rossen *et. al.* (21) were able to apply their direct ribotyping method to an outbreak of *C. difficile* RT 017 which correctly identified related clusters, which is a proof-of-principle lacking in the method described here.

Identification and characterization of mixed *C. difficile* by direct stool ribotyping in this chapter was a novel finding compared to the previous direct ribotyping studies (19,21). Mixed *C. difficile* infection has been previously reported with frequencies ranging from 7-16% (13,16,23–25), which was consistent in this study with a rate of 7.5% (of which 6.4% could be confirmed through isolation of two unique ribotypes). Generally, mixed *C. difficile* infection has been found to have two toxigenic strains or a mix of toxigenic and non-toxigenic strains (13,23), and in this study, mixed cases containing binary toxin positive strains were identified (**Table 3.6**), which are rarely reported (16). One sample had a total of three ribotypes with one toxigenic and two non-toxigenic strains (**Appendix F** and **Table 3.6,** Sample 5). Stools containing 3 ribotypes have been reported previously in literature (13) but are exceptionally rare.

While mixed *C. difficile* cases have been previously reported, the proportion of each individual genotype present in mixed *C. difficile* infection remains relatively unknown. Published studies have shown that the population of the minor strain represented 1-26% (13,26) of the colonies on the culture plate. In this study, sample 1 (**Appendix F** and **Table 3.6**) had a total of 25 isolates picked and only 4 isolates were toxigenic ribotype 014 (16%), which matched the direct

ribotype. Generally, it was anticipated that the result of the direct ribotype would be a reflection of the predominant strain present in the sample, but this was not the case for this particular sample. There is also the possibility that culture and broth enrichment likely alters the proportions of each strain and must be considered when deciding the number of colonies to be picked in order to recover the minor strain (13). It's likely that different strains favor *in vivo* compared to *in vitro* growth, which may account for the difference in proportions of each strain after culture.

Another novel aspect of this study compared to those previously published (13,16,23,24,27) was the identification of mixed *C. difficile* populations prior to culture by comparing Ct values between target genes and from composite direct ribotypes. Most samples had a ~3 Ct difference between 16S and toxin genes. However, the stool (and broths) in Sample 5 (**Appendix F**) have 16S Cts ~6 cycles lower than *tcdA/tcdB* and this sample contained both toxigenic and non-toxigenic strains. Similar observations were made with the *cdtB* target as shown in Sample 4 from the BHI and BB broth (**Appendix F**). As 16S is present in multiple copies across the genome, it can be difficult to interpret whether a low 16S Ct, relative to the toxin genes, is reflective of >1 strain as the 16S Ct value is generally lower than the toxin genes in most cases due to the difference in gene copy number between the 16S and toxin genes. A *tpi* qPCR assay was developed which may provide a remedy to this issue. The *tpi* gene is present in only a single copy in the *C. difficile* genome, similar to the toxin genes, and thus mixed *C. difficile* may be more readily identified by comparing *tpi* and toxin gene Cts; however, this has yet to be demonstrated. Another technique has been developed with the potential to identify mixed *C. difficile* without culture. This method uses cell sorting to isolate *C. difficile* bacteria directly from stool followed by sequencing to characterize both single and multiple strain infections directly from the stool

(28). However, this method may be difficult to implement in a molecular microbiology laboratory due to the instrumentation required.

Several questions remain unresolved pertaining to mixed *C. difficile* infection such as the clinical significance of having CDI with >1 toxigenic strain. Do both toxigenic strains produce toxin *in vivo* and in greater amounts resulting in a more severe disease with a worse outcome? Does coinfection with *cdtB* positive strains increase morbidity? It has been suggested that mixed toxigenic strains both produce toxins *in vitro*; however, it was not possible to distinguish toxin production between strains (29,30). Non-toxigenic *C. difficile* has been shown to be protective against infection (31–33) and is associated with lower disease recurrence (34) but is still frequently isolated from CDI patients. Other published studies, using animal models, have shown that concurrent challenge with toxigenic and non-toxigenic strains offers little protection (33). It is possible that toxigenic and non-toxigenic *C. difficile* are equally able to colonize the gut after antibiotic exposure and such cases still result in CDI due to the absence of a pre-existing non-toxigenic population. Lastly, the proportion of each strain in mixed *C. difficile* cases is largely unknown. One study observed, using an *in vitro* gut model, two unique RT 001 strains (differing in antimicrobial susceptibilities) produced similar growth patterns and bacterial loads (29,30) suggesting non-competitive growth between different strains.

While the direct ribotyping method is useful for identifying mixed infections, its primary purpose is *C. difficile* surveillance. A potential direct ribotyping algorithm (**Figure 3.9**) was developed for implementation alongside routine *C. difficile* diagnostics in a clinical molecular microbiology laboratory for *C. difficile* cluster detection. Toxin positive stools are likely to produce a DFS ribotype while toxin negative samples require confirmation of the presence of the toxin gene. Detection of *tcdB* by GeneXpert® or qPCR targeting the toxin genes and the resulting

Ct value generated can be used to stratify samples into those most likely to be directly ribotyped and those requiring enrichment based on pre-determined Ct cut off values (**Figure 3.9**). This proposed algorithm would minimize turnaround time for direct ribotyping and reduce the cost of multiple qPCRs and unnecessary broth enrichment. Using BB for enrichment is beneficial in that it can be used aerobically without the need for anaerobic equipment (35). While BHI broth is more economical ($0.73 per BHI broth vs. $5 per broth for BB), BB is likely the more optimal broth due to its selectivity for *C. difficile*. A previous study suggested that the optimal *C. difficile* culture method involves a heat shock pre-treatment, inoculation into *C. difficile* specific broth and followed by recovery of isolates on either *C. difficile* specific or non-specific agar (36). In some cases an isolate may not be available, such as the "No isolate" sample in **Figure 3.5** for which no isolate could be recovered but direct ribotyping identified RT 076, and direct ribotyping may be the only opportunity for genotyping of such samples.

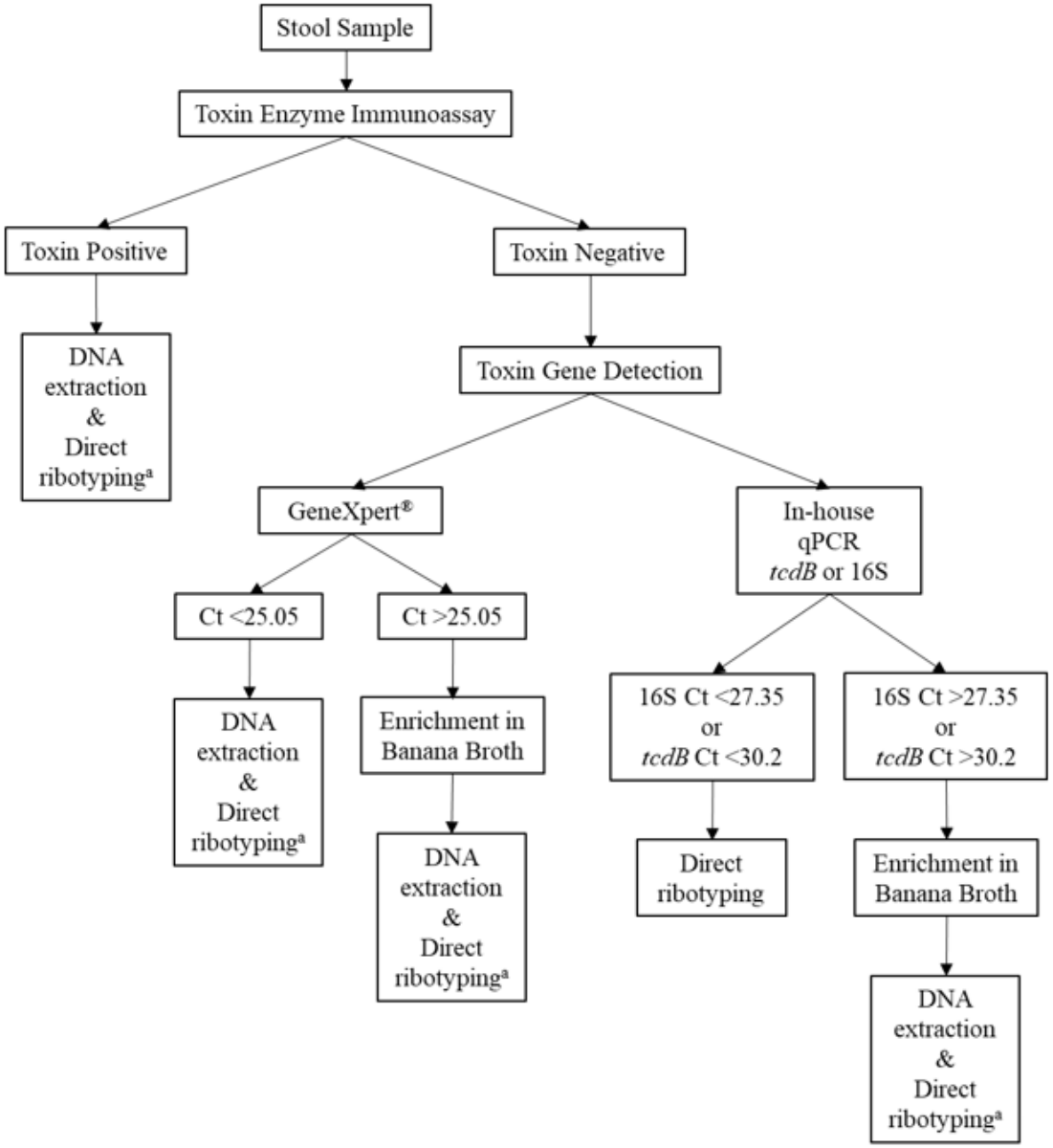

Figure 3.9. Proposed algorithm for direct stool ribotyping of toxigenic *C. difficile* stools in a microbiology laboratory using a two-step algorithm for *C. difficile* detection. [a]In these cases, additional qPCR testing (as shown above) on the extracted DNA from stool or broth is required for standardization prior to direct ribotyping.

Previous studies have shown that toxin EIA positive patients have higher *C. difficile* loads (37–39), greater risk for CDI complications, and are more likely to require CDI treatment (37,40) compared to toxin EIA negative, PCR positive patients. Results from this study have shown that

C. difficile toxin positive patients have greater success for DFS ribotyping. Thus, the patients at greatest risk of CDI complications can be ribotyped with a rapid turnaround time, and if clusters are identified infection control measures can be implemented immediately to prevent further transmission.

Another advantage of this direct ribotyping method is its application for epidemiological studies of environmental contamination. Swabs are frequently utilized in this setting and enrichment can be performed in broth to increase the bacterial load as part of the screening process (35,41). Direct ribotyping from the broth can identify ribotypes from patient environments, and these ribotypes can then be linked to patients for contact tracing within a very short timeframe; isolates recovered from the enrichment broth can then be used for next generation sequencing to investigate potential transmission events (42).

The GeneXpert® *C. difficile* and *C. difficile/Epi* are frequently used commercial molecular diagnostic assays for *C. difficile* and both were compared to the singleplex qPCR reactions for 16S, and *tcdB*. For direct ribotyping, GeneXpert® *tcdB* Ct provides the most utility as it is routinely performed in *C. difficile* diagnoses; however, the 16S and *tcdB* qPCR Ct better correlated with direct ribotyping compared to GeneXpert® (**Figure 3.7**). While the 16S gene was chosen for direct ribotyping standardization because it is the same target as the ribotyping assay, it's also possible that a "Ct key" could be developed using *tcdB* (including GeneXpert® *tcdB*) for direct ribotyping standardization. There was discordance in *tcdB* detection for 11 samples between the qPCR panel and GeneXpert®, which may be due to differences in DNA extraction protocols and sample amount (~100 mg/250 µL stool vs. a stool coated swab) between the two assays.

The GeneXpert® *C. difficile/Epi* presumptively identifies RT 027 based on *cdtA* and Δ117 *tcdC* detection; however, previous studies have shown false positive results with non-RT 027

strains using this assay (39,43), and was further confirmed in this study. The two discordant cases were *cdtB* positive isolates of RTs 011 and ns64, matching the direct ribotype, suggesting likely false positive identification by GeneXpert® and not a mixed infection.

When comparing the adult *C. difficile* isolate ribotypes identified in 2015 (**Chapter 2**) to isolates identified in 2018 (**Chapter 3**) there was a marked decrease in ribotype 027 prevalence, which was 44.3% in the adult CDI cases from **Chapter 2** and was only 7% prevalent in the isolates recovered in this chapter. This trend was also observed in a Canada-wide surveillance study of PCR ribotypes recovered from CDI cases between 2013 and 2017 (4). Increases in prevalence for ribotypes 106, 020, and 002 (**Figure 3.8**) are also in agreement with the national study (4).

There are several limitations to this direct ribotyping method. Stools with low amounts of *C. difficile* may not generate a complete ribotype. *C. difficile* isolation remains necessary in several scenarios such as: newly identified ribotypes, direct ribotypes matching non-toxigenic strains (eg: RT 039 in Samples 5 and 6, **Table 3.6**), or if >1 strain is presumed. Mixed *C. difficile* can also cause incorrect direct ribotype assignment (Samples 2 and 12, **Table 3.6** and Sample 15 **Table 3.7**). Only stools that were presumptively mixed or had failed to generate ribotypes directly were enriched which potentially missed additional mixed *C. difficile* cases due to minor populations that were not detected. An extensive curated database of ribotype patterns and toxin genes associated with each ribotype is advantageous with direct ribotyping to ensure accurate pattern assignment and identification of novel or mixed ribotypes.

The direct PCR ribotyping method is robust and can identify ribotypes from stool within 24 hours if enrichment is not required. The decreased turnaround time allows for faster identification of *C. difficile* clusters leading to earlier implementation of infection control and prevention measures. In addition, this method can occasionally detect cohabiting *C. difficile* strains. The direct

ribotyping method is versatile and can be easily implemented into a molecular microbiology laboratory for surveillance purposes.

3.5 References

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Chapter 4 Discussion, Significance, and Future Directions.

4.1 Discussion

Clostridioides difficile remains a significant cause of morbidity particularly in the elderly and those receiving antibiotic treatment with frequent hospital exposure. In the current antibiotic era of medicine, this bacterium contributes a substantial burden and cause of infection particularly in the hospital with cases also occurring in the community (1,2). However, novel therapies have been developed to improve *C. difficile* patient outcomes for recurrent *C. difficile* infection (CDI) in particular. Such novel therapeutics are at the forefront of medicine including fecal microbiota transplantation (3) and microbial ecosystem therapy (4). In addition, there have been newly developed treatments such as fidaxomicin, an antibiotic which is less disruptive to the host microbiome than vancomycin (5), non-toxigenic *C. difficile* colonization for recurrent CDI (6) and monoclonal TcdB antibody therapies (7). The development of potential *C. difficile* vaccines (8) will also help to lessen the burden of disease.

Prior to the emergence of the fluoroquinolone resistant epidemic ribotype (RT) 027, several pre-epidemic RT 027 isolates, lacking the *gyrA* Thr82Ile mutation conferring fluoroquinolone resistance, were identified in geographically diverse isolates (9). It has been hypothesized that the emergence of RT 027 was in part due to its fitness for the hospital environment with such traits as fluoroquinolone resistance (9) and increased spore production (10). Several recent studies have reported that the prevalence of RT 027 is declining in Canada, the U.S., and England (11–13) but with a corresponding increase in RT 106 in both Canada and the U.S. (11,12), which suggests the molecular epidemiology of *C. difficile* may currently be shifting. As ribotype 027 is no longer predominant in Canada and the U.S. (11,12), *C. difficile* surveillance is essential, which includes monitoring changes in molecular epidemiology, antibiotic susceptibility, antibiotic usage patterns,

and detection of emerging novel virulence factors, such as antibiotic resistance, in order to identify and prevent the emergence of another epidemic strain.

As *C. difficile* is both spore-forming and contains resistances to several antibiotics, *C. difficile*'s relevance as a One Health pathogen is immense. In Knight *et al.*'s study (14) several antibiotic resistance markers were identified in both human and non-human sequence type 11 (RT 078) isolates, and in some cases, isolates originating from humans and animals were closely genotypically related suggestive of inter-species transmission. Another study (15) suggested agricultural tetracycline use has likely led to the acquisition, clonal expansion, and widespread dissemination of *C. difficile* RT 078 carrying tetracycline resistance genes, which may have contributed to an increase in CDI cases associated with this ribotype. Knight *et al.* (14) observed tetracycline resistance in 70.0% of RT 078 and 77.6% of RT 126 isolates, of which RT 126 is genetically descendant from RT 078 (15). Both studies observed a high degree of sequence similarity between genetic determinants of tetracycline resistance identified in RT 078 and those present in zoonotic pathogens (14,15). The linkages between fluoroquinolone use and the emergence of RT 027 (9) and, more recently, tetracycline use and the potential emergence of RT 078 (15) suggests that antibiotic selective pressure in both human and non-human *C. difficile* reservoirs may contribute to enhanced virulence of *C. difficile*.

While many genotypic studies have focused on CDI cases, there is increasing interest in *C. difficile* present outside of the hospital. As demonstrated by Eyre *et al.*'s (16) study, only a minority of CDI cases are potentially attributable to transmission between CDI patients or the hospital environment and there is a considerable reservoir of diverse strains that are identified in CDI cases. *C. difficile* reservoirs in the community are abundant with *C. difficile* identified in the environment and colonized humans and animals (17). Many questions remain regarding *C. difficile* transmission

and the interrelationship between hospital and community environments, and a study with sufficient recruitment, sampling, and contact tracing to assess the interrelation and flow of *C. difficile* between the different *C. difficile* reservoirs and CDI cases would prove challenging.

C. difficile genotypes present in colonized children have been reported in cohorts from Europe (18–20) and Asia (21); however, there are only very few published studies that report the genotypes present in colonized children from North America (22). In **Chapter 2,** the most prevalent *C. difficile* genotypes were identified in a cohort of children from the community in Alberta, Canada. Using PCR ribotyping, *C. difficile* ribotype 106 was identified as the most prevalent genotype (20.8%) in this cohort, and isolates from the children were compared to the ones from pediatric and adult CDI cases with respect to the ribotype and toxin genes. The pediatric CDI cohort and child cohort contained predominantly $tcdA^+/tcdB^+/cdtB^-$ isolates (88.1-94.1%) (**Table 2.3**), whereas adult CDI isolates were predominantly $tcdA^+/tcdB^+/cdtB^+$ (53.2%) (**Table 2.3**), largely due to the prevalence of RT 027 (44.3%) in this patient population (**Table 2.4**). Similar to the APPETITE children, pediatric CDI cases were primarily RT 106 (27.8%) (**Table 2.4**). Strains containing the binary toxin, such as RT 027, 078, and 019, were identified in children as well as the CDI cases (**Appendix C**). However, it is notable that RT 027 had not been reported in the previous studies investigating colonized children (18,19). It has been previously suggested that RT 027 might be identified in colonized children from North America due to the diffusion of this strain into the community, whereas RT 027 was absent in children from France and the United Kingdom (18,19). Thirty-four ribotypes were identified in isolates from both the colonized children and adult and pediatric CDI cohorts (**Table 2.4**), which confirms the observations of previous studies that the *C. difficile* genotypes observed in colonized children are similar to those identified in adult CDI cases (18,19). In some cases, isolates from the colonized children and CDI

cases shared both ribotype and pulsotype suggesting clonal relatedness of these strains (**Figure 2.2**). Generally, these isolates with matching ribotype and pulsotype between cohorts corresponded to the most prevalent ribotypes in their respective cohorts which were RT 106 & 020. Interestingly, isolates of ribotypes 027 and 078, as well as their corresponding pulsed-field gel electrophoresis patterns, were shared between children and adult CDI cases (**Figure 2.2**). The major findings of **Chapter 2** is that *C. difficile* isolates present in children are similar to those identified in pediatric and adult CDI cases and ribotype 106 may play a large role in pediatric colonization in the community as well as pediatric CDI.

Current *C. difficile* genotyping methods are limited in that they require an isolate for characterization, which is labor intensive and time consuming. In **Chapter 3**, a direct *C. difficile* ribotyping method was developed along with a proposed testing algorithm (**Figure 3.9**) for implementation in the clinical molecular microbiology laboratory for rapid identification of *C. difficile* clusters. Using 187 *C. difficile* positive stools included in the validation for direct ribotyping, ribotypes were obtained in 66.8% of the stools directly whereas the remaining 33.2% required an intermediate broth enrichment before a ribotype was generated (**Figure 3.5**). This discrepancy was due to differences in the *C. difficile* bacterial load between stool samples and was determined based on the qPCR cycle threshold (Ct) values targeting the 16S and *tcdB* genes. A lower Ct value indicates a higher organism burden and results in greater success with direct ribotyping (**Figure 3.6**). For laboratories that utilize molecular assays detecting the toxin genes, Ct value thresholds can be used to differentiate stool samples most likely to be successful using direct ribotyping from those that require enrichment (**Figure 3.9**). In this study, 16S and *tcdB* Ct values determined by singleplex qPCR assay were found to be more predictive than *tcdB* Ct values from the commercial GeneXpert® assay for identifying samples that could be directly ribotyped

(**Figure 3.7**). For laboratories that utilize toxin enzyme immunoassays (EIA), most toxin EIA positive samples were significantly more likely to be directly ribotyped ($p<0.001$, **Figure 3.5**), whereas toxin EIA negative samples may require enrichment.

There was good correlation (94.7%) between the direct and isolate ribotyping methods as nearly all samples matched and those that didn't either contained mixed infections, with >1 ribotype (4.8%) or an isolate was not recoverable for comparison (0.5%) (**Figure 3.5**). An unexpected finding was the identification of mixed *C. difficile*. These mixed cases were identified in several different ways including: discordance in the toxin gene profiles and ribotypes detected between stool, broth and isolate; composite direct ribotype patterns indicating a mixture of two unique ribotype patterns, and through identification of a second ribotype when selecting colonies for isolate ribotyping (**Table 3.6** and **Appendix F**). In addition, direct ribotypes were compared to isolate ribotypes generated at the Public Health Agency of Canada - National Microbiology Laboratory (PHAC-NML) for 23 stools, and there was an 87% (20/23) agreement between laboratories (**Table 3.7**). In the three discordant cases, two were due to mixed infections, whereas the other was due to differences in ribotype assignment between highly related strains. A marked decrease in the proportion of RT 027 was noted in isolates identified from adult CDI cases in 2015 (**Chapter 2 Table 2.4**) as compared to the isolates in 2018 (**Chapter 3 Figure 3.8**).

Lastly, in order to develop a qPCR assay for simultaneous detection of toxigenic and non-toxigenic *C. difficile*, a multiplex qPCR assay was developed targeting *tpi* and *tcdB,* which was able to correctly discriminate toxigenic strains, $tpi^{+}/tcdB^{+}$, from non-toxigenic strains, which were $tpi^{+}/tcdB^{-}$ (**Table 3.3**). The multiplex *tpi* and *tcdB* assay was compared to singleplex qPCR assays for 16S, *tcdA, tcdB,* and *cdtB,* and the results showed similar Ct values with respect to the toxin genes (**Table 3.4)**.

4.2 Significance

The findings of this thesis are significant in several respects. It is important to determine which *C. difficile* strains are present in children as well as in CDI cases. As children primarily acquire *C. difficile* from the environment (18,23) it's likely that the strains identified in children are primarily reflective of those circulating in the community. In addition, genotyping of CDI cases is essential for monitoring emerging clones, identifying transmission events and potential outbreak clusters. Characterizing local *C. difficile* epidemiology has further implications for vaccine development as the prevailing *C. difficile* strains differ worldwide, and pharmaceutical companies may aim to ensure that any vaccine being developed can provide sufficient coverage against the most prevalent strains in their target markets. Several studies have investigated *C. difficile* genotypes in children (18–21,24), but what makes this study significant is it reports the genotypes from a cohort of children that have not been previously described.

The development of a direct PCR ribotyping algorithm in **Chapter 3** is a significant contribution to the clinical microbiology laboratory as well as for studies investigating *C. difficile* molecular epidemiology. Confirmation of *C. difficile* outbreaks can be lengthy as culture is not routinely performed in frontline clinical microbiology laboratories. If a potential outbreak is suspected in a hospital in Alberta, the stool samples must be referred to the PHAC-NML for culture and genotyping with a turnaround time for reporting of around 7 to 10 days. In contrast, direct ribotyping can potentially identify the ribotype(s) locally and report results to infection prevention and control departments to implement infection control measures within 24 hours. There are several other benefits to the direct ribotyping method such as reducing the time required to ribotype large numbers of samples, such as in retrospective surveillance studies, as well as potentially identifying mixed infections. Mixed *C. difficile* cases have been a confounding factor of *C. difficile*

genotyping methods, including whole genome sequencing, as often only a single isolate is subject to characterization (25,26). Selecting only a single isolate can miss potential mixed infections, which can have implications for case clustering if the outbreak strain has not been selected for characterization. However, such cases may be identified by direct ribotyping as demonstrated in **Chapter 3**. By identifying mixed *C. difficile* cases, the direct ribotyping method can potentially assist in answering questions regarding *C. difficile* infection. There is no data regarding clinical outcomes of having >1 toxigenic *C. difficile* strain (26) largely due to the rarity of detecting multiple strain infections. It is unclear as to whether toxin titers are greater in multi-strain infections compared to single strain infections (27). Of the mixed *C. difficile* cases identified in **Chapter 3**, several also contained binary toxin positive isolates (**Table 3.6**), which have been associated with increased severity of disease and poorer disease outcomes (28). Mixed *C. difficile* cases containing non-toxigenic *C. difficile* (NTCD) were also identified, which is interesting as NTCD can be protective against recurrent CDI (6).

4.3 Future Directions

Several interesting findings were identified in the current investigation into *C. difficile* molecular epidemiology and direct ribotyping, but there remains other interesting avenues to pursue. Ribotyping and PFGE have been discussed as typing tools for *C. difficile*, but whole genome sequencing (WGS) has provided numerous insights into *C. difficile* transmission and is the optimal tool for investigating relatedness between *C. difficile* isolates. Direct ribotyping can rapidly identify *C. difficile* clusters and further investigation using WGS can be performed to determine transmission based on single nucleotide polymorphism (SNP) differences between isolates. Previously defined SNP thresholds could be used to determine if matching ribotypes are

related in a manner suggestive of transmission (≤2 SNPs) or shared exposure (≤10 SNPs) (16,18,29). In addition, patient contact tracing data can provide support and evidence for potential transmission events identified by WGS. Using ribotyping along with WGS to assess transmission has been described previously in the literature by Widmer *et al.* (29). In this study, 6 pairs of index and contact patients, having shared a hospital room, were identified to have matching ribotypes, and 2 index and contact patient pairs with the same ribotype were confirmed, using WGS, to have transmitted *C. difficile* as the isolates differed by only 1 SNP. Widmer *et al.* (29) demonstrated that ribotyping combined with WGS is a useful algorithm for identifying transmission cases, as potential transmissions can be narrowed down to patients with the same ribotype and WGS can be used to determine whether the isolates with related ribotypes are clonally related or genetically distinct. In addition to determining the clonal relatedness between strains, genomic data generated by WGS can also be used to study virulence factors, antibiotic resistance markers, and genetic mutations.

Direct ribotyping can be used as a rapid screen to investigate recurrent CDI cases longitudinally. It is essential to perform genotyping for recurrent CDI patients in order to differentiate relapse of disease attributable to the original strain from reinfection with another strain. For cases of reinfection, it may be possible to determine, using direct ribotyping, if the loss of the original strain is gradual over time or if there is a sudden shift in ribotype with the acquisition of a new strain. As non-toxigenic *C. difficile* colonization appears to be protective against recurrent CDI (6), it would be interesting to compare the number of recurrences and disease severity in CDI cases that contain: a single toxigenic strain, concurrent toxigenic and non-toxigenic strains, and cases that become subsequently colonized with a non-toxigenic strain following CDI treatment.

The application of novel *C. difficile* assays for routine screening plays a major role in the diagnosis of this nosocomial infection. Using single molecule array (SIMOA) technology, new ultrasensitive toxin detection platforms have been developed, such as the Singulex Clarity C. diff toxins A/B assay (Singulex, Alameda, California, USA), for detection and quantification of the *C. difficile* toxins. SIMOA assays are highly sensitive with limits of detection for TcdA and TcdB ranging from 0.45 to 2.0 pg/mL in stool (30,31). The principle behind the SIMOA assay uses antibody coated beads that bind target antigen molecules which form complexes with a second detection antibody conjugated to a fluorescent reporter, which is similar to standard enzyme linked immunosorbent assays (ELISA) (32). The most unique feature of SIMOA (also referred to as digital ELISA) is that fluorescence due to individually-bound target molecules can be detected when capture beads are sorted into femtoliter-volume wells, and the concentration of target protein can be extrapolated by analyzing the fraction of bound (fluorescent) to unbound (non-fluorescent) beads detected in the wells (32).

SIMOA assays have the potential to replace both nucleic acid amplification tests (NAAT) and toxin EIA as the primary *C. difficile* diagnostic tests in the clinical microbiology laboratory (31,33). The toxin levels present in CDI patient stool is relatively unknown as the cell cytotoxicity neutralization assay (CCNA) is not routinely used in diagnoses (33). Additionally, *C. difficile* culture (CC) and nucleic acid amplification tests (NAAT) may lack clinical specificity as detection of the organism does not always correlate with disease particularly if there is no detectable toxin production in patient stool or if a non-toxigenic strain is recovered by culture (2). Studies using SIMOA TcdA and TcdB assays have shown high (>95%) sensitivity and specificity with this assay compared to several different reference methods including CC, NAAT, and CCNA (30,31,34). Interestingly, in one study (31), using a SIMOA assay developed by Quanterix Corp. (Billerica,

Massachusetts, USA), 22 and 25% of patients positive by CC (only toxigenic strains were considered for the sample to be positive) and NAAT respectively were classified as TcdB negative by the SIMOA assay due to low TcdB production, which provides further evidence questioning the ability of these two assays to accurately identify all cases of infection. Similarly, in another study comparing NAAT to the Singulex Clarity C. diff toxins A/B assay (35), CDI over diagnosis, determined as diarrhea attributable to both infectious and non-infectious etiologies other than CDI, was tripled among $NAAT^{+}/Clarity^{-}$ patients compared to $NAAT^{+}/Clarity^{+}$ patients.

Validating SIMOA assays for use in the clinical microbiology laboratory will be of great value to the clinical microbiology laboratory and can help to determine whether toxin concentration correlates with the bacterial load for direct stool ribotyping. Previous studies have shown that the level of toxin detected using SIMOA toxin assays correlates with the Ct values reported from the GeneXpert® assay (30); which suggests toxin concentration, as determined by SIMOA assay, may also correlate with direct ribotyping from stool. By pairing SIMOA toxin assays with direct ribotyping, toxin levels can be quantified between patients with single and multiple toxigenic strain infections, identified through direct ribotyping, making it possible to determine if there is a correlation between toxin burden, clinical severity and outcome in such cases.

Based on the developed SIMOA assays, it is also feasible to develop a SIMOA toxin assay for detection and quantification of the binary toxin, TcdA, and TcdB toxins to correlate toxin production between ribotypes and disease outcome. It will be interesting to investigate if binary toxin is produced in cases of mixed *C. difficile* infections containing toxigenic and binary toxigenic strains as in the mixed infections identified in **Chapter 3** (**Table 3.6**). By studying the disease outcomes in CDI cases that have mixed infection with or without binary toxigenic strains will be

a new avenue in the field of *C. difficile* diagnosis. There have been reports of CDI cases due to TcdA positive, TcdB negative strains (36) in addition to TcdA and TcdB negative, binary toxin positive strains such as ribotype 033 (37) that may not be detected by current NAAT and EIA based multi-step algorithms, but presumably would be identified using SIMOA toxin assays that include testing for TcdA, TcdB, and the binary toxin. A potential all-in-one *C. difficile* diagnostic package could include TcdA, TcdB, and binary toxin SIMOA assays which would provide great utility to routine *C. difficile* diagnostic testing.

In conclusion, direct ribotyping is a valuable tool to the diagnostic microbiology laboratory, bridging the gap between *C. difficile* identification by routine diagnostics and further genomic investigation using WGS, by providing essential rapid cluster detection and identification of mixed infections. Rapid case clustering with direct ribotyping complimented with high-resolution genomic data generated by WGS will be able to better support *C. difficile* outbreak investigations.

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Appendix

Appendix A Capillary electrophoresis for PCR ribotyping assay

Table A.1. Capillary electrophoresis settings used in the PCR ribotyping assay

Setting	Value
Oven Temperature	60°C
Poly Fill Vol	4840 steps
Current Stability	5 uAmps
PreRun Voltage	15 kVolts
Pre Run Time	180 sec
Injection Voltage	5 kVolts
Injection Time	5 sec
Voltage Number of Steps	20 nk
Voltage Step Interval	15 sec
Data Delay Time	60 sec
Run Voltage	6.5 kVolts
Run Time	6180 sec

Appendix B Calculation of Simpson's index of diversity

The formula for Simpson's index of diversity is shown below in which D symbolizes the diversity index, n is equal to the total number of isolates for a particular ribotype, and N is the total number of isolates in the population.

$$\boldsymbol{D = 1 - \left(\frac{\sum n(n-1)}{N(N-1)}\right)}$$

Calculations for the ribotypes identified in each cohort in **Table 2.4** are shown below using Microsoft Excel and ribotypes with a single isolate are omitted as they do not contribute to the diversity calculation e.g. (1(1-1))=0). Using the symptomatic children as an example, the numerator was calculated as the sum of the n(n-1) values for each ribotype which is 2720 and the denominator was calculated to be 35156 as 188 isolates were included in the symptomatic children and D was calculated: 1-(2720÷35156)=0.92

Table B.1 Calculation of Simpson's index of diversity for symptomatic children

SC (n=188)	(N(N-1))=35156		
Ribotype	**No. Isolates**	**n(n-1)**	**D**
106	42	1722.0	**0.92**
014	18	306.0	
020	16	240.0	
629	16	240.0	
002	7	42.0	
076	7	42.0	
077	6	30.0	
010	5	20.0	
039	4	12.0	
056	4	12.0	
325	4	12.0	
019	3	6.0	
057	3	6.0	
ns180	3	6.0	
296	3	6.0	
005	2	2.0	
027	2	2.0	
046	2	2.0	
511	2	2.0	
ns205	2	2.0	
012	2	2.0	
ns113	2	2.0	
ns164	2	2.0	
ns70	2	2.0	

Table B.2 Calculation of Simpson's index of diversity for healthy children

HC (n=120)	(N(N-1))=14280		
Ribotype	**No. Isolates**	**n(n-1)**	**D**
106	22	462.0	**0.93**
020	13	156.0	
629	13	156.0	
076	8	56.0	
014	6	30.0	
009	4	12.0	
077	3	6.0	
010	3	6.0	
056	3	6.0	
137	3	6.0	
103	3	6.0	
328	3	6.0	
154	3	6.0	
325	2	2.0	
057	2	2.0	
ns180	2	2.0	
296	2	2.0	
027	2	2.0	
012	2	2.0	
072	2	2.0	
017	2	2.0	

Table B.3 Calculation of Simpson's index of diversity for pediatric CDI

PC (n=18)	(N(N-1))=306		
Ribotype	**No. Isolates**	**n(n-1)**	**D**
106	5	20.0	**0.91**
014	2	2.0	
039	2	2.0	
057	2	2.0	
629	2	2.0	

Table B.4 Calculation of Simpson's index of diversity for adult CDI.

AC (n=79)	(N(N-1))=6162		
Ribotype	**No. Isolates**	**n(n-1)**	**D**
027	35	1190.0	**0.80**
106	5	20.0	
056	4	12.0	
002	2	2.0	
014	2	2.0	
020	2	2.0	
072	2	2.0	
075	2	2.0	
076	2	2.0	
078	2	2.0	
629	2	2.0	
ns70	2	2.0	

Appendix C. Toxin profiles for ribotypes shared between children and CDI cases

The toxin profiles are shown for isolates with ribotypes that were shared between cohorts and also had toxin gene PCR amplification performed (**Figure 2.1**). Symptomatic children (SC), healthy children (HC), adult CDI (AC) cases, and pediatric CDI (PC) cases. Ribotype 009 had isolates with differing toxin profiles identified (indicated in bold).

Table C.1 Toxin profiles for ribotypes shared between children and CDI cases included in the ribotyping validation.

Shared Ribotype	Toxin Profile	SC	HC	AC	PC
106	*tcdA*+/*tcdB*+/c*dtB*-	15	6	5	5
027	*tcdA*+/*tcdB*+/*cdtB*+	1		35	
629	*tcdA*+/*tcdB*+/c*dtB*-	4	1	2	2
020	*tcdA*+/*tcdB*+/c*dtB*-	6	2	2	1
014	*tcdA*+/*tcdB*+/c*dtB*-	3		2	2

076	*tcdA+/tcdB+/cdtB-*	2	2	2	
056	*tcdA+/tcdB+/cdtB-*	1		4	
002	*tcdA+/tcdB+/cdtB-*	1		2	
010	*tcdA-/tcdB-/cdtB-*	1		1	
039	*tcdA-/tcdB-/cdtB-*	3		1	2
072	*tcdA+/tcdB+/cdtB-*			2	1
009	***tcdA+/tcdB+/cdtB-***	**1**			
	tcdA-/tcdB-/cdtB-			**1**	
ns70	*tcdA+/tcdB+/cdtB-*	1		2	
137	*tcdA+/tcdB+/cdtB-*		1	1	
019	*tcdA+/tcdB+/cdtB+*	1		1	
046	*tcdA+/tcdB+/cdtB-*	1		1	
078	*tcdA+/tcdB+/cdtB+*		1	2	
ns205	*tcdA+/tcdB+/cdtB-*	1	1		
015	*tcdA+/tcdB+/cdtB-*	1		1	
ns123	*tcdA+/tcdB+/cdtB-*	1		1	

Appendix D. P-values of pairwise Fisher's Exact tests comparing the proportion of isolates between cohorts for shared ribotypes identified in Table 2.4.

Comparisons with p-values <0.01 were considered significant, and values that are bolded were either statistically significant or approached statistical significance (p-values: 0.0178-0.0878). Symptomatic children (SC), healthy children (HC), adult CDI (AC), and pediatric CDI (PC). Areas with hyphens indicate shared ribotypes were not identified between these cohorts and comparison was not possible.

Table D.1 Fisher's Exact test p-values comparing cohorts for shared ribotypes

		Fisher's Exact Test p-value for each pairwise cohort comparison					
		SC vs. HC	SC vs. AC	SC vs. PC	HC vs. AC	HC vs. PC	AC vs. PC
Ribotype	106	0.472	**0.0013**	0.5662	**0.0191**	0.3481	**0.0178**
	027	0.6443	**<0.0001**	-	**<0.0001**	-	-
	629	0.5504	0.1068	0.6612	**0.0309**	>0.9999	0.1555
	020	0.5504	0.1068	>0.9999	**0.0309**	0.6938	0.4637
	014	0.1913	**0.0711**	0.6887	0.4817	0.2797	0.1555
	076	0.2823	>0.9999	-	0.3207	-	-
	056	>0.9999	0.2413	-	0.4388	-	-
	002	0.1561	>0.9999	-	0.564	-	-
	010	>0.9999	0.6734	-	>0.9999	-	-
	077	>0.9999	-	-	-	-	-
	039	-	>0.9999	**0.0878**	-	-	**0.0875**
	057	>0.9999	-	**0.0617**	-	**0.0825**	-
	072	0.5626	0.2099	0.1675	0.65	0.3447	0.4637
	009	**0.0776**	0.505	-	0.65	-	-
	325	>0.9999	-	-	-	-	-
	ns180	>0.9999	-	0.3083		0.3447	
	ns70	>0.9999	0.5841	-	0.564	-	-
	137	0.303	0.505	-	>0.9999	-	-
	296	>0.9999	-	-	-	-	-
	012	0.6443	>0.9999	-	>0.9999	-	-
	019	-	>0.9999	-	-	-	-
	046	>0.9999	>0.9999	-	>0.9999	-	-
	511	>0.9999	>0.9999	-	>0.9999	-	-
	103	0.303	-	-	-	-	-
	328	0.303	-	-	-	-	-
	078	-	-	-	0.564	-	-
	ns205	>0.9999	-	-	-	-	-
	017	-	-	-	>0.9999	-	-

075	-	-	-	0.564	-	-
015	-	0.505	-	-	-	-
ns123	-	0.505	-	-	-	-
354	>0.9999	-	-	-	-	-
207	>0.9999	-	-	-	-	-
ns103	>0.9999	-	-	-	-	-

Appendix E APPETITE ribotypes identified in children from Calgary and Edmonton.

Twenty-three ribotypes were identified in children from both cities, whereas 35 were unique to children from Calgary and 6 were unique to children from Edmonton.

Table E.1 APPETITE ribotypes in Alberta

Ribotype	Calgary	Edmonton
106	48 (20.4%)	16 (21.9%)
020	22 (9.4%)	7 (9.6%)
629	22 (9.4%)	7 (9.6%)
014	18 (7.7%)	6 (8.2%)
076	11 (4.7%)	4 (5.5%)
010	7 (3.0%)	1 (1.4%)
077	6 (2.6%)	3 (4.1%)
002	5 (2.1%)	3 (4.1%)
056	5 (2.1%)	2 (2.7%)
009	4 (1.7%)	1 (1.4%)
ns180	4 (1.7%)	1 (1.4%)
039	3 (1.3%)	1 (1.4%)
057	3 (1.3%)	2 (2.7%)
103	3 (1.3%)	1 (1.4%)
137	3 (1.3%)	1 (1.4%)

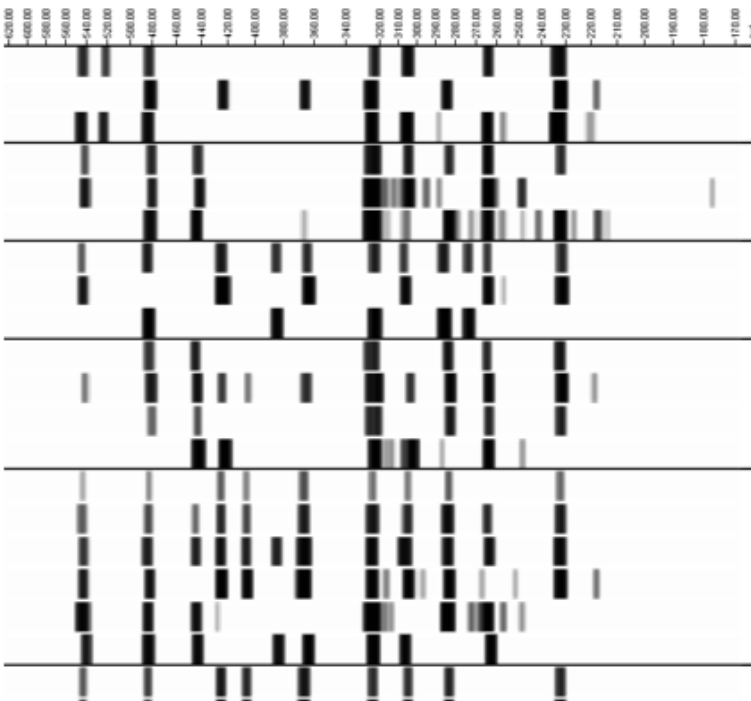
019	2 (0.9%)	1 (1.4%)
027	2 (0.9%)	2 (2.7%)
046	2 (0.9%)	1 (1.4%)
328	2 (0.9%)	2 (2.7%)
ns205	2 (0.9%)	1 (1.4%)
005	1 (0.4%)	1 (1.4%)
354	1 (0.4%)	1 (1.4%)
511	1 (0.4%)	2 (2.7%)
325	6 (2.6%)	
296	5 (2.1%)	
012	4 (1.7%)	
072	3 (1.3%)	
154	3 (1.3%)	
ns70	3 (1.3%)	
017	2 (0.9%)	
207	2 (0.9%)	
ns103	2 (0.9%)	
ns113	2 (0.9%)	
ns164	2 (0.9%)	
015	1 (0.4%)	
023	1 (0.4%)	
075	1 (0.4%)	
078	1 (0.4%)	
085	1 (0.4%)	
097	1 (0.4%)	
153	1 (0.4%)	
293	1 (0.4%)	
351	1 (0.4%)	
530	1 (0.4%)	
ns138	1 (0.4%)	

ns165	1 (0.4%)	
ns195	1 (0.4%)	
ns202	1 (0.4%)	
ns203	1 (0.4%)	
ns204	1 (0.4%)	
ns223	1 (0.4%)	
ns23	1 (0.4%)	
ns235	1 (0.4%)	
ns281	1 (0.4%)	
ns326	1 (0.4%)	
ns293	1 (0.4%)	
ns	1 (0.4%)	
ns296	1 (0.4%)	
ns123		1 (1.4%)
ns145		1 (1.4%)
ns166		1 (1.4%)
ns184		1 (1.4%)
ns267		1 (1.4%)
ns107		1 (1.4%)

Appendix F. Ribotype and toxin genes detected for mixed *C. difficile* cases.

Data for the samples included in Table 3.6 is shown in the figure. Numbers represent cycle threshold values for each target gene and +/- indicates gene presence or absence. NT denotes new type as this ribotype was not present in the reference collection. For Sample 4 the stool did not generate a direct ribotype. Sample 2 and 4 isolate ribotype 024 was produced using a 1:100 dilution of amplicon. Ribotypes from Brain Heart Infusion (BHI) and Banana Broth™ (BB) enrichment are shown with the abbreviation. BB samples in bold indicate ribotype patterns resulting from a subculture of the unbolded Banana Broth™.

Figure F.1 Ribotype and toxin genes detected for mixed *C. difficile* cases.



Sample Number	Sample Type	Ribotype	16S	*tcdA*	*tcdB*	*cdtB*
1	Stool	014	27.67	30.74	30.85	-
	Isolate	084	+	-	-	-
	Isolate	014	+	+	+	-
2	Stool	043	22.59	26.29	26.84	-
	Isolate	ns366	+	+	+	-
	Isolate	024	+	+	+	-
3	Stool	Composite	19.97	-	26.12	-
	Isolate	017	+	-	+	-
	Isolate	NT	+	-	-	-
4	BHI	024	23.99	27.49	25.79	34.8
	BB	Composite	23.16	26.58	28.6	32.98
	Isolate	024	+	+	+	-
	Isolate	075	+	+	+	+
5	Stool	039	29.29	35.47	36.01	-
	BB	Composite	19.26	25.68	27.26	-
	BB	Composite	20.45	27.65	29.13	-
	Isolate	039	+	-	-	-
	Isolate	106	+	+	+	-
	Isolate	009	+	-	-	-
6	Stool	039	14.77	27.22	27.68	-
	BB	039	18.61	32.70	33.9	-
	BB	103	19.1	21.53	22.42	-
	Isolate	039	+	-	-	-
	Isolate	103	+	+	+	-
7	Stool	Composite	20.07	23.68	24.78	26.3
	BB	056	20.65	22.36	24.23	23.14
	Isolate	056	+	+	+	-
	Isolate	ns290	+	+	+	-
8	Stool	014	20.21	24.14	24.41	35.33
	BB	Composite	21.37	24.31	24.95	27.82
	Isolate	014	+	+	+	-
	Isolate	019	+	+	+	+
9	Stool	Composite	20.84	23.2	23.19	23.59
	Isolate	020	+	+	+	-
	Isolate	176	+	+	+	+
10	Stool	Composite	21.22	24.75	25.57	-
	Isolate	629	+	+	+	-
	Isolate	ns113	+	-	-	-
11	BHI	Composite	18.46	22.9	22.65	-
	Isolate	153	+	+	+	-
	Isolate	005	+	+	+	-
12	BHI	011	22.92	26.15	27.05	-
	Isolate	103	+	+	+	-
	Isolate	001	+	+	+	-
13	BB	Composite	20.49	23.71	24.95	-
	Isolate	ns107	+	+	+	-
14	Stool	Composite	16.87	20.19	21.07	-
	BB	057	18.99	21.49	22.42	-
	Isolate	057	+	+	+	-