A *Clostridioides difficile* surveillance study of Canadian retail meat samples from 2016-2018: a possible source of human clinical infections?

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Abstract

Introduction: *C. difficile* spores are dispersed throughout the environment and can asymptomatically colonize and/or infect animals. Previous studies have shown that *C. difficile* spores can be isolated from commercially available beef, veal, pork, vegetables, and seafood. However, a definitive link has yet to have been made between food contamination and hospitalized cases. This study aims to isolate *C. difficile* from retail meat samples and compare them to human isolates.

Methods: Frozen retail pork, beef, and veal samples were obtained from the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) program and FoodNet Canada. These samples were analyzed for *C. difficile* contamination by direct plating on selective media and by inoculation in enrichment broth. Suspected *C. difficile* colonies were confirmed by polymerase chain reaction (PCR). Toxigenic *C. difficile* isolates were molecularly characterized by ribotyping and pulsed-field gel electrophoresis (PFGE). Antibiotic susceptibility was determined by ETEST® strips. Whole genome sequencing (WGS) was performed on all *C. difficile* isolates from retail meats and from select human cases.

Results: Overall, toxigenic *C. difficile* was isolated from 10 of 644 retail meat samples (1.6%). All 10 isolates were A/B toxin positive. Additionally, 2 isolates were found to harbor binary toxin. All retail meat isolates were susceptible to vancomycin, metronidazole, tigecycline, rifampin, and clindamycin, excluding 1 NAP1 isolate that was resistant to moxifloxacin. Molecular typing revealed strain types commonly found in human clinical isolates (e.g. NAP1 (RT027), NAP4 (RTNS195), and NAP11 (RT106)). The closest related human clinical isolate to the *C. difficile* isolates from retail meats differed by 8-34 SNVs when analyzed at the highest resolution (84.54%-95.02% core genome).

Conclusion: A low percentage of retail meats (1.6%) were contaminated by *C. difficile*. All ribotypes and NAP types of *C. difficile* isolates from retail meats have been previously identified in human cases. Through WGS, half of the *C. difficile* isolates from retail meats were determined to be closely related to a *C. difficile* isolate from a human case (≤10 SNVs), while the other half were genetically distinct (>10 SNVs), suggesting that retail meats may be a vector of dissemination for *C. difficile* in the community.

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List of Abbreviations

BHI	Brain-Heart Infusion
bp	Base Pair
BRU	Brucella Blood Agar
CA-CDI	Community Associated *Clostridioides difficile* Infection
Ca-DPA	Calcium Dipicolinic Acid
CCFA	Cycloserine-Cefoxitin Fructose Agar
CCNA	Cell Cytotoxicity Neutralization Assay
CDAD	*Clostridioides difficile* Associated Diarrhea
CDC	Centers for Disease Control and Prevention
CDI	*Clostridioides difficile* Infection
CDMN	*Clostridium difficile* Moxalactam Norfloxacin
CDT	*C. difficile* Binary Toxin
CdtLoc	*C. difficile* Binary Toxin Locus
CIPARS	Canadian Integrated Program for Antimicrobial Resistance Surveillance
CLE	Cortex Lytic Enzyme
CNISP	Canadian Nosocomial Infection Surveillance Program
CIPARS	Canadian Integrated Program for Antimicrobial Resistance Surveillance
DDD	Defined Daily Doses
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EIA	Enzyme Immunoassay
EPA	Environmental Protection Agency
FMT	Fecal Microbiota Transplantation
GDH	Glutamate Dehydrogenase
GRDI	Genomics Research and Development Initiative
HA-CDI	Healthcare Associated *Clostridioides difficile* Infection
ISR	Intergenic Spacer Region
M	Molar

mM	Millimolar
MAL	Muramic-δ-Lactam
mg	Milligram(s)
MLST	multi-locus sequence typing
MP1	Multiplex 1
MP2	Multiplex 2
MRSA	Methicillin-Resistant Staphylococcus aureus
NAM	N-Acetylmuramic Acid
NAP	North American Pulsed-Field Type
NML	National Microbiology Lab
NS	No Standard
PaLoc	*C. difficile* Pathogenicity Locus
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
PHAC	Public Health Agency of Canada
PMC	Pseudomembranous Colitis
RCM	Reinforced Clostridial Medium
rDNA	Ribosomal Deoxyribonucleic Acid
REA	Restriction Endonuclease Analysis
RNA	Ribonucleic Acid
RPM	Revolutions per Minute
SASP	Small Acid-Soluble Proteins
SNV	Single Nucleotide Variant
TBE	Tris/Borate/Ethylenediaminetetraacetic Acid (EDTA)
TcdA	*C. difficile* Toxin A
TcdB	*C. difficile* Toxin B
TEM	Transmission Electron Microscopy
TRIS-EDTA	Tris Ethylenediaminetetraacetic Acid (EDTA)

TSA	Trypticase Soy Agar
µL	Microlitre
u	Units
UV	Ultraviolet
V/cm	Volts per Centimeter
VRE	Vancomycin-Resistant Enterococcus
WGS	Whole Genome Sequencing
x g	Times Gravity

Contributions of Authors

Experiments, validation, analysis, writing (original draft, review, and editing) performed by Derek Tan. Conceptualization, funding, project administration, resources, and writing (review and editing) performed and provided by Dr. George Golding for Chapters 1-4. Conceptualization and writing (review and editing) by Dr. George Zhanel for Chapters 1-4. Conceptualization and writing (review and editing) by Dr. Michael Mulvey for Chapters 1-4. Conceptualization and writing (review and editing) by Dr. Denice Bay for Chapters 1-4. Isolation of *C. difficile* from retail meat methodology provided by Dr. Scott Weese (Chapter 2). Retail meat collection performed by the retail samplers of FoodNet Canada and of the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) (Chapter 2 and Chapter 3). NAP typing of the majority of the *C. difficile* isolates from human clinical cases was completed prior to this study by the Antimicrobial Resistance and Nosocomial Infections (ARNI) division of the National Microbiology Laboratory (NML) (Chapter 3).

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CHAPTER 1: INTRODUCTORY CHAPTER

1.1. History and Discovery of *Clostridioides difficile*

Clostridioides difficile was first described and isolated by Ivan Hall and Elizabeth O'Toole in 1935 while investigating the bacterial flora of infants [1]. Since its discovery, *C. difficile* has risen to become the most notorious nosocomial pathogen responsible for severe hospital-acquired diarrhea [2]. Due to the technological limitations of the 1930's, Hall's newly identified bacterium was solely classified based upon its phenotypic characteristics. What is known today as *C. difficile* was first described by Hall and O'Toole as a "hitherto undescribed obligate anaerobic pathogen" that was "a large Gram-positive rod with elongate subterminal spores of about the same width as the rods" [1]. As *C. difficile* is an obligate anaerobe that is extremely sensitive to environmental oxygen, isolating and culturing the bacteria from human feces was met with great difficulty [3]. For this reason, Hall and O'Toole named their new discovery *Bacillus difficilis* [4].

Today, *Clostridioides difficile* is commonly known as "C. diff" *or C. difficile*. Since *C. difficile* was first described, its name has changed twice. When originally named *Bacillus difficilis* in 1935, the bacterium was not known to cause any kind of disease in humans [1]. It wasn't until 1978 that *C. difficile* was determined to be a potential cause pseudomembranous colitis [5]. With this revelation, *Bacillus difficilis* was reclassified as *Clostridium difficile* and remained as such until 2016 [5]. *Clostridioides difficile* experienced a second reclassification in 2016 when the bacterium was reclassified from *Clostridium difficile* to *Clostridioides difficile*. In 2015, the *Clostridium* genus was restricted to include only *Clostridium butyricum* and closely related species [4]. Through bioinformatic analysis, *Clostridioides difficile* was determined to be divergent enough from *Clostridium butyricum* to warrant a reclassification [4]. In the early stages of reclassifying *Clostridium difficile*, *Peptoclostridium* was considered as a potential new genus [6]. However, disapproval from the scientific community prompted re-evaluation of the new genus name as it would have caused widely known acronyms such as CDI (*Clostridioides difficile* infection), CDAD (*Clostridioides difficile* associated diarrhea), "C. diff", and "*C. difficile*" to become obsolete [4]. In the end, the new genus of *Clostridioides* was created. Although incorrect, *Clostridioides difficile*

is often still referred to as *Clostridium difficile*. There are currently species that populate the *Clostridioides* genus: *Clostridioides difficile* and *Clostridioides mangenotii*.

1.2. Lifecycle of *C. difficile*

C. difficile follows an oral-fecal lifecycle. Similar to other spore formers, *C. difficile* exists in 2 life stages: vegetative and spore (Figure 1) [7]. A *C. difficile* spore's primary purpose is to survive environmental stresses and colonize a new host. Spores act as latent bodies of the bacteria, expelled from the original host. These spores are extremely robust as they are resistant to oxygen, heat, cold, and common cleaners [8]. It has been shown that raising the temperature of meat to the recommended normal cooking temperature of 71°C is insufficient at inactivating *C. difficile* spores [9]. Furthermore, it is speculated that cooking at temperatures below 71°C may enhance the reactivation of latent spores [9]. In contrast to spores, vegetative *C. difficile* cells are metabolically active and reproduce.

Being an obligate anaerobe, vegetative *C. difficile* cells can not survive in oxygenated environments [7]. Because of this restriction, when being cultured or isolated, *C. difficile* must be incubated in anaerobic chambers which are free of oxygen. Generally, these chambers contain a mix of non-oxygenated gases such as nitrogen, hydrogen, and carbon dioxide.

To become infected by *C. difficile*, *C. difficile* spores must first enter the gastrointestinal tract of a potential host. Upon ingestion, the spores move along the gastrointestinal tract until they reach the small intestine. It is in the small intestine where the spores begin to germinate and become vegetative *C. difficile* cells. Once germinated, the newly formed cells attempt to colonize the gut. Due to the weak competitive nature of *C. difficile*, within normal healthy individuals, the gastrointestinal microbiota is usually more than capable of preventing colonization. However, when the gut microbiota is disrupted, the risk of *C. difficile* colonization is increased substantially [10,11]. Disease begins when the colonized *C. difficile* cells begin to produce toxins. Symptoms occur when cells of the gastrointestinal tract react with *C. difficile*'s toxin A, toxin B, and/or binary toxin.

When *C. difficile* must leave the anaerobic environment of the colon to find a new host, *C. difficile* colonies produce oxygen tolerant spores. Thus, vegetative *C. difficile* cells can be found

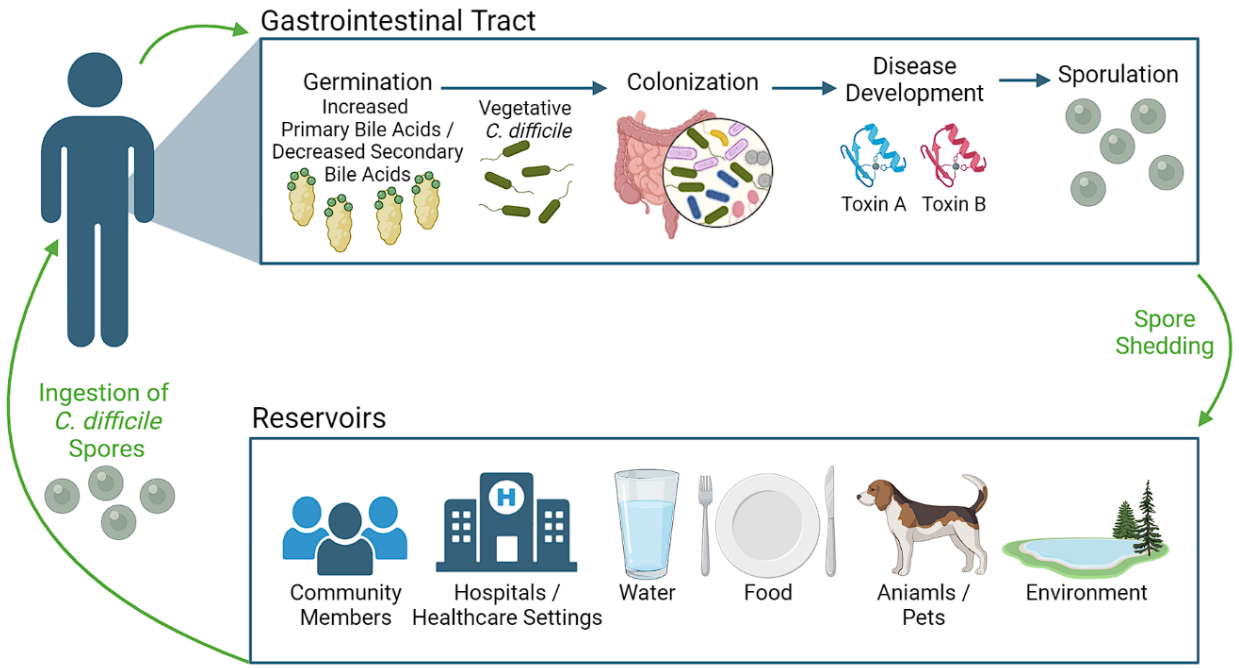


Figure 1. Vegetative Cell and Spore Lifecycle of *C. difficile*. Vegetative cell portion of *C. difficile*'s life cycle occurs in the gastrointestinal tract of a host. Spore portion of *C. difficile*'s lifecycle occurs after spore shedding. Adapted from Seekatz et al. [16].

only inside of a host while *C. difficile* spores are found both inside and outside a host [12]. Vegetative *C. difficile* cells begin to sporulate by producing an endospore at their subterminal end [13]. Various factors are involved in the sporulation of *C. difficile*. Potential sporulation signals include a combination of environmental stimuli, nutrient limitation, quorum sensing, and other stresses [7]. *B. subtilis* possess a sporulation signalling cascade which results in the activation of 4 RNA polymerase sigma factors [12]. Although these 4 RNA polymerase sigma factors are conserved in *C. difficile*, the connected cascade between the sigma factors is not present within *C. difficile* [12].

Upon being shed into the environment from the host via feces, the *C. difficile* spores disseminate across the environment [14]. Although *C. difficile* is commonly viewed as being a nosocomial infection, *C. difficile* spores can be found spread across the community and thus, can be acquired outside of hospital settings. Several vectors of spore dissemination exist which include direct contact with infected feces (either animal or human), contaminated food or water, and surface contamination. It is notable that within healthcare facilities, many infections arise where healthcare workers unknowingly distribute spores around a facility on their hands [15]. Once disseminated across the environment, these spores persist waiting to enter a new host [14].

1.2.1. Spores

C. difficile exists in 2 different life stages: active vegetative cells and latent spores. Following an oral fecal lifecycle, to disseminate, *C. difficile* eventually leaves one host for another host [17]. While in the host transition and searching phase, spores must be capable of surviving in aerobic conditions [18]. The production of aerotolerant spores, which are shed into the environment via host fecal excretions, is one evolutionary solution to the anaerobic nature of *C. difficile*. *C. difficile* produces spores not only to disseminate into the environment but also as a defence mechanism when exposed to stresses [19]. Besides oxygen, nutrient deficiencies are other stress that may prompt the bacteria to sporulate [19]. Spores are latent bodies of the pathogen which are extremely robust to a variety of stresses. Due to the metabolic latency in spores, *C. difficile* is only able to cause disease as vegetative cells and not as spores [7]. They can

survive in classically inhospitable environments that may have extreme temperatures, humidity variation, oxygen, pH, and even in common disinfectants such as ethanol [20,21].

The deactivation of spores requires sporicidal compounds capable of deactivating spores; some of which are listed in the United States by the United States Environmental Protection Agency (EPA). The EPA's List K lists registered antimicrobial products proven to be effective against *Clostridium difficile* spores. Chlorine based disinfectants are commonly used to inactivate *C. difficile* spores [22]. One example of a chlorine-based disinfectant would be sodium hypochlorite, commonly known as bleach.

In infected patients, spores are responsible for relapses. This often occurs since *C. difficile* spores are resistant against antibiotics [23]. Although antibiotics are effective against vegetative *C. difficile* cells, spores are unharmed and will germinate once the antibiotic regimen has ended [23].

1.2.1.1. *C. difficile* Spore Structure

The unique structure and physiology of spores are what allows them to be ultra resistant against a multitude of stresses [20]. Bacterial spores possess multiple layers of various substances (Figure 2). Collectively, the spore's anatomy is responsible for its extreme robustness against multiple stresses.

Contained within the spore core, at the center of the spore, is the bacterial DNA [20]. The DNA is bound and supercoiled to various proteins [20]. The bacterial DNA is not free floating but is supercoiled and bound to small acid-soluble proteins (SASPs) [24]. In this conformation, DNA damage can be minimized over time. Additionally, supercoiled DNA bound to SASPs inhibits transcription from initiating as transcription factors are unable to bind to the DNA [24]. This is desirable as the spore is without access to nutrients that would be required to support bacterial replication. Germination when not located in the gastrointestinal tract would ultimately lead to cell death. Other replication proteins are also contained within the spore core [24]. These proteins are ready to perform their specific tasks once the spore beings to germinate [24]. Compared to vegetative cells, spores possess an extremely low water content [25]. The water

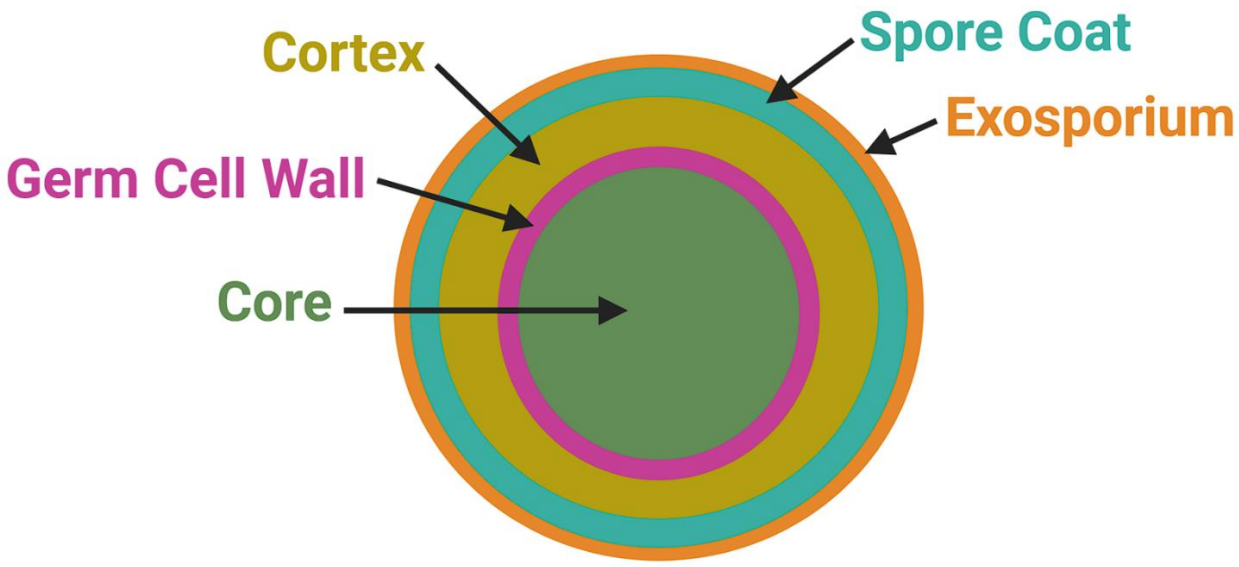


Figure 2. Anatomical cross section of a *C. difficile* spore. *C. difficile* spores are formed of multiple layers which contributes to their robustness. Adapted from Permpoonpattana et al. [26]

content within the spore core is associated with a decrease in resistance to some stresses such as wet heat, hydrogen peroxide and formaldehyde [25].

To protect the core from harmful chemicals, the core is surrounded by an inner membrane [26]. The inner membrane is composed of a peptidoglycan layer which possesses low permeability to chemicals [24]. Besides potentially harmful chemicals, the inner membrane also keeps the core well dehydrated by preventing water from entering the core [24].

The next layer of the spore is the germ cell wall. The germ cell wall provides another barrier of protection for the core and is also incorporated into the cell wall of the bacteria once germination occurs [26]. During spore formation, the germ cell wall is derived from the mother vegetative cell's cell wall [26]. The germ cell wall is the only part of the mother cell which is fully incorporated into the newly germinated vegetative cell.

The next layer surrounding the germ cell wall is the cortex. The cortex is composed of a thick layer of modified peptidoglycan [24]. Compared to normal peptidoglycan which makes up the germ cell wall, the cortex's peptidoglycan is modified by replacing side chains [26]. By removing the peptide side chain, a lactam ring forms, resulting in 50% of the N-acetylmuramic acid being converted to muramic-δ-lactam (MAL) [24]. This process is to ensure that cortex lytic enzymes (CLEs) degrade only the spore's cortex during germination and not the germ cell wall [27]. Unintentionally degrading the germ cell wall would result in cell death. Additionally, 25% of the cortex's N-acetylmuramic acid (NAM) residues are substituted with short peptides [24]. This provides the cortex with a lower degree of crosslinking compared the germ cell wall [24].

The cortex is surrounded by another layer of peptidoglycan, the outer membrane, and the spore coat. The outer membrane region of the spore is where the cortex lytic enzymes involved in germination are located [27].

The second-last layer of the spore is the spore coat. The spore coat is another layer of protection made of dense protein located on the outside of the outer membrane [26]. This layer is composed from a dense layer of various proteins [24].

The last layer of the *C. difficile* spore is the exosporium. This layer is loose fitting and made from a highly permeable complex of carbohydrates [26].

1.2.1.2. Factors of *C. difficile* Germination

For *C. difficile* spores to reproduce, they must first germinate and become vegetative cells. Germination is the transition of a latent spore into an active vegetative cell. While vegetative *C. difficile* cells produce toxins, and are thus are responsible for causing diseases, spores have no such pathogenesis. Depending on the species of the spore former, spores require different signals to begin germinating. These signals are generally based upon environmental and nutrient conditions [27]. While some aspects of germination are known, such as some germination signals and pathways, many are not. *C. difficile* lacks highly conserved germination receptors when compared to other bacteria that germinate from spores. For example, GerA, a transmembrane germination receptor is relatively highly conserved among spore formers [24]. However, *C. difficile* does not possess GerA let alone any known transmembrane germination receptor [24]. More research is required to fully understand the germination pathway of *C. difficile*. It is believed that bile salts and amino acids play an active role in in *C. difficile* spore germination signals [24].

1.2.1.2.1. Germination Signals

Bile salts and amino acids are thought to play vital roles in *C. difficile* spore germination by acting as germination signals.

1.2.1.2.1.1. Bile Salts

One requirement for the germination of spores is the presence of cholate-derived bile salts [28]. Such compounds, present within the gastrointestinal tract, are exclusively produced in the mammalian gut as by-products of digestion [28].

Of all the cholate-derived bile salts, taurocholate is the most effective at promoting germination [24]. For *C. difficile,* the presence of taurocholate indicates that the spore is in the correct environment to germinate: the colon. Taurocholate is a secondary bile acid which are produced by bacterial processes in the colon [2]. Taurocholate levels in the colon also increase when there is dysbiosis in the gut resulting from activities such as antibiotic use [24]. Antibiotic use further increases the odds of successful colonization of the newly produced vegetative cell as *C. difficile* is an opportunistic pathogen. While taurocholate is a promoter of germination,

chenodeoxycholate is the opposite. Chenodeoxycholate acts as an efficient competitive inhibitor of taurocholate [10]. Chenodeoxycholate is a primary bile acid produced by the liver and present in the earlier parts of the gastrointestinal tract [28]. Evolutionarily, the combination of taurocholate and chenodeoxycholate act as geographical markers for the pathogen as it moves through the gastrointestinal tract. The presence of chenodeoxycholate would signify that the spore has yet to reach its ideal environment. It should also be noted that taurocholate alone is not enough at initiating germination alone [24].

In vitro, it has been shown that the bile salt cholate and its derivatives, taurocholate in particular, are efficient at promoting spore germination [29]. Though bile salts promote the germination of spores, they are insufficient alone to cause germination [24].

1.2.1.2.1.2. Amino Acids

Requiring a co-stimulant, amino acids are one type of co-germinant to bile salts that can initiate germination. Unfortunately, the pathway which amino acids act upon during germination are widely unknown. It has been shown that glycine is the most effective amino acid at stimulating germination in combination with bile salts [24]. However, other amino acids have been shown to work which include L-alanine, L-histidine and L-serine [24]. D-Alanine and D-serine can also act as co-germinants but require a racemase dependant process [24]. The other co-germinants to taurocholate to initiate germination are divalent cations; specifically, magnesium or calcium [24]. The presence of such cations has been shown to be a suitable substitute for the aforementioned amino acids [30]. Once again, the pathway in which these divalent cations act upon during germination are widely unknown. There does however exist synergy between the 2 pathways. When in the presence of amino acids and divalent cations, the required concentration of either substrate is reduced by 10-fold [24]. The interaction between the pathways indicates that a variety of signals is required for germination to commence. These signals all ensure that the spore is in the gastrointestinal tract. Germinant requirements, bile salts, calcium and glycine, are all present in the gastrointestinal tract [24].

1.2.1.2.2. Mechanisms of *C. difficile* Germination

Although not completely understood, there are many players thought to be involved in the germination of *C. difficile* spores. The germination signalling pathway of *C. difficile* is not fully understood.

1.2.1.2.2.1. Csp Germination Cascade

C. difficile lacks a transmembrane germinant receptor present in many other spores. When detecting bile salts, *C. difficile* possesses a unique signalling pathway that is composed of the Csp family of proteins located on the spore's spore coat [31]. CspC, a pseudoprotease, has been identified as a major *C. difficile* germinant receptor [31]. When CspC detects the presence of bile acids, such as taurocholate, it initiates downstream signalling [31]. CspC first activates CspB. CspB then activates SleC-zymogen by cleavage [31]. Activated SleC, a cortex lytic enzyme (CLE), is then able to begin enzymatically degrading the cortex layer of the spore. Degradation of the cortex layer is one factor which reactivates the spore allowing it to resume metabolism and grow [26]. One physiological change to the spore due to cortex degradation is the rehydration of the core [27]. While the core rehydrates, calcium dipicolinic acid (Ca-DPA) is expelled from the core via SpoVA [32].

While some parts are known, the link between CspC and CspB activation remain unclear. The first model relies on a yet to be identified protein that acts an intermediate between CspC and CspB [24]. Here, all components are proposed to form a complex. The complex is thought to be composed of CspC, CspA as a chaperone, CspB, GerS and pro-SleC. Activation of the complex would thus occur when taurocholate and co-germinate factors interact with the complex [24]. The second model foregoes the concept of a complex and instead recognizes CspC as a gatekeeper. Through some unknown pathway, it believes that CspC interaction with taurocholate facilitates the passage of co-germinate factors into the spore [24]. These co-germinate factors are then believed to move deeper into the cell to uncharacterized receptors located on the inner membrane which then activates CspB [24]. This model attempts to explain how the co-germinant amino acids and divalent cations enter the spore in the first place.

1.2.1.2.2.2. Other Germination Factors

GerG is a lipoprotein that is highly expressed during sporulation [31]. Studies have shown that GerG mutant *C. difficile* strains are unable to initiate cortex hydrolysis [31]. It is hypothesized that GerG additionally modifies the cortex during germination [24].

GerS is a protein which has been shown to affect germination. However, it is thought to affect the spore during sporulation [24]. GerS mutant *C. difficile* strains are also unable to initiate cortex hydrolysis [24]. GerS is thought to transport Csp proteins to the cortex during sporulation [24].

CD630_32980 encodes for an ATPase. However, since ATP is not required during germination, it is most likely active during sporulation but affects germination [24]. *CD630_32980* mutants are deficient in both Ca-DPA and calcium. These mutants can respond and germinate when in the presence of taurocholic acid and amino acids but not when in the presence of taurocholic acid and calcium without amino acids [24].

1.2.1.3. Sporulation

While germination is the transition of a latent spore into an active vegetative cell, sporulation is the opposite. Sporulation produces a latent spore from an active vegetative cell. As with germination, the pathways which involve sporulation are not all completely understood. Like with other spore forming bacteria, *C. difficile* retained the conserved sporulation factor Spo0A and its spore formation process [33]. Vegetative *C. difficile* cells replicate via binary fission producing more vegetative cells. It should be noted that sporulation is a defence mechanism against stresses. While binary fission produces more bacterial cells, spore formation kills the mother cell in the process inhibiting replication [34]. When Spo0A is activated, the sporulation process begins [33].

The first step of sporulation begins with the formation of a polar septum [33]. This asymmetric division produces a larger mother cell and a smaller forespore [33]. Eventually the mother cell fully engulfs the forespore in its entirely [33]. The mother cell's sole purpose is to complete the construction of the spore while sacrificing itself. Calcium dipicolinic acid (Ca-DPA) is synthesized by the mother cell and transported to the forespore [33]. When the Ca-DPA enters

the spore, it is exchanged for water dehydrating the core of the spore [33]. The cortex and spore coat are then formed. Following the completion and maturation of the spore, the mother cell lyses which releases the spore [33]. This spore is intended to exit the host so that it can find a fresh host to colonize and reproduce in.

1.2.1.3.1. Mechanisms of *C. difficile* Sporulation

Although *C. difficile* sporulation is not completely understood, there are many players which are proposed to be involved in regulating sporulation.

1.2.1.3.1.1. Spo0A Activation

Regulation of sporulation is regulated by many different factors. Activation of Spo0A via phosphorylation is what ultimately initiates sporulation [33]. Across spore formers, while Spo0A may be conserved, the histidine kinases which act upon Spo0A vary across species [35]. The main histidine kinases in *C. difficile* that phosphorylated Spo0A are CD1579 and CD2492 [33]. It has been shown that knocking out *CD1579* or *CD2492* results in a 3-fold reduction in spore production [33]. On the other hand, CD1492 is a histidine kinase which is believed to dephosphorylate Spo0A, thus negatively regulating sporulation [33]. It has been shown that a CD1492 knockout increased sporulation by 4-fold [33].

1.2.1.3.1.2. Other Sporulation Factors

RstA, regulator of sporulation and toxins, an RRNPP family orthologue, is one of those factors that also regulates Spo0A [33]. *rstA* knockout mutants produce 20-fold fewer spores compared to their wild type counterparts [33]. RstA homologues in other spore forming bacteria are regulated by Opp and App, conserved oligopeptide permeases that often interact with quorum-sensing peptide genes [33]. However, is has not been established if such quorum-sensing peptides affect *C. difficile*'s RstA.

CcpA is another regulator of *C. difficile* sporulation but who detects carbon availability [33]. Since sporulation is a defence mechanism, to guarantee survival, it is in the best interest of the bacteria to sporulate only during nutrient deprivation. Low carbon availability is one such

stress. *ccpA* knockout mutants have been shown to produce 10-fold more spores [33]. CcpA represses Spo0A directly, Opp and SinR [33].

sinR encodes sporulation enhancing products [33]. CodY detects nutrients like CcpA but detects amino acid levels instead of carbon [33]. Additionally, CodY supresses SinR and Opp [33].

Overall, there is more than a single factor that regulates the sporulation of vegetative *C. difficile* cells. An intertwined network of various receptors exists which comes together to decide if the cell is going to being sporulation or not.

1.2.2. Oxygen Tolerance

C. difficile incubation requires special conditions since the bacteria is strictly anaerobic. While *C. difficile* bacterial spores can survive in normal atmospheric oxygen levels, *C. difficile* vegetative cells can not. Within the gastrointestinal tract, the natural environment of *C. difficile*, oxygen concentrations are low [36]. With varying concentrations of oxygen throughout the gastrointestinal tract, *C. difficile* finds microenvironments which are exceptionally low in oxygen. Gastrointestinal microenvironments with exceptionally low oxygen include crypts [36]. The lumen is a gastrointestinal microenvironment with higher oxygen concentrations [36].

It has been demonstrated that the slightest concentration of oxygen impacts the growth of *C. difficile*. Grown in brain-heart infusion (BHI) broth, a 2018 study by Giordano et al. monitored the growth of *C. difficile* over the course of 8 hours in environments with varying oxygen concentrations. The different oxygen concentrations tested were 0%, 1%, 2%, 3% and 5% [37]. As expected, growth was negatively correlated to the concentration of oxygen in the incubation chamber [37]. Compared to the culture grown in 0% oxygen concentration, the culture grown with 1% oxygen concentration began to show decreased growth [37]. The culture grown in 2% oxygen concentration saw an even larger decrease in growth proportionally compared to the 1% oxygen concentration culture [37]. At 3% oxygen concentration, the OD600 fell as much as the 2% oxygen concentration vs the 1% oxygen concentration [37]. Finally, the culture grown in 5% oxygen concentration exhibited no growth past the 4-hour mark [37]. Inhibition of growth at 5% makes microaerophilic incubators who produce an environment containing ≥5% oxygen concentration such as CO_2 incubators who have an oxygen concentration

of 15%, and aerobic incubators unsuitable for *C. difficile* incubation, unsuitable for *C. difficile* incubation [38]. Atmospheric oxygen concentration is approximately 21%, which is well above the threshold for viable vegetative *C. difficile* growth. Bacterial RNA was also recovered from bacteria growing in environments with oxygen concentrations of 0% and 2%. It was shown that an environment containing 2% oxygen is enough oxygen to cause *C. difficile* RNA levels to decrease by approximately 50% [37].

1.3. Physical Characteristics of *C. difficile*

C. difficile is a Gram-positive, spore forming, anaerobic bacteria. Depending on the strain, certain mutations can alter the physical characteristics of *C. difficile*. Interestingly, one hallmark of *C. difficile* is its distinct scent. When smelt, *C. difficile* possesses a distinct and potent manure smell [39].

1.3.1. Colony Morphology

The morphology of *C. difficile* differs based upon its current lifecycle stage. When *C. difficile* is in its vegetative cell form, the bacteria possess a rod-shaped appearance [40]. Most *C. difficile* strains, in their vegetative cell form, are peritrichously flagellated [41]. However, strains can have flagella that is monotrichous or non- flagellated [41]. The level of adherence of peritrichously flagellated *C. difficile* cells to the epithelium is 10-fold greater than that of non-flagellated *C. difficile* [42]. Sporulating *C. difficile* cells form oval shaped spores at their subterminal ends which are eventually released from the cell [13].

C. difficile colonies exhibit 2 distinct morphologies. One colony morphology type appears circular, convex, and smooth [43]. The other colony morphology type appears non-circular with jagged edges, occasionally with hyphae like extensions, flat, and rough [43]. The exhibited morphology type is controlled by a phase-variable signal transduction system [43]. This system is composed histidine kinase and 2 response regulators [43]. By possessing multiple morphology types, colonies are more tolerable towards various environmental stresses [43].

1.3.2. Colony Fluorescence when Exposed to Near UV Light

When colonies are isolated, their morphology varies depending on the type of media used. When grown on blood agar-based plates, *C. difficile* colonies possess a whiteish grey colour, are non-hemolytic, and do not alter the colour or opacity of the blood agar [39]. Additionally, through an unknown mechanism, *C. difficile* colonies fluoresce when exposed to longwave UV / UVA / near UV light [44]. Depending on the growth medium utilized to for culture *C. difficile*, the colour of fluorescence may vary. Examples of medium that produces *C. difficile* colonies that fluoresce yellow-green when exposed to near UV light include brucella blood agar (BRU), *Clostridioides difficile* moxalactam norfloxacin agar (CDMN), and cycloserine-cefoxitin fructose agar (CCFA) [44,45]. This fluorescence is the most intense in fresh cultures under 48 hours old; after which, florescence begins to wain. Examples of medium that produces *C. difficile* colonies that fluoresce golden-yellow when exposed to near UV light include cycloserine fructose agar (CFA) [45].

1.3.3. Vegetative Cell and Spore Staining

C. difficile vegetative cells and spores can be visualized via optical light microscopy once prepared and stained. Without staining, bacteria appear translucent when observed by light microscopy. The preparation of the bacterial sample is determined by the phase of the *C. difficile* being visualized.

1.3.3.1. Gram-Stain

Gram-staining bacteria aids in the visual identification of *C. difficile*. As *C. difficile* is a Gram-positive bacillus, crystal violet dye used in the Gram-stain will stain the thick layer of bacterial peptidoglycan purple. The red safranin counterstain of the method will therefore not be observable. Under a microscope, after Gram-staining, the stained bacteria will appear as purple rods [3]. Although Gram-staining can help visualize *C. difficile* once isolated, it “is not an effective strategy to identify *C. difficile* directly from stool samples” as stool samples are composed a variety of bacterial species which may possess similar morphology [3].

1.3.3.2. Spore Stains

Because *C. difficile* is one of few bacteria that produce spores, *C. difficile* endospores can be visualized by microscopy if spore stained. Common spore staining techniques include Schaeffer-Fulton staining and Moeller staining. The Schaeffer-Fulton stain utilizes heat to penetrate malachite green into the spores. Since malachite green is water soluble, it does not remain in vegetative cells but will remain in spores. Safranin is then used to dye the vegetative cells pinkish red in contrast to the green spores when visualized under a microscope. Moeller staining utilizes the same principles as Moeller staining but exchanges malachite green for carbolfuchsin as a spore stain, water for acid-alcohol as a decolourizer, and safranin for methylene blue as a counter stain. The Schaeffer-Fulton spore stain is often the preferred method due to its quicker staining time [46].

1.3.4. Flagella

The gastrointestinal tract is a hostile environment, even for pathogens. With an extensive and diverse population, there is constant competition for space and nutrients. As waste moves through the digestive tract, it dislodges and carries bacteria through and eventually out of the system. One of *C. difficile*'s virulence factors, which also acts as an adherence factor, is its flagella [27].

A 2013 study investigated the importance of flagella and their effect on the adherence of *C. difficile* to the intestinal wall. While previous *C. difficile* flagella studies have utilized 630Δerm, a peritrichously flagellated strain of *C. difficile* in their research, this study utilized R20291, a monoflagellated strain [41]. The researchers compared wildtype R20291 *C. difficile*, a *filC*-mutant, and a *motB*-mutant [41]. Flagellin, also known as FilC, is a major structural protein of flagella that is encoded by *filC*. MotB, encoded by *motB*, is involved in flagellar rotation. Therefore, *filC*-mutants do not possess any flagella while *motB*-mutants posses a flagellum but who are paralyzed. In their mouse model, bacterial adherence was measured after 7 days, after which the mouse's caecum was collected and analyzed [41]. This experiment showed that flagellum motility is not a factor of adherence [41]. The researchers next coinfected mice with both wildtype R20291 *C. difficile* and *filC*-mutant non-flagellated *C. difficile* to investigate whether the presence

of flagellum influenced intestinal adherence. Their findings demonstrated that the wildtype strain, that possessed a flagellum, exhibited greater adherence compared to the non-flagellated mutant [41]. Finally, the researchers coinfected mice with both the *motB*-mutant paralyzed *C. difficile* and *filC*-mutant non-flagellated *C. difficile*. This experiment showed that the flagellated, but paralyzed *C. difficile* mutant, had greater adherence than the non-flagellated mutant [41]. From this series of experiments, researchers concluded that in some uncharacterized mechanism, flagella, but not its motility, affects the adhesion of *C. difficile* in the gastrointestinal tract [41].

1.3.5. Type IV Pili

One known *C. difficile* colonization factor is the type IV pili (TFP) [47]. Type IV pili are involved in cell adherence by interacting mucosal surfaces [48]. The *C. difficile*'s type IV pili's synthesis is regulated by c-di-GMP [47]. c-di-GMP is also involved in the regulation of multiple other *C. difficile* cellular pathways such as controlling flagellar biosynthesis and motility, biofilm formation, and toxin synthesis [47].

1.4. Isolation and Incubation of *C. difficile*

The isolation and lab culture of *C. difficile* is an integral part in researching the pathogen. While the anaerobic nature of *C. difficile* makes the pathogen difficult to culture without special equipment, the robust nature of *C. difficile* spores and its intrinsic antimicrobial resistance aids in the isolation of the pathogen.

1.4.1. Isolation of *C. difficile* from Mixed Species Sample

Isolation of a pathogen is an integral part of research. When a sample is taken from patients or from the environment, it contains a dynamic population of organisms. One gram of soil contains up to 10^{10} bacterial cells composed of an estimated 4×10^3 to 5×10^4 unique species [49]. The colon is even more bacterially dense, containing approximately 10^{11} bacterial cells per gram [50]. Isolating pathogens from a sample confirms the presence of the pathogen and allows for research to occur on the specific organism of interest.

Following the lifecycle of *Clostridioides difficile*, the bacterium produces spores within the gastrointestinal tract. A strict anaerobe, *C. difficile* is extremely sensitive to oxygen and will not survive in normal atmospheric oxygen concentrations [17]. Spores also allow the bacteria to survive in oxygenated environments outside of the gastrointestinal tract. Once produced, the spores are excreted from the host's gastrointestinal tract via the feces [17]. While the feces of a colonized individual will contain *C. difficile* spores, the feces also contain other biological matter. Of the total weight of feces, approximately 75% is composed of water [51]. The remaining portion of the feces, the dry solids, are composed of bacterial biomass, undigested carbohydrates, fiber, proteins, and fats [51]. The quantity of each component largely depends on the diet of the individual. Of the dry fecal mass, between 25% and 54% is composed of both life and dead bacterial biomass [51]. Separating such biologicals can prove to be challenging.

1.4.1.1. *C. difficile* Isolation by Ethanol Shock

Inactivation of *C. difficile* spores requires special sporicidal cleaners. A list of such sporicidal cleaners is listed by the EPA under List K: EPA's registered antimicrobial products effective against *Clostridium difficile* spores [52]. Most of these disinfectants, such as bleach, are chlorine based. However, using bleach as a common disinfectant has many disadvantages such as strong fumes irritating the airway, skin, and mucous membranes; having a strong odour; decomposing under heat and light; and easily reacting with other chemicals [53].

C. difficile in spore form is resistant to many commonly used disinfectants. One of those commonly used disinfectants, ethanol, is also utilized in the isolation of bacterial spores [20]. Ethanol shocking is a technique where samples are submerged with 95% to 100% ethanol for a prolonged period [20]. The purpose of the ethanol shock is to kill all vegetative cells that are present in the sample. As a result, it leaves spores as the only viable biological matter in the sample. *C. difficile* spores are more than capable at surviving submerged for prolonged periods in ethanol. However, that is not the case for other cells. At 50% ethanol, most cells can be eradicated in only a few minutes of exposure [54]. Upon centrifuging the sample-ethanol mix to separate the solid matter from the ethanol, the supernatant can be decanted before the

remaining pellet is spread on agar media. With all other bacterial cells rendered dead, growth should be restricted to only *C. difficile* spores.

1.4.1.2. Selective Growth Medium for *C. difficile*

Various aspects of *C. difficile* makes it an extremely robust pathogen. The bacterium is known for being resistant to multiple classes of antibiotics which include aminoglycosides, lincosamides, tetracyclines, erythromycins, penicillins, cephalosporins, and fluoroquinolones [55]. It should be noted that antibiotic resistance varies across different strains of *C. difficile*. While one strain may be resistant to all antibiotics listed above, other strains may only be resistant to a few or none. Quite often, antibiotic resistance is viewed as a potential threat to human health. However, with *C. difficile*, it can be used to select for the bacteria from a mixed sample.

Although *C. difficile* is culturable on many different types of media, a few different types have been conventionally used for *C. difficile* isolation [56]. The first selective media was reinforced clostridial medium (RCM) developed in 1976 [13]. This media utilized 0.2% phenol or 0.2% cresol to inhibit the growth of other non-*C. difficile* organisms [13]. A new and improved selective *C. difficile* media was created in 1979 [45]. Cycloserine-Cefoxitin fructose agar (CCFA) was created which utilizes cycloserine and cefoxitin as selective agents [45]. Modifications to CCFA media have also produced improved *C. difficile* recovery. One such variant is CCFA-HT made by adding horse blood and taurocholate to CCFA media [57]. The third milestone in *C. difficile* selective medias occurred in 1992 when *Clostridium difficile* moxalactam norfloxacin (CDMN) selective supplement was created. [58]. This supplement utilized the antibiotics moxalactam and norfloxacin as a selective agent for *C. difficile*. Compared to its predecessor CCFA, the CDMN supplement has been shown to improve *C. difficile* recovery by 20% [58].

Specialty growth medium do also exist for culturing *C. difficile*. The aim of these specialty selective *C. difficile* mediums is to provide a selective growth culture with improved recovery rates that also helps identify *C. difficile* colonies. CHROMID *C. difficile*, sold by bioMérieux, is one such example. CHROMID *C. difficile*, is a chromogenic agar which not only selects for *C. difficile*, but also turns the typically white/grey colonies on blood agar black [59]. While useful for

identification, such specialty medium cost come at an increased cost than conventional agar plates. Although the colonies will not change colour, phenol red, when added to CCFA, can cause agar to turn yellow in the presence of *C. difficile* [57]. Phenol red, a pH indicator, changes colour from a pink-orange to yellow as the bacteria releases acids as a by-product of breaking down peptones in the media [57].

1.4.2. Anaerobic Incubation and Reduction Environment

Due to the strict anerobic nature of *C. difficile*, it must be cultured in anerobic conditions. In large labs, the main method of incubation is achieved by using anaerobic chambers. All chambers are composed of a main compartment and a pass box. The main compartment is filled with gases, free of oxygen, such as hydrogen, nitrogen, and carbon dioxide. Oxygen monitors may be present within the chamber to alert of abnormally high oxygen levels. These oxygen indicators may be electronic or chemical. Chemical indicators include methylene blue and resazurin. Additionally, oxygen absorbers may also be placed inside the chamber to remove any oxygen that manages to make its way through the pass box. The pass box is how items are transported from the oxygenated environment outside of the main chamber into the anerobic chamber. Pass boxes may utilize different techniques to remove the oxygen of the outside air depending on their make and model. Techniques that are commonly used include creating a vacuum inside of the pass box before filling it with non-oxygenated mixed gases and pumping in an anerobic gas mixture into the pass box to displace any oxygen within the pass box.

Anaerobic chambers are also manufactured gloved and gloveless. Gloved anaerobic chambers create a sealed environment inside the main chamber with the pass box being the only opening and exit. To work in a gloved chamber, one simply puts their hands into the thick rubber gloves which are attached to the chamber walls. On the other hand, gloveless chambers eliminate the need for thick rubber gloves by allowing the user to insert their arms into the chamber. To prevent oxygen from entering the chamber via the arm holes, rubber gaskets seal against the user's arms. Additionally, the chamber maintains positive pressure. This causes the anaerobic gases to flow out of the chamber whenever there is a breach.

For labs without access to anaerobic chambers, many alternative methods do also exist. Container based anaerobic chambers eliminate oxygen by a variety of techniques. The method utilized depends on the manufacturer and the model of the system in question. Oxygen may be aspirated out by attaching an external vacuum pump or by manually pumping an integrated pump. These containers may also contain chemical pouches which remove oxygen and produce other gases. Bag systems that follow the same principles as the containers also do exist. These systems are useful for when supplies or facilities are not readily available.

Primitive anaerobic containers can also be created by burning a candle inside of a sealed container. The flame of the candle consumes the available oxygen in the sealed container while releasing CO_2 [60]. This technique is almost instantaneous and produces an environment containing only 1% to 2% oxygen within the container [60].

1.4.3. Anaerobic Transport Environment

For the transport of clinical samples, special transport devices have been produced. Original transport devices reacted environmental oxygen with hydrogen over a palladium catalyst to create water within a tube [61]. This effectively lowered the oxygen concentration to a maximum of 2% [61]. Modern anerobic transport medium are tubes with hungate caps. Hungate caps are screw caps with a rubber septum which allows for sample inoculation without having to open the lid.

1.5. Typing Methods Utilized on *C. difficile*

Since *C. difficile* was first isolated and described in 1935 by Ivan Hall and Elizabeth O'Toole, various typing techniques have been employed to group various strains of the Gram-positive, spore forming, anaerobic bacteria together [1]. This task, known as typing, can serve as a useful resource for researchers as closely related strains can be grouped together.

Across the scientific community, researchers have yet to establish a standardized typing technique regarding *C. difficile*. However, some methods are more prevalent than other such as PCR ribotyping and pulsed-field electrophoresis [62]. There are a multitude of different *C. difficile* typing techniques available for use. *C. difficile* typing techniques include restriction endonuclease

analysis (REA), multi-locus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), repetitive-element PCR typing, toxinotyping, PCR ribotyping, serotyping, surface layer protein A typing, and whole genome typing (WGS) [63]. However, the most common typing methods are PCR ribotyping and PFGE.

An additional factor of complexity across typing methods is that they are not interchangeable. Types are specific to the typing method used to generate them. Although there may be some strong correlations between typing methods, results are not necessarily identical. The techniques each target different aspects of the *C. difficile* genome, thus creating different types. Types can be thought of as groups where member strains share a specified characteristic depending on the selected parameter. This lack of standardization greatly complicates the typing of *C. difficile* as one strain of the bacteria will have multiple types associated with it. An example would include the commonly known and hypervirulent, NAP1/B1/027 *C. difficile* [64]. Instead of being identified by one type, it is known by 3 commonly used types: North American pulsed-field gel electrophoresis type 1 (NAP1), restriction endonuclease analysis type B1 (B1), and polymerase chain reaction ribotype 027 (027) [64].

Inferences can be made on an isolate when grouped with other well studied and closely related strains. For clinicians, typing information may be used to guide their treatment of patients by predicting characteristics of the *C. difficile* type. *C. difficile* typing of strains can also be used to investigate the evolution of the bacteria [65]. Additionally, they can be used to detect and follow outbreaks [65]. Typing can also fill in missing info in the event that epidemiological data is missing [65].

The typing method employed depends on the purpose of the research, goal of the research, and technological access. Of all the typing methods, any method that utilizes agarose gels intrinsically possesses the difficulty of data portability and machine variability. Overall, whole genome sequencing is the best typing method. Possession of the complete genomic sequence allows for deduction of other types. Additionally, genomes must only be sequenced once before the whole genomes can be analyzed in various ways. Many pipelines can be executed if the genome is available, thus allowing unlimited methods of typing to grow from one procedure. Additionally, online libraries for whole genome sequencing data also provides an easily accessible

platform for data sharing, gathering, and analysis. However, the cost and materials required for whole genome sequencing are significantly higher than other typing methods.

As *C. difficile* research has been occurring for more than 80 years, preferences have changed. However, selecting a single typing method proves to be a challenge. Previously produced literature will always contain their respective typing methods and nomenclature. Standardization would only affect the typing of *C. difficile* moving forward. As prevalence of each technique varies geographically, another challenge is gathering scientists globally to agree on one single system. Without an overseeing body regarding the issue, it is highly unlikely that a gold standard for *C. difficile* typing can be established.

1.5.1. Multi-Locus Sequence Typing

Multi-Locus sequencing typing (MLST) is a typing technique that employs the variability in housekeeping genes [66]. Housekeeping genes are targeted due to their reliable presence in various forms across different strains of *C. difficile*. MLST first requires that the housekeeping genes be amplified via PCR [67]. This newly amplified gene product is then sequenced. Obtaining the sequence of multiple housekeeping gene variations allows differentiation of types. Every sequence type contains a unique combination of gene variations [66].

The original proposed MLST scheme was proposed for *C. difficile* in 2004 [68]. The 7 targets were *aroE*, *ddl*, *dutA*, *gmk*, *recA*, *sodA,* and *tpi* [68]. However, this scheme was not widely adopted as the *ddl* locus failed to amplify in some strains [66]. Additionally, an online database of MLST types was not made available [66]. The loci were updated in 2010 to instead include *adk*, *atpA*, *dxr*, *glyA*, *recA*, *sodA*, and *tpi* [66]. Additionally, types can be found in an online library at pubmlst.org.

Since MLST deals with sequences instead of gels, a major advantage is that the data can be easily shared between researchers. Scientists across the world can upload their data into the *C. difficile* MLST database or download reference data from previously performed experiments. However, a downside of MLST is the need for advanced materials. Multiple primers and sequencing reagents are required for this technique which may act as a barrier for smaller labs.

1.5.2. Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis is a method that is similar in nature to restriction endonuclease analysis (REA). While REA utilizes restriction endonucleases, such as *Hind*III, that cleave the bacterial genome at multiple points producing small fragments, pulsed-field gel electrophoresis (PFGE) utilizes restriction endonucleases that cleave the bacterial genome in fewer locations [69]. Examples of restriction endonucleases used for pulsed-field gel electrophoresis include *Sma*I and *Sac*II [70]. The reduced number of generated fragments results in larger DNA fragments. While normal gel electrophoresis is not able to resolve large fragments of DNA, PFGE is capable of such a task. In normal gel electrophoresis, the electric field is unidirectional the entire duration of the cycle. Large fragments of DNA can not be resolved by such a technique. However, in PFGE, the electric field produced by the machine periodically changes directions throughout the duration of the cycle [71]. Larger DNA fragments align slower to the change of the electric field while smaller DNA fragments align quicker to the change in the electric field [71].

PFGE cycles last longer compared to normal gel electrophoresis and generates more heat. This adds to the technique's limitations as it requires a cooling module to ensure that the gel does not overheat. Additional limitations of PFGE are similar to those of REA since this technique also relies on the resolution of DNA fragments on agarose gel. Additionally, data portability is an issue unless the gel is digitized. The added complexity of an electric field that changes directions also produces potential consistency issues where reproducibility may vary depending on the equipment used. Slight differences may exist in the brand, model or age of the equipment available.

1.5.3. PCR Ribotyping

PCR ribotyping is a typing technique that investigates the 16S-23S intergenic spacer region (ISR) [72]. This is currently one of the most used typing techniques by scientists worldwide, making it an unofficial standard [63]. Between different strains, there is significant variability in the size and repeats in the 16S-23S intergenic spacer region. It is this variability in the genome that makes it a suitable candidate for typing *C. difficile*. PCR ribotyping first arose as a technique

to type many clinical isolates. 4 regions of the rRNA operon were initially amplified [73]. It was discovered that the 16S-23S spacer region between alleles varied. From the initial publication which outlined the concept of PCR ribotyping, 24 strains were analyzed yielding 14 ribotypes [73].

Analysis of the PCR products may occur by many techniques. Firstly, the product can be resolved by typical gel electrophoresis. As with all conventional gel electrophoresis techniques, the limitations occur due to nature of agarose gels. Increased resolution may be obtained using capillary gel electrophoresis [74].

Sequencer based capillary gel electrophoresis makes the PCR ribotyping an even more powerful version of PCR ribotyping. Although the cost of such techniques is significantly higher than that of gel electrophoresis, there are many benefits of the variation. Electronic data has much better data portability and distribution. Similar to MLST, this has allowed the creation of an open-source library of data where researchers can use and contribute information.

It has been shown that capillary sequencing, such as the Qiagen QIAxcel CGE (QCGE), platform is superior to other cheaper techniques [75]. Compared to the QIAxcel, sequencer-based gel electrophoresis can resolve DNA bands more accurately [75]. In an experiment conducted by Xiao et al., Qiagen's QIAxcel was shown to produce bands which deviated by 4.2 base-pairs to 9.5 base-pairs [75]. On the other hand, sequencer based capillary gel electrophoresis only deviated by between 0 base-pairs and 0.2 base-pairs [75].

1.5.4. Whole Genome Sequencing

Whole genome sequencing is the most powerful discriminatory method currently available for *C. difficile* analysis and typing. The first *C. difficile* genome was published in 2006 [76]. Since then, many other strains of *C. difficile* have been sequenced and whose genome have been made available to the public. The technique's power comes from producing the genomic sequence of the isolate in question. Instead of targeting a small specific localized area, whole genome sequencing provides all the above information and more. Once an isolate has been sequenced, multiple pipelines can be executed to type the isolates. Various aspects of the genome, such as single nucleotide polymorphisms, deletions, insertions, inversions, and other

genetic variations can be detected and analyzed. This provides a base to produce multiple typing methods while eliminating the need for multiple techniques to type *C. difficile* in different ways.

Besides typing, whole genome sequencing is a valuable tool in determining the phylogeny of the bacteria. This allows researchers to track the evolution and distribution of different *C. difficile* strains. Once again, this digital data can be shared and easily accessed with other researchers using online databases. Because of the versatility of this technique, the entire field is moving towards whole genome sequencing instead of other typing methods.

Limitations behind whole genome sequencing primarily involves cost. The specialized equipment required to sequence a genome may not be available to smaller labs where funds are limited. However, as technology advances, the method has been made accessible to many more users as the cost of whole genome sequencing has decreased substantially. As whole genome sequencing generates a great amount of data, this method may generate too much data depending on the type of research being conducted. With a specific question and target to be addressed, one of the more specific methods may be preferable.

1.5.5. Antiquated Typing Methods

Typing *C. difficile* aims to group strains based upon a certain characteristic. Although other typing methods have been previously used on *C. difficile*, many of these methods are no longer in use today. Low discriminatory power, difficulty of use, inconsistent results, and lack of widespread adoption are all reasons for why a typing method may be abandoned.

1.5.5.1. Restriction Endonuclease Analysis

Restriction endonuclease analysis (REA) is a technique that uses restriction enzymes to cleave DNA at specific restriction sites. Restriction endonuclease analysis of *C. difficile* utilizes the restriction endonuclease *Hind*III to cleave the bacterial genome into fragments [69]. The generated fragments are then resolved by gel electrophoresis [69]. The DNA fragment profile generated by this technique is then analyzed and compared to reference sets. Differentiation of *C. difficile* strains is determined by the presence or absence of restriction sites. Each restriction site creates a DNA fragment which are then visualized by agarose gel electrophoresis [69].

Mutations in the genome may add or remove restriction sites thus altering the length/presence of fragments. This results in observable differences in banding. Although REA typing can produce distinguishable types of *C. difficile*, limitations do exist. Data portability is difficult as generated data is on gels and is not digital. Furthermore, the use of gel electrophoresis creates opportunity for inconsistencies as technical variables may differ depending on the facility and equipment used.

C. difficile typing via restriction endonuclease analysis has been used since 1987 when first proposed by Kujiper et al. [77]. These researchers were investigating the endonuclease profile of *C. difficile* isolated from 2 patients who had developed pseudomembranous colitis after surgery [77]. Samples from the patients' environment were also taken and analyzed. Besides HindIII, the restriction endonucleases *Eco*RI, *Sau*3A, *Hpa*I, *Apa*I, *Pst*I, and *Bam*HI were also tested [77]. However, besides *Hind*III, all of the other restriction endonucleases produced profiles that were not significantly different from one another [77]. Too many restriction sites result in low resolution while too few bands result in low discrimination. Restriction endonuclease analysis was further developed in 1993 by Calbots et al. [78]. These researchers analyzed 1965 *C. difficile* clinical isolates to determine their REA profiles [78]. Of the 1965 isolates, the researchers further refined the REA profiles and determined that they had identified 206 unique REA types [78]. From the 206 identified REA types, they were further organized into 75 groups [78].

1.5.5.2. Serotyping

Serotyping is a technique first investigated in the early 1980's [79]. When serotyping *C. difficile*, researchers look for slide agglutination when the bacteria is exposed to antisera [79]. Different serotypes agglutinate to different anti-sera. Although more serogroups have since been added, there were initially only 6 serogroups: A, B, C, D, F, and G [80]. These groups were relatively distinct, strains isolated from antibiotic-associated diarrhea were found to all be a part of either serogroup A, C, or D [80]. Serogroups B, F, and G only contained strains isolated from stools of asymptomatic neonates or young children [80]. Although this way of typing *C. difficile* may have correlations to certain phenotypic aspects of strains, this does not mean that they will always react identically towards different stimuli. Another issue with serotyping is that not all *C.*

difficile strains may agglutinate anti-sera. Of 315 strains of *C. difficile* investigated by Delmee et al., only 312 of the 315 strains agglutinated anti-sera [80].

1.5.5.3. Surface-Layer Protein-A Typing

Related to *C. difficile* serotypes is surface-layer protein-A typing. Surface-layer protein-A is a *C. difficile* protein located in the S-layer of the bacteria [81]. Surface-layer protein-A is encoded by the *slpA* gene which varies across *C. difficile* strains [81]. When typing based on *slpA*, the groupings are almost identical to those of serotyping [63]. Serotype A is the only exception where the nucleic sequence of *slpA* varies [63].

As serotyping is based on the reaction of the bacteria to anti-sera, surface-layer protein-A may possess clinical relevance. As there are no vaccines currently available to combat *C. difficile*, surface-layer protein-A could be a viable candidate for vaccine development. If a vaccine were to be developed against surface-layer protein-A, protection could be restricted to a certain surface-layer protein-A type.

1.5.5.4. Toxinotyping

Compared to pulsed-field gel electrophoresis and restriction endonuclease analysis which utilize the entire bacterial genome, toxinotyping does not. Toxinotyping only utilizes the pathogenicity locus (PaLoc) portion of the *C. difficile* genome [82]. The pathogenicity locus contains 2 important genes for *C. difficile* pathogenicity: *tcdA*, which encodes *C. difficile* toxin A, and *tcdB,* which encodes *C. difficile* toxin B [83]. Primers specific for different portions of each gene are used to amplify the gene via PCR. The generated DNA product is then digested using the restriction endonucleases *Hind*III, *Hinc*II, and *Pst*I [82]. Restriction sites exist within the 2 genes and elsewhere in the pathogenicity locus [82]. However, DNA outside of the primer targeted region will not be amplified [82]. The generated DNA fragments are resolved by gel electrophoresis before being visualized and analyzed. Variations in the toxin genes can be the result of, but not limited to mutations, insertions, deletions, and invasions. By using PCR and gel electrophoresis to investigate deviations in *tcdA* and *tcdB*, the limitations associated with this type of typing are similar to those of REA and PFGE.

Alternatively, the *C. difficile* isolate's genome can be partially sequenced to identify any DNA deviations [84]. Instead of digesting the DNA using restriction endonuclease, the fragments can instead be sequenced. The sequence can then be used to reconstruct the PaLoc using bioinformatics [84]. Sequencing this specific region of the genome may give more insight into other mutations in the PaLoc and not only mutations that affect the restriction site.

1.6. Mechanisms of Pathogenicity

While *C. difficile* is known for causing disease, not all *C. difficile* strains are pathogenic. Pathogenic *C. difficile* strains are strains that produce toxins which affect the host. *C. difficile* toxin production is encoded on a section of the genome known as the pathogenicity locus (PaLoc) [85].

1.6.1. Pathogenicity Locus

The primary pathogenesis factor of *C. difficile* is its ability to form toxins, *namely C. difficile* toxin A and *C. difficile* toxin B. These 2 toxins, which are responsible for the bulk of *C. difficile*'s pathogenicity, are encoded by within a 19.6kb genomic DNA region known as the pathogenicity locus (PaLoc) (Figure 3) [2,86]. *C. difficile* toxin A (TcdA) is encoded by *tcdA*, and *C. difficile* toxin B (TcdB) is encoded by *tcdB* [19].

The *C. difficile* pathogenicity locus is composed of 5 units, respectively ordered *tcdR*, *tcdB*, *tcdE*, *tcdA*, and *tcdC*. *tcdR* encodes for a sigma factor that acts as a positive regulator of *tcdA* and *tcdB* (Figure 3). Therefore, *tcdR* activation stimulates the production of toxin A and toxin B. *tcdE* encodes a holin-like protein involved in the secretion of toxins [87]. *tcdC* encodes an anti-sigma factor meant to inhibit the products of *tcdR* and thus, inhibits toxin production [88]. However, the function of *tcdC* has yet to be fully understood and is debated amongst researchers [88].

Some toxigenic *C. difficile* strains may produce a third toxin, binary toxin (CDT) [29]. Although *C. difficile* toxin A and *C. difficile* toxin B were identified to cause disease in 1978, it was not until 1987 that binary toxin was characterized [18]. Binary toxin is composed of 2 separate subunits, CDTa (subunit A) and CDTb (subunit B), respectively encoded by *cdtA* and *cdtB*. *cdtA* and *cdtB* are located elsewhere in the genome outside of the pathogenicity locus [83]. Combined, the genomic region which contains *cdtA* and *cdtB* is referred to as the Cdt locus (CdtLoc) [18].

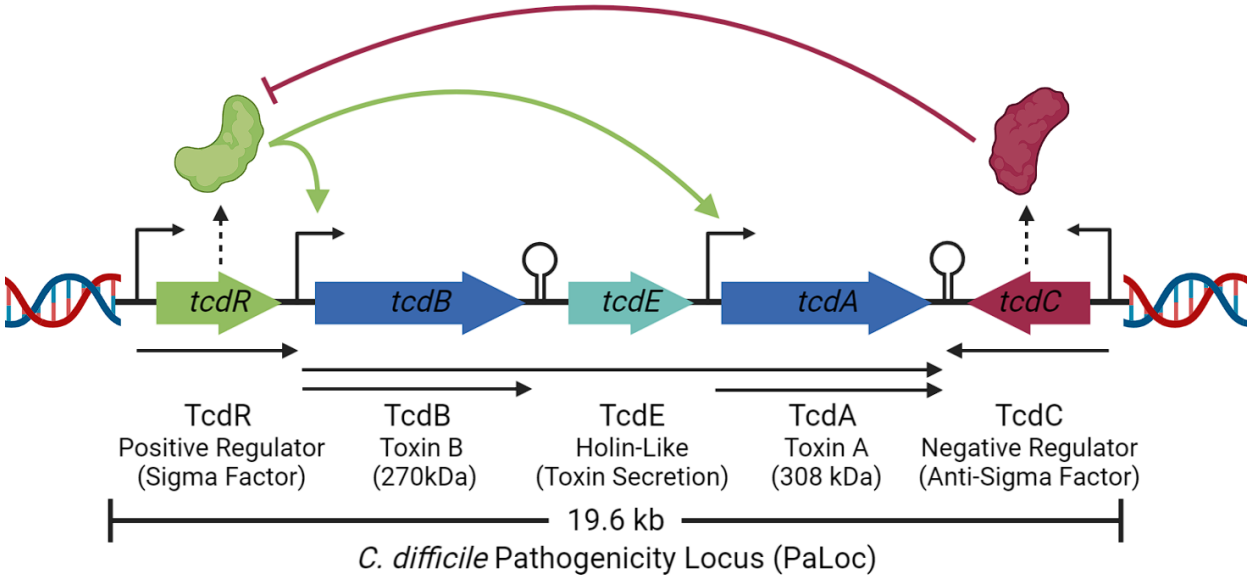


Figure 3. Gene Organization of *C. difficile's* Pathogenicity Locus (PaLoc). The *C. difficile* pathogenicity locus is a 19.6kp segment of DNA which is composed of 5 units, respectively ordered *tcdR, tcdB, tcdE, tcdA, and tcdC*. *tcdR* is a positive regulator of toxin production who produces a sigma factor (TcdR) for transcription initiation. *tcdB* encodes *C. difficile* toxin B. *tcdE* encodes a holin-like protein involved in the secretion of toxins. *tcdA* encodes *C. difficile* toxin A. *tcdC* is a negative regulator of toxin production who produces an anti-sigma factor (TcdC) which inhibits TcdR. Adapted from Fortier et al. [89].

1.6.2. *C. difficile* Toxin A, Toxin B, and Binary Toxin

C. difficile strains are often characterized by their toxin production profile. While some strains produce toxin A, toxin B, and binary toxin, some produce none. Mutations within the pathogenicity locus may disrupt the transcription or translation of the toxins [29]. A strain that produces toxin A but that does not produce toxin B is characterized as being "toxin A positive, toxin B negative". The opposite is also possible, a strain that doesn't produces toxin A but produces toxin B is characterized as being "toxin A negative, toxin B positive". If a strain does not produce neither toxin A, toxin B or binary toxin, then the strain is characterized as being non-toxigenic [18].

1.6.2.1. Toxin Expression

The signals involved in *C. difficile* toxin gene expression and toxin production remain unclear. However, it has been hypothesized that stresses are involved in the process [83]. An early study investigating toxin expression first looked at the possible role of subinhibitory concentrations of antibiotics. With *C. difficile* causing diseases such as pseudomembranous colitis after the use of antibiotics, researchers reasoned that perhaps the residual concentrations of antibiotics were a trigger of toxin production [83]. Growing *C. difficile* in continuous media with trace amounts of antibiotics, it was found that toxin levels increased when challenged with vancomycin and penicillin, but not clindamycin [83]. Little other research has been done to described toxin levels in relation to subinhibitory concentrations of antibiotics. This may be one investigation area of interest since many members of the human gut microbiota also produce various antimicrobial peptides. With some evidence, it would not be unreasonable to assume that there may be a correlation.

Another investigated angle of toxin expression is nutrient stress. An early study investigating toxin expression also looked at toxin expression when *C. difficile* was grown in media with limited amino acids [90]. The research showed that biotin was an amino acid that significantly increased toxin production by the pathogen [90]. When grown in biotin deficient media, containing 0.05nM of biotin, *C. difficile* toxin A production increased 35-fold [90]. Simultaneously *C. difficile* toxin B production increased 64-fold [90]. However, it must be noted

that this increase in toxin production was not reproduceable when different amino acids are limited in the growth medium [27,83].

1.6.2.2. *C. difficile* Toxin A and B Activity

C. difficile toxin A is a 308-kDa enterotoxin while *C. difficile* toxin B is a 270-kDa cytotoxin [85]. Although cytotoxic, TcdB is not immediately cytotoxic towards cells [91]. Upon exposure, cell rounding can be seen as early as 2 hours post-exposure [83]. However, it can be upwards of 24-hours before cell death occurs [83].

For TcdA and TcdB to cause any harm to cells, they must first be internalized into the host cell [18]. Once endocytosed and inside of the endosome, an acidic environment is required for the toxins to be activated. The acidic environment causes the toxins to undergo a conformational change exposing a hydrophobic domain [83]. The toxin is then able to translocate the enzymatic domain portion of itself into the cell [83]. Without an acidic endosome, the toxin is unable to undergo the conformational change required to enter the cytoplasm and thus, will have no effect on the cell.

TcdA and TcdB both work as glucosyltransferases inside their target cells inactivating GTPases [18]. One protein which is affected is Rho. TcdA and TcdB are able to target all variations of Rho in the human body which include RhoA, RhoB, and RhoC [18]. It is by inactivating Rho that the actin cytoskeleton is can be disrupted. Rho acts as the actin cytoskeleton's main regulator [83]. The toxins also target Rac and Cdc42, both GTPase proteins which are involved with the cell actin cytoskeleton [18]. Besides being responsible for the actin cytoskeleton, Rho, Rac, and Cdc42 have other functions in the cell. Consequently, TcdA and TcdB also disrupt many other cellular processes [18]. Programmed cell death, apoptosis, is partially inhibited by the inactivation of these GTPases. However, there are other pathways to cell death, such as via caspases, that can be activated in response to TcdA or TcdB detection [18,83].

TcdA and TcdB both cause major inflammation. This occurs when the tight junctions of the epithelium are destroyed by the toxins [92]. The disruption of tight junctions also results in constant diarrhea [17]. Although TcdA and TcdB have the capability of causing a substantial amount of pathology, there are other factors that contribute as well. Other events that

contribute to disease progression, such as in pseudomembranous colitis, include severe inflammation, neutrophil infiltration, activation of submucosal neurons, production of reactive oxygen intermediates, mast cell activation, substance P production and direct intestinal mucosa damage [83].

1.6.2.3. Binary Toxin

Strains which produce binary toxin are often hypervirulent compared to non-binary toxin producing strains [64]. Overall, it is estimated that 17%-37% of strains produce binary toxin [93]. Although the role of binary toxin in pathogenicity is not completely understood, one of its mechanisms of action is thought to target microfilaments disrupting the actin cytoskeleton [94].

Binary toxin is composed of 2 separate subunits, CDTa and CDTb, respectively encoded by *cdtA* and *cdtB*. *cdtA* and *cdtB* are located elsewhere in the genome outside of the pathogenicity locus [83]. Combined, the genomic region which contains *cdtA* and *cdtB* is referred to as the *Cdt* locus (CdtLoc) [18].

1.6.3. Tests to Confirm Presence of *C. difficile* Toxins

Following the isolation of *C. difficile* colonies, researchers must determine if the *C. difficile* strain in question produces toxins or not. Toxin producing strains are known as toxigenic and are capable of causing disease. On the other hand, non-toxin producing strains are known as non-toxigenic, not capable of causing disease.

1.6.3.1. Cell Cytotoxicity Neutralization Assay

One method of detection of *C. difficile* toxin detection is the cell cytotoxicity neutralization assay (CCNA) [95]. The cytotoxicity neutralization assay, when done on stool, takes filtrate from a sample and inoculates it onto a tissue bacterial culture that is sensitive to the toxins [95]. Typically, human fibroblast cells are used as the test tissue. If toxins are present in the filtrate, the cells will start to round. Cell rounding is a typical symptom of cytotoxicity [83]. Because other factors in the sample may be causing the rounding of cells, a second tissue bacterial culture that contains *C. difficile* toxin anti-toxin is run in parallel. Cells in this culture are

not expected to round due to the anti-toxin neutralizing the toxin. These exact results would confirm that *C. difficile* toxins are present. Although this method is the most sensitive method for the detection of *C. difficile* toxins in stools, it requires the use of expensive and difficult to handle bacterial cultures, at least 48 hours of incubation time, and special reagents. Additionally, it requires technicians to manually interpret the presence of cytopathic effects on the bacterial culture [95]. However, automated CCNAs have been in development [95].

1.6.3.2. Polymerase Chain Reaction

Targeting the toxin genes located within the pathogenicity locus (PaLoc) via polymerase chain reaction (PCR) is another way at detecting *C. difficile* toxins. First, using primers specific for the toxin genes, the toxin genes are amplified using PCR. The generated fragments are then separated by gel electrophoresis, stained, and visualized. The presence of amplified fragments confirms the presence of toxin genes. This method is much more efficient than a CCNA but is less sensitive. Additionally, detection of the gene does not confer production of the gene product. There may be underlying factors that prohibit the expression of the toxin gene and/or transcript. Depending on the testing facility, the special equipment required for PCR may also not be available. In such circumstances, a simpler and quicker method of toxin detection required may be more suitable.

1.6.3.3. Enzyme Immunoassay

Enzyme immunoassay (EIA) utilize antibodies that target and bind *C. difficile* targets. One aspect of *C. difficile* that can be targeted are their toxins. With this approach, binding of the toxin by the antibodies allows the complex to be visualized. However, toxins are often not the targe of choice since not all strains of *C. difficile* produce toxins.

Antibodies can also be designed to target glutamate dehydrogenase (GDH). GDH is an enzyme associated with the cell wall of *C. difficile*. The enzyme is encoded separately from the pathogenicity locus (PaLoc) and conserved across all *C. difficile* strains. For quick detection, it has been recommended that a two-step approach should be used to improve accuracy. EIA-GDH tests are commercially available such as the VIDAS *C. difficile* Panel (BioMérieux, France) and the

C. diff Quik Chek Complete (TechLab, United States). C. diff Quik Chek Complete 2-in-1 test was shown to accurately screen 88% of samples within 30 minutes [96]. However, screening accuracy was improved to 100% when PCR was used to further investigate inconclusive tests [97].

1.7. Risks of Contracting a *C. difficile* Infection

Most *Clostridioides difficile* infections occur in parallel with the use of antibiotics. However, several other risk factors also exist. Major risk factors include being 65 years of age or older, recent prolonged hospitalizations, having a weakened immune system, and previously being infected by *C. difficile* [98]. Interestingly, there does not appear to be a disparity among sexes as CNISP surveillance data shows that both men and women are equally represented by Canadian HA-CDI cases [99]. The Centers for Disease Control and Prevention (CDC) reports that 1 in 6 people who have contracted a CDI will contract it again within 2-8 weeks [100]. During the 1970's *C. difficile* infections increased exponentially due to the liberal use of clindamycin among healthcare practitioners. The frequent association of clindamycin usage and pseudomembranous colitis resulted in the condition being known as "clindamycin colitis" [101].

The elderly, aged 65 and older, are considered to be the most at risk of contracting a CDI [19,99,100]. CNISP reports that from 2009-2015, the average age of an adult Canadian HA-CDI patient was 69.2 years old [99]. The bulk of the HA-CDI cases, 65.8%, were from patients aged 65 years of age and older [99]. Only 34.2% of reported CA-CDI cases were from patients aged between 18 and 64 years old [99]. Additionally, the elderly are the most at risk of death due to their already weakened immune system and other co-morbidities [19]. 1 in 11 of those aged 65 and older are expected to die within a month of diagnosis of a *C. difficile* healthcare-associated infection [100]. With 500 000 annual reports of *C. difficile* infections across the United States of America, the CDC estimates that *C. difficile* related deaths of those aged 65 and older represent 80% of annual *C. difficile* related death [100]. In Canada, CNISP reports that from 2009-2015, 13.4% of cases where the patient contracted a HA-CDI resulted in a fatality [99]. However, only 1/3 of the fatalities were attributable directly to the HA-CDI [99].

1.8. Disease and Complications Caused by *C. difficile*

A *C. difficile* infection is when *C. difficile*, colonized within an individual's digestive tract, affects the gastrointestinal tract by producing toxins. Symptoms of an infection vary case to case, ranging from abdominal cramping and watery diarrhea in mild cases, to pseudomembranous colitis and toxic megacolon in severe cases [102]. The severity of symptoms during a CDI depends on a multitude of factors which include the bacterial strain involved and the immunological strength of the patient. However, due to unknown reasons, some individuals remain asymptomatic while infected [14]. Fortunately, not everyone who ingests spores will develop a CDI. *C. difficile* is an opportunistic bacterium. The immune system and gut microbiota of a normal healthy adult can prevent vegetative *C. difficile* cells from colonizing in the gut [11]. An issue arises when healthcare practitioners prescribe patients broad spectrum antibiotics. While the antibiotic may kill what they were originally prescribed for, collateral damage occurs on commensal gut bacteria resulting in a disrupted gut microbiota [11]. It is these commensal gut bacteria which compete with *C. difficile*, effectively preventing *C. difficile* from establishing itself in the gut. However, with a void in the gut microbiota, *C. difficile* spores are free to germinate and flourish in the newly less competitive environment [103].

While *Clostridioides difficile* is known for causing disease, not all *Clostridioides difficile* strains are pathogenic. Pathogenic *C. difficile* strains must produce toxins which affect the host. The 2 main types of toxins produced by toxigenic *C. difficile* are *C. difficile* toxin A (TcdA) and *C. difficile* toxin B (TcdB) [93]. *C. difficile* toxin A is an enterotoxin while *C. difficile* toxin B is a cytotoxin [83]. Not all *C. difficile* strains produce both TcdA and TcdB. Mutations in *tdcA* and *tcdB* the *C. difficile* pathogenicity locus (PaLoc), which encodes TcdA and TcdB respectively, may inhibit their production. It is also possible for mutations to cause *C. difficile* strains to produce only one toxin, either TcdA or TcdB [82]. These non-toxigenic strains of *C. difficile* do not cause disease when colonized in the gut [86]. The use of non-toxigenic *C. difficile* strains as a therapeutic or prophylaxis against toxigenic *C. difficile* strains is currently being explored [86].

The third type of toxin produced by *C. difficile*, binary toxin (CDT), is produced by approximately 17%-37% of strains; many of which are hypervirulent [93]. Although the role of

binary toxin in pathogenicity is not completely understood, one of its mechanisms of action is thought to target microfilaments, thus disrupting the actin cytoskeleton [94].

1.8.1. Pseudomembranous Colitis

Typical *C. difficile* infections may result in complications such severe diarrhea, dehydration due to severe diarrhea, and pseudomembranous colitis (PMC). Severe cases may result in sepsis, toxic megacolon, and death [8]. Pseudomembranous colitis is a condition where inflammation of the colon is accompanied by elevated levels of yellow-white plaques that coagulate to form pseudomembranes on the mucosa [83]. Although most cases of pseudomembranous colitis are *C. difficile* related, physicians must nonetheless confirm the presence of *C. difficile* as there is a possibility that the condition is caused by other factors [83].

1.8.2. Toxic Megacolon

Toxic megacolon is a lethal CDI associated complication where “inflammation spreads into deeper layers of the colon” [104]. This results in the colon widening and potentially stop working. At this level of inflammation, there is a high risk of internal bleeding and perforation of the colon [104]. Such complications may progress and cause shock and or sepsis [104]. For cases where treatment is ineffective, represented by 5%-20% of patients, surgical intervention may be necessary to prevent further progression of toxic megacolon [101]. While a normal colon’s diameter ranges from 6.5cm to 10.5cm, a colon with toxic megacolon reaches a diameter of 12cm and greater [105]. Colonized toxigenic *C. difficile* continue to produce toxins resulting in severe damages to the gastrointestinal tract of the patient during both pseudomembranous colitis and toxic megacolon [106].

1.9. Treatment of a *C. difficile* Infection

Traditionally, *C. difficile* infections are treated by prescribing antibiotics. However, alternative treatments are also available to patients depending on their circumstances. In the event of watery diarrhea, it is imperative that fluid and electrolyte levels are maintained in the

body [107]. Even if the symptoms of infection dissipate after treatment, the chances of *C. difficile* colonization remain high [108].

1.9.1. Antibiotics

As the gut microbiota can prevent *Clostridioides difficile* from colonizing in a healthy gut, discontinuing the antibiotic taken prior to the onset of infection may resolve the infection. It has been reported that 20% of patients can resolve an infection by simply discontinuing the aforementioned antibiotics alone [109].

In cases where antibiotics must be prescribed, other antibiotics such as vancomycin and fidaxomicin may be administered [55]. Rifampin was previously a widely administered treatment but is currently no longer recommended. Metronidazole is another antibiotic that was widely used to treat CDIs in the past [110]. However, research has shown that when the effectiveness of metronidazole and vancomycin are compared, vancomycin possesses increased activity is more effective [110].

1.9.1.1. Antimicrobial Resistance

With the use of antibiotics becoming increasingly liberal in the modern world, antimicrobial resistance is on the rise. *C. difficile* is notorious for being resistant to a multitude of antibiotics including aminoglycosides, lincomycin, tetracyclines, erythromycin, clindamycin, penicillins, cephalosporins, and fluoroquinolones [111]. While other bacteria may be killed off during antibiotic treatment, the opposite frequently occurs with *C. difficile*. Consequently, *C. difficile* begins to fill the newly created void in the gut microbiota while remaining unaffected by the drug [39]. Although effective antibiotic treatments for *C. difficile* currently exist, the potential for *C. difficile* to gain resistance to current treatment antibiotics is a dangerous possibility.

1.9.2. *C. difficile* Toxin Targeting Monoclonal Antibodies

The administration of monoclonal antibodies is being investigated as a potential preventative CDI treatment. Bezlotoxumab is an IgG immunoglobulin, administered intravenously, which binds to *C. difficile* toxin B [112]. The binding of *C. difficile* toxin B by

Bezlotoxumab results in the neutralization of *C. difficile* toxin B's effect on gastrointestinal cells [112]. Merck has run 2 phase 3 trials of ZINPLAVA, their brand name of bezlotoxumab, where patients received bezlotoxumab in addition to conventional standard of care. The study demonstrated a 4.8% and 14.6% increase in clinical response when treated with the monoclonal antibody vs conventional standard of care [112].

Another monoclonal antibody developed by Merck is actoxumab. Originally part of bezlotozumab's early development stages, actoxumab was ultimately discontinued by Merck due to its lack of proven efficacy [113]. Actoxumab targeted *C. difficile* toxin A in a similar fashion to how bezlotoxumab targets *C. difficile* toxin B [114].

1.9.3. Fecal Microbiota Transplantation

Another treatment of recurrent *C. difficile* is fecal microbiota transplantation (FMT) [17]. Since *C. difficile* is an opportunistic bacterium which thrives in guts with disrupted microbiotas, the rationale behind fecal transplantation is to recolonize a patient's gastrointestinal microbiota with the fecal matter of a healthy individual [17]. The newly introduced bacteria would then cause nutrient and spatial competition similar to that of a normal healthy microbiota. The rescued competition thus limits *C. difficile* colonization and prevents future infections. The treatment involves delivering a donor's feces, mixed in saline, to the recipient's colon via colonoscopy, nasogastric tube, or enema [17]. Fecal transplantation is an extremely effective method of preventing recurring CDIs. Depending on the method of delivery, fecal microbiota transplantation has between a 76% and 95% effectiveness [17].

1.10. *C. difficile* Infection Prevention

It is estimated that 1 in 5 patients who contract a CDI will experience a second episode. The risk continues to increase after each subsequent CDI episode. It is reported that there is a "35%-65% risk of reinfection after the first recurrent episode" [115]. With respect to recurrent CDIs, a relapse is when a patient is infected again with the original *C. difficile* strain [108]. On the other hand, reinfection is when a patient is infected again with a different *C. difficile* strain when

compared to the original [108]. Some *C. difficile* treatments, such as fecal microbiota transplantation also act as methods to prevent recurrent infections.

1.10.1. Antimicrobial Stewardship

The best way to decreases the chances of acquiring a *C. difficile* infection is by taking only antibiotics when needed. Reducing unnecessary antibiotic exposure reduces the risk of contracting a CDI while preventing antimicrobial resistance [101]. The Cleveland VA Medical Center, a 215-bed hospital and a 250-bed long-term care facility, is one of many facilities that has seen a decrease in CDI since the inception of stewardship intervention programs. Fluroquinolone use is one driver of CDI, exemplified by fluoroquinolone resistant NAP1/RT027. From 2009 to 2018, the Cleveland VA Medical Center was able to reduce inpatient fluroquinolone prescriptions by 43% [116]. The result of the stewardship initiative, in conjunction with improved cleaning, was a 50% reduction in HA-CDI incidences [116]. Additionally, the percentage of HA-CDI incidences caused by NAP1/RT027 *C. difficile* fell from 65% to 10% [116]. Other studies have also shown that CDI incidence rates can be reduced by limiting fluroquinolone use. Parallel results have been reported in England, where CDI incidences caused by fluroquinolone susceptible strains has remained constant while CDI incidences caused by fluroquinolone resistant strains has decreased [117].

1.10.2. Hand Hygiene

While antimicrobial stewardship may decrease the chances of *C. difficile* colonizing in the gut, it doesn't prevent the bacteria from entering one's body to begin with. While an individual may not have come in direct contact with an infected patient, the individual may have touched something that another individual who did have contact with an infected patient contaminated. It is as simple as grabbing a doorknob or using a toilet that an infected, or contaminated individual, has used. Touching one's mouth succeeding such an event allows spores to enter the gastrointestinal tract.

Proper hand hygiene is one of the most effective methods at preventing the ingestion of *C. difficile* spores [23]. Although alcohol-based hand sanitizers are often used to disinfect hands,

they are ineffective against *C. difficile* spores [118]. Washing hands with soap and water remains the most effective way of removing *C. difficile* from one's bare hands [118]. Banks et al. demonstrated that within a healthcare setting, without making changes to environmental cleaning or antibiotic stewardship, increased hand washing compliance can decrease a facility's *C. difficile* incidence rate [119]. Using electronic trackers, Banks et al. were able to increase hand hygiene compliance among staff which resulted in the facility's annual *C. difficile* incident rate falling from 9.5 to 3.7 incidents per 10,000 patient-days. [119].

1.10.3. Disinfection of Surfaces

Killing *C. difficile* in its vegetative state is not a difficult task, common bactericidal cleaners, such as ethanol and isopropyl, are able to do the job [20]. *C. difficile* spores on the other hand, are much more difficult to kill as they are resistant to many common cleaners [20]. It is recommended that people prone to potential contact with spores use appropriate personal protective equipment, such as gloves and gowns, when working with potentially infected people or material [17]. Special sporicidal cleaners are required to deactivate spores in the environment. Generally, such products contain sodium hypochlorite (bleach), chlorine bases at high concentration, or hydrogen peroxide [20].

Products that are recognized as being effective against *C. difficile* spore are listed by the United States Environmental Protection Agency (EPA). *C. difficile* sporicidal products can be found on the EPA's List K: EPA's Registered Antimicrobial Products Effective against *Clostridium difficile* Spores [52].

1.10.4. Government Policy and Regulations

In 2013, the Public Health Agency of Canada (PHAC) released guidance information for infection prevention and control of *C. difficile* for both acute- and long-term care facilities [120,121]. These guidelines were created to provide guidance to help both acute- and long-term care facilities reduce the risk of patients contracting CDIs from their facilities.

1.10.5. Infectious Disease Surveillance

Surveillance also plays a large role in preventing CDIs. The Canadian Nosocomial Infection Surveillance Program (CNISP) was established in 1994 as a collaborative effort between the Public Health Agency of Canada (PHAC) and 18 hospitals across Canada [122]. Since being established in 1994, surveilled infections are reported by 90 Canadian member acute-care hospitals [122]. Aside from *C. difficile* infections, CNISP also tracks other nosocomial infections across Canada such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) [122]. Data generated by CNISP helps public health "reduce the impact of healthcare-associated and antimicrobial resistant infections" [122]. By monitoring potential outbreaks across the country, a quicker mobilization of a response to contain and solve the outbreak can occur.

1.11. *C. difficile*'s Burden on Healthcare

It is estimated that there are between 400,000 and 500,000 annual *C. difficile* infections (CDI) in the United States [123]. CDC surveillance data shows that overall rate of American CDI incidences has decreased from 141.61 CDI cases per 100,000 residents in 2014 to 121.2 CDI cases per 100,000 residents [124]. Combined, these *C. difficile* infections are estimated to have resulted in the fatalities of 30,000 individuals [123]. The situation is similar in Canada where 2012 CDI surveillance data estimates there to have been approximately 37,900 annual episodes, 10,900 of which were episodes of recurrence [125]. These 37,900 CDI cases consumed CAD 272 million of annual Canadian healthcare spending, resources that could have been utilized elsewhere [125].

1.11.1. Hospital Associated *C. difficile* Infections (HA-CDI)

A hospital-associated CDI (HA-CDI) case is defined by CNISP as a patient who at the onset of symptoms, has been admitted to the hospital for more than 72-hours or has been discharged for less than 4 weeks [99]. However, other studies have defined a HA-CDI as a patient who at the onset of symptoms, has been admitted to the hospital for more than 48-hours or has been discharged for less than 4 weeks [126]. An intermediate CDI case is defined as a patient who at

the onset of symptoms, has been discharged from the hospital for more then 4 but less than 12 weeks [126].

Surveillance data has shown that the incidence rate of HA-CDI have been on the decline year after year [99,127–129]. CNISP reports that from 2009 to 2019, the Canadian incidence rate of HA-CDI decreased from 5.9 HA-CDI cases per 10,000 patient-days to 3.62 HA-CDI cases per 10,000 patient-days, peaking at 6.7 HA-CDI cases per 10,000 patient-days in 2011 [99]. Unfortunately, for the first time in a decade, the incidence rate of HA-CDI increased in 2020 to 3.8 Canadian HA-CDI cases per 10,000 patient-days.

Canadian CDI surveillance groups CDI cases into 3 distinct geographic regions [128,129]. The Western region, composed of British Columbia, Alberta, Saskatchewan, and Manitoba; the Central region, composed of Ontario and Quebec; and the Eastern region, composed of Nova Scotia, New Brunswick, Prince Edward Island, and Newfoundland and Labrador [128,129]. The geographical distribution of HA-CDI incidence rates across Canada varies depending on the region [99,128,129]. HA-CDI incidence rates are generally highest in the Central region and lowest in the Eastern region [99,127–129]. The majority (97.3%) of HA-CDI cases reported to CNISP were received from large hospitals containing more than 200 beds [99]. 53.2% of reported HA-CDI cases originated from hospitals containing 200-500 beds [99]. Additionally, 44.1% of reported HA-CDI cases originated from hospitals containing more than 500 beds [99].

HA-CDI has also been on the decline in the United States. CDC surveillance data from 2014-2019 reveals a similar trend of decreasing HA-CDI incidences year over year [124]. In 2014, the American HA-CDI incidence rate was 83.78 per 100,000 residents [124]. 5 years later, the incidence American HA-CDI incidence rate decreased by 30% to 57.9 per 100,000 residents [124]. A meta-analysis of *C. difficile* publications from between 2005 and 2015 by Balsells et al. uncovered that the incidence rate of HA-CDI are higher in North America when compared to Europe or the Western Pacific [130]. The calculated incidence rate of HA-CDI was 7.03 per 10,000 patient-days, 95% CI [5.23, 9.44] in North America (n=12); 3.14 per 10,000 patient-days, 95% CI [2.80, 3.53] in Europe (n=72); and 3.45 per 10,000 patient-days, 95% CI [2.41, 4.95] in the Western Pacific (n=5) [130].

1.11.2. Community Associated *C. difficile* Infections (CA-CDI)

A community-acquired CDI case is defined as a patient who at the onset of symptoms, had been admitted to the hospital for less than 48-hours or more than 12 weeks after discharge [126]. It should be noted that fewer hospitals report CA-CDI when compared to HA-CDI [127].

Historically, *C. difficile* was commonly viewed as strictly being a nosocomial infection. However, *C. difficile* spores are ubiquitous in nature and can thus be acquired outside of hospital settings. In the United States, CA-CDI cases have been increasing while HA-CDI cases have been decreasing [124]. In 2018, the American incidence rate of CA-CDI, 65.93 CA-CDI per 100,000 residents, surpassed the incidence rate of HA-CDI, 64.18 HA-CDI per 100,000 residents [124].

In Canada, approximately 1/3 of CDI infections in patients admitted to hospitals are community-acquired [128,129]. Contrary to the American CA-CDI trend, HA-CDI incidence rates have been decreasing slowly while CA-CDI incidence rates have been relatively stable [128,129]. However, as with HA-CDI, CA-CDI incidence rates and proportions vary province to province [127]. A surge of CA-CDI appeared across Canada in the 2010s, where at its peak, some provinces reported more than 50% of their CDI cases were community acquired [127]. While incidences of HA-CDI declined throughout the late 2010s, incidences of CA-CDI did not decline at the same rate [127,128]. From between 2015 and 2020, CA-CDI incidence rates have remained relatively stable between 1.5 and 1.17 ($\bar{x}$=1.34) cases per 1,000 patient admissions [127,128]. The Canadian CA-CDI incidence rate is low when compared to the estimated North American average of 2.36-5.59 CA-CDI (n=2) incidents per 1,000 patient admissions [130]. However, the Canadian CA-CDI incidence rate is high when compared to the European incidence rate of 0.23, 95% CI [0.18,0.29] [130].

With CA-CDI cases presenting in patients at a steady rate while HA-CDI cases decline, a community reservoir *C. difficile* must exist. Although community reservoirs such as animals, water, and food have been proposed, a definitive link between a community *C. difficile* reservoir and human clinical infections has yet to be identified [131].

1.12. Asymptomatic Zoonotic *C. difficile* Infection

Aside from humans, *C. difficile* can be found colonizing or infecting animals. Unfortunately, detection can be difficult as these animals may be colonized by toxigenic strains without any visible symptoms. Food production animals such as pigs, cattle, sheep, and goats; horses; and household pets such as cats and dogs, are all susceptible to *C. difficile* colonization and infection [132]. There have been reports that *C. difficile* is even capable of infecting wild bush animals such as white-tailed deer, birds, non-human primates, zebras, and elephants [132].

In food production farm animals, *C. difficile* carriage rates are highest in neonatal animals and declines as animals age [132]. These animals are frequently infected asymptomatically and colonized by *C. difficile*. A 2010 study found that 40% of sows were colonized with *C. difficile* prior to farrowing [133]. Due to the high *C. difficile* colonization rate in sows, nearly 75% of the piglets born were found to be carrying *C. difficile* at 2 days of age [133]. As these animals are being raised for food purposes, there is a zoonotic potential regarding *C. difficile*.

Previous studies surveying the prevalence of *C. difficile* contamination on commercially available meats have yielded varying rates of contamination [19,85,125,134–138]. Such results suggests that retail meats may be a vector of dissemination for *C. difficile* which is responsible for CA-CDI incidences. Contamination rates of retail meats are not static and significantly vary across studies. When investigating commercially available ground pork and ground beef, one Canadian study reported a *C. difficile* contamination rate of 6.3% [136]. Additionally, all *C. difficile* isolates recovered from the ground retail meats were toxin A and toxin B positive [136]. Another Canadian study conducted by Rodriguez-Palacios et al. reported a *C. difficile* retail meat contamination rate of 20% [134]. However, only 3 of the 12 *C. difficile* isolates were of a ribotype previously isolated from a human clinical case [134]. A 2007 American study reported a *C. difficile* contamination rate of 42% [138]. Interestingly, *C. difficile* was not only recovered from raw uncooked retail meat products, but also from cooked ready to eat products. The rate of retail meats contaminated by *C. difficile* has also been reported to be as low as 1.98% [135]. Studies have also shown that *C. difficile* is more commonly found in young animals such as piglets and calves [19]. Rates of *C. difficile* contamination in such young animals has been frequently reported at being over 90% [19].

1.13. Thesis Rationale

The rationale behind this study is that toxigenic *C. difficile* is ubiquitous in nature and is not only found and disseminated within healthcare facilities. In previous studies, it has been shown that *C. difficile* spores can be isolated from commercial beef, veal, pork, vegetables, and seafood [19,85,125,134–138]. However, these studies have not linked the *C. difficile* strains isolated from the food samples in any meaningful way to *C. difficile* strains from human clinical cases.

As *C. difficile* is capable of colonizing and infecting food production animals, there is a potential that *C. difficile* may be present on retail food products. Numerous opportunities exist where *C. difficile* may contaminate commercial raw meats. Contamination points include, but are not limited to processing, butchering, and packaging of the animal. This study does not aim to uncover specific sources of contamination along the continuum, but to link commercially prepared meat in Canada as a possible vector of *C. difficile* transmission. The consumption of such contaminated products may result in CDI development and provide novel information of potential community sources of CDI for future mitigation studies.

1.14. Thesis Hypothesis

The hypothesis of this study is that *Clostridioides difficile* spores are found on raw retail beef, pork, and veal samples and that the *C. difficile* found on raw retail meat samples are genetically similar to *C. difficile* isolates collected by the Canadian Nosocomial Infection Surveillance Program (CNISP) originating from human clinical CDI patients of similar geographic and temporal origins.

CHAPTER 2: MATERIALS AND METHODS

In this study (Figure 4), frozen retail pork, beef, and veal samples were obtained from the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) program and FoodNet Canada. These samples were analyzed for *C. difficile* contamination by direct plating on selective media and by inoculation in enrichment broth. Suspected *C. difficile* colonies were confirmed by polymerase chain reaction (PCR). Antibiotic susceptibility was determined by ETEST® strips. Toxigenic *C. difficile* isolates were molecularly characterized by ribotyping and pulsed-field gel electrophoresis (PFGE). Whole genome sequencing (WGS) was performed on all *C. difficile* isolates from retail meats and from select human clinical cases.

2.1. Retail Meat Sample Collection

Retail pork, beef, and veal samples were obtained from the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) program and FoodNet Canada. Retail meat samplers collected fresh retail meat samples (n = 644) from various provinces across Canada. Samples were sourced from Alberta (n = 179), British Columbia (n = 218), and Ontario (n = 247) from between 2016 and 2018. The retail meat samples were repackaged in Whirl-Pak bags (Whirl-Pak, United States), inventoried, and frozen at -80°C for storage. When requested, the retail meat samples were shipped frozen on dry ice directly from the University of Guelph (Guelph, Ontario, Canada) to the National Microbiology Lab (Winnipeg, Manitoba, Canada) overnight. Once received, the samples were kept frozen at -80°C for storage until processed.

2.2. Isolation of *C. difficile* Spores from Retail Meats

This isolation of *C. difficile* spores from retail meats protocol was adapted from the lab of Dr. Scott Weese at the University of Guelph. Only *C. difficile* spores are recoverable from retail meats as *C. difficile* vegetative cells are strict anaerobes and would thus not be able to survive on the surface of retail meats. The utilized protocol was determined to have a limit of detection of 7.5 spores per mL of generated phosphate-buffered saline (PBS) rinsate (Appendix B). The ethanol shock and centrifugation aspects of this isolation *of C. difficile* from retail meat sample

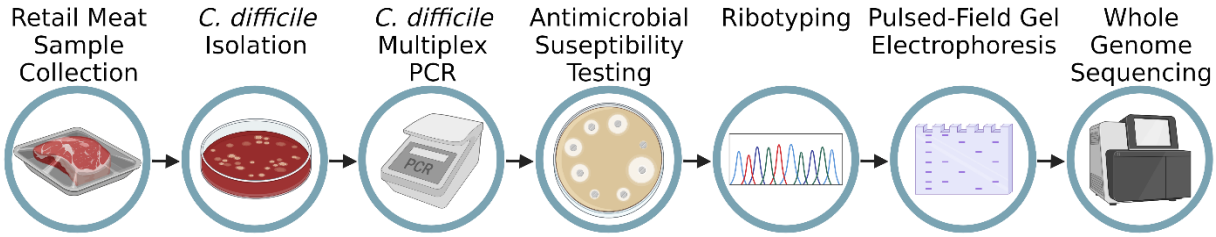


Figure 4. Methods Flowchart of Study. Frozen retail meat samples, collected from across Canada between 2016-2018, were screened for *C. difficile* contamination. Suspected *C. difficile* isolates were confirmed by *C. difficile* multiplex PCR. The *C. difficile* isolates from retail meats were then subjected to antimicrobial susceptibility testing, and molecular typing by ribotyping and pulsed-field gel electrophoresis. Finally, the *C. difficile* isolates from retail meats underwent whole genome sequencing to compare their genomes to *C. difficile* isolates from human clinical cases.

protocol were also validated with no concerns (Appendix B).

The frozen retail meat samples were first thawed in their received Whirl-Pak bag (Whirl-Pak, United States) at room temperature until completely defrosted. 50mL of sterile PBS was then aseptically pumped from a 1L glass bottle directly into the Whirl-Pak bag containing the defrosted retail meat sample. The Whirl-Pak bag, retail meat sample, and 50mL of PBS were then hand massaged for 60 seconds to dislodge surface contaminants into the PBS.

For direct plating of the PBS rinsate, a 0.1mL aliquot of the PBS wash was aseptically transferred onto *C. difficile* moxalactam norfloxacin (CDMN) agar and spread across the plate using a disposable plastic plate spreader. These plates were incubated anaerobically at 37°C for 5-7 days.

For the broth enrichment of the PBS rinsate, a 1mL aliquot of the PBS rinsate was added to 9mL of pre-reduced CDMN enrichment broth, contained within a 16mm straight cap glass test tube, before being incubated anaerobically at 37°C for 5-7 days. Post incubation, after being vortexed to homogenize the culture, a 2mL broth sample was subjected to a 50% v/v ethanol shock. To ethanol shock, 2mL of anhydrous ethanol was first dispensed into a sterile 15mL conical tube. 2mL of broth was then aseptically transferred to the anhydrous ethanol containing conical tube. The broth-ethanol mixture was then incubated at room temperature for 60 minutes. After 60 minutes, the broth-ethanol mixture was centrifuged for 10 minutes at 4000g to pellet the contents. The supernatant was then removed by decanting. Any supernatant remaining post decantation was removed by aspiration. Approximately 50µL: (1 drop) of PBS was then added to the pellet containing conical tube. A 1mL pipette with filtered tips was then used to homogenize the pellet and newly added PBS. Using the same pipette and tip, the resulting mixture was then aspirated out of the conical tube, ensuring to aspirate all pellet pieces, and dispensed onto brucella blood agar (BRU). The mixture was then spread across the BRU agar's surface using a disposable plastic plate spreader and incubated anaerobically at 37°C for 5-7 days.

For both direct plating and enrichment culture a positive and negative control were processed along with each batch of retail meat samples. For the positive control, a 10µL loop full of 48–72-hour old 11ACD0075 *C. difficile* culture was then inoculated into 50 mL of PBS. The inoculated PBS tube was then vortexed, for 5 seconds at maximum speed, to homogenize the

culture. For the negative control, sterile PBS, devoid of sample, was processed along with each batch of retail meat samples Positive and negative controls were run in parallel to the retail meat isolations.

All anaerobic incubation was conducted in an environment consisting of 5% H_2, 10% CO_2, and 85% N_2. The anerobic environment was maintained by the use of a palladium catalyst in either a gloveless anerobic chamber (Anaerobe Systems, United States) or vinyl gloved anaerobic chamber (Coy, United States) and monitored with resazurin based anaerobic indicator strips (Fisher Scientific, United States). Incubation at 37°C occurred in a Model 2000 incubator (Coy Laboratory Products, United States) placed inside the anerobic chambers. Prior to incubation, petri plates were stacked inside plastic sleeves in groups of 12 or 18 plates to prevent the agar from prematurely drying.

2.2.1. Visual Selection of Potential *C. difficile* Isolates

All agar plates were inspected manually for growth of any suspected *C. difficile* colonies throughout the incubation period. 11ACD0075 *C. difficile* streaked on brucella blood agar (BRU) and incubated anaerobically for 24-48 hours served as a positive control plate. The positive control plate was used as a visual reference for colony selection. Colonies, based upon having a similar morphology and fluorescence profile to *C. difficile* were selected for further testing.

When grown on blood-based agar and incubated anaerobically for a minimum of 24 hours, *C. difficile* colonies exhibit one of 2 distinct morphologies. One colony morphology type appears circular, convex, and smooth [43]. The other colony morphology type appears non-circular with jagged edges, occasionally with hyphae like extensions, flat, and rough [43]. Additionally, since grown on blood plates, *C. difficile* colonies will possess a whiteish grey colour while exhibiting no signs of hemolysis [39].

The use of a longwave UV / UVA / near UV light (365nm) was employed to assist in the preliminary identification of any suspected *C. difficile* colonies. The UV light was produced by a handheld UV lamp (Analytik Jena). When exposed to long-wave UV light on blood plates, *C. difficile* colonies instantly fluoresce bright green while non-*C. difficile* colonies will not. All suspected *C. difficile* colonies were picked and then individually streaked on Brucella Blood agar

(BRU) to further isolate the colonies. Colonies which exhibited *C. difficile*'s morphological and fluorescence profile were further confirmed by PCR (Chapter 2.2.2).

2.2.2. Multiplex Polymerase Chain Reaction (PCR)

C. difficile multiplex 1 (MP1) targets *tcdA* (*C. difficile* toxin A), *tcdB* (*C. difficile* toxin B), and *cdtB* (binary toxin) [139]. *C. difficile* multiplex 2 (MP2) targets *tcdC* (PaLoc negative regulator) and *tpi* (triose phosphate isomerase) [139].

A 1µL loop full of each suspected *C. difficile* colony from the BRU agar plate was resuspended individually into 200µL of InstaGene Matrix (Bio-Rad, United States) in a 1.5mL capacity pop-top conical tube. The culture was homogenized by vortexing for 5 seconds at maximum speed. The mixture was then heated on a heat block at 95°C for 30 minutes to lyse the *C. difficile* cells. The lysate tubes were centrifuged at 21,100 x g for 2 minutes to pellet the cellular debris, leaving crude genomic DNA suspended in the supernatant. The DNA was stored at 4°C until used. The MP1 master mix, MP2 master mix, control DNAs, and H_2O were defrosted at room temperature (Table 1). 22.5µL of MP1 master mix and MP2 master mix (Table 2), per sample, were dispensed into separate 200µL capacity PCR tubes or into separate 200µL capacity wells of a 96-well plate. 2.5µL of crude extract sample *C. difficile* DNA was then individually added to the master mix. Positive and negative controls were included for each PCR run (Table 3). 2 positive control strains were included in MP1 while 3 controls strains were included in MP2 (Table 4). Distilled water (Qiagen, Germany) was used as a negative control for both MP1 and MP2.

Tube lids were closed, and plates were sealed with a Microseal B PCR Plate Sealing Film (Bio-Rad, United States) before being centrifuged for 5 seconds at 1,278 x g to remove bubbles.

The samples were placed into a thermocycler and the *C. difficile* multiplex PCR cycle was started (Table 4). The samples were processed by conventional gel electrophoresis following *C. difficile* multiplex PCR.

A stock culture of isolates that were determined to be *C. difficile* by *C. difficile* multiplex PCR were frozen in CryoBank cryobeads (Copan Diagnostics, United States) at -80°C until required.

Table 1. *C. difficile* Multiplex PCR Master Mix Composition

Multiplex	PCR Mix Ingredients	Volume	Description
MP1[1]	2X Taq PCR Master Mix (Qiagen)	12.5 µL	*Taq* DNA Polymerase + dNTPs
	cdtB-F1/R1 (10µM)	0.75 µL	*cdtB* Primer
	tcdA-F/A3B (10µM)	0.75 µL	*tcdA* Primer
	tcdB-3/4 (10µM)	0.5 µL	*tcdB* Primer
	ddH_2O	8.5 µL	Filler
MP2[2]	2X Taq PCR Master Mix (Qiagen)	12.5 µL	*Taq* DNA Polymerase + dNTPs
	pal-15/16 (10µM)	0.5 µL	*tcdC* Primer
	tpi-F/R (10µM)	0.5 µL	*tpi* Primer
	ddH_2O	9.5 µL	Filler

[1] MP1: Multiplex 1

[2] MP2: Multiplex 2

Table 2. PCR Primers Utilized in *C. difficile* Multiplex 1 and *C. difficile* Multiplex 2

Multiplex	Primer	Sequence (5'-3')	Size	Target Gene
MP1[1]	*cdtB*-F1	TGGACAGGAAGAATAATTCCTTC	23bp	*cdtB*
	cdtB-R1	TGCAACTAACGGATCTCTTGC	21bp	(Binary toxin subunit B)
	tcdA-F	AGATTCCTATATTTACATGACAATAT	26bp	*tcdA*
	A3B	ACCATCAATCTCGAAAAGTCCAC	26bp	(*C. difficile* toxin A)
	tcdB-3	AATGCATTTTTGATAAACACATTG	24bp	*tcdB*
	tcdB-4	AAGTTTCTAACATCATTTCCAC	22bp	(*C. difficile* toxin B)
MP2[2]	*paL*-15	TCTCTACAGCTATCCCTGGT	20bp	*tcdC*
	paL-16	AAAAATGAGGGTAACGAATTT	21bp	(PaLoc negative regulator)
	tpi-F	AAAGAAGCTACTAAGGGTACAAA	23bp	*tpi*
	tpi-R	CATAATATTGGGTCTATTCCTAC	23bp	(Triose phosphate isomerase)

[1] MP1: Multiplex 1

[2] MP2: Multiplex 2

Table 3. *C. difficile* Multiplex PCR Control Profiles

Control Strain	Multiplex	Target Gene	Result Profile[1]
11ACD0075 NAP1	MP1[2]	*cdtB*	Positive
		tcdA	Positive (420bp)
		tcdB	Positive
	MP2[3]	*tcdC*	Positive (18bp Deletion)
		tpi	Positive
N07-01533	MP1[2]	*cdtB*	Negative
		tcdA	Positive (150bp)
		tcdB	Positive
	MP2[3]	*tcdC*	Positive (No Deletion)
		tpi	Positive
11ACD0028 NAP7	MP2[3]	*tcdC*	Positive (>18bp Deletion)
		tpi	Positive
Distilled Water	MP1[2]	N/A	Negative Control
	MP2[3]	N/A	Negative Control

[1] Profile defined as the expected result for the target. PCR products are only produced by a positive profile

[2] MP1: Multiplex 1

[3] MP2: Multiplex 2

[4] N/A: Non-Applicable

Table 4. *C. difficile* Multiplex PCR Cycle Conditions

Number of Cycles	Cycle Temperature	Cycle Duration	Purpose of Cycle
1	95°C	15 Minutes	Initial Denaturization
30	94°C	30 Seconds	Denaturization
	57°C	90 Seconds	Annealing
	72°C	60 Seconds	Extension
1	72°C	7 Minutes	Final Extension
1	4°C	Unlimited	Storage

2.2.3. Gel Electrophoresis of *C. difficile* PCR Amplified Gene Products

The PCR amplified gene products from section 2.2.2 were visualized by gel electrophoresis. The Owl gel electrophoresis system (ThermoFisher, United States) was prepared by filling with 0.5X Tris/Borate/ethylenediaminetetraacetic acid (TBE) (Sigma-Aldrich, United States) to the fill line. 1.5% weight/volume agarose gels were made by combining 100mL of 0.5X TBE (Sigma-Aldrich, United States) and 1.5g of Froggarose LE (Froggabio, Canada) in a 500mL bottle. The mixture was then microwaved for 75 seconds, swirled to homogenize, and poured into a gel mould. A 20 well comb was then inserted into the before the gel was allowed to cool and solidify at room temperature.

The well comb was removed from the solidified gel and the gel was removed from the mould. The gel was placed into the gel electrophoresis system. The samples were centrifuged for 5 seconds at 1,278 x g before the plate seal was removed from the 96-well plate. Approximately 2µL of gel loading dye, purple 6X (New England BioLabs, United States) was added to each PCR product and homogenized by pipetting. 10µL of PCR product, either MP1 or MP2, was loaded into a well on the gel. 10µL of 100 base pair ladder (New England BioLabs, United States) was added to each well flanking the PCR products. The power supply was set to 8 V/cm, 60 minutes at 120V for MP1 and 80 minutes at 120V for MP2.

2.2.4. Visualization of Gel Electrophoresis Separated DNA Products

Gel Red was prepared by combining 150µL of 10,000X Gel Red (Biotium, United States) with 500µL of dH_2O. The separated PCR products were stained in the diluted Gel Red for 30 minutes on an automatic tilting plate. The gel was removed form the Gel Red and UV imaged at 302nm and at 365nm on an Azure C200 gel imaging system (Azure Biosystems, United States). Automatic colour correction was performed on the imager before the imaged was saved digitally and printed (Figure 5).

2.3. *C. difficile* Molecular Typing

Positive confirmed *C. difficile* isolates from retail meats were further characterized by PCR ribotyping and pulsed-field gel electrophoresis (PFGE).

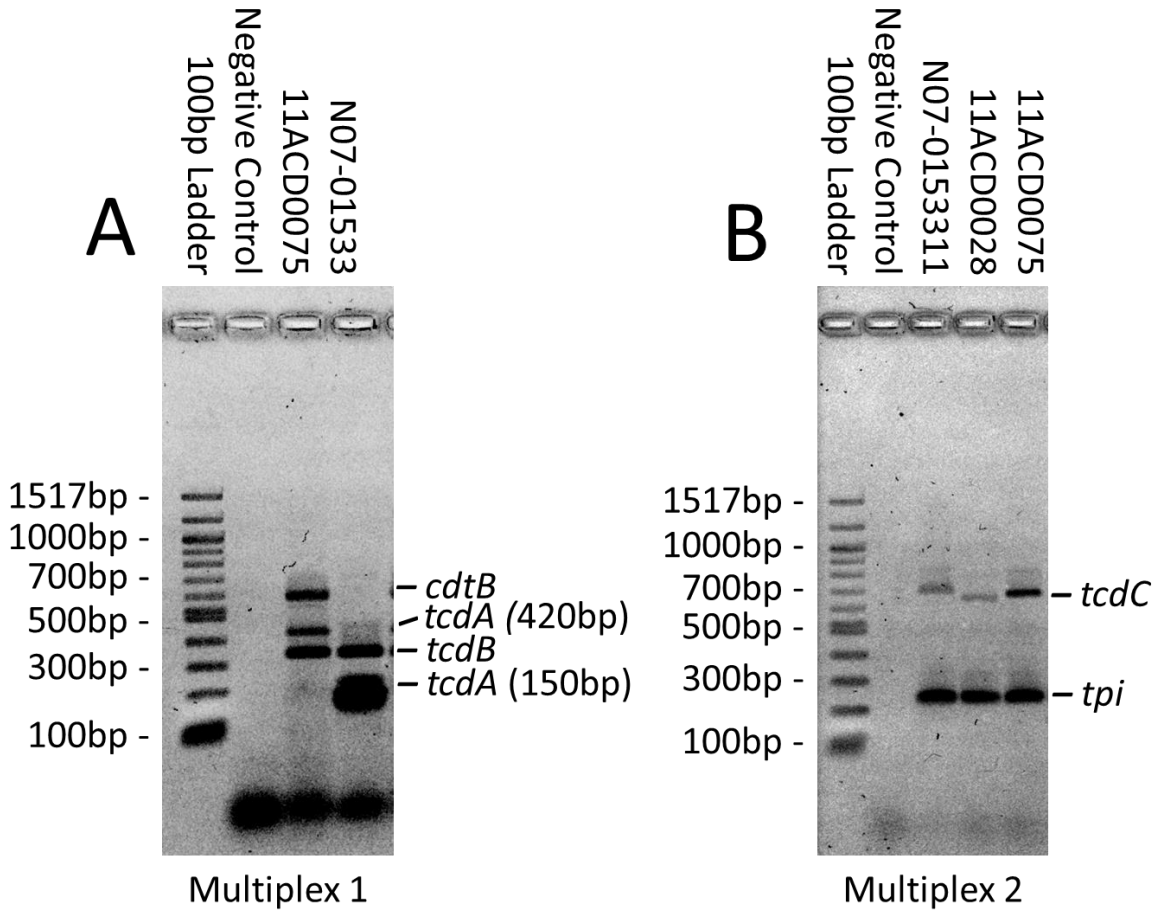


Figure 5. Digital image of *C. difficile* multiplex PCR MP1 and MP2 gels. A) *C. difficile* multiplex 1 PCR (MP1). *cdtB* encodes for binary toxin; *tcdA* encodes for *C. difficile* toxin A; and *tcdB* encodes for *C. difficile* toxin B. ddH_2O used as sample for the negative control. B) *C. difficile* multiplex 2 PCR (MP2). *tcdC* encodes for PaLoc's negative regulator and *tpi* encodes for triose phosphate isomerase. ddH_2O used as sample for the negative control.

2.3.1. PCR Ribotyping

PCR ribotyping, based on the 16S-23S intergenic spacer region of ribosomal deoxyribonucleic acid (rDNA), is completed by first amplifying the genes by PCR [139]. The amplified genes are then sequenced and analyzed.

C. difficile isolates were first spread on BRU agar and incubated anaerobically at 37°C for 24-48 hours. A 1µL loop full of *C. difficile*, from the BRU agar plate, was resuspended into 200µL of InstaGene Matrix (Bio-Rad, United States) in a 1.5mL tube. The culture was homogenized via vortexing for 5 seconds at maximum speed. The mixture was then heated on a heat block at 95°C for 30 minutes to lyse the cells. The tubes were then centrifuged at 16,000 x g for 2 minutes to pellet the cellular debris.

C. difficile ribotyping primers (Table 5) and PCR master mix (Table 6) was defrosted at room temperature. Once defrosted, 24.5µL of the *C. difficile* ribotyping PCR master mix was dispensed for each sample into the wells of a 96-well plate or into 200µL PCR tubes. 0.5µL of template DNA (supernatant) was then added to the master mix and mixed by pipetting up and down. The plates were sealed with a Microseal B PCR Plate Sealing Film (Bio-Rad, United States) before being centrifuged for 5 seconds at 1,278 x g to remove bubbles.

The samples were placed into a thermocycler and the *C. difficile* ribotyping PCR cycle was started (Table 7). The samples were processed by capillary electrophoresis following *C. difficile* ribotyping PCR.

The PCR products were analyzed by capillary electrophoresis. The samples were centrifuged for 5 seconds at 1,278 x g and the plate seal removed from the 96-well plate. 9µL of *C. difficile* ribotyping capillary gel electrophoresis master mix (Table 8) was added to wells of new 96-well plate in columns. An even number of columns, with a minimum of 2 columns, of a 96-well plate is required by the 3130XL Genetic Analyzer (Applied Biosystems). If less than 16 samples were part of a given run, wells contained duplicated samples or were filled with 10µL of water to ensure the condition was met. 1µL of amplified PCR product was added to the wells of the 96-well plate and mixed by pipetting up and down. The plate was centrifuged for 5 seconds at 1,278 x g to remove bubbles. Samples were denatured at 95°C for 2 minutes in the thermocycler and immediately placed on a metal cooling block.

Table 5. *C. difficile* Ribotyping PCR Primers

Primer	Sequence (5'-3')	Size	Gene Target
16S-FAM Forward	GTGCGGCTGGATCACCTCCT	20bp	16S
23S Reverse	CCC TGCACCCTTAATAACTTGACC	24bp	23S

Table 6. *C. difficile* Ribotyping PCR Master Mix Composition

Ingredient	Volume	Purpose
HotStar Taq Master Mix[1]	12.5 µL	HotStarTaq DNA Polymerase + dNTPs
16S-FAM Forward (10µM)	0.5 µL	16S Forward Primer
23S Reverse (10µM)	0.5 µL	23S Reverse Primer
H_2O	11.0 µL	Filler

[1] Qiagen, Germany

Table 7. *C. difficile* Ribotyping PCR Cycle Conditions

Cycles	Temperature	Time	Purpose
1	95°C	15 Minutes	Initial Denaturization
24	95°C	60 Seconds	Denaturization
	57°C	60 Seconds	Annealing
	72°C	60 Seconds	Extension
1	72°C	30 Minutes	Final Extension
1	4°C	Unlimited	Storage

Table 8. *C. difficile* Ribotyping Capillary Gel Electrophoresis Master Mix Composition

Ingredient	Volume	Purpose
Hi-Di Formamide[1]	8.5 µL	Samples Resuspension
GeneScan 1200 LIZ	0.5 µL	Size Standard

[1] Applied Biosystems, United States

The plate was then loaded into a 3130XL Genetic Analyzer (Applied Biosystems) according to the instructions for the sequencer. Sample injection was set to 5kV for 5 seconds. Run time was set to 103 minutes with 6.5kV separation.

The raw data produced by the 3130XL Genetic Analyzer (Applied Biosystems) was exported as a ".fsa" file. This file was processed by gating all PCR fragments to within the size standard of GeneScan 1200 LIZ; only samples of interest were included. Furthermore, only the blue channel was selected. The resulting sizing table, containing all samples of interest, was then exported for analysis.

The sizing table was imported into Bionumerics version 7.6.3 (Applied Maths, Belgium) for analysis. A digital gel image was produced by the software to visualize the PCR products. Although band calling was automatically performed by the software, bands were manually adjusted if needed. The banding pattern was comparted to known ribotype banding patterns in our database. Depending on the corresponding banding pattern, size and quantity, the isolate was either assigned a standard ribotype or considered a "No Standard" (NS) ribotype.

2.3.2. Pulsed-Field Gel Electrophoresis (PFGE)

We utilized pulsed-field gel electrophoresis (PFGE) typing based off the *Sma*1 digestion pattern of *C. difficile* [140].

C. difficile isolates were first spread on BRU agar from a defrosted CryoBank bead (Copan Diagnostics, United States) and incubated anaerobically at 37°C for 24-48 hours. Per sample, 5mL of brain heart infusion (BHI) broth in 13mm screw top test tubes were anaerobically reduced at 37°C for 24-48 hours. Post incubation of the BRU agar plates, half of a 10µL loop of culture was taken from the plate and inoculated into the BHI broth. The inoculated broth was then incubated anaerobically at 37°C for 6-8 hours.

In a 250mL bottle, 0.5g of SeaKem Gold Agarose (Lonza Bioscience, Switzerland), 5mL of 10% sodium dodecyl sulfate (SDS) (Sigma Aldrich, United States), and 45mL of 1X Tris-Ethylenediaminetetraacetic Acid (TRIS-EDTA) buffer (Sigma Aldrich, United States) was combined. The mixture was microwaved for 20 seconds and swirled gently to mix. The mixture was kept in a 67°C bead bath (Lab Armor, United States) to keep the agarose liquid.

400µL of the BHI broth culture was transferred into a 1.5mL tube. The culture was centrifuged at 21,100 x g for 2 minutes to pelletize the contents. The supernatant was then aspirated and discarded. The pellet was then resuspended in 150µL of *C. difficile* lysis buffer (Table 9). 150µL of liquid SeaKem Gold Agarose was then added to each suspension and mixed by pipetting up and down. The mixture was then dispensed evenly between 2 disposable plug moulds (BioRad, United States). The plugs were then incubated at room temperature until the plugs solidified.

For each sample, 1mL of *C. difficile* lysis buffer (Table 9) was dispensed into a 1.5mL tube. RNase A (Qiagen, Germany), lysozyme, and mutanolysin were added to each lysis buffer tube (Table 10). Corresponding agarose plug pairs were then placed into a lysis tube and incubated overnight (~18 hours) in a 37°C bead bath (Lab Armor, United States).

Following incubation, all lysis buffer was aspirated from the tubes and discarded. 1mL of PK buffer and 10µL of PKA (20mg/ml) were then dispensed into each plug containing tube and mixed via pipetting up and down (Table 11). The plug containing tubes were then incubated for 2 hours in a 55°C bead bath (Lab Armor, United States). Additional incubation time was given to plugs that were not clear after the initial 2-hour incubation.

Following incubation, all PK buffer was aspirated from the tubes and discarded. Plug pairs were rinsed with wash buffer (Table 12) prior to being washed 4 times each. To rinse, 1mL of wash buffer was dispensed into each tube. The tubes were inverted 5 times before the wash buffer was aspirated out and discarded. To wash, 1mL of wash buffer was dispensed into each tube. The tubes were then placed on a shaking plate for 5 minutes before the wash buffer was aspirated out and discarded. The wash cycle was repeated 3 times but were kept on the shaking plate for 15 minutes each cycle.

C. difficile DNA captured in the agarose plugs was digested by *Sma*1. 1 of the 2 plugs per sample were cut into thirds using a scalpel blade. Each sample's plug thirds were then placed into a new 1.5mL tube with 150µL of 1X CutSmart buffer (New England Biolabs, United States). 1X CutSmart buffer was produced by diluting 1mL of 10X CutSmart buffer with 9mL of H_2O. Samples were allowed to equalize by incubating the tubes at room temperature for 10 minutes. The CutSmart buffer was then aspirated and discarded. 150µL of 1X CutSmart buffer and 3µL of *Sma*1

Table 9. *C. difficile* PFGE Cell Lysis Buffer Composition

Ingredient	Final Concentration
Tris-HCl pH 8.0	6mM
NaCl	1.0M
EDTA	100mM
Deoxycholate	0.2%
N-Laurylsarcosine	0.5%
Brij 58[1]	0.5%

[1] Sigma Aldrich, United States

Table 10. *C. difficile* PFGE Cell Lysis Enzymatic Mix Composition

Ingredient	Final Concentration
RNase	20µg/mL
Lysozyme	2.0mg/mL
Mutanolysin	12.5u/mL

Table 11. *C. difficile* PFGE PK Buffer Composition

Ingredient	Final Concentration
EDTA pH 8.0	500mM
N-Laurylsarcosine	1%

Table 12. *C. difficile* PFGE Wash Buffer Composition

Ingredient	Final Concentration
Tris-HCl pH8.0	10mM
EDTA	0.1mM

were then added to the plug thirds and mixed by pipetting up and down. The plug thirds were then incubated at 25°C for 2-3 hours.

1.0% weight/volume agarose gels were made by combining 1.5g of Pulsed Field Certified Agarose (BioRad, United States) with 150mL of 0.5X TBE (Tris/Borate/EDTA) (Sigma-Aldrich, United States) in a 500mL bottle. The mixture was then microwaved for 75 seconds and swirled to homogenize. Digested plugs were placed on the tip of a 15-well comb and placed into a PFGE casting mould. 2 control H9812 plugs were placed on comb teeth each flanking the *C. difficile* sample plugs. Additional control H9812 plugs were placed on comb teeth if more than 3 *C. difficile* sample plugs were in a row. The plugs were pushed down so that they were in contact with the metal bottom plate. Excess liquid was aspirated from the plugs and absorbed with a Kimwipe (Kimberly-Clark, United States). The liquid Pulsed-Field Certified Agarose was then carefully poured into the PFGE casting mould. The gel was incubated at room temperature until hardened. Once hardened, the well comb was carefully removed from the gel and the gel was removed from the casting mould.

The PFGE chamber was prepared by adding 2L of tris-borate-EDTA (Sigma Aldrich, United States) and 1mL of thiourea. The connected cooling module was turned on and set to 14°C. The gel was then placed into the chamber and the run started (Table 13).

Gel Red was prepared by combining 150µL of 10,000X Gel Red (Biotium, United States) with 500µL of dH_2O. The separated PCR products were then stained in the Gel Red for 30 minutes on an automatic tilting plate. The gel was then removed form the Gel Red and imaged at 302nm and at 365nm on an Azure C200 gel imaging system (Azure Biosystems, United States). Automatic colour correction was performed on the imager before the imaged was saved digitally and printed.

The digital gel image was imported into BioNumerics version 7.6.3 (Applied Maths, Belgium) for analysis. Although band calling was automatically performed by the software, bands were manually adjusted if needed. The banding pattern was then comparted to known NAP type banding patterns. Depending on the corresponding banding pattern, size and quantity, the isolate was either assigned a standard NAP type or considered a non-NAP type.

Table 13. *C. difficile* PFGE Electrophoresis Run Conditions

Condition	Setting
Initial Switch Time	1 Second
Final Switch Time	40 Seconds
Voltage	6 V/cm
Included Angle	120°
Run Time	21 Hours

Salmonella enterica, serotype Braenderup H9812, was used as a positive control and marker for *C. difficile* pulsed-field gel electrophoresis. H9812 control plugs were produced in bulk and stored at 4°C until needed. The H9812 control plugs followed the same procedure as the *C. difficile* samples with a few modifications. The initial growth was performed on Trypticase soy agar (TSA) plates instead of on BRU, and the incubations occurred aerobically instead of anaerobically. Additionally, the digestion of H9812's DNA in the agarose plugs was performed by 1.5µL of *Xba*1 instead of 3µL *Sma*1, and incubated 37°C instead of at 25°C.

2.4. Antimicrobial Susceptibility Testing (AST)

Antimicrobial susceptibility testing (AST) was performed the use of ETEST® strips (bioMérieux, France) following manufacturer instructions. Breakpoints from The Clinical and Laboratory Standards Institute's (CLSI) M100 and Canadian Nosocomial Infection Surveillance Program (CNISP) were used for interpretation [141]. The minimum inhibitory concentration (MIC) of the isolates to clindamycin, metronidazole, moxifloxacin, rifampin, tigecycline, and vancomycin were assessed.

C. difficile was first spread on BRU agar and incubated anaerobically at 37°C for 24-48 hours. The preparation of the AST plates was performed under anaerobic conditions. 1mL of brucella broth was dispensed into a glass test tube. A sterile wooden dowel was used to inoculate the *C. difficile* samples into the brucella broth from the BRU plates. The tube was then vortexed and compared to a 1.0 McFarland unit turbidity standard. Depending on the turbidity, brucella broth was added with a dropper or more culture was added with the dowel to alter the turbidity of the suspension. Once a 1.0 McFarland unit turbidity was achieved, a sterile cotton swab was dipped into the suspension. Excess liquid was then removed from the cotton swab by pressing it against the side of the tube. A 15mm x 150mm brucella blood agar (BRU) plate with 5% v/v laked sheep blood and vitamin K was the placed on a plate spinner. With the agar plate spinning, the cotton swab was spread from edge to center twice over the surface the agar plates.

ETEST® strips (bioMérieux, France) were placed evenly spaced on the agar by a Simplex C76 (Biomérieux, France). The plates were then incubated anaerobically at 37°C for 48 hours. The MIC breakpoint for any given antimicrobial was determined to at where the zone of inhibition

intersected the ETEST® strip. MIC breakpoints were then compared to those established by CLSI's M100 and CNISP (Table 14).

2.4.1. Antimicrobial Susceptibility Testing Control Strain Resistance Profiles

ATCC 700057 (*C. difficile*) and ATCC 25285 (*Bacteroides fragilis*) were used as controls of for antimicrobial susceptibility testing. The test was validated if the control strains' MICs fell within CLSI's established acceptable MIC range (Table 15). ATCC 700057 was only tested against vancomycin. ATCC 25285 was tested against clindamycin, metronidazole, moxifloxacin, rifampin, tigecycline, and vancomycin.

2.5. Human Clinical Case Isolate Selection

Human clinical case isolates were selected based upon case characteristics. Isolates were selected if they were from between 2016 and 2018; from Alberta, British Columbia, or Ontario; and were of matching ribotype, PF type, or NAP type as any of the *C. difficile* isolates from retail meats.

2.6. *C. difficile Genomic* DNA Extraction via Qiagen DNeasy Blood & Tissue Kit

Qiagen's DNeasy Blood & Tissue Kit (Qiagen, Germany) was used in accordance with the Gram-positive bacteria extraction protocol established by the manufacturer with some modifications. Upon comparison of several genomic DNA extraction methods and materials, Qiagen's DNeasy Blood & Tissue Kit was selected for the extraction of *C. difficile* genomic DNA (Appendix C). Qiagen's DNeasy Blood & Tissue Kit, when used with our modified protocol, extracted high quality genomic DNA from *C. difficile* isolates (Appendix C). Additionally, the extracted genomic DNA produced high quality assemblies once the genomic DNA was sequenced (Appendix C, Appendix D).

2.6.1. Bacterial Culture Growth

C. difficile isolates were first spread on BRU agar and incubated anaerobically at 37°C for 24-48 hours. Per sample, 5mL of brain heart infusion (BHI) broth in 13mm screw top test tubes

Table 14. Minimum Inhibitory Concentration (MIC) Breakpoints for *C. difficile*

	Susceptible (µg/mL)	**Intermediate (µg/mL)**	**Resistant (µg/mL)**
Clindamycin[1]	≤ 2	4	≥ 8
Metronidazole[1]	≤ 8	16	≥ 32
Moxifloxacin[1]	≤ 2	4	≥ 8
Rifampin[2]	≤ 1	2	≥ 4
Tigecycline[1]	≤ 4	8	≥ 16
Vancomycin[2]	≤ 4	8	≥ 32

[1] CLSI Breakpoint

[2] CNISP Breakpoint

Table 15. Acceptable Minimum Inhibitory Concentrations (MIC) in µg/mL of ATCC 700057 and ATCC 25285

	ATCC 700057[1]	**ATCC 25285[2]**
Clindamycin	N/A	0.5 – 2.0
Metronidazole	N/A	0.25 – 1.0
Moxifloxacin	N/A	0.125 – 0.5[3]
Rifampin	N/A	0.064 – 0.094
Tigecycline	N/A	0.12 – 1.0[3]
Vancomycin	0.5 – 4.0	24 – 32

[1] ATCC 7000571: *Clostridioides difficile*

[2] ATCC 25285: *Bacteroides fragilis*

[3] Non-CLSI established breakpoint. CNISP established breakpoint utilized.

were anaerobically reduced. Anerobic reduction occurred at 37°C for 24-48 hours. Post incubation of the BRU agar plates, half of a 10µL loop of culture was taken from the plate and inoculated into the BHI broth. The inoculated broth was then incubated for anaerobically at 37°C for 18 hours.

2.6.2. Vegetative *C. difficile* Cell Lysis

Culture tubes were vortexed to mix before 1mL of culture was transferred into a sterile 96 deep-well block. The deep-well block was sealed with a Microseal B PCR Plate Sealing Film (Bio-Rad, United States) and centrifuged at 1,278 x g for 10 minutes to pelletize the contents. The deep-well block was then unsealed. The supernatant was aspirated and discarded. The remaining pellets were then resuspended in 180µL of *C. difficile* lysis buffer (Table 16). The deep-well block was resealed and incubated for 60 minutes in a 37°C bead bath (Lab Armor, United States). The deep-well block was unsealed and 5µL of 100mg/mL RNase A (Qiagen, Germany) and 25µL of protein kinase A (Qiagen, Germany) was then added to each sample. Each culture was homogenized by pipetting up and down. The deep-well block was resealed and incubated for 60 minutes in a 56°C bead bath (Lab Armor, United States). The deep-well block was unsealed and 200µL of buffer AL (Qiagen, Germany) was mixed into each sample. The deep-well block was resealed and incubated for 60 minutes in a 56°C bead bath (Lab Armor, United States). The deep-well block was unsealed and 200µL of anhydrous undenatured ethanol (Greenfield Global, Canada) was mixed into each sample.

2.6.3. DNA Purification and Elution

The contents of each well were then transferred into a 96-well DNeasy filter block (Qiagen, Germany) placed on a deep well block. The plate was sealed with an Airpore tape sheet (Qiagen, Germany) and centrifuged in a heated centrifuge set at 37°C for 10 minutes at 3720 x g. The waste collected in the deep well block was discarded. The plate was unsealed to add 500µL of buffer AW1 with ethanol (Qiagen, Germany) to each sample. The plate was resealed and centrifuged in a heated centrifuge set at 37°C for 5 minutes at 3720 x g. The waste collected in the deep well block was discarded. The plate was unsealed to add 500µL of buffer AW2 with

Table 16. *C. difficile* Lysis Buffer for Genomic DNA Extraction

Ingredient	Concentration
TRIS	20mM
EDTA	2mM
Triton X	1.2%
Lysozyme From Chicken Egg White[1]	N/A

[1] Sigma-Aldrich, United States

ethanol (Qiagen, Germany) to each sample. Without resealing, the plate was centrifuged in a heated centrifuge set at 37°C for 15 minutes at 3720 x g. The waste collected in the deep well block was discarded. The plate was then transferred onto racked elution microtubes (Qiagen, Germany). 55µL of 37°C 10mM TRIS-HCl was then added to each sample. The plate was resealed and centrifuged in a heated centrifuge set at 37°C for 2 minutes at 3720 x g. The collected DNA elution was transferred to a sterile 96-well plate. The sample plate was unsealed and the elution process was repeated. 55µL of 37°C 10mM TRIS-HCl was added to each sample, the plate was resealed and centrifuged in a heated centrifuge set at 37°C for 2 minutes at 3720 x g. The collected DNA elution was transferred to the previous elution and the 96-well plate was sealed with an aluminium plate seal (Axygen Scientific, United States). The genomic DNA was stored at 4°C until its quality was assessed (Chapter 2.7).

2.7. Genomic DNA Quality Assessment

Genomic *C. difficile* DNA was quantified and assessed by Qubit (Invitrogen, United States), gel electrophoresis, and Tapestation (Agilent, United States).

2.7.1.1. Qubit Fluorometric Genomic DNA Quantification

Genomic DNA concentrations of all *C. difficile* isolates were determined by Invitrogen's Qubit dsDNA broad range quantification kit (Invitrogen, United States) per the manufacturer's instructions. A stock solution of buffer and dye was first created and vortexed to mix. The stock solution contained 199µL of buffer and 1µL of dye per sample, plus an additional 398µL of buffer and 2µL of dye. For the standards, 190µL of stock solution was dispenses in 2 Qubit tubes (Invitrogen, United States) each. 198µL of stock solution was dispenses into a Qubit tube for each sample. 10µL of each standard was then dispensed into their respective standard tube. Genomic DNA samples were then centrifuged for 5 seconds at 1,278 x g and the plate seal removed from the 96-well plate. 2µL of genomic DNA were added to their respective Qubit tube. The Qubit tubes were then vortexed for 3 minutes each to mix and incubated for 2 minutes at room temperature.

The Qubit reader was calibrated using the 2 standard tubes. Each sample tube was then analyzed. The DNA concentration for each sample was recorded in ng/µL.

2.7.1.2. Gel Electrophoresis of Genomic DNA

The Owl gel electrophoresis system (ThermoFisher, United States) was prepared by filling with 0.5X (Tris/Borate/EDTA (TBE) (Sigma-Aldrich, United States) to the fill line. 0.8% agarose gels were made by combining 100mL of 0.5X TBE (Sigma-Aldrich, United States)) and 0.8g of Froggarose LE (Froggabio, Canada) in a 500mL bottle. The mixture was then microwaved for 75 seconds, swirled to homogenize, and poured into a gel mould. A 20 well comb was then inserted into the before the gel was allowed to cool and solidify at room temperature.

The genomic DNA was visualized by gel electrophoresis. The well comb was removed from the solidified gel and the gel was removed from the mould. The gel was then placed into the gel electrophoresis system. A stock solution of 6µL dH_2O and 2µL of gel loading dye, purple 6X (New England BioLabs, United States) per sample was created. 8µL of the solution was dispensed into sterile PCR tubes. The samples were then centrifuged for 5 seconds at 1,278 x g and the plate seal removed from the 96-well plate. Approximately 2µL of genomic DNA was transferred to the PCR tubes and homogenized by pipetting. The entire contents of the tube was then loaded into a well on the gel. 4µL of 1,000 base pair ladder (New England BioLabs, United States) was added to each well flanking the genomic DNA. The power supply was set to 120 minutes at 80V. View Chapter 2.2.4 for methods pertaining to the visualization of gel electrophoresis separated products.

2.7.1.3. Tapestation Genomic DNA Quality Assessment

Agilent's 4150 Tapestation (Agilent, United States) was used to analyze all genomic *C. difficile* DNA. Stored at 4°C, Agilent Genomic DNA ScreenTapes (Agilent, United States) were incubated at room temperature for 30 minutes. Each lane of the Screentape was then checked for bubbles. If bubbles were present in lanes, the Screentape was flicked multiple times. The tape was then inserted into the machine. The ladder and samples were then added to a tube strip. The one ladder well contained 1µL of ladder and 8µL of buffer. Sample wells contained 2µL of

sample genomic DNA and 6µL of buffer. The tube strip was then quickly centrifuged to eliminate any air bubbles before being loaded into the machine. Using Agilent TapeStation software (Agilent, United States), the ladder and samples were identified. With the analysis run setup complete, the Tapestation analysis was commenced.

2.8. Illumina NextSeq Whole Genome Sequencing

All Genomic DNA sequencing was performed by the National Microbiology Laboratory's DNA core using Illumina NextSeq (Illumina, United States). Library preparation was performed using Nextera XT (Illumina, United States). Size selection was then performed to gate between 600 to 1,000 base-pairs on a BluePippin (Sage Science, United States). The DNA sample was then cleaned up with PCRClean DX (Aline Bioscience, United States). Library quantification was performed using a QuBit dsDNA High-Sensitivity (Invitrogen, United States) kit. Average DNA fragment size was determined by running the sample on a Tapestation (Agilent, United States). Finally, the sample was sequenced using a 300 cycle Mid Output Kit (Illumina, United States) for 150 base-pair paired end reads on a NextSeq 500/550 (Illumina, United States).

Gating the library between 300 to 1,000 base pairs was also evaluated (Appendix E). To evaluate the differences between gating the library between 600 to 1,000 base-pair and 300 to 1,000 base pairs, a *C. difficile* DNA elution was split into 2 aliquots. One aliquot was sequenced using a 600 to 1,000 base-pair library, while the other aliquot was sequenced using a 300 to 1,000 base-pair library (Appendix E). DNA sequence files were uploaded onto the NML's server for storage until use. The quality of DNA sequences was analyzed based upon its depth of coverage and uniformity. Coverage uniformity was analyzed at a threshold of ≥40X and of ≥50X coverage.

2.8.1. Short Read DNA Sequence Assembly

The short read DNA sequencing file collections, obtained from Illumina NextSeq (Illumina, United States) whole genome sequencing, were imported into Galaxy from the National Microbiology Laboratory (Winnipeg, Canada) database via IRIDA. *De novo* assemblies of the short reads of all isolates were performed using Shovill (Galaxy Version 1.0.4). Genomes

assembly was set to require a minimum contig length of 300 base-pairs and a minimum contig coverage of 2.

Assembly quality was determined by analyzing the DNA sequence files with FastQC (Galaxy Version 1.0.0) and Panel Coverage Report (Galaxy Version 0.0.4). Genome uniformity was determined by QualiMap BamQC. DNA whose DNA short read sequence files produced poor quality assemblies with excessive contigs (>350) were discarded and repeated with freshly extracted DNA.

2.8.2. Single Nucleotide Variant (SNV) Analysis

The short read DNA sequencing file collections were imported into Galaxy from the National Microbiology Laboratory's (Winnipeg, Canada) database via IRIDA. SNV analysis was performed on all isolates using the SNVPhyl pipeline V1.1 [142]. The percentage of core genome included in the analysis and quantity of SNVs were obtained by extracting raw data from the pipeline analysis.

2.8.3. Phylogenetic Tree Building

The ".phylip" SNV alignment file was extracted from each SNV analysis. The file was run through PhyML 3.0, set to default parameters with the addition of 1,000 standard bootstrap replicates, to generate a phylogenetic tree [143]. Phylogenetic trees were visualized and edited in PRESTO [143]. Linear dendrograms were created with isolates possessing the most branches at the bottom. Branches corresponding to *C. difficile* isolates from retail meat samples were highlighted in red while branches corresponding to *C. difficile* isolates from human CDI cases were left in black (View Figures 10 – 19).

CHAPTER 3: RESULTS

3.1. Retail Meat Sample Collection

In total, 644 retail meat samples were analyzed for *C. difficile* contamination. Fresh retail meat samples were sourced between 2016 and 2018 from Alberta, British Columbia, and Ontario (Table 17). 179 retail meat samples were sourced from Alberta and were comprised of 140 pig samples, 12 beef samples, and 27 veal samples. 218 retail meat samples were sourced from British Columbia and were comprised of 164 pig samples, 6 beef samples, and 48 veal samples. 247 retail meat samples were sourced from Ontario and were comprised of 196 pig samples, 10 beef samples, and 41 veal samples. Retail meat samples were skewed towards pig samples as pigs are known to commonly be asymptomatically infected by *C. difficile* [133].

3.2. Isolation of *C. difficile* from Retail Meats

Overall, *C. difficile* was isolated from 10/644 (1.6%) available retail meat samples (Table 18). Of the 644 samples, no *C. difficile* isolates were obtained from 28 beef samples, 4 isolates (3.4%) were obtained from 116 veal samples, and 6 (1.2%) isolates were identified from 500 pork samples (Table 19). All 10 *C. difficile* isolates were recovered by enrichment broth. Additionally, 2 *C. difficile* isolates, N20-00231 and N20-00441, were also recovered by direct plating. Retail meat samples contaminated with *C. difficile* were collected from across Canada. 3 positive samples came from Alberta, 2 positive samples came from British Columbia, and 5 positive samples came from Ontario (Table 18). The limit of detection of the isolation of *C. difficile* from retail meat samples, from both direct plating and by enrichment broth, was determined to be 7.5 spores/mL (Appendix B). The ethanol shock and centrifugation aspects of this isolation *of C. difficile* from retail meat sample protocol were also validated with no concerns (Appendix B).

3.3. Multiplex PCR of *C. difficile* Isolates from Retail Meat Samples

All 10 *C. difficile* isolates were toxin A and B positive determined by *C. difficile* multiplex PCR (Table 20, Figure 6, Figure 7). Additionally, 2 of the 10 isolates, 1 veal sample from Ontario and 1 pork sample from Alberta, were binary toxin positive.

Table 17. Retail Meat Sample Commodity Type, Province of Collection, and Year of Collection

		Pig	Beef	Veal	Total
Alberta	2016	56	0	0	56
	2017	0	12	27	39
	2018	84	0	0	84
	Total	**140**	**12**	**27**	**179**
British Columbia	2016	48	0	0	48
	2017	0	6	48	54
	2018	116	0	0	116
	Total	**164**	**6**	**48**	**218**
Ontario	2016	114	0	0	114
	2017	0	10	41	51
	2018	82	0	0	82
	Total	**196**	**10**	**41**	**247**
		500	**28**	**116**	**644**

Table 18. *C. difficile* Isolates from Retail Meats

Isolate Number	Sample Number	Year	Commodity	Processing	Province	Isolation Method
N20-00231	CE-R2-17-0239	2017	Veal	Nuggets	ON	Direct Plating + Broth
N20-00438	CE-R2-17-0120	2017	Veal	Red Veal	ON	Broth
N20-00439	CE2-R2-17-3130	2017	Veal	Cutlet	BC	Broth
N20-00440	CE2-R2-17-2054	2017	Veal	Ground	BC	Broth
N20-00441	CE-R2-18-0102	2018	Pig	Sausage	ON	Direct Plating + Broth
N20-00442	CE-R2-18-0014	2018	Pig	Sausage	ON	Broth
N20-00443	CE3-R2-18-4030	2018	Pig	Sausage	AB	Broth
N20-02756	CE-R2-16-0258	2016	Pig	Ground	ON	Broth
N20-03010	CE3-R2-16-5048	2016	Pig	Ground	AB	Broth
N21-00109	CE3-R2-16-5061	2016	Pig	Ground	AB	Broth

Table 19. Quantity of Retail Meat Commodity Types where *C. difficile* was Recovered in Respect to Sampling Origin

	Beef	**Pork**	**Veal**	**Total**
Alberta	0/12 (0.0%)	3/140 (2.2%)	0/27 (0.0%)	3/179 (1.7%)
British Columbia	0/6 (0.0%)	0/164 (0.0%)	2/48 (4.2%)	2/218 (0.9%)
Ontario	0/10 (0.0%)	3/196 (1.5%)	2/41 (4.9%)	5/247 (2.0%)
Total	0/28 (0.0%)	6/500 (1.2%)	4/116 (3.5%)	10/644 (1.6%)

Positive samples / Total samples processed (Positivity rate %)

Table 20. Multiplex PCR Profiles of *C. difficile* Isolated from Retail Meats

Isolate Number	*cdtB*	*tcdA*	*tcdB*	*tcdC*	*tpi*
N20-00231	+	+ (420bp)	+	+ (18bp Deletion)	+
N20-00438	-	+ (420bp)	+	+	+
N20-00439	-	+ (420bp)	+	+	+
N20-00440	-	+ (420bp)	+	+	+
N20-00441	-	+ (420bp)	+	+	+
N20-00442	-	+ (420bp)	+	+	+
N20-00443	-	+ (420bp)	+	+	+
N20-02756	-	+ (420bp)	+	+	+

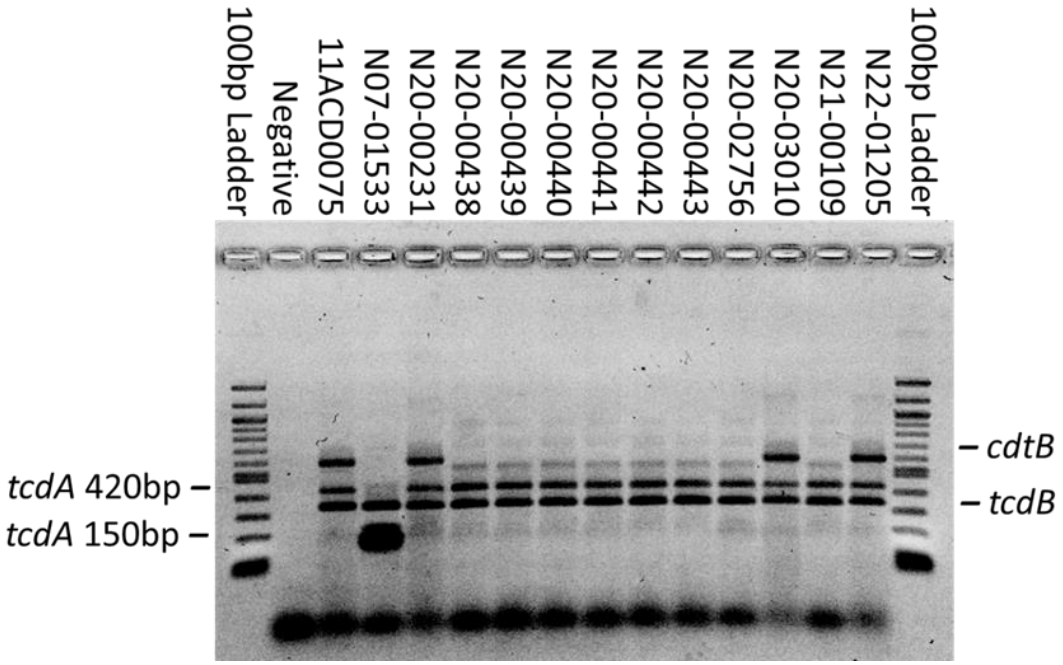


Figure 6. Image of Gel Electrophoresis separated *C. difficile* Multiplex 1 (MP1) PCR Products from *C. difficile* Isolated from Retail Meats

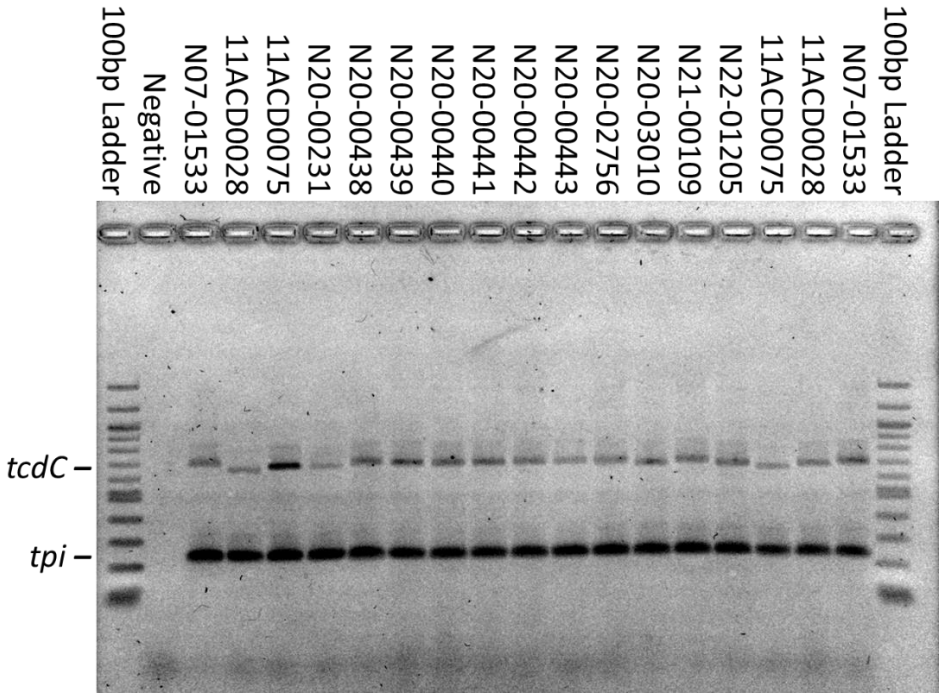


Figure 7. Image of Gel Electrophoresis separated *C. difficile* Multiplex 2 (MP2) PCR Products from *C. difficile* Isolated from Retail Meats

3.4. Ribotypes of *C. difficile* Isolates from Retail Meats

All *C. difficile* isolates from retail meat were ribotyped (Figure 8), which revealed 5 different ribotypes – 027 (n=1; pork), 106 (n=4; pork/veal), 131 (n=1; veal), NS110 (n=3; pork/veal), and NS195 (n=1; pork). All ribotypes isolated from retail meats have been previously identified in Canadian human clinical cases acquired through the Canadian Nosocomial Infection Surveillance Program (CNISP) [128].

3.5. Pulsed-Field Gel Electrophoresis Types of *C. difficile* Isolates from Retail Meats

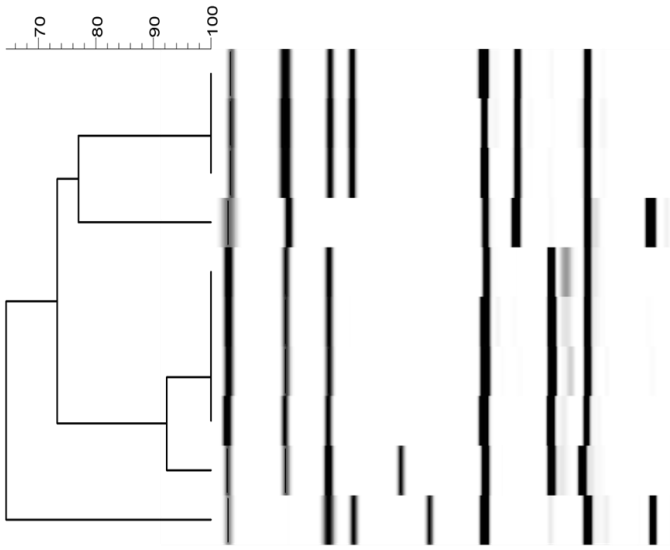
All *C. difficile* retail meat isolates were assigned NAP types (North American pulsed-field type) and PF types (pulsed-field type) by pulsed-field gel electrophoresis (Figure 9). PFGE analysis revealed 4 defined NAP types – NAP1 (n=1; pork), NAP4 (n=1; pork), NAP10 (n=1; veal), and NAP11 (n=4; pork/veal) (Figure 9). Additionally, there were 3 PF types that did not cluster within a pre-defined NAP type (Figure 9). Although N20-00439, N20-00440, and N20-00441 were designated as non-NAP types, their banding pattern were all indistinguishable (PF type 225). The banding pattern of N21-00109, N20-02756, and N20-00442 were indistinguishable and were all determined to be NAP11/PF1191. The banding pattern of N20-00438 was similar to that of N21-00109, N20-02756, and N20-00442 but contained an extra band. However, N20-00438 was still determined to be a NAP11 but of PF type 46.

3.6. Antimicrobial Susceptibility Profile of *C. difficile* Isolates from Retail Meats

All 10 *C. difficile* isolates from retail meats were susceptible to all tested antimicrobials apart from N20-03010 (Table 21). N20-03010 is a NAP1 (RT027) strain type and was found to be resistant to moxifloxacin with an MIC of >32 μg/mL (Table 21).

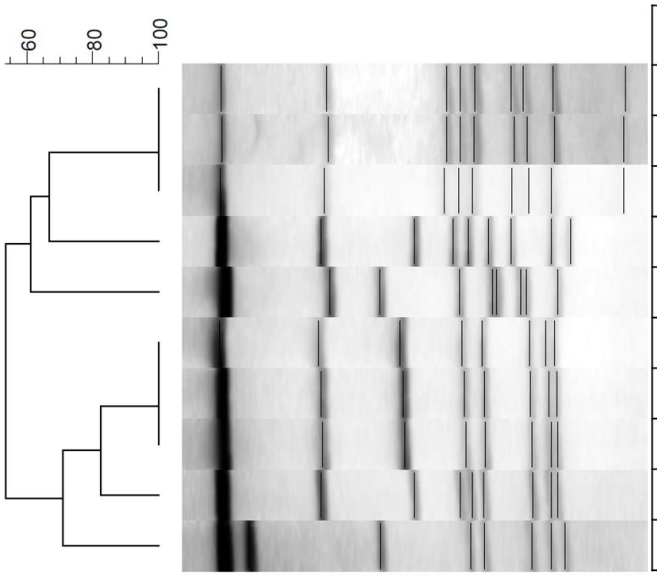
3.7. Human Clinical Case Isolate Selection

174 human clinical isolates were selected for whole genome sequencing and genomic comparison based on their year of infection, NAP type, and PFGE type (Table 22). Selection parameters of the human clinical *C. difficile* isolates were set based on matching characteristics to the *C. difficile* isolates from retail meat samples. Selected human clinical isolates were



Isolate	Year	Commodity	Province	NAP Type	Ribo Type
N20-00439	2017	Veal	BC	Non-Nap	NS110
N20-00440	2017	Veal	BC	Non-Nap	NS110
N20-00441	2017	Pork	ON	Non-Nap	NS110
N20-00443	2018	Pork	AB	NAP4	NS195
N20-00438	2017	Veal	ON	NAP11	106
N20-00442	2018	Pork	ON	NAP11	106
N20-02756	2016	Pork	ON	NAP11	106
N21-00109	2016	Pork	AB	NAP11	106
N20-00231	2017	Veal	ON	NAP10	131
N20-03010	2016	Pork	AB	NAP1	027

Figure 8. Ribotyping Dendrogram of *C. difficile* Isolates from Retail Meats



Isolate	Year	Commodity	Province	NAP Type	PF Type
N20-00439	2017	Veal	BC	Non-Nap	225
N20-00440	2017	Veal	BC	Non-Nap	225
N20-00441	2017	Pork	ON	Non-Nap	225
N20-03010	2016	Pork	AB	NAP1	1
N20-00443	2018	Pork	AB	NAP4	33
N21-00109	2016	Pork	AB	NAP11	1191
N20-02756	2016	Pork	ON	NAP11	1191
N20-00442	2018	Pork	ON	NAP11	1191
N20-00438	2017	Veal	ON	NAP11	46
N20-00231	2017	Veal	ON	NAP10	663

Figure 9. Pulsed-Field Gel Electrophoresis Dendrogram of *C. difficile* Isolates from Retail Meats

Table 21. Minimum Inhibitory Concentrations (MIC) in µg/mL of Clindamycin, Metronidazole, Moxifloxacin, Rifampin, Tigecycline, and Vancomycin on C. difficile Isolates from Retail Meats

	N20-00231	**N20-00438**	**N20-00439**	**N20-00440**	**N20-00441**	**N20-00442**	**N20-00443**	**N20-02756**	**N20-03010**	**N21-00109**
Clindamycin	0.5	0.75	0.5	0.38	0.5	0.5	0.38	0.75	1.0	1.0
Metronidazole	3.0	2.0	2.0	1.0	1.5	1.5	1.5	2.0	1.0	1.5
Moxifloxacin	0.5	0.5	0.75	0.75	0.5	0.5	0.38	0.75	0.38	0.5
Rifampin	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.002
Tigecycline	1.5	1.5	1.5	1.0	1.5	1.0	1.5	1.5	>32*	1.0
Vancomycin	0.094	0.094	0.094	0.125	0.125	0.125	0.094	0.125	0.094	0.064

* N20-03010 found to be resistant to moxifloxacin

Table 22. Quantity of C. difficile Isolates Selected for Whole Genome Sequencing and Genomic Comparison in Respect to NAP Type

	NAP1	**NAP4**	**NAP10**	**NAP11**	**Non-NAP**	**Total**
Human Isolates	37	32	6	96	3	174
Retail Meat Isolates	1	1	1	4	3	10

collected between 2015-2018 in Alberta, British Columbia, or Ontario, and determined to be a NAP1 (n=37), NAP4 (n=32), NAP10 (n=6), NAP11 (n=96), PFGE type 225 (n=2) (Table 22), or ribotype NS110 (n=1).

3.8. Genomic Comparison of *C. difficile* Isolates from Retail Meats and *C. difficile* Isolates from Human Clinical Cases

The genomes of *C. difficile* isolates from retail meat samples and human clinical cases were compared to establish relatedness. 10 *C. difficile* isolates from retail meats were analyzed against 174 select *C. difficile* isolates from human clinical cases (Figure 10 and Appendix A). N20-00231 served as the internal reference. Despite a low core genome percentage of 15.16% included in the analysis, the *C. difficile* isolates clustered into their respective NAP types. Analysis was conducted on each NAP type and their respective *C. difficile* isolate from each retail meat to increase the core genome coverage. Core genome was increased by between 69.38% and 78.14% at the highest analysis resolution. Maximizing core genome coverage increases the discriminatory power of the SNV analysis.

3.8.1. NAP1 *C. difficile* Isolates

One NAP1 *C. difficile* isolate (N20-03010) from retail meats was analyzed against 37 NAP1 *C. difficile* isolates from human clinical cases (Figure 11 and Appendix A). N20-03010 served as the internal reference for the analysis. At 79.99% core genome, the range of SNVs amongst all NAP1 isolates was 0-63 SNVs. The range of SNVs when comparing N20-03010 to the NAP1 isolates from human clinical cases was 23-55 SNVs. The average number of SNVs when comparing N20-03010 to the NAP isolates from human clinical cases was 36.2 SNVs ($\sigma = 7.4$).

To continue increasing the core genome included in the analysis, the clade of *C. difficile* NAP1 human clinical isolates clustered around the *C. difficile* NAP1 isolate from retail meats (N20-03010) was then reanalyzed together.

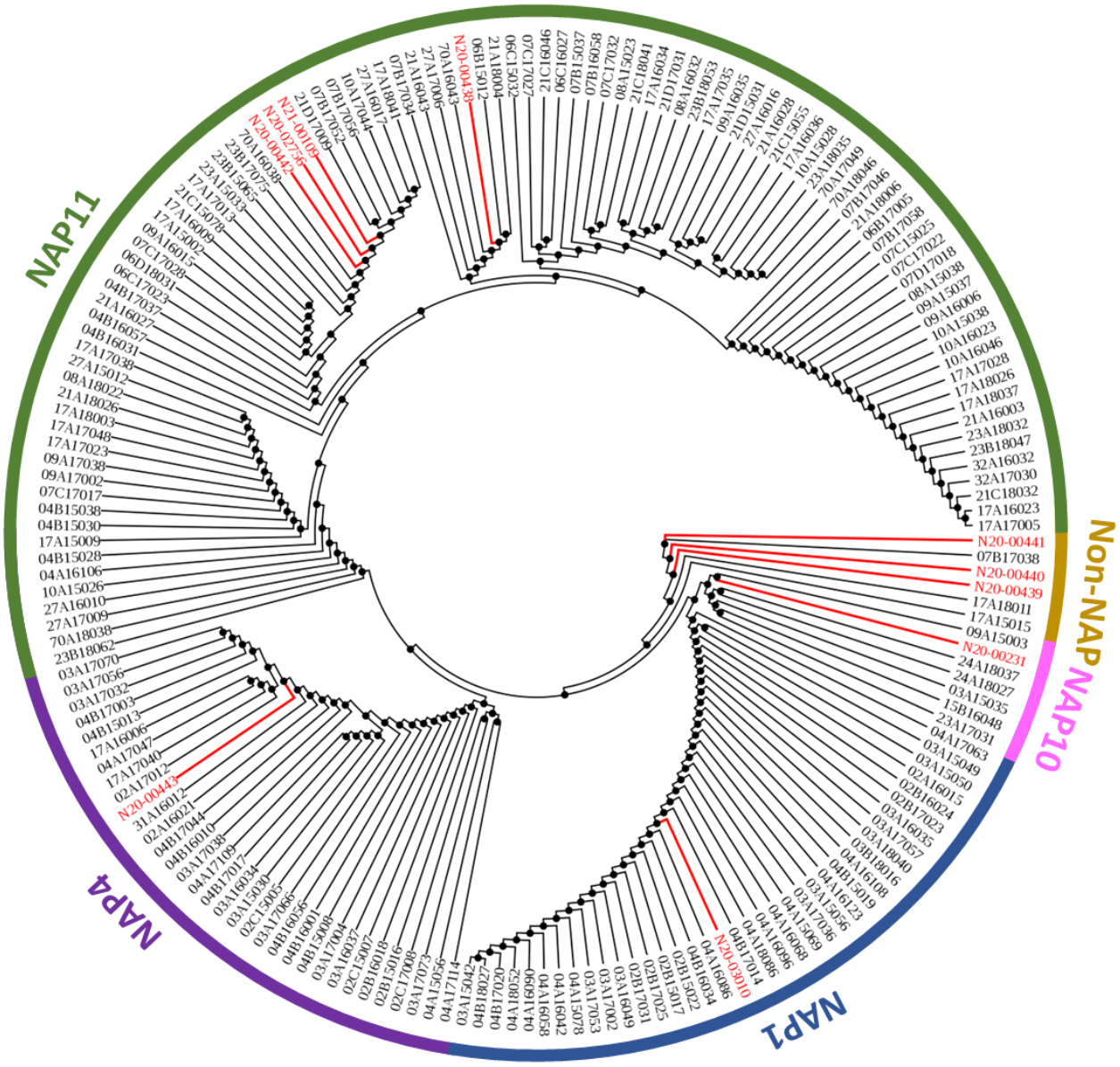


Figure 10. Radial Dendrogram of all *C. difficile* Isolates from Retail Meat Samples and Human Clinical Cases. *C. difficile* isolates from retail meat samples are represented by red branches. *C. difficile* isolates from human clinical cases are represented by black branches. N20-03010 served as an internal reference for the analysis with 15.16% of the core genome included.

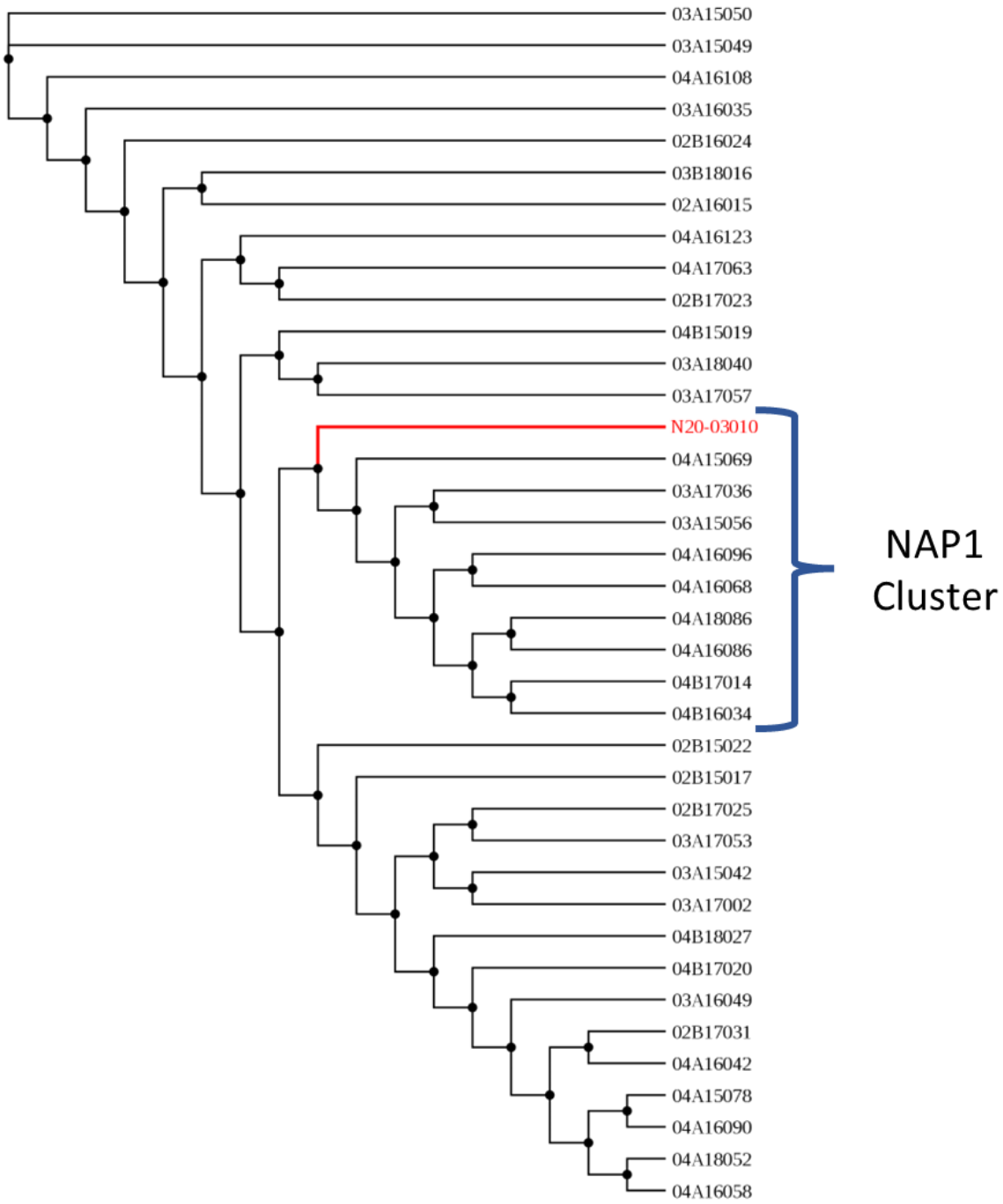


Figure 11. Linear Dendrogram of NAP1 *C. difficile* Isolates from Retail Meat Samples and Human Clinical Cases. The *C. difficile* isolate from retail meat samples is represented by a red branch. *C. difficile* isolates from human clinical cases are represented by black branches. N20-03010 served as an internal reference for the analysis with 79.99% of the core genome included.

3.8.1.1. NAP1 *C. difficile* Clade

To increase the core genome included in the analysis, a NAP1 clade of 9 clinical isolates containing N20-03010 (Figure 11 and Figure 12) was reanalyzed together. As N20-03010 was part of this NAP1 clade, N20-03010 continued to serve as the internal reference for the analysis. The inclusion of core genomes in the analysis improved from 79.99% to 84.54%. In this smaller defined clade, at 84.54% core genome included in the analysis, the range of SNPs when comparing the retail meat NAP1 isolate (N20-03010) to human clinical NAP1 was 24-35 SNVs ($\bar{x}$ = 29.2, σ = 4.9). The closest related human *C. difficile* clinical isolates to N20-03010 in this clade were 4 isolates from Alberta (03A15056, 04A15069, 04A16068, and 04A16096). Isolates 03A15056 and 04A15069 are both 2015 healthcare-associated isolates, and they differ from N20-03010 (2016 ground pig isolate) by 24 SNVs each. While 04A16068 (2016 community-associated) and 04A16096 (2016 healthcare-associated) differ from N20-03010 by 25 SNVs each (Table 23).

3.8.2. NAP4 *C. difficile* Isolates

1 NAP4 *C. difficile* isolate from retail meats (N20-00443) was analyzed against 32 *C. difficile* NAP4 isolates from human clinical cases (Figure 13 and Appendix A). N20-00443 served as the internal reference for the analysis. At 80.54% core genome, the range of SNVs among all NAP4 isolates was 0-107. The range of SNVs when comparing N20-00443 to the NAP4 isolates from human clinical cases was 7-92 SNVs. The average number of SNVs when comparing N20-00443 to the NAP4 isolates from human clinical cases was 40.0 SNVs (σ = 21.4).

To continue increasing the core genome included in the analysis, the clade of *C. difficile* NAP4 human clinical isolates clustered around the *C. difficile* NAP4 isolate from retail meats (N20-00443) was then reanalyzed together.

3.8.2.1. NAP4 *C. difficile* Clade

To increase the core genome core genome included in the analysis, a NAP4 clade of 8 clinical isolates containing N20-00443 (Figure 13 and Figure 14) was reanalyzed together. N20-00441 continued to serve as the internal reference for the analysis. The inclusion of core genomes in the analysis improved from 80.54% to 90.39%. In this smaller defined clade, at 90.39% core

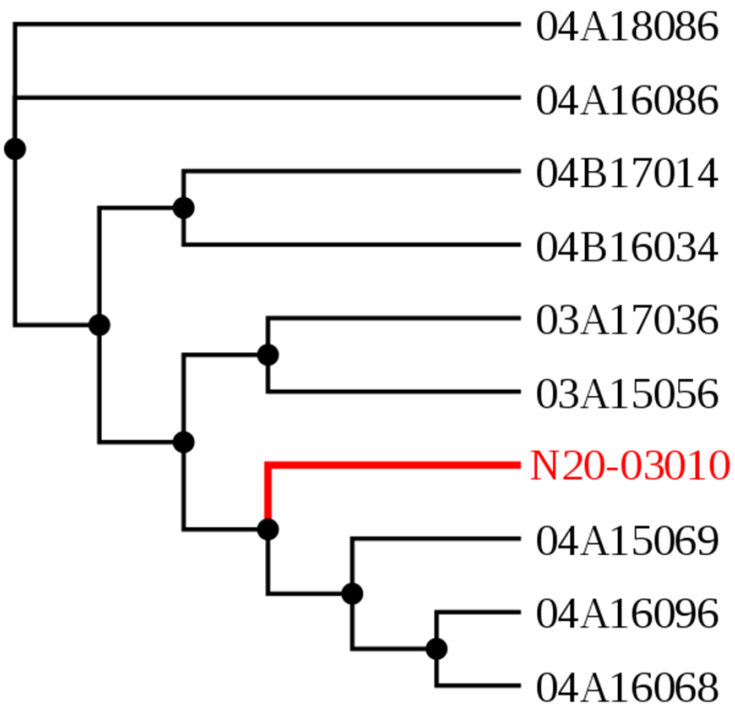


Figure 12. Linear Dendrogram of a Clade of a NAP1 *C. difficile* Isolates from Retail Meat Samples and Human Clinical Cases. The *C. difficile* isolate from retail meat samples is represented by a red branch. *C. difficile* isolates from human clinical cases are represented by black branches. N20-03010 served as an internal reference for the analysis with 84.54% of the core genome included.

Table 23. Single Nucleotide Variant (SNV) Matrix of a Clade of NAP1 *C. difficile* Isolates from Retail Meat Samples and Human Clinical Cases

Strain	04A1 6086	N20-03010	04A1 8086	04B1 7014	04B1 6034	03A1 7036	04A1 6068	04A1 6096	03A1 5056	04A1 5069
N20-03010	35	0	35	34	33	28	25	25	24	24
03A15056	13	24	13	12	11	4	3	3	0	2
04A15069	13	24	13	12	11	6	3	3	2	0
04A16068	14	25	14	13	12	7	0	0	3	3
04A16096	14	25	14	13	12	7	0	0	3	3
03A17036	17	28	17	16	15	0	7	7	4	6
04B16034	18	33	18	13	0	15	12	12	11	11
04B17014	19	34	19	0	13	16	13	13	12	12
04A16086	0	35	14	19	18	17	14	14	13	13
04A18086	14	35	0	19	18	17	14	14	13	13

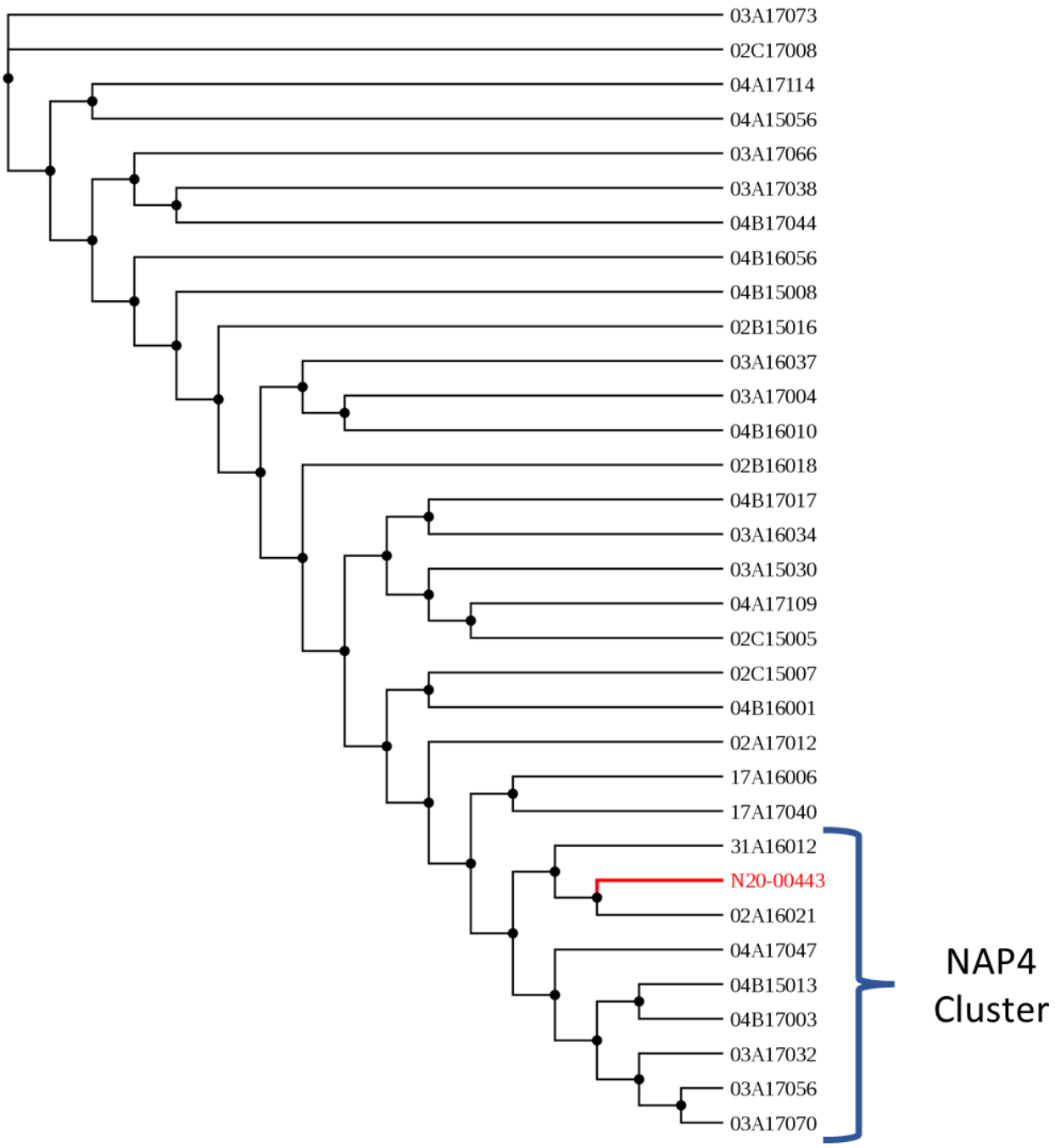


Figure 13. Linear Dendrogram of NAP4 *C. difficile* Isolates from Retail Meat Samples and Human Clinical Cases. The *C. difficile* isolate from retail meat samples is represented by a red branch. *C. difficile* isolates from human clinical cases are represented by black branches. N20-00443 served as an internal reference for the analysis with 80.54% of the core genome included.

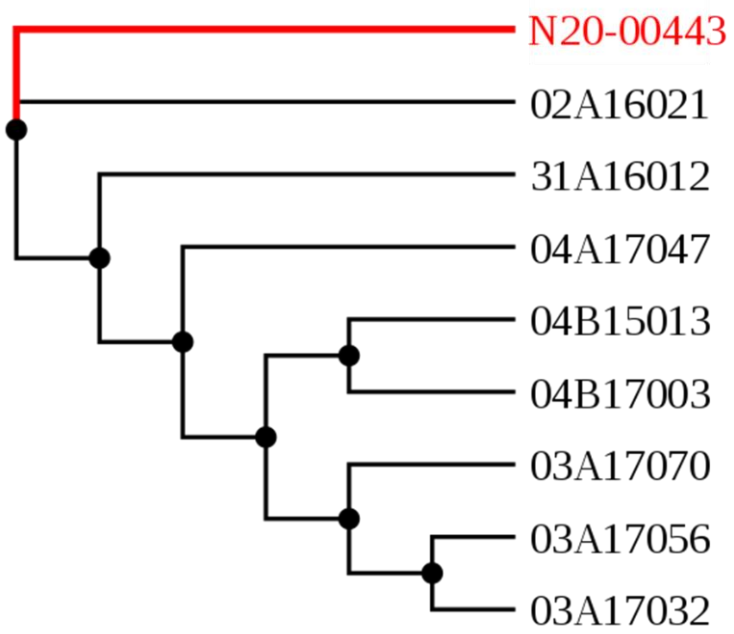


Figure 14. Linear Dendrogram of a Clade of NAP4 *C. difficile* Isolates from Retail Meat Samples and Human Clinical Cases. The *C. difficile* isolate from retail meat samples is represented by a red branch. *C. difficile* isolates from human clinical cases are represented by black branches. N20-00443 served as an internal reference for the analysis with 90.39% of the core genome included.

genome included in the analysis, the range of SNVs when comparing the retail meat NAP4 isolate (N20-00443) to human clinical NAP4 cases was 13-44 SNVs ($\bar{x}$ = 27.9, σ = 10.0). The closest related human clinical isolate to N20-00443 in this clade were 02A16021 and 31A16012. Isolates 02A16021 (2016 healthcare-associated clinical case from Alberta) and 31A16012 (2016 community-associated clinical case from Quebec) respectively differed from N20-00443 (2018 pig sausage from Alberta) by 13 and 15 SNVs (Table 24).

3.8.3. NAP10 *C. difficile* Isolates

One NAP10 *C. difficile* isolate (N20-00231) from retail meat was analyzed against 6 available *C. difficile* isolates from human clinical cases (Figure 15 and Appendix A). N20-00231 served as the internal reference for the analysis. At 88.04% core genome, the range of SNVs when comparing N20-00231 to the NAP10 isolates from human clinical cases was 3-28 SNVs. The average number of SNVs when comparing the N20-00231 to the NAP10 isolates from human clinical cases was 19.3 SNVs (σ = 3.4).

A clade of *C. difficile* NAP10 human clinical isolates clustered around the *C. difficile* NAP10 isolate from retail meats (N20-00231) was then reanalyzed together. To increase the core genome core genome included in the analysis, a NAP10 clade of 3 clinical isolates containing N20-00231 (Figure 15) was reanalyzed together. N20-00231 continued to serve as the internal reference for the analysis. When N20-00231 is analyzed with 09A15003, 24A18027, and 24A18037, the included core genome increases from 88.04% to 93.33%. Overall, the range of SNVs when comparing the retail meat NAP10 isolate (N20-00231) to the 3 aforementioned human clinical NAP10 cases in this smaller defined clade was 15-24 SNVs ($\bar{x}$ = 20.8, σ = 3.9). These results were similar as previously noted in the larger dataset with the exception of 23A17031. Isolate 09A15003 (2015 healthcare-associated from Ontario) differs from N20-00231 (2017 veal nugget from Ontario) by 15 SNVs while isolate 24A18027 (2018 healthcare-associated from Quebec) and isolate 24A18037 (2018 healthcare-associated from Quebec) each differ from N20-00231 by 17 SNVs each (Table 25).

Table 24. Single Nucleotide Variant (SNV) Matrix of a Clade of NAP4 *C. difficile* Isolates from Retail Meat Samples and Human Clinical Cases

Strain	04A1 7047	N20-00443	02A1 6021	04B1 7003	03A1 7032	03A1 7056	03A1 7070	31A1 6012	04B1 5013
N20-00443	44	0	13	32	31	31	31	15	26
02A16021	43	13	0	31	30	30	30	14	25
31A16012	37	15	14	25	24	24	24	0	20
04B15013	35	26	25	9	12	12	12	20	0
03A17032	39	31	30	17	0	0	0	24	12
03A17056	39	31	30	17	0	0	0	24	12
03A17070	39	31	30	17	0	0	0	24	12
04B17003	40	32	31	0	17	17	17	25	9
04A17047	0	44	43	40	39	39	39	37	35

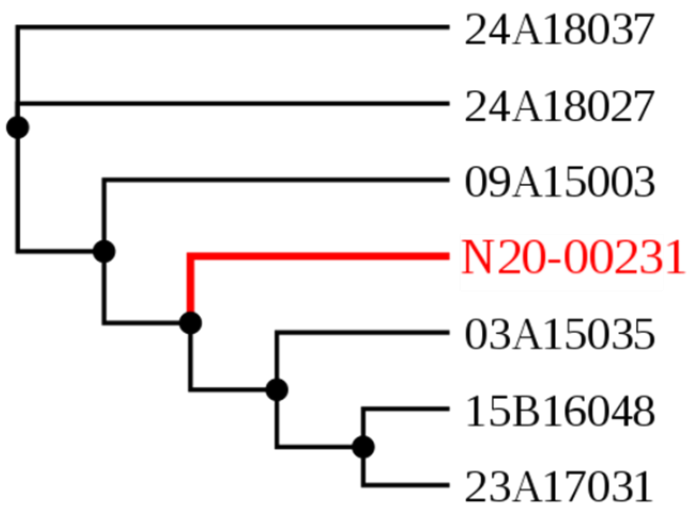


Figure 15. Linear Dendrogram of NAP10 *C. difficile* Isolates from Retail Meat Samples and Human Clinical Cases. The *C. difficile* isolate from retail meat samples is represented by a red branch. *C. difficile* isolates from human clinical cases are represented by black branches. N20-00231 served as an internal reference for the analysis with 93.33% of the core genome included.

Table 25. Single Nucleotide Variant (SNV) Matrix of a Clade of NAP10 *C. difficile* Isolates from Retail Meat Samples and Human Clinical Cases

Strain	23A17031	24A18027	24A18037	03A15035	15B16048	09A15003	N20-00231
N20-00231	23	17	17	22	22	15	0
09A15003	26	12	12	25	25	0	15
24A18027	28	0	0	27	27	12	17
24A18037	28	0	0	27	27	12	17
03A15035	5	27	27	0	4	25	22
15B16048	3	27	27	4	0	25	22
23A17031	0	28	28	5	3	26	23

3.8.4. NAP11 *C. difficile* Isolates

4 NAP11 *C. difficile* isolate (N20-00438, N20-00442, N20-02756, and N21-00109) from retail meats was analyzed against 96 *C. difficile* isolates from human clinical cases (Figure 16 and Appendix A). N20-00438 served as the internal reference for the analysis.

At 78.48% core genome, the range of SNVs amongst all NAP11 isolates was 0-84 SNVs. The range of SNVs when comparing the *C. difficile* isolates from retail meats to the NAP11 isolates from human clinical cases was 5-55 SNVs. The average number of SNVs when comparing the *C. difficile* isolates from retail meats to the NAP11 isolates from human clinical cases was 23.3 SNVs (σ = 9.0).

To continue increasing the core genome included in the analysis, 2 clades of *C. difficile* NAP11 human clinical isolates clustered around the *C. difficile* NAP11 isolates from retail meats (N20-00438, N20-00442, N20-02756, and N21-00109) was then reanalyzed together.

3.8.4.1. NAP11 *C. difficile* Clade 1

To increase the core genome core genome included in the analysis, the NAP11 clade 1, containing 4 human clinical isolates and the retail meat isolate N20-00438, were reanalyzed together (Figures 16 and 17). N20-00438 continued to serve as the internal reference for the analysis. The inclusion of core genomes in the analysis improved from 78.48% to 92.55%. In this smaller defined clade, at 92.55% core genome included in the analysis, the range of SNVs when comparing the retail meat NAP11 isolate N20-00438 to human clinical NAP11 cases was 34-52 SNVs ($\bar{x}$ = 40.8, σ = 7.9). The closest related human clinical isolates in this clade to N20-00438 (2017 red veal from Ontario) were both 2016 community-associated cases from Ontario, which included isolate 21C16046 (34 SNVs) and isolate 07C17027 (37 SNVs) (Table 26).

3.8.4.2. NAP11 *C. difficile* Clade 2

To increase the core genome core genome included in the analysis, NAP11 clade 2, containing 4 human clinical isolates and 3 retail meat isolates (N20-00442, N20-02756, and N21-00109), were reanalyzed together (Figure 16 and 18). N20-00442 served as the internal reference for the analysis. The inclusion of core genomes in the analysis improved from 78.48% to 88.80%.

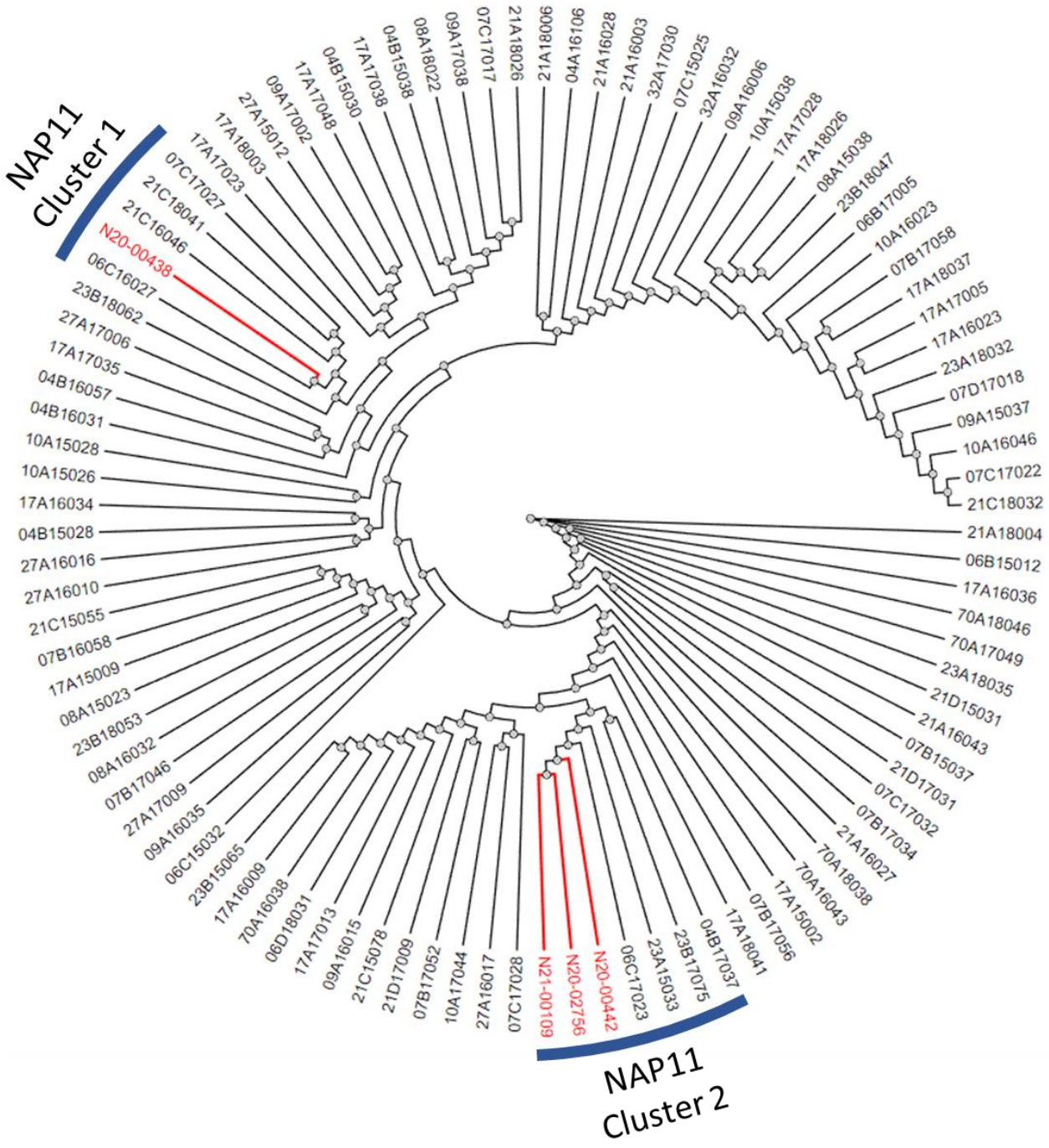


Figure 16. Linear Dendrogram of NAP11 *C. difficile* Isolates from Retail Meat Samples and Human Clinical Cases. *C. difficile* isolates from retail meat samples are represented by red branches. *C. difficile* isolates from human clinical cases are represented by black branches. N20-00438 served as an internal reference for the analysis with 78.48% of the core genome included.

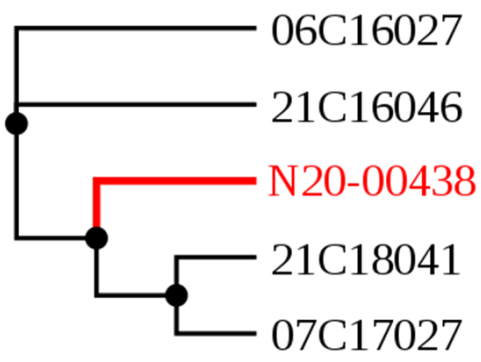


Figure 17. Linear Dendrogram of a Clade of a NAP11 *C. difficile* Isolate from Retail Meat Samples and Human Clinical Cases. The *C. difficile* isolate from retail meat samples is represented by a red branch. *C. difficile* isolates from human clinical cases are represented by black branches. of reference core genome included. N20-00438 continued to serve as an internal reference for the analysis with 92.55% of the core genome included.

Table 26. Single Nucleotide Variant (SNV) Matrix of NAP11 Clade 1

Strain	06C16027	N20-00438	21C18041	07C17027	21C16046
N20-00438	52	0	40	37	34
21C16046	32	34	18	15	0
07C17027	35	37	11	0	15
21C18041	38	40	0	11	18
06C16027	0	52	38	35	32

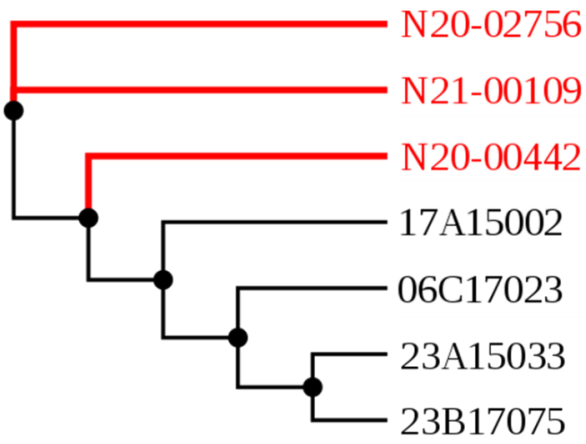


Figure 18. Linear Dendrogram of NAP11 Clade 2 of *C. difficile* Isolate from Retail Meat Samples and Human Clinical Cases. The *C. difficile* isolate from retail meat samples is represented by a red branch. *C. difficile* isolates from human clinical cases are represented by black branches. of reference core genome included. N20-00442 served as an internal reference for the analysis with 88.80% of the core genome included.

In this smaller defined clade, at 88.80% core genome included in the analysis, the range of SNPs when comparing the retail meat NAP11 isolates N20-00442, N20-02756, and N21-00109 to human clinical NAP11 cases was 8-13 SNVs ($\bar{x}$ = 10.3, σ = 1.6). The 3 retail meat isolates were all closely related, as N20-00442 differs from N20-02756 and N21-00109 by only 1 SNV.

The closest related human clinical isolate in this clade to N20-00442 (2018 pig sausage from Ontario) is isolate 17A15002 (2016 intermediate clinical isolate from Ontario) which differs by 9 SNPs (Table 27). The closest related human clinical isolate in this clade to N20-02756 (2016 ground pig from Ontario) and N21-00109 (2016 ground pig from Alberta) is also isolate 17A15002 which differs by 8 SNPs (Table 27).

3.8.5. Pulsed-Field Type 225 & NS110

3 PF225/NS110 *C. difficile* retail meat isolates (N20-00439, N20-00440, and N20-00440) was analyzed against 3 *C. difficile* isolates from human clinical cases (Figure 19 and Appendix A). N20-00441 served as the internal reference for the analysis. 93.00% of the reference core genome was included in the analysis. The range of SNVs when comparing the *C. difficile* isolates from retail meats to the PF225 and NS110 isolates from human clinical cases was 8-33 SNVs. The average number of SNVs when comparing the *C. difficile* isolates from retail meats to the human clinical cases isolates was 23.3 SNVs (σ = 9.0).

While N20-00439 and N20-00440 share 0 SNVs, they both differ from N20-00441 by 11 SNVs (Table 38). The closest human clinical isolate to N20-00439 (2017 veal cutlet from British Columbia) and N20-00440 (2017 ground veal from British Columbia) is isolate 17A18011 (2018 intermediate clinical isolate from Ontario), which differs by 8 SNVs. The closest human clinical isolate to N20-00441 (2018 pig sausage from Ontario), aside from the other 2 *C. difficile* isolates from retail meats, is also isolate 17A18011, which differ by 19 SNVs (Table 28).

Table 27. Single Nucleotide Variant (SNV) Matrix of NAP11 Clade 2 of *C. difficile* Isolates from Retail Meat Samples and Human Clinical Cases

Strain	06C17023	23A15033	N20-00442	23B17075	N20-02756	N21-00109	17A15002
N20-00442	11	13	0	11	1	1	9
N20-02756	10	12	1	10	0	0	8
N21-00109	10	12	1	10	0	0	8
17A15002	10	4	9	2	8	8	0
06C17023	0	14	11	12	10	10	10
23B17075	12	6	11	0	10	10	2

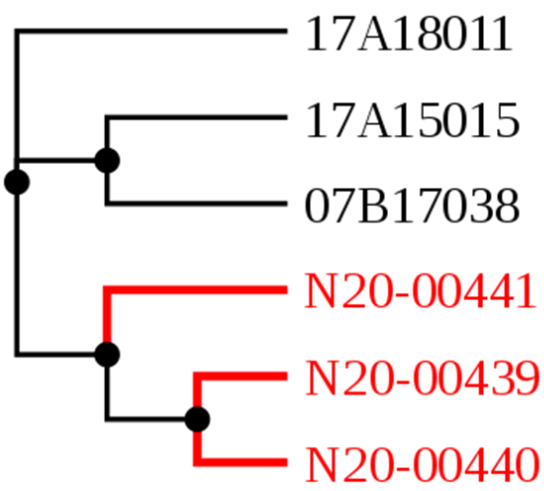


Figure 19. Linear Dendrogram of Pulsed-Field Type 225 *C. difficile* Isolates from Retail Meat Samples and Human Clinical Cases. The *C. difficile* isolate from retail meat samples is represented by a red branch. *C. difficile* isolates from human clinical cases are represented by black branches. N20-00441 served as an internal reference for the analysis with 93.00% of the core genome included.

Table 28. Single Nucleotide Variant (SNV) Matrix of Pulsed-Field Type 225 *C. difficile* Isolates from Retail Meat Samples and Human Clinical Cases

Strain	07B17038	N20-00441	17A15015	17A18011	N20-00439	N20-00440
07B17038	0	33	16	28	22	22
N20-00441	33	0	31	19	11	11
17A15015	16	31	0	26	20	20
17A18011	28	19	26	0	8	8
N20-00439	22	11	20	8	0	0
N20-00440	22	11	20	8	0	0

CHAPTER 4: DISCUSSION

In this study, we show that a small percentage of retail meats are contaminated with *C. difficile* (1.6%). Furthermore, the *C. difficile* isolates from retail meats share identical ribotypes, NAP types, and pulsed-field types to *C. difficile* isolated from human clinical cases (Figure 8, Figure 9, Appendix A). In addition, through whole genome sequencing (WGS), 50% of the *C. difficile* isolates recovered from retail meats were genetically similar (<10 SNVs) to *C. difficile* isolates from human clinical cases, suggesting that raw retail meats may pose as a community source of *C. difficile*.

4.1. *C. difficile* from Retail Meats

In this study, *C. difficile* was isolated from 10 of 644 retail meat samples. Among all samples processed, this represents a contamination rate of 1.6% (Table 19). Previous studies surveying the prevalence of *C. difficile* contamination on commercially available meats have yielded varying rates of contamination [19,85,125,134–138]. An American study by Songer et al. reported *C. difficile* isolation rates on retail meat as high as 42% [138]. However, while this study examined retail meat samples collected between 2016-2018, Songer et al. used samples collected in 2007 [138]. The disparity in reported contamination rates could be partially attributed to an increased sanitation and food safety practices. The contamination rate is slightly lower when compared to previous locally conducted studies. In 2012, Visser et al. reported a contamination rate of 6.3% across 48 samples collected in 2007 from Winnipeg, Canada [136]. Although both studies were conducted in the same location, the sampling parameters and techniques were different. While the retail meat samples utilized by Visser et al. originated from Winnipeg, the retail meat samples used in this study originated from other provinces across Canada (Table 19) [136]. Compared to other studies, no other study surpassed the number of retail meat samples processed in the present study (n=644) (Table 19). The majority of past studies utilized between 50-100 retail meat samples [134,136–138]. Variations in sampling techniques, sampling locations, isolation methods, transportation, handling, storage, and commodity type may have all had an influence on the contamination rates reported by these studies.

Although this study reports a lower *C. difficile* contamination prevalence than those previously reported in North America, the results are similar to findings in other international studies which utilized retail samples from the same time period [135,144]. The results of this study results align with a 2018 Turkish study that reported a retail meat contamination rate of 1.98% [135].

Past studies have identified *C. difficile* as a bacterium that commonly colonizes live veal calves and piglets [133,145]. Antibiotics can affect the gastrointestinal microbiota of humans and animals to the point where the altered microbiota mimics the gut of neonates [146]. The gut microbiota of neonates is primitive when compared to that of a healthy adult [146]. Therefore, an adult who possess a gut microbiota that mimics the gut microbiota of a neonate is at an increased risk of *C. difficile* colonization. While the beef samples were differentiated by the relative age of the animal into beef and veal, the pork samples were not (Table 17). This is important as it has been reported that *C. difficile* can be recovered from nearly all piglets (96%) at least once within the first 62 days of their life [133]. As piglets ages, the number of piglets colonized or infected with *C. difficile* decreases [133]. It would be ideal if the animal's age of all retail meat samples was known. If the source of *C. difficile* contamination on retail meats was due to infected and/or colonized animals, it would be expected that retail meat samples originating from infant animals would harbour a higher rate of *C. difficile*. This could possibly explain why we observed no *C. difficile* on beef products (0%) and the highest recovery from veal (3.5%) (Table 19).

4.1.1. Antimicrobial Susceptibility Profile of *C. difficile* Isolates from Retail Meats

All 10 *C. difficile* isolates from the retail meat samples were susceptible to all tested antimicrobials apart from the NAP1/RT027 isolate (N20-03010) that was resistant to moxifloxacin. In the early 2000s, nearly all NAP1/RT027 *C. difficile* acquired resistance against fluoroquinolones [117]. The widespread use of fluoroquinolones has led to multiple outbreaks of NAP1/RT027 *C. difficile* across the world, most notably across North America and Europe in the early 2000s [117]. The antimicrobial susceptibility profile of the *C. difficile* isolates from retail meat samples (Table 21) largely reflects antimicrobial susceptibility patterns of human clinical

cases. Canadian *C. difficile* surveillance data, produced by the Canadian Nosocomial Infection Surveillance Program (CNISP), shows that from 2015-2018, no tested CDI isolates were resistant to either tigecycline or vancomycin [129]. Rifampin resistance was low as only 1.4% of tested isolates were resistant to this drug [129]. The percentage of moxifloxacin resistant isolates, 13.1%, is comparable to the results of this study where 10% of *C. difficile* isolates from retail meats were resistant to moxifloxacin [129]. With respect to clindamycin resistance, the resistance profile of *C. difficile* isolates from human clinical cases and *C. difficile* isolates from retail meat samples varied greatly. 30.6% of tested *C. difficile* isolates from human clinical cases were resistant to clindamycin [129]. Interestingly, none of the *C. difficile* isolates from retail meat samples from this study were resistant against clindamycin. From between 2011-2017, American resistance to moxifloxacin and rifampin among *C. difficile* from CDI incidences has been decreasing [147]. However, in the same timeframe, resistance to tetracycline was on the rise [147]. Most concerning is vancomycin resistance in *C. difficile* as vancomycin is a widely used first-line CDI antibiotic [148]

Administration of any antibiotic that disrupts the gastrointestinal flora can lead to a *C. difficile* infection [10,11,103,149,150]. Antibiotics most notable for distributing the microbiota include moxifloxacin, clindamycin, ampicillin, amoxicillin, and cephalosporins [150]. One possible explanation for the discrepancy in clindamycin resistance could be related to its use. In humans, clindamycin is a frequently prescribed broad-spectrum antibiotic to treat minor infections [92]. However, when it comes to administering antibiotics to food-producing animals in North America, clindamycin is not an approved drug [151]. The only approved veterinary use of clindamycin is for cats and dogs [151]. One preliminary Spanish study by Andrés-Lasheras et al. investigated *C. difficile* from dogs and exotic pets [152]. Amazingly, 50% of the *C. difficile* isolates from dogs colonized by *C. difficile* were resistant to clindamycin [152]. Although Andrés-Lasheras et al. established a potential reservoir of clindamycin resistant *C. difficile*, their sample size was small (n=6) [152].

With antibiotic stewardship at the forefront of global antimicrobial resistance prevention, the reduced use of antibiotics, such as fluoroquinolones since the early 2000s, has resulted in decreased pressure on *C. difficile* strains to develop resistance to these drug classes [116].

Fluoroquinolone-based selective pressure favours the colonization and infection of global hypervirulent fluoroquinolone resistant strains such as NAP1/RT027 *C. difficile* [116]. Institutions who have implemented antimicrobial stewardship programs aimed at decreasing the usage of fluoroquinolones have seen a significant decrease in NAP1/RT027 incidences in their institutions [116]. For example, from 2009 to 2018, the Cleveland VA Medical Center was able to reduce inpatient fluroquinolone prescriptions by 43% [116]. The result of the stewardship, in conjunction with improved cleaning of surfaces, was a 50% reduction in HA-CDI incidences [116]. Additionally, in this study, the percentage of their HA-CDI caused by NAP1/RT027 *C. difficile* fell from 65% to 10% [116]. The reduction in fluroquinolone prescriptions was therefore correlated with a decrease in HA-CDI incidences [116].

The decline of CDI incidences due to the restriction of fluroquinolone is not exclusive to North America. Similar results have been reported from the Czech Republic. Through an effective antibiotic stewardship campaign, the University Hospital Hradec Králové was able to reduce CDI incidences by 23.2% and lower the identification rate of hypervirulent ribotypes responsible for HA-CDI incidences [153]. Between 2014 and 2018, their fluroquinolone consumption fell from 103.3 defined daily doses (DDD) per 1,000 patient days to 34.0 DDD per 1,000 patient days [153]. In the same period, CDI incidences fell from 4.6 cases per 1,000 patient days to 4.3 cases per 1,000 patient days [153].

4.1.2. Typing *C. difficile* Isolates from Retail Meats and Human Clinical Cases

In this study, all *C. difficile* PFGE strain types and ribotypes isolated from retail meats have been previously identified in Canadian human clinical cases acquired through the Canadian Nosocomial Infection Surveillance Program (CNISP) [128]. Through WGS we identified that each NAP type (and non-NAP types) formed distinct clades (Figure 10). This suggests PFGE designation of NAP types is a reliable typing method for examining molecular epidemiology at a wide scale. However, in this thesis, we demonstrated and utilized the higher discriminatory power of WGS for identifying genomic differences between the isolates. In this study, 7 of the 10 *C. difficile* isolates from retail meats all were of established NAP types, encompassing NAP1, NAP4, NAP10, and NAP11.

Since its peak in the early 2000s, the prevalence of hospitalized NAP1/RT027 CDI cases have been continuously decreasing in Canada. In this study, the PFGE pattern (PF 001) of the single NAP1 pork isolate (N20-03010) matches the most commonly observed PFGE pattern amongst human clinical NAP1 CDI cases [128]. NAP1 *C. difficile* is often associated with increased odds of severe disease when compared to other strains of *C. difficile* [154]. From 2009 to 2020, Canadian HA-CDI cases associated to NAP1/RT027 *C. difficile* has decreased from 47% to 6.2% [99,129]. The decreasing trend in NAP1/RT027 is similar in the United States. Over the 2010s, NAP1/RT027 CDI cases have decreased from 31% in 2011, to 14% in 2017 [147]. From 2012 to 2013, European NAP1/RT027 prevalence was an estimated 19% [155].

Among the 10 *C. difficile* isolates from the retail meat samples used in this study (Figure 9), only 1 isolate (N20-00443) was identified as a NAP4 *C. difficile*. Throughout the 2010s, NAP4 *C. difficile* was an emerging NAP type responsible for HA-CDIs. Canadian surveillance data by CNISP shows that NAP4 *C. difficile* HA-CDI rates are currently on the rise. In 2009, NAP4 *C. difficile* was responsible for 8.7% of HA-CDI incidences [99]. By 2015, the rate had more than doubled to 18.5% of HA-CDI cases [99]. In 2011, American CDI incidences attributed to NAP4 *C. difficile* is approximately 10.9% [123].

In this thesis, 1 of 10 *C. difficile* isolates from the retail meat samples (Figure 9) was a NAP10 *C. difficile* (N20-00231). NAP10 *C. difficile* is not a common CDI causing strain in Canada. Canadian surveillance data by CNISP shows that NAP10 *C. difficile* was responsible for only 3.7% of HA-CDI incidences from between 2009-2015 [99]. The incidence rate of CDI associated with NAP10 *C. difficile* is quite similar in the United States were NAP10 *C. difficile* was responsible for 2.9% of CA-CDI incidences and 2.4% of HA-CDI incidences, representing 2.6% of total American CDI incidences [123].

In this study, the most common NAP type of *C. difficile* isolated from retail meat was NAP11/RT106 represented by isolates N20-00438, N20-00442, N20-02756, and N21-00109 (Figure 8 and Figure 9). NAP11/RT106 is currently an emerging NAP type in human Canadian clinical cases [128]. In 2016, NAP11/RT106 surpassed NAP1/RT027 as the predominant type and is currently the most common *C. difficile* strain type identified in hospitalized patients [128,129]. In 2020, 15% of all *C. difficile* isolates in CNISP were NAP11/RT106 [129]. CDI incidences caused

by NAP11/RT106 *C. difficile* is also a prevailing strain in the United States. Between 2014 and 2018, NAP1/RT027 *C. difficile* was the most prevalent American HA-CDI strain, responsible for 15.8% of all HA-CDI incidences [156–160]. NAP11/RT106 was the second most common American HA-CDI associated strain, responsible for 10.8% of HA-CDI incidences [156–160]. Additionally, NAP11/RT106 was the most common CA-CDI strain during the same period, responsible for 12.4% of CA-CDI incidences [156–160]. Interestingly, in the late 2010s, NAP11/RT106 *C. difficile* was prevalent in Europe by being associated with 20-26% of CDI cases in England [161]. However, by 2013, NAP11/RT106 *C. difficile* prevalence had fallen to 0.6% across Europe.

A substantial portion of CDI causing *C. difficile* strains are not a member of any predefined NAP type. 3 of the 10 *C. difficile* isolates from the retail meat samples fall into this unassigned category (Figure 9). CNISP surveillance data reports that the strain type of 20-27% of HA-CDI causing *C. difficile* are unassigned [99]. A slightly higher percentage of American CDI *C. difficile* isolates, approximately 32.2%, are unassigned [123]. Interestingly, a higher proportion of CA-CDI incidences (1/3) are caused by unassigned *C. difficile* than HA-CDI incidences (1/4) [123].

It should be noted that 2 frequently documented ribotypes of *C. difficile* found on raw meat products and animals, RT078 and RT126, were not identified in this study [137,138,144]. However, from 2015-2020, RT078 and/or RT126 were only identified in 2.0-3.7% of Canadian HA-CDI patients and in 0.6-4.6% of CA-CDI patients [128,129]. In addition, emerging CDI ribotypes RT056 and RT002 were also not identified in this study [147].

Overall, the presence of ribotypes and NAP types previously described from human clinical cases suggests that retail meats could be an infrequent, but potential, source of CA-CDIs.

4.2. Genomic Comparison of Isolates from Retail Meats and Human Clinical Cases

When the SNV analysis was performed on the *C. difficile* isolates from retail meat samples and *C. difficile* isolates from human clinical cases, some isolates appeared genetically related. The closest related human clinical isolate when compared to *C. difficile* isolates from retail meats was between 8-34 SNVs when analyzed at the highest resolution (84.54%-95.02% core genome). The evolutionary rate of *C. difficile* is estimated to be 3.2×10^{-7} mutations per site per year 95% CI [1.3×10^{-7}, 5.3×10^{-7}] [162], which translates into approximately 1.4 SNVs per year [162]. In

hospital outbreak scenarios, *C. difficile* isolates have been proposed to be clonal if isolates differ by ≤2 SNVs [162–164]. Isolates that differ by <10 SNVs are considered genetically similar, while isolates that differ by >10 SNVs are considered genetically distinct [162–164].

Of the 10 *C. difficile* isolates recovered from retail meats, 5/10 (50%) are considered genetically related to a selected *C. difficile* isolate from a human clinical case that is a part of CNISP's collection. This included 3 NAP11 pig isolates (N20-00442, N20-02756, and N21-00109) that differed by 8-10 SNVs from 2 Ontario human clinical cases (17A15002 and 23B17075) (Table 27), and 2 non-NAP (PFGE type 225/RTNS110) (N20-00439 and N20-00440) veal isolates that differed by 8 SNVs from a clinical case in Ontario (Table 28). While the remaining 5 retail meat isolates could not be definitely linked genetically to a human clinical case, the number of SNV differences fell within the natural diversity of SNVs found within each NAP clade which were selected based on PFGE, ribotype, and geospatial biases. The location of the identified SNVs on the *C. difficile* genome was not determined in this thesis.

While this study provides some additional evidence to that *C. difficile* disseminates in the community via retail meats, a definitive link can not be made between retail meats and the infection of hospitalized individuals as this study does not establish the point source of contamination; only that the retail meat samples were contaminated by *C. difficile*.

4.3. Limitations of the Study

A total of 644 retail meat samples from between 2016 and 2018 were analyzed as part of this project. Although the samples were kept under normal storage conditions, the amount of time that the samples spent frozen at -80°C could affect spore viability. Ideally, samples would be processed in their fresh state shortly after sample collection.

The method utilized tin this study to isolate *C. difficile* from retail meat samples as a sensitive technique. The limit of detection of the technique was determined to be 7.5 spores per mL of PBS rinsate (Appendix B). The use of less PBS to create the initial rinsate could have been implemented to obtain a more concentrated rinsate. This would have thus increased the chances of isolating *C. difficile* from retail meat samples with low spore contamination.

Although 5 of the 10 *C. difficile* isolates from retail meat samples were determined to be genetically similar (<10 SNVs), the core genome included in the analysis was not 100%. Additional SNVs may be identified if 100% of the core genome was included in the analysis.

Overall, 174 *C. difficile* isolates from human clinical cases were selected for sequencing (Table 22 and Appendix A). Restricting human clinical isolates to those with a matching ribotype, NAP type, and/or pulsed-field type excluded many related isolates. Additionally, these samples were then filtered by year and location thus, further eliminating additional isolates from human clinical cases available through CNISP. Location and timeframe were chosen as factors due to the likelihood that the end consumer of the product would reside around the collection location. However, retail meats move through national and global supply chains thus, masking the origin of the retail meat samples. Expanding the location and timeframe parameters for selecting *C. difficile* isolates from human clinical cases could lead to the inclusion of additional related *C. difficile* isolates. For future studies, obtaining supply chain information regarding the retail meat samples would aid in tracing potential points of contamination of the retail meats.

Another limitation of this study, due to the use of CNISP *C. difficile* isolates from human clinical cases, is that the CNISP collection only represents a fraction of the human CDI cases in Canada [128,129]. Collection of *C. difficile* isolates from human clinical cases is limited to only Canadian hospitals who participate in CNISP. Additionally, the travel history of patients is not taken into consideration, and adult human clinical isolates are not collected year-round. CNISP collects *C. difficile* isolates from CDI cases from March 1st through April 30th for adult CDI cases [128,129]. However, CNISP collects *C. difficile* isolates from pediatric CDI cases year-round. Throughout the years, Canadian *C. difficile* surveillance has improved as more hospitals have joined the CNISP program. Between 2015 and 2020, the number of hospitals reporting HA-CDI to CNISP increased from 66 to 82. However, less hospitals report CA-CDI to CNISP. Between 2015 and 2020, the number of hospitals reporting CA-CDI to CNISP increased from only 53 to 71.

Another limitation of this project is that while *C. difficile* contamination of retail meats is established, the source of contamination is not. Throughout the supply chain, from farm to retail store, many *C. difficile* contamination opportunities exist. Possible contamination sources include the slaughterhouse where the animal is butchered and processed; the retail store where the

carcasses are divided and packages; and the samplers from their hands and materials. Additional sources of contamination exist when the food is prepared for consumption. The food preparation area or anyone handling the food is a potential source of *C. difficile* contamination. Gaining a better understanding of the source of contamination, would allow for improved knowledge of *C. difficile* transmission in the community, the role of the food-chain, and potential intervention points should they be required.

4.4. Future Works

Additional frozen rinsates of retail meats are currently available for screening though CIPARS. Prospective sampling of fresh samples from 2022-2026 is ongoing through a Genomics Research and Development Initiative (GRDI), which could alleviate some of the potential limitations of using older frozen rinsates. In addition to retail meat, this GRDI project, in collaboration with CIPARS and other government organizations, will also be sampling environmental, on farm, abattoir, and packaging facilities for *C. difficile*. Genomic comparisons of *C. difficile* across the farm continuum should provide some additional evidence of Agri-food sources of *C. difficile* to human populations. As CNISP moves to sequencing all *C. difficile* isolates, further analysis of this complete dataset with retail meat isolates would remove the geospatial bias this study was limited to. Aside from *C. difficile*, the presence of additional pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant Enterococcus (VRE), and carbapenemase producing pathogens could also be investigated in tandem using the same samples.

4.5. Conclusion

Overall, 10 of 644 retail meat samples (1.6%) tested positive for toxigenic *C. difficile*. All ribotypes and NAP types of isolated *C. difficile* from retail meat have been identified in human clinical isolates. Through WGS, half of the *C. difficile* isolates from retail meats were determined to be closely related to *C. difficile* isolates from Canadian human clinical cases (≤10 SNVs), while the other half were genetically distinct with >10 SNVs. Although *C. difficile* is known as a prominent nosocomial pathogen, this study has shown that retail meats may act as a minor vector of dissemination. This study is corroborated by previous research that has shown that *C.*

difficile spores can be isolated from commercially available beef, veal, pork, vegetables, and seafood. While this study provides some additional evidence, a definitive link remains to be elicited between food contamination and hospitalized human CDI cases.

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The Following Figures are Created with BioRender.com: Figure 1, Figure 2, Figure 3, and Figure 4.

APPENDIX

Appendix A – *C. difficile* Isolate and Retail Meat Sample Metadata

Table 29. *C. difficile* Isolates from Retail Meats

Submission Number	Isolate	Year	Species	Processing	Province	Source	Ribotype	Tox A	Tox B	Binary	PF Type	NAP Type
CE-R2-17-0239	N20-00231	2017	Veal	Nuggets	ON	Plate & Broth	131	+	+	+	663	NAP10
CE-R2-17-0120	N20-00438	2017	Veal	Red Veal	ON	Broth	106	+	+	-	46	NAP11
CE2-R2-17-3130	N20-00439	2017	Veal	Cutlet	BC	Broth	NS110	+	+	-	225	N/A
CE2-R2-17-2054	N20-00440	2017	Veal	Ground	BC	Broth	NS110	+	+	-	225	N/A
CE-R2-18-0102	N20-00441	2018	Pig	Sausage	ON	Plate & Broth	NS110	+	+	-	225	N/A
CE-R2-18-0014	N20-00442	2018	Pig	Sausage	ON	Broth	106	+	+	-	1191	NAP11
CE3-R2-18-4030	N20-00443	2018	Pig	Sausage	AB	Broth	NS195	+	+	-	33	NAP4
CE-R2-16-0258	N20-02756	2016	Pig	Ground	ON	Broth	106	+	+	-	1191	NAP11
CE3-R2-16-5048	N20-03010	2016	Pig	Ground	AB	Broth	27	+	+	+	1	NAP1
CE3-R2-16-5061	N21-00109	2016	Pig	Ground	AB	Broth	106	+	+	-	1191	NAP11

Table 30. *C. difficile* Isolates from Human Clinical Cases

Sample ID	Case Year	Province	Source	NAP Type	Ribotype	PF Type
02A16015	2016	AB	Healthcare-Associated	NAP1	27	251
02A16021	2016	AB	Healthcare-Associated	NAP4	14	33
02A17012	2017	AB	Healthcare-Associated	NAP4	NS195	33
02B15016	2015	AB	Healthcare-Associated	NAP4	20	33
02B15017	2015	AB	Healthcare-Associated	NAP1	27	1
02B15022	2015	AB	Healthcare-Associated	NAP1	27	251
02B16018	2016	AB	Healthcare-Associated	NAP4	NS206	33
02B16024	2016	AB	Healthcare-Associated	NAP1	27	251
02B17023	2017	AB	Healthcare-Associated	NAP1	27	18
02B17025	2017	AB	Healthcare-Associated	NAP1	27	256
02B17031	2017	AB	Healthcare-Associated	NAP1	27	1
02C15005	2015	AB	Healthcare-Associated	NAP4	76	33
02C15007	2015	AB	Healthcare-Associated	NAP4	20	33
02C17008	2017	AB	Healthcare-Associated	NAP4	20	33
03A15030	2015	AB	Healthcare-Associated	NAP4	76	33
03A15035	2015	AB	Healthcare-Associated	NAP10	131	633
03A15042	2015	AB	Healthcare-Associated	NAP1	27	607
03A15049	2015	AB	Healthcare-Associated	NAP1	27	251
03A15050	2015	AB	Healthcare-Associated	NAP1	27	251
03A15056	2015	AB	Healthcare-Associated	NAP1	27	607
03A16034	2016	AB	Healthcare-Associated	NAP4	76	33
03A16035	2016	AB	Healthcare-Associated	NAP1	27	251
03A16037	2016	AB	Healthcare-Associated	NAP4	20	33
03A16049	2016	AB	Healthcare-Associated	NAP1	27	1
03A17002	2017	AB	Healthcare-Associated	NAP1	27	256

03A17004	2017	AB	Healthcare-Associated	NAP4	76	33
03A17032	2017	AB	Healthcare-Associated	NAP4	296	33
03A17036	2017	AB	Healthcare-Associated	NAP1	27	256
03A17038	2017	AB	Healthcare-Associated	NAP4	511	33
03A17053	2017	AB	Healthcare-Associated	NAP1	27	1
03A17056	2017	AB	Healthcare-Associated	NAP4	296	33
03A17057	2017	AB	Healthcare-Associated	NAP1	27	18
03A17066	2017	AB	Healthcare-Associated	NAP4	20	33
03A17070	2017	AB	Healthcare-Associated	NAP4	NS298	33
03A17073	2017	AB	Healthcare-Associated	NAP4	20	33
03A18040	2018	AB	Healthcare-Associated	NAP1	27	N/A
03B18016	2018	AB	Healthcare-Associated	NAP1	27	N/A
04A15056	2015	AB	Community-Associated	NAP4	20	33
04A15069	2015	AB	Healthcare-Associated	NAP1	27	1
04A15078	2015	AB	Community-Associated	NAP1	27	1
04A16042	2016	AB	Healthcare-Associated	NAP1	27	607
04A16058	2016	AB	Healthcare-Associated	NAP1	27	256
04A16068	2016	AB	Community-Associated	NAP1	27	256
04A16086	2016	AB	Community-Associated	NAP1	27	475
04A16090	2016	AB	Healthcare-Associated	NAP1	27	256
04A16096	2016	AB	Healthcare-Associated	NAP1	27	256
04A16106	2016	AB	Community-Associated	NAP11	106	91
04A16108	2016	AB	Community-Associated	NAP1	27	18
04A16123	2016	AB	Healthcare-Associated	NAP1	27	6
04A17047	2017	AB	Community-Associated	NAP4	20	33
04A17063	2017	AB	Healthcare-Associated	NAP1	27	251
04A17109	2017	AB	Healthcare-Associated	NAP4	76	33
04A17114	2017	AB	Community-Associated	NAP4	296	33

04A18052	2018	AB	Community-Associated	NAP1	27	N/A
04A18086	2018	AB	Healthcare-Associated	NAP1	27	N/A
04B15008	2015	AB	Community-Associated	NAP4	20	33
04B15013	2015	AB	Community-Associated	NAP4	629	33
04B15019	2015	AB	Healthcare-Associated	NAP1	27	18
04B15028	2015	AB	Community-Associated	NAP11	106	46
04B15030	2015	AB	Community-Associated	NAP11	106	612
04B15038	2015	AB	Community-Associated	NAP11	106	46
04B16001	2016	AB	Healthcare-Associated	NAP4	14	33
04B16010	2016	AB	Healthcare-Associated	NAP4	20	33
04B16031	2016	AB	Community-Associated	NAP11	106	46
04B16034	2016	AB	Community-Associated	NAP1	27	256
04B16056	2016	AB	Healthcare-Associated	NAP4	76	33
04B16057	2016	AB	Community-Associated	NAP11	106	499
04B17003	2017	AB	Community-Associated	NAP4	77	33
04B17014	2017	AB	Community-Associated	NAP1	27	251
04B17017	2017	AB	Indeterminate	NAP4	76	33
04B17020	2017	AB	Healthcare-Associated	NAP1	27	256
04B17037	2017	AB	Indeterminate	NAP11	106	1249
04B17044	2017	AB	Indeterminate	NAP4	20	33
04B18027	2018	AB	Healthcare-Associated	NAP1	27	N/A
06B15012	2015	ON	Community-Associated	NAP11	106	925
06B17005	2017	ON	Community-Associated	NAP11	106	618
06C15032	2015	ON	Community-Associated	NAP11	106	46
06C16027	2016	ON	Indeterminate	NAP11	106	612
06C17023	2017	ON	Community-Associated	NAP11	106	1033
06D18031	2018	ON	Community-Associated	NAP11	106	N/A
07B15037	2015	ON	Community-Associated	NAP11	106	443

07B16058	2016	ON	Community-Associated	NAP11	106	499
07B17034	2017	ON	Community-Associated	NAP11	106	557
07B17038	2017	ON	Community-Associated	Unassigned	5	225
07B17046	2017	ON	Community-Associated	NAP11	106	612
07B17052	2017	ON	Community-Associated	NAP11	106	686
07B17056	2017	ON	Community-Associated	NAP11	106	686
07B17058	2017	ON	Community-Associated	NAP11	106	618
07C15025	2015	ON	Community-Associated	NAP11	106	612
07C17017	2017	ON	Community-Associated	NAP11	106	46
07C17022	2017	ON	Community-Associated	NAP11	106	618
07C17027	2017	ON	Community-Associated	NAP11	106	950
07C17028	2017	ON	Community-Associated	NAP11	106	686
07C17032	2017	ON	Community-Associated	NAP11	106	1197
07D17018	2017	ON	Community-Associated	NAP11	106	618
08A15023	2015	ON	Community-Associated	NAP11	106	46
08A15038	2015	ON	Community-Associated	NAP11	106	618
08A16032	2016	ON	Community-Associated	NAP11	106	46
08A18022	2018	ON	Community-Associated	NAP11	106	N/A
09A15003	2015	ON	Healthcare-Associated	NAP10	131	633
09A15037	2015	ON	Indeterminate	NAP11	106	612
09A16006	2016	ON	Community-Associated	NAP11	106	1075
09A16015	2016	ON	Community-Associated	NAP11	106	1033
09A16035	2016	ON	Community-Associated	NAP11	106	46
09A17002	2017	ON	Community-Associated	NAP11	106	46
09A17038	2017	ON	Indeterminate	NAP11	106	46
10A15026	2015	ON	Community-Associated	NAP11	106	46
10A15028	2015	ON	Community-Associated	NAP11	106	612
10A15038	2015	ON	Community-Associated	NAP11	106	618

10A16023	2016	ON	Community-Associated	NAP11	106	618
10A16046	2016	ON	Indeterminate	NAP11	106	618
10A17044	2017	ON	Community-Associated	NAP11	106	686
15B16048	2016	NS	Community-Associated	NAP10	131	633
17A15002	2015	ON	Indeterminate	NAP11	106	686
17A15009	2015	ON	Indeterminate	NAP11	106	46
17A15015	2015	ON	Indeterminate	Unassigned	5	225
17A16006	2016	ON	Indeterminate	NAP4	NS195	581
17A16009	2016	ON	Indeterminate	NAP11	106	686
17A16023	2016	ON	Indeterminate	NAP11	106	618
17A16034	2016	ON	Indeterminate	NAP11	106	46
17A16036	2016	ON	Indeterminate	NAP11	106	925
17A17005	2017	ON	Indeterminate	NAP11	106	618
17A17013	2017	ON	Indeterminate	NAP11	106	686
17A17023	2017	ON	Indeterminate	NAP11	106	612
17A17028	2017	ON	Indeterminate	NAP11	106	618
17A17035	2017	ON	Indeterminate	NAP11	106	499
17A17038	2017	ON	Indeterminate	NAP11	118	46
17A17040	2017	ON	Healthcare-Associated	NAP4	NS195	581
17A17048	2017	ON	Indeterminate	NAP11	106	46
17A18003	2018	ON	Indeterminate	NAP11	106	N/A
17A18011	2018	ON	Indeterminate	Unassigned	NS110	N/A
17A18026	2018	ON	Indeterminate	NAP11	106	N/A
17A18037	2018	ON	Indeterminate	NAP11	106	N/A
17A18041	2018	ON	Indeterminate	NAP11	106	N/A
21A16003	2016	ON	Community-Associated	NAP11	106	46
21A16027	2016	ON	Community-Associated	NAP11	106	557
21A16028	2016	ON	Community-Associated	NAP11	106	46

21A16043	2016	ON	Community-Associated	NAP11	106	557
21A18004	2018	ON	Community-Associated	NAP11	106	N/A
21A18006	2018	ON	Indeterminate	NAP11	106	N/A
21A18026	2018	ON	Community-Associated	NAP11	106	N/A
21C15055	2015	ON	Community-Associated	NAP11	106	46
21C15078	2015	ON	Community-Associated	NAP11	106	686
21C16046	2016	ON	Community-Associated	NAP11	106	46
21C18032	2018	ON	Community-Associated	NAP11	106	N/A
21C18041	2018	ON	Community-Associated	NAP11	106	N/A
21D15031	2015	ON	Community-Associated	NAP11	106	443
21D17009	2017	ON	Indeterminate	NAP11	106	1033
21D17031	2017	ON	Indeterminate	NAP11	106	557
23A15033	2015	ON	Community-Associated	NAP11	106	686
23A17031	2017	ON	Community-Associated	NAP10	131	633
23A18032	2018	ON	Community-Associated	NAP11	106	N/A
23A18035	2018	ON	Community-Associated	NAP11	106	N/A
23B15065	2015	ON	Community-Associated	NAP11	106	686
23B17075	2017	ON	Community-Associated	NAP11	106	686
23B18047	2018	ON	Community-Associated	NAP11	106	N/A
23B18053	2018	ON	Community-Associated	NAP11	106	N/A
23B18062	2018	ON	Community-Associated	NAP11	106	N/A
24A18027	2018	QC	Healthcare-Associated	NAP10	131	N/A
24A18037	2018	QC	Healthcare-Associated	NAP10	131	N/A
27A15012	2015	ON	Community-Associated	NAP11	106	968
27A16010	2016	ON	Community-Associated	NAP11	106	46
27A16016	2016	ON	Community-Associated	NAP11	106	46
27A16017	2016	ON	Community-Associated	NAP11	106	686
27A17006	2017	ON	Community-Associated	NAP11	106	499

27A17009	2017	ON	Community-Associated	NAP11	106	46
31A16012	2016	QC	Community-Associated	NAP4	NS195	760
32A16032	2016	ON	Community-Associated	NAP11	106	618
32A17030	2017	ON	Community-Associated	NAP11	106	91
70A16038	2016	ON	Indeterminate	NAP11	106	1033
70A16043	2016	ON	Indeterminate	NAP11	106	46
70A17049	2017	ON	Community-Associated	NAP11	106	557
70A18038	2018	ON	Community-Associated	NAP11	106	N/A
70A18046	2018	ON	Community-Associated	NAP11	106	N/A

Table 31. Retail Meat Sample Information

Submission Number	Year	Commodity	Processing	Province	Isolate
CE-R2-17-0239	2017	Veal	Nuggets	ON	N20-00231
CE-R2-17-0120	2017	Veal	Red Veal	ON	N20-00438
CE2-R2-17-3130	2017	Veal	Cutlet	BC	N20-00439
CE2-R2-17-2054	2017	Veal	Ground	BC	N20-00440
CE-R2-18-0102	2018	Pig	Sausage	ON	N20-00441
CE-R2-18-0014	2018	Pig	Sausage	ON	N20-00442
CE3-R2-18-4030	2018	Pig	Sausage	AB	N20-00443
CE-R2-16-0258	2016	Pig	Ground	ON	N20-02756
CE3-R2-16-5048	2016	Pig	Ground	AB	N20-03010
CE3-R2-16-5061	2016	Pig	Ground	AB	N21-00109
CE2-R-17-1067	2017	Beef	Ground (Lean)	BC	N/A
CE2-R-17-1069	2017	Beef	Ground (Lean)	BC	N/A
CE2-R-17-1071	2017	Beef	Ground (Lean)	BC	N/A
CE2-R-17-2061	2017	Beef	Ground (Angus)	BC	N/A
CE2-R-17-2063	2017	Beef	Ground (Regular)	BC	N/A
CE2-R-17-3075	2017	Beef	Ground (Lean)	BC	N/A
CE2-R2-16-1032	2016	Pig	Ground-Lean	BC	N/A
CE2-R2-16-1034	2016	Pig	Ground-Lean	BC	N/A
CE2-R2-16-1036	2016	Pig	Ground-Lean	BC	N/A
CE2-R2-16-1040	2016	Pig	Ground	BC	N/A
CE2-R2-16-1041	2016	Pig	Ground	BC	N/A
CE2-R2-16-1042	2016	Pig	Ground	BC	N/A
CE2-R2-16-1044	2016	Pig	Ground	BC	N/A
CE2-R2-16-1046	2016	Pig	Ground	BC	N/A
CE2-R2-16-1048	2016	Pig	Ground	BC	N/A
CE2-R2-16-1055	2016	Pig	Ground	BC	N/A

CE2-R2-16-1057	2016	Pig	Ground	BC	N/A
CE2-R2-16-1061	2016	Pig	Ground	BC	N/A
CE2-R2-16-1063	2016	Pig	Ground	BC	N/A
CE2-R2-16-1065	2016	Pig	Ground	BC	N/A
CE2-R2-16-1067	2016	Pig	Ground	BC	N/A
CE2-R2-16-2044	2016	Pig	Ground	BC	N/A
CE2-R2-16-2046	2016	Pig	Ground	BC	N/A
CE2-R2-16-2048	2016	Pig	Ground	BC	N/A
CE2-R2-16-2050	2016	Pig	Ground	BC	N/A
CE2-R2-16-2052	2016	Pig	Ground	BC	N/A
CE2-R2-16-2054	2016	Pig	Ground	BC	N/A
CE2-R2-16-2062	2016	Pig	Ground	BC	N/A
CE2-R2-16-2064	2016	Pig	Ground	BC	N/A
CE2-R2-16-2066	2016	Pig	Ground	BC	N/A
CE2-R2-16-2067	2016	Pig	Ground	BC	N/A
CE2-R2-16-3034	2016	Pig	Ground	BC	N/A
CE2-R2-16-3035	2016	Pig	Ground-Lean	BC	N/A
CE2-R2-16-3036	2016	Pig	Ground-Lean	BC	N/A
CE2-R2-16-3059	2016	Pig	Ground-Lean	BC	N/A
CE2-R2-16-3079	2016	Pig	Ground-Lean	BC	N/A
CE2-R2-16-3080	2016	Pig	Ground-Lean	BC	N/A
CE2-R2-16-3081	2016	Pig	Ground-Lean	BC	N/A
CE2-R2-16-3086	2016	Pig	Ground	BC	N/A
CE2-R2-16-3088	2016	Pig	Ground-Lean	BC	N/A
CE2-R2-16-3090	2016	Pig	Ground-Regula	BC	N/A
CE2-R2-16-3091	2016	Pig	Ground	BC	N/A
CE2-R2-16-3092	2016	Pig	Ground	BC	N/A
CE2-R2-16-3093	2016	Pig	Ground	BC	N/A

CE2-R2-16-3099	2016	Pig	Ground	BC	N/A
CE2-R2-16-3104	2016	Pig	Ground	BC	N/A
CE2-R2-16-3106	2016	Pig	Ground	BC	N/A
CE2-R2-16-3108	2016	Pig	Ground	BC	N/A
CE2-R2-16-3110	2016	Pig	Ground	BC	N/A
CE2-R2-16-3112	2016	Pig	Ground	BC	N/A
CE2-R2-16-3114	2016	Pig	Ground	BC	N/A
CE2-R2-16-3124	2016	Pig	Ground	BC	N/A
CE2-R2-16-3125	2016	Pig	Ground	BC	N/A
CE2-R2-16-3126	2016	Pig	Ground	BC	N/A
CE2-R2-17-1031	2017	Veal	Scallopini	BC	N/A
CE2-R2-17-1033	2017	Veal	Ground	BC	N/A
CE2-R2-17-1035	2017	Veal	Ground	BC	N/A
CE2-R2-17-1039	2017	Veal	Cutlet	BC	N/A
CE2-R2-17-1041	2017	Veal	Scallopini	BC	N/A
CE2-R2-17-1061	2017	Veal	Scallopini Red Veal	BC	N/A
CE2-R2-17-1063	2017	Veal	Cutlet Red Veal	BC	N/A
CE2-R2-17-1065	2017	Veal	Veal	BC	N/A
CE2-R2-17-1067	2017	Veal	Scallopini	BC	N/A
CE2-R2-17-1069	2017	Veal	Ground	BC	N/A
CE2-R2-17-1071	2017	Veal	Ground Red Veal	BC	N/A
CE2-R2-17-2034	2017	Veal	Ground	BC	N/A
CE2-R2-17-2036	2017	Veal	Cutlet	BC	N/A
CE2-R2-17-2038	2017	Veal	Stew Chunks	BC	N/A
CE2-R2-17-2040	2017	Veal	Ground	BC	N/A
CE2-R2-17-2042	2017	Veal	Cutlet	BC	N/A
CE2-R2-17-2050	2017	Veal	Ground	BC	N/A
CE2-R2-17-2052	2017	Veal	Cutlet	BC	N/A

CE2-R2-17-2056	2017	Veal	Ground	BC	N/A
CE2-R2-17-2058	2017	Veal	Scallopini	BC	N/A
CE2-R2-17-2060	2017	Veal	Cutlet	BC	N/A
CE2-R2-17-2062	2017	Veal	Cutlet	BC	N/A
CE2-R2-17-2064	2017	Veal	Ground	BC	N/A
CE2-R2-17-2066	2017	Veal	Ground	BC	N/A
CE2-R2-17-3056	2017	Veal	Scallopini	BC	N/A
CE2-R2-17-3058	2017	Veal	Ground	BC	N/A
CE2-R2-17-3062	2017	Veal	Ground	BC	N/A
CE2-R2-17-3064	2017	Veal	Ground	BC	N/A
CE2-R2-17-3066	2017	Veal	Cutlet	BC	N/A
CE2-R2-17-3070	2017	Veal	Other Cut Boneless	BC	N/A
CE2-R2-17-3074	2017	Veal	Cutlet	BC	N/A
CE2-R2-17-3076	2017	Veal	Scallopini	BC	N/A
CE2-R2-17-3078	2017	Veal	Stew Chunks (Milk)	BC	N/A
CE2-R2-17-3080	2017	Veal	Ground	BC	N/A
CE2-R2-17-3082	2017	Veal	Scallopini	BC	N/A
CE2-R2-17-3084	2017	Veal	Cutlet	BC	N/A
CE2-R2-17-3110	2017	Veal	Ground	BC	N/A
CE2-R2-17-3112	2017	Veal	Scallopini	BC	N/A
CE2-R2-17-3114	2017	Veal	Stew Chunks	BC	N/A
CE2-R2-17-3116	2017	Veal	Other	BC	N/A
CE2-R2-17-3118	2017	Veal	Ground	BC	N/A
CE2-R2-17-3122	2017	Veal	Cutlet	BC	N/A
CE2-R2-17-3124	2017	Veal	Chops Red Veal	BC	N/A
CE2-R2-17-3126	2017	Veal	Ground	BC	N/A
CE2-R2-17-3128	2017	Veal	Scallopini	BC	N/A
CE2-R2-17-3132	2017	Veal	Chops White Veal	BC	N/A

CE2-R2-18-1002	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1004	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1006	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1008	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1010	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1012	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1014	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1016	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1018	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1026	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1030	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1032	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1036	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1038	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1040	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1042	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1044	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1046	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1048	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1056	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1058	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1060	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1062	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1064	2018	Pig	Sausage	BC	N/A
CE2-R2-18-1066	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2002	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2008	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2010	2018	Pig	Sausage	BC	N/A

CE2-R2-18-2012	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2014	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2016	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2018	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2020	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2022	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2024	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2026	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2028	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2030	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2032	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2034	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2036	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2040	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2042	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2044	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2046	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2048	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2050	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2052	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2054	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2056	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2058	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2060	2018	Pig	Sausage	BC	N/A
CE2-R2-18-2066	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3002	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3004	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3006	2018	Pig	Sausage	BC	N/A

CE2-R2-18-3008	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3010	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3012	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3014	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3016	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3018	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3022	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3024	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3026	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3028	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3030	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3032	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3034	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3036	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3038	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3042	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3044	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3046	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3048	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3050	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3052	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3054	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3056	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3058	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3060	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3062	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3064	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3066	2018	Pig	Sausage	BC	N/A

CE2-R2-18-3068	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3070	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3072	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3074	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3076	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3078	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3080	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3082	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3084	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3086	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3088	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3090	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3092	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3094	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3096	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3098	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3100	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3102	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3104	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3106	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3108	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3110	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3112	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3114	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3116	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3118	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3120	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3124	2018	Pig	Sausage	BC	N/A

CE2-R2-18-3126	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3128	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3130	2018	Pig	Sausage	BC	N/A
CE2-R2-18-3132	2018	Pig	Sausage	BC	N/A
CE3-R-17-4125	2017	Beef	Ground (Lean)	AB	N/A
CE3-R-17-4181	2017	Beef	Ground (Lean)	AB	N/A
CE3-R-17-4183	2017	Beef	Ground (Extra-Lean)	AB	N/A
CE3-R-17-4185	2017	Beef	Ground (Lean)	AB	N/A
CE3-R-17-4187	2017	Beef	Ground (Regular)	AB	N/A
CE3-R-17-4189	2017	Beef	Ground (Extra-Lean)	AB	N/A
CE3-R-17-4191	2017	Beef	Ground (Lean)	AB	N/A
CE3-R-17-4195	2017	Beef	Ground (Regular)	AB	N/A
CE3-R-17-4197	2017	Beef	Ground (Extra-Lean)	AB	N/A
CE3-R-17-5047	2017	Beef	Ground (Regular)	AB	N/A
CE3-R-17-5056	2017	Beef	Ground (Lean)	AB	N/A
CE3-R-17-5057	2017	Beef	Ground (Lean)	AB	N/A
CE3-R2-16-4110	2016	Pig	Ground-Lean	AB	N/A
CE3-R2-16-4112	2016	Pig	Ground-Lean	AB	N/A
CE3-R2-16-4114	2016	Pig	Ground-Lean	AB	N/A
CE3-R2-16-4116	2016	Pig	Ground-Lean	AB	N/A
CE3-R2-16-4118	2016	Pig	Ground-Lean	AB	N/A
CE3-R2-16-4122	2016	Pig	Ground	AB	N/A
CE3-R2-16-4124	2016	Pig	Ground-Lean	AB	N/A
CE3-R2-16-4126	2016	Pig	Ground-Lean	AB	N/A
CE3-R2-16-4128	2016	Pig	Ground	AB	N/A
CE3-R2-16-4130	2016	Pig	Ground	AB	N/A
CE3-R2-16-4132	2016	Pig	Ground	AB	N/A
CE3-R2-16-4134	2016	Pig	Ground	AB	N/A

CE3-R2-16-4136	2016	Pig	Ground	AB	N/A
CE3-R2-16-4138	2016	Pig	Ground	AB	N/A
CE3-R2-16-4140	2016	Pig	Ground	AB	N/A
CE3-R2-16-4142	2016	Pig	Ground	AB	N/A
CE3-R2-16-4144	2016	Pig	Ground	AB	N/A
CE3-R2-16-4146	2016	Pig	Ground	AB	N/A
CE3-R2-16-4148	2016	Pig	Ground	AB	N/A
CE3-R2-16-4150	2016	Pig	Ground	AB	N/A
CE3-R2-16-4158	2016	Pig	Ground	AB	N/A
CE3-R2-16-4160	2016	Pig	Ground	AB	N/A
CE3-R2-16-4162	2016	Pig	Ground	AB	N/A
CE3-R2-16-4166	2016	Pig	Ground	AB	N/A
CE3-R2-16-4168	2016	Pig	Ground	AB	N/A
CE3-R2-16-4172	2016	Pig	Ground	AB	N/A
CE3-R2-16-4174	2016	Pig	Ground	AB	N/A
CE3-R2-16-4176	2016	Pig	Ground	AB	N/A
CE3-R2-16-4178	2016	Pig	Ground	AB	N/A
CE3-R2-16-4184	2016	Pig	Ground	AB	N/A
CE3-R2-16-4188	2016	Pig	Ground	AB	N/A
CE3-R2-16-4190	2016	Pig	Ground	AB	N/A
CE3-R2-16-4192	2016	Pig	Ground	AB	N/A
CE3-R2-16-4194	2016	Pig	Ground	AB	N/A
CE3-R2-16-4196	2016	Pig	Ground	AB	N/A
CE3-R2-16-4198	2016	Pig	Ground	AB	N/A
CE3-R2-16-4200	2016	Pig	Ground	AB	N/A
CE3-R2-16-4202	2016	Pig	Ground	AB	N/A
CE3-R2-16-4204	2016	Pig	Ground	AB	N/A
CE3-R2-16-5025	2016	Pig	Ground	AB	N/A

CE3-R2-16-5027	2016	Pig	Ground-Regula	AB	N/A
CE3-R2-16-5029	2016	Pig	Ground-Lean	AB	N/A
CE3-R2-16-5039	2016	Pig	Ground-Lean	AB	N/A
CE3-R2-16-5041	2016	Pig	Ground-Lean	AB	N/A
CE3-R2-16-5043	2016	Pig	Ground-Regula	AB	N/A
CE3-R2-16-5050	2016	Pig	Ground	AB	N/A
CE3-R2-16-5052	2016	Pig	Ground	AB	N/A
CE3-R2-16-5053	2016	Pig	Ground	AB	N/A
CE3-R2-16-5054	2016	Pig	Ground	AB	N/A
CE3-R2-16-5062	2016	Pig	Ground	AB	N/A
CE3-R2-16-5063	2016	Pig	Ground	AB	N/A
CE3-R2-16-5075	2016	Pig	Ground	AB	N/A
CE3-R2-16-5076	2016	Pig	Ground	AB	N/A
CE3-R2-16-5077	2016	Pig	Ground	AB	N/A
CE3-R2-17-4065	2017	Veal	Liver	AB	N/A
CE3-R2-17-4071	2017	Veal	Stew Chunks	AB	N/A
CE3-R2-17-4073	2017	Veal	Chops	AB	N/A
CE3-R2-17-4075	2017	Veal	Ground	AB	N/A
CE3-R2-17-4091	2017	Veal	Ground (Lean)	AB	N/A
CE3-R2-17-4093	2017	Veal	Steak	AB	N/A
CE3-R2-17-4095	2017	Veal	Cutlet	AB	N/A
CE3-R2-17-4097	2017	Veal	Stew Chunks	AB	N/A
CE3-R2-17-4106	2017	Veal	Cutlet	AB	N/A
CE3-R2-17-4108	2017	Veal	Ground	AB	N/A
CE3-R2-17-4110	2017	Veal	Ground	AB	N/A
CE3-R2-17-4118	2017	Veal	Liver	AB	N/A
CE3-R2-17-4120	2017	Veal	Cutlet	AB	N/A
CE3-R2-17-4122	2017	Veal	Scallopini	AB	N/A

CE3-R2-17-4167	2017	Veal	Ground Red Veal	AB	N/A
CE3-R2-17-4169	2017	Veal	Stew Chunks Red	AB	N/A
CE3-R2-17-4184	2017	Veal	Scallopini	AB	N/A
CE3-R2-17-4186	2017	Veal	Cutlet Red Veal	AB	N/A
CE3-R2-17-4188	2017	Veal	Ground	AB	N/A
CE3-R2-17-4190	2017	Veal	Cutlet	AB	N/A
CE3-R2-17-4192	2017	Veal	Stew Chunks	AB	N/A
CE3-R2-17-4194	2017	Veal	Cutlet Red Veal	AB	N/A
CE3-R2-17-4196	2017	Veal	Ground	AB	N/A
CE3-R2-17-4198	2017	Veal	Cutlet	AB	N/A
CE3-R2-17-4200	2017	Veal	Cutlet Red Veal	AB	N/A
CE3-R2-17-5022	2017	Veal	Scallopini	AB	N/A
CE3-R2-17-5038	2017	Veal	Scallopini White Veal	AB	N/A
CE3-R2-18-4002	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4004	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4006	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4008	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4010	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4012	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4014	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4016	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4018	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4020	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4022	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4024	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4026	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4028	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4032	2018	Pig	Sausage	AB	N/A

CE3-R2-18-4034	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4036	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4038	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4040	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4042	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4044	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4046	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4048	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4049	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4051	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4054	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4062	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4064	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4066	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4068	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4070	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4072	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4073	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4074	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4075	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4076	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4077	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4080	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4082	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4084	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4086	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4092	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4094	2018	Pig	Sausage	AB	N/A

CE3-R2-18-4096	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4097	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4102	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4105	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4107	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4111	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4113	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4115	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4117	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4119	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4121	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4123	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4125	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4127	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4128	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4129	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4130	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4131	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4132	2018	Pig	Sausage	AB	N/A
CE3-R2-18-4133	2018	Pig	Sausage	AB	N/A
CE3-R2-18-5004	2018	Pig	Sausage	AB	N/A
CE3-R2-18-5005	2018	Pig	Sausage	AB	N/A
CE3-R2-18-5006	2018	Pig	Sausage	AB	N/A
CE3-R2-18-5010	2018	Pig	Sausage	AB	N/A
CE3-R2-18-5011	2018	Pig	Sausage	AB	N/A
CE3-R2-18-5016	2018	Pig	Sausage	AB	N/A
CE3-R2-18-5017	2018	Pig	Sausage	AB	N/A
CE3-R2-18-5018	2018	Pig	Sausage	AB	N/A

CE3-R2-18-5022	2018	Pig	Sausage	AB	N/A
CE3-R2-18-5024	2018	Pig	Sausage	AB	N/A
CE3-R2-18-5025	2018	Pig	Sausage	AB	N/A
CE3-R2-18-5026	2018	Pig	Sausage	AB	N/A
CE3-R2-18-5027	2018	Pig	Sausage	AB	N/A
CE3-R2-18-5034	2018	Pig	Sausage	AB	N/A
CE3-R2-18-5035	2018	Pig	Sausage	AB	N/A
CE3-R2-18-5036	2018	Pig	Sausage	AB	N/A
CE3-R2-18-5040	2018	Pig	Sausage	AB	N/A
CE3-R2-18-5041	2018	Pig	Sausage	AB	N/A
CE3-R2-18-5042	2018	Pig	Sausage	AB	N/A
CE-R-17-0113	2017	Beef	Ground (Lean)	ON	N/A
CE-R-17-0241	2017	Beef	Ground (Lean)	ON	N/A
CE-R-17-0243	2017	Beef	Ground (Lean)	ON	N/A
CE-R-17-0245	2017	Beef	Ground (Lean)	ON	N/A
CE-R-17-0247	2017	Beef	Ground (Lean)	ON	N/A
CE-R-17-0249	2017	Beef	Ground (Lean)	ON	N/A
CE-R-17-0251	2017	Beef	Ground	ON	N/A
CE-R-17-0253	2017	Beef	Ground (Lean)	ON	N/A
CE-R-17-0255	2017	Beef	Ground (Lean)	ON	N/A
CE-R-17-0257	2017	Beef	Ground (Lean)	ON	N/A
CE-R2-16-0014	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0018	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0028	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0032	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0034	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0038	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0040	2016	Pig	Ground-Regula	ON	N/A

CE-R2-16-0042	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0044	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0046	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0048	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0050	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0052	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0054	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0056	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0058	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0060	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0062	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0064	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0066	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0068	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0070	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0072	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0074	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0076	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0078	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0080	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0082	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0084	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0086	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0088	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0090	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0092	2016	Pig	Ground	ON	N/A
CE-R2-16-0094	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0096	2016	Pig	Ground-Lean	ON	N/A

CE-R2-16-0098	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0100	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0102	2016	Pig	Ground-Regula	ON	N/A
CE-R2-16-0104	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0106	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0108	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0110	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0112	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0114	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0116	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0118	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0120	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0122	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0124	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0126	2016	Pig	Ground-Regula	ON	N/A
CE-R2-16-0128	2016	Pig	Ground-Regula	ON	N/A
CE-R2-16-0130	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0132	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0134	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0136	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0138	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0140	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0142	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0144	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0146	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0148	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0150	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0152	2016	Pig	Ground-Lean	ON	N/A

CE-R2-16-0154	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0156	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0158	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0160	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0162	2016	Pig	Ground-Lean	ON	N/A
CE-R2-16-0164	2016	Pig	Ground	ON	N/A
CE-R2-16-0166	2016	Pig	Ground	ON	N/A
CE-R2-16-0168	2016	Pig	Ground	ON	N/A
CE-R2-16-0170	2016	Pig	Ground	ON	N/A
CE-R2-16-0172	2016	Pig	Ground	ON	N/A
CE-R2-16-0174	2016	Pig	Ground	ON	N/A
CE-R2-16-0178	2016	Pig	Ground	ON	N/A
CE-R2-16-0180	2016	Pig	Ground	ON	N/A
CE-R2-16-0184	2016	Pig	Ground	ON	N/A
CE-R2-16-0186	2016	Pig	Ground	ON	N/A
CE-R2-16-0188	2016	Pig	Ground	ON	N/A
CE-R2-16-0190	2016	Pig	Ground	ON	N/A
CE-R2-16-0192	2016	Pig	Ground	ON	N/A
CE-R2-16-0194	2016	Pig	Nuggets	ON	N/A
CE-R2-16-0196	2016	Pig	Ground	ON	N/A
CE-R2-16-0198	2016	Pig	Ground	ON	N/A
CE-R2-16-0200	2016	Pig	Ground	ON	N/A
CE-R2-16-0202	2016	Pig	Ground	ON	N/A
CE-R2-16-0204	2016	Pig	Ground	ON	N/A
CE-R2-16-0206	2016	Pig	Ground	ON	N/A
CE-R2-16-0208	2016	Pig	Ground	ON	N/A
CE-R2-16-0210	2016	Pig	Ground	ON	N/A
CE-R2-16-0212	2016	Pig	Ground	ON	N/A

CE-R2-16-0214	2016	Pig	Ground	ON	N/A
CE-R2-16-0216	2016	Pig	Ground	ON	N/A
CE-R2-16-0220	2016	Pig	Ground	ON	N/A
CE-R2-16-0224	2016	Pig	Ground	ON	N/A
CE-R2-16-0226	2016	Pig	Ground	ON	N/A
CE-R2-16-0228	2016	Pig	Ground	ON	N/A
CE-R2-16-0230	2016	Pig	Ground	ON	N/A
CE-R2-16-0232	2016	Pig	Ground	ON	N/A
CE-R2-16-0234	2016	Pig	Ground	ON	N/A
CE-R2-16-0238	2016	Pig	Ground	ON	N/A
CE-R2-16-0240	2016	Pig	Ground	ON	N/A
CE-R2-16-0242	2016	Pig	Ground	ON	N/A
CE-R2-16-0244	2016	Pig	Ground	ON	N/A
CE-R2-16-0246	2016	Pig	Ground	ON	N/A
CE-R2-16-0248	2016	Pig	Ground	ON	N/A
CE-R2-16-0250	2016	Pig	Ground	ON	N/A
CE-R2-16-0252	2016	Pig	Ground	ON	N/A
CE-R2-16-0254	2016	Pig	Ground	ON	N/A
CE-R2-16-0256	2016	Pig	Ground	ON	N/A
CE-R2-16-0260	2016	Pig	Ground	ON	N/A
CE-R2-16-0262	2016	Pig	Ground	ON	N/A
CE-R2-16-0264	2016	Pig	Ground	ON	N/A
CE-R2-17-0114	2017	Veal	Ground	ON	N/A
CE-R2-17-0116	2017	Veal	Stew Chunks	ON	N/A
CE-R2-17-0118	2017	Veal	Scallopini	ON	N/A
CE-R2-17-0124	2017	Veal	Red Veal	ON	N/A
CE-R2-17-0126	2017	Veal	Scallopini Red Veal	ON	N/A
CE-R2-17-0128	2017	Veal	Scallopini Red Veal	ON	N/A

CE-R2-17-0130	2017	Veal	Red Veal	ON	N/A
CE-R2-17-0132	2017	Veal	Scallopini Red Veal	ON	N/A
CE-R2-17-0134	2017	Veal	Scallopini Red Veal	ON	N/A
CE-R2-17-0136	2017	Veal	Red Veal	ON	N/A
CE-R2-17-0140	2017	Veal	Scallopini Red Veal	ON	N/A
CE-R2-17-0142	2017	Veal	Scallopini Red Veal	ON	N/A
CE-R2-17-0145	2017	Veal	Scallopini Red Veal	ON	N/A
CE-R2-17-0147	2017	Veal	stew chunks Red Veal	ON	N/A
CE-R2-17-0150	2017	Veal	stew chunks Red Veal	ON	N/A
CE-R2-17-0152	2017	Veal	stew chunks Red Veal	ON	N/A
CE-R2-17-0207	2017	Veal	Red Veal	ON	N/A
CE-R2-17-0209	2017	Veal	Ground Red Veal	ON	N/A
CE-R2-17-0211	2017	Veal	stew chunks Red Veal	ON	N/A
CE-R2-17-0212	2017	Veal	Scallopini Red Veal	ON	N/A
CE-R2-17-0214	2017	Veal	Scallopini Red Veal	ON	N/A
CE-R2-17-0216	2017	Veal	Scallopini White Veal	ON	N/A
CE-R2-17-0218	2017	Veal	Ground Red Veal	ON	N/A
CE-R2-17-0220	2017	Veal	Scallopini Red Veal	ON	N/A
CE-R2-17-0223	2017	Veal	Ground Red Veal	ON	N/A
CE-R2-17-0225	2017	Veal	Scallopini Red Veal	ON	N/A
CE-R2-17-0227	2017	Veal	stew chunks Red Veal	ON	N/A
CE-R2-17-0229	2017	Veal	Ground Red Veal	ON	N/A
CE-R2-17-0231	2017	Veal	stew chunks Red Veal	ON	N/A
CE-R2-17-0233	2017	Veal	stew chunks Red Veal	ON	N/A
CE-R2-17-0235	2017	Veal	stew chunks Red Veal	ON	N/A
CE-R2-17-0237	2017	Veal	Ground Red Veal	ON	N/A
CE-R2-17-0240	2017	Veal	stew chunks Red Veal	ON	N/A
CE-R2-17-0242	2017	Veal	Ground Red Veal	ON	N/A

CE-R2-17-0245	2017	Veal	Scallopini White Veal	ON	N/A
CE-R2-17-0247	2017	Veal	Ground Red Veal	ON	N/A
CE-R2-17-0249	2017	Veal	Stew Chunks Red Veal	ON	N/A
CE-R2-17-0251	2017	Veal	Scallopini Red Veal	ON	N/A
CE-R2-17-0253	2017	Veal	Scallopini Red Veal	ON	N/A
CE-R2-18-0002	2018	Pig	Sausage	ON	N/A
CE-R2-18-0004	2018	Pig	Sausage	ON	N/A
CE-R2-18-0006	2018	Pig	Sausage	ON	N/A
CE-R2-18-0008	2018	Pig	Sausage	ON	N/A
CE-R2-18-0010	2018	Pig	Sausage	ON	N/A
CE-R2-18-0012	2018	Pig	Sausage	ON	N/A
CE-R2-18-0016	2018	Pig	Sausage	ON	N/A
CE-R2-18-0018	2018	Pig	Sausage	ON	N/A
CE-R2-18-0020	2018	Pig	Sausage	ON	N/A
CE-R2-18-0022	2018	Pig	Sausage	ON	N/A
CE-R2-18-0024	2018	Pig	Sausage	ON	N/A
CE-R2-18-0026	2018	Pig	Sausage	ON	N/A
CE-R2-18-0028	2018	Pig	Sausage	ON	N/A
CE-R2-18-0030	2018	Pig	Sausage	ON	N/A
CE-R2-18-0032	2018	Pig	Sausage	ON	N/A
CE-R2-18-0034	2018	Pig	Sausage	ON	N/A
CE-R2-18-0036	2018	Pig	Sausage	ON	N/A
CE-R2-18-0038	2018	Pig	Sausage	ON	N/A
CE-R2-18-0040	2018	Pig	Sausage	ON	N/A
CE-R2-18-0042	2018	Pig	Sausage	ON	N/A
CE-R2-18-0044	2018	Pig	Sausage	ON	N/A
CE-R2-18-0046	2018	Pig	Sausage	ON	N/A
CE-R2-18-0048	2018	Pig	Sausage	ON	N/A

CE-R2-18-0050	2018	Pig	Sausage	ON	N/A
CE-R2-18-0052	2018	Pig	Sausage	ON	N/A
CE-R2-18-0056	2018	Pig	Sausage	ON	N/A
CE-R2-18-0058	2018	Pig	Sausage	ON	N/A
CE-R2-18-0060	2018	Pig	Sausage	ON	N/A
CE-R2-18-0062	2018	Pig	Sausage	ON	N/A
CE-R2-18-0064	2018	Pig	Sausage	ON	N/A
CE-R2-18-0066	2018	Pig	Sausage	ON	N/A
CE-R2-18-0068	2018	Pig	Sausage	ON	N/A
CE-R2-18-0070	2018	Pig	Sausage	ON	N/A
CE-R2-18-0072	2018	Pig	Sausage	ON	N/A
CE-R2-18-0074	2018	Pig	Sausage	ON	N/A
CE-R2-18-0076	2018	Pig	Sausage	ON	N/A
CE-R2-18-0078	2018	Pig	Sausage	ON	N/A
CE-R2-18-0080	2018	Pig	Sausage	ON	N/A
CE-R2-18-0082	2018	Pig	Sausage	ON	N/A
CE-R2-18-0086	2018	Pig	Sausage	ON	N/A
CE-R2-18-0088	2018	Pig	Sausage	ON	N/A
CE-R2-18-0090	2018	Pig	Sausage	ON	N/A
CE-R2-18-0092	2018	Pig	Sausage	ON	N/A
CE-R2-18-0094	2018	Pig	Sausage	ON	N/A
CE-R2-18-0096	2018	Pig	Sausage	ON	N/A
CE-R2-18-0097	2018	Pig	Sausage	ON	N/A
CE-R2-18-0099	2018	Pig	Sausage	ON	N/A
CE-R2-18-0100	2018	Pig	Sausage	ON	N/A
CE-R2-18-0104	2018	Pig	Sausage	ON	N/A
CE-R2-18-0106	2018	Pig	Sausage	ON	N/A
CE-R2-18-0108	2018	Pig	Sausage	ON	N/A

CE-R2-18-0112	2018	Pig	Sausage	ON	N/A
CE-R2-18-0114	2018	Pig	Sausage	ON	N/A
CE-R2-18-0116	2018	Pig	Sausage	ON	N/A
CE-R2-18-0118	2018	Pig	Sausage	ON	N/A
CE-R2-18-0120	2018	Pig	Sausage	ON	N/A
CE-R2-18-0121	2018	Pig	Sausage	ON	N/A
CE-R2-18-0122	2018	Pig	Sausage	ON	N/A
CE-R2-18-0123	2018	Pig	Sausage	ON	N/A
CE-R2-18-0124	2018	Pig	Sausage	ON	N/A
CE-R2-18-0125	2018	Pig	Sausage	ON	N/A
CE-R2-18-0126	2018	Pig	Sausage	ON	N/A
CE-R2-18-0128	2018	Pig	Sausage	ON	N/A
CE-R2-18-0132	2018	Pig	Sausage	ON	N/A
CE-R2-18-0134	2018	Pig	Sausage	ON	N/A
CE-R2-18-0136	2018	Pig	Sausage	ON	N/A
CE-R2-18-0138	2018	Pig	Sausage	ON	N/A
CE-R2-18-0140	2018	Pig	Sausage	ON	N/A
CE-R2-18-0142	2018	Pig	Sausage	ON	N/A
CE-R2-18-0144	2018	Pig	Sausage	ON	N/A
CE-R2-18-0145	2018	Pig	Sausage	ON	N/A
CE-R2-18-0146	2018	Pig	Sausage	ON	N/A
CE-R2-18-0147	2018	Pig	Sausage	ON	N/A
CE-R2-18-0149	2018	Pig	Sausage	ON	N/A
CE-R2-18-0150	2018	Pig	Sausage	ON	N/A
CE-R2-18-0152	2018	Pig	Sausage	ON	N/A
CE-R2-18-0154	2018	Pig	Sausage	ON	N/A
CE-R2-18-0156	2018	Pig	Sausage	ON	N/A
CE-R2-18-0160	2018	Pig	Sausage	ON	N/A

CE-R2-18-0162	2018	Pig	Sausage	ON	N/A

Appendix B - Method Validation of Isolation of *C. difficile* from Retail Meats

Validation of Isolation of *C. difficile* from Retail Meats Method

Validation experiments concluded that the isolation protocol was a suitable method to isolate *C. difficile* from retail meat. All tested aspects did not hinder the recovery of *C. difficile* from the samples. Several aspects of the isolation method were tested to parameters beyond those of the protocol with no detriment to isolation. The *C. difficile* spores were able to endure an ethanol shock of 3 times (180 minutes) the duration used in the protocol (60 minutes. Additionally, the *C. difficile* spores were able to endure centrifugation for 6 times (60 minutes) the duration used in the protocol (10 minutes). These results are not surprising as method has been used in the past under similar circumstances [136,137].

The limit of detection of the isolation of *C. difficile* from retail meat protocol was determined to be approximately 7.5 spores/mL. This result is similar to that of a previously published limit of detection of a variation of the isolation method used in this study, determined to be 3.2 spores/mL [136]. However, the method utilized by Visser et al. to determine the limit of detection was different than the one used in this study. Instead, they inoculated a nonsterile meat sample to serve as a test sample.

Ethanol Shock Validation

C. difficile growth was detected after 30, 60, 90, 120, 150, and 180 minutes of ethanol shocking in 50% v/v anhydrous ethanol. Mixture was spread on BRU and incubated anaerobically at 37°C for 5 days. Plates were observed after 3 and 5 days of incubation (Table 32).

Spin / Centrifugation Validation

C. difficile growth was not detected in the supernatant after 10, 20, 30, 40, 50, and 60 minutes of centrifugation at 4000g. *C. difficile* growth was detected in the generated pellet after 10, 20, 30, 40, 50, and 60 minutes of centrifugation at 4000g (Table 33). Supernatant and mixture were spread on BRU and incubated anaerobically at 37°C for 5 days. Plates were observed after 3 and 5 days of incubation.

Table 32. *C. difficile* Growth After Various Ethanol Shock Exposure Times

	Day 3	Day 5
30 Minutes	TMTC	TMTC
60 Minutes	TMTC	TMTC
90 Minutes	TMTC	TMTC
120 Minutes	TMTC	TMTC
150 Minutes	TMTC	TMTC
180 Minutes	TMTC	TMTC

TMTC: too many to count

Table 33. *C. difficile* Growth After Various Centrifugation Times

	Supernatant		Pellet	
	Day 3	Day 5	Day 3	Day 5
10 Minutes	No Growth	No Growth	TMTC	TMTC
20 Minutes	No Growth	No Growth	TMTC	TMTC
30 Minutes	No Growth	No Growth	TMTC	TMTC
40 Minutes	No Growth	No Growth	TMTC	TMTC
50 Minutes	No Growth	No Growth	TMTC	TMTC
60 Minutes	No Growth	No Growth	TMTC	TMTC

Limit of Detection

The limit of detection for direct plating was determined to be 7.5 spores/mL, 95% CI [4.01, 10.8] (Table 34). The limit of detection for the enrichment broth was determined to be 7.5 spores/mL, 95% CI [4.4, 11.1] (Table 34).

Table 34. Limit of Detection of the Isolation of *C. difficile* from Retail Meats Via Direct Plating and Enrichment Broth

	Direct Plating (spores/mL)	Enrichment Broth (spores/mL)
RT027 (NAP1)	6	2.5
RT131 (NAP10)	13	12
RT106 (NAP11)	3.5	8
Average	**7.5**	**7.5**

Appendix C - Additional Extraction Methods

Multiple *C. difficile* genomic DNA extraction methods were evaluated (Table 35). 3 *C. difficile* isolates were utilized for each method. N20-00231 (NAP10/RT131) isolated from a retail meat sample, N20-00442 (NAP11, RT106) isolated from a retail meat sample, and 11ACD0075 (NAP1/RT027) isolated from a human clinical case.

The best determined growth conditions and sequencing parameters was determined to be *C. difficile* grown for 18 hours in 5mL of pre-reduced brain heart infusion (BHI) broth extracted using Qiagen's DNeasy whole genome DNA extraction kit (Qiagen, Germany) with a modified procedure. Modifications include incubating Rnase A and protein kinase A together for an hour without buffer AL (Qiagen, Germany). This effectively doubled solution's incubation time with protein kinase A. Additionally, it was determined that the buffer Al (Qiagen, Germany), composed of 30% - 50% guanidine hydrochloride inhibited the enzymatic activity of RNase A. Although it is hypothesized that long incubation in BHI broth may permit the formation of spores in the culture instead of only vegetative cells, growth and extraction during the hours of a single working day is not commercially viable. It would lead to a workday far beyond the 8-hour standard. One alternative is switching the BHI broth incubation time and lysozyme incubation time. This condition was tested as condition E2 but produced inferior assemblies when compared.

The timing of the extraction procedure allows for an optimized technique for high-throughput processing in the workplace. The incubation of *C. difficile* in BHI broth is completed overnight. The culture is inoculated at the end of the workday, producing a ready to be extracted culture at the beginning of the following workday. The extraction protocol can be efficiently performed in a single day with the use of a multi-channel pipette and multi-dispensing pipette.

When tested on the 3 test strains [N20-00231 (NAP10/RT131), N20-00442 (NAP11, RT106), and 11ACD0075 (NAP1/RT027)], this method consistently produced DNA that when sequenced, produced some of the best assemblies. Assemblies were judged upon their mean contig length, number of contigs, and number of contigs in the N50. Additionally, the coverage of the sequences was also some of the best when compared to other compared extraction methods. The percentage of the genome covered 50X or more was consistently above 80% with mean coverage of approximately 140X.

Table 35. *C. difficile* Extraction Optimization Naming Suffix Key

Suffix	Condition
①	6h Growth + Same-Day Extraction
②	6h Growth + Overnight Extraction
③	18h Growth + Same-Day Extraction
④	Plate → Broth 18h → Broth 18h
A	DNeasy 2mL
B	Masterpure
C	QIAamp via DNeasy Protocol
D	DNeasy Protocol (Rnase Pre PK/AL)
E	DNeasy (Rnase with PK then AL)
F	DNeasy (Rnase Post PK then AL)
G	QIAsymphony
H	DNeasy Paper Extraction without Rnase
I	DNeasy Paper Extraction with Rnase
J	MicroGEM PDQex 18h BHI
K	MicroGEM PDQex 18h BRU
L	MicroGEM PDQex 48h TSB
M	MicroGEM PDQex 6h BHI
N	Dneasy AVL Lysis
P	Dneasy Rnase w/Lysozyme (8 Day Spore)
Q	Dneasy 18h Same-Day Rnase w/Lysozyme
R	Dneasy Rnase w/Lysozyme + 2h PKA
S	Dneasy Rnase+Mutanolysin(0.5µL/Sample) w/Lysozyme

Qiagen DNEasy

Bacterial culture Growth Variations (Qiagen DNEasy)

Several bacterial culture growth modifications were trialed. Starting with *C. difficile* isolates first spread on BRU agar and incubated anaerobically at 37°C for 24-48 hours. *C. difficile* DNA extraction using a BHI culture that had been incubated anaerobically at 37°C for 6-8 hours was tested. Additionally, *C. difficile* DNA extraction using 1mL of the 18-hour incubated broth inoculated into 9mL of fresh BHI and incubated anaerobically at 37°C for 18 hours was also trialed. Another method trialed was the growth of *C. difficile* from inoculation by a defrosted CryoBank bead (Copan Diagnostics, United States) directly into 1.5mL of BHI in deep well blocks.

Cell Lysis Modifications (Qiagen DNEasy)

Several cell lysis modifications were trialed. The original protocol called for the use of 5µL of 100mg/mL RNase A (Qiagen, Germany) after and 25µL of protein kinase A (Qiagen, Germany) and 200µL of buffer AL (Qiagen, Germany) had been added.

Different *C. difficile* lysis buffer incubation times were tested. From a BHI culture that had been growing for 6 hours, incubation in *C. difficile* lysis buffer for 2 hours or 18 hours was evaluated.

Another trial decoupled the 25µL of protein kinase A and 200µL of buffer AL incubation. Instead, 5µL of 100mg/mL RNase A was added with 25µL of protein kinase A was incubated at 60 minutes in a 56°C bead bath (Lab Armor, United States). Upon adding 200µL of buffer AL, the culture was again incubated at 60 minutes in a 56°C bead bath. This variation was also tested where 5µL of 100mg/mL RNase A was added after the 60 minutes in a 56°C bead bath for 25µL of protein kinase A.

Cell lysis where the *C. difficile* lysis buffer was exchanged for the use of buffer AVL (Qiagen, Germany) was also trialed.

4 trials modified the *C. difficile* lysis buffer composition. 3 trials modified the *C. difficile* lysis buffer by adding 5µL of 100mg/mL RNase A to the *C. difficile* lysis buffer. The first trial only exchanged *C. difficile* lysis buffer with *C. difficile* lysis buffer containing RNase A. Another trial

incubated the 25µL of protein kinase A and 200µL of buffer AL for 2 hours in a 56°C bead bath in addition to the modified *C. difficile* lysis buffer. The third trial used 8-day old *C. difficile* spores as a culture with the modified *C. difficile* lysis buffer. The fourth *C. difficile* lysis buffer modification trial modified the *C. difficile* lysis buffer by adding 0.5µL of 5KU/mL mutanolysin.

Qiagen's QIAmp

Qiagen's QIAmp DNA Kit (Qiagen, Germany) was used in accordance with the gram-positive bacteria extraction protocol established by the manufacturer with some modifications. Bacterial culture growth and cell lysis followed the same procedure as performed with Qiagen's DNEasy genomic DNA extraction method.

The contents of each well were then transferred into QIAmp columns (Qiagen, Germany) secured in collection tubes. The QIAmp column caps were closed and centrifuged for 10 minutes at 21,100 x g. The waste collected in the collection tube was discarded. 500µL of buffer AW1 with ethanol (Qiagen, Germany) was to each sample. The QIAmp columns were replaced into their collection tube, their caps closed, and centrifuged for 5 minutes at 21,100 x g. The waste collected in the collection tube was discarded. 500µL of buffer AW2 with ethanol (Qiagen, Germany) to each sample. The QIAmp columns were replaced into their collection tube, with open caps, and centrifuged for 15 minutes at 21,100 x g. The waste collected in the collection tube was discarded. The QIAmp columns were then placed into DNA collection tubes and 55µL of 37°C 10mM TRIS-HCl was then added to each sample. The samples were centrifuged for 2 minutes at 21,100 x g. The collected DNA elution was transferred to a sterile 96-well plate. The elution process was repeated by once again by replacing the QIAmp columns back into the DNA elution tubes and adding 55µL of 37°C 10mM TRIS-HCl was then added to each sample. The samples were centrifuged for 2 minutes at 21,100 x g. The collected DNA elution was transferred to the previous elution.

Qiagen QIAmp Optimized by Sim et al. [165]**.**

C. difficile isolates were first spread on BRU agar and incubated anaerobically at 37°C for 24-48 hours. 12 ml of anaerobically reduced TSB broth was then dispensed into 15mL conical

tubes. The broth was then inoculated with *C. difficile* to a turbidity of 3-4 McFarland. The cultures were then incubated anaerobically at 37°C for 48 hours. Post incubation, the cultures were centrifuged for 15 minutes at 4000g. The supernatant then discarded by being decanted and aspirated. The pellet was then resuspended in 180µl of buffer ATL (Qiagen, Germany) and 20µL of protein kinase A (Qiagen, Germany). The suspensions were transferred to 1.5mL tubes and vortexed for 15 seconds every 10 minutes during a 1-hour incubation 56°C in a bead bath (Lab Armor, United States). This protocol was tested with and without the addition of 5µL of 100mg/mL RNase A (Qiagen, Germany) added to each sample, mixed, and incubated for 5 minutes at room temperature. 200µL of buffer AL (Qiagen, Germany) was then added to each sample, mixed, and incubated for 10 minutes at 70°C in a bead bath. 200µL of anhydrous undenatured ethanol (Greenfield Global, Canada) was mixed into each sample.

DNA Purification and Elution followed the standard QIAmp protocol. View "DNA Purification and Elution" 3.10.2.2.

The optimized protocol produced by Sim et al. was extremely laborious and added multiple days to the already long extraction process [165]. The main benefit of the Sim et al. protocol is that it provided DNA that could be sequenced to produce high-quality assemblies. It could be a viable option for research that does not require a large amount of *C. difficile* genomes to be extracted and who are not under any time constraint. However, the method is not suitable for larger labs as the method can not be adapted into a method that can handle high throughput.

Qiagen QIAsymphony

Qiagen's QIAsymphony (Qiagen, Germany) automated DNA extraction was used in accordance with the gram-positive bacteria extraction protocol established by the manufacturer.

Epicentre's MasterPure Complete DNA and RNA Purification Kit

Epicentre's MasterPure Complete DNA and RNA Purification Kit (Epicentre, United States) was used in accordance with the gram-positive bacteria extraction protocol established by the manufacturer. Bacterial culture growth followed the same procedure as performed with Qiagen's DNEasy genomic DNA extraction method

1mL of culture was transferred to a 1.5mL screw-top tube. The cap was secured and the culture was centrifuged for 2 minutes at 21,100 x g. The supernatant was aspirated and discarded. The pellets were then resuspended in 150µL of *C. difficile* lysis buffer (Table 36) and incubated for 2 hours in a 37°C bead bath (Lab Armor, United States). 150µL of 2X Epicentre T&C Lysis Solution (Epicentre, United States) and 10µL of 20mg/mL Protinase K were then added to the samples and vortexed briefly to homogenize. The cultures were then incubated for 15 minutes on a ThermoMixer (Eppendorf, Germany) set to 1,000RPM at 65°C. During the incubation, the cultures were vortexed every 5 minutes to mix.

The cultures were placed on ice to cool for at least 5 minutes. 175µL of Epicentre's MPC Protein Precipitation (Epicentre, United States) was then added to each culture and vortexed for 10 seconds. The cultures were then centrifuged in 4°C environment for 2 minutes at 21,100 x g. The supernatant was then collected and transferred to a sterile 1.5mL pop-top tube. 1µL of Rnase A (10µg/mL) was then added to each culture and inverted to mix. The cultures were then incubated for 30 minutes in a 37°C bead bath (Lab Armor, United States). 500µL of 2-propanol was then added to each culture and inverted 40 times to mix. The cultures were then centrifuged in 4°C environment for 10 minutes at 21,100 x g. The supernatant was aspirated and discarded. The pellet was then rinsed twice in 70% ethanol. With tube caps open, the pellets were allowed to air dry for 5 minutes at room temperature. The pellets were then resuspended in 50µL of 10mM TRIS-HCl.

Table 36 *C. difficile* Cell Lysis Buffer for Epicentre's MasterPure Complete DNA and RNA Purification Kit

Ingredient	Concentration	Quantity
Lysozyme	20mg/mL	50 µL
Mutanolysin	5KU/mL	10 µL
RNase A	10µg/mL	1 µL
Tris-EDTA Buffer	1X	89 µL

Robotic Extraction

Robotic genomic DNA extraction utilized both LuminUltra genomic DNA extraction kits (LuminUltra, Canada) and Omega Bio-tek genomic DNA extraction kits (Omega Bio-tek, United States) according to manufacture instructions.

The use of robotic extraction method was explored in an attempt to eliminate the laborious process of *C. difficile* genomic DNA extraction. Unfortunately, the method proved to not eliminate much of the tedious aspects of a *C. difficile* genomic DNA extraction. Only the washes were automated which did not significantly simplify the protocol. Additionally, the assemblies produced from genomic DNA extracted via robotics was extremely fragmented. Robotic extracted DNA increased the number of contigs per assembly by 280%. While the same samples possessed assemblies that averaged 101.86 contigs per assembly, the robotic extracted DNA averaged 386.95 contigs per assembly. The effect on the average number of contigs in the N50 was even more apparent. The average number of contigs in the N50 increased 13 times when compared to the DNeasy from 14.1 contigs in the N50 to 197.72 contigs in the N50. A more complete assembly provides a more complete image of the genome and thus increases the quality of the analysis.

MicroGEM PDQeX

MicroGEM's PDQeX (MicroGEM, United States) was used in accordance with the gram-positive bacterial extraction protocol established by the manufacturer. The MicroGEM's PDQeX is a streamlined process.

Several growth conditions were tested. For growth on agar, *C. difficile* isolates were spread on BRU agar and incubated anaerobically at 37°C for 18 hours prior to PDQeX (MicroGEM, United States) DNA extraction. For growth in broth, *C. difficile* isolates were first spread on BRU agar and incubated anaerobically at 37°C for 24-48 hours. 5mL of anaerobically preproduced BHI broth or TSB was then inoculated with half of a 10µL loop of culture. BHI cultures were incubated anaerobically at 37°C for 6 or 18 hours prior to PDQeX DNA extraction. TSB cultures were incubated anaerobically at 37°C for 48 hours prior to PDQeX DNA extraction.

DNA Extraction (MicroGEM PDQeX)

400µL of 1X WASH+ (MicroGEM, United States) was dispensed into a 1.5mL pop-top tube. A 1µL loop of culture or 100µL of broth culture was taken from the plate and inoculated into the 1X WASH+. The tubes were vortexed to homogenize the culture before being centrifuged for 5 minutes at 21,100 x g. The supernatant was aspirated and discarded. 10µL of 10X GREEN+ (MicroGEM, United States), 2µL of PDQeX prepGEM (MicroGEM, United States), 2µL of lysozyme, and 88µL of dH_2O were added to each pellet and vortexed to mix. PDQeX HC cartridges (MicroGEM, United States) and PCR collection tubes were loaded into the machine. The PDQeX HC cartridges were loaded with the entire contents of the cultures and sealed with their cap. The machine was run on the gram-positive bacteria setting.

Appendix D - Sequencing Optimization

Sequencing optimization aimed to determine the best extraction and sequencing method to obtain the highest quality assembly and coverage. A high-quality assembly allows for improved SNV annotation and analysis.

N20-00231 Trials

N20-00231 is a *C. difficile* isolate from retail meats. It is a NAP10, ribotype 131, PF type 663. The best 10 assemblies (Table 37) and 10 best sequence coverage trials (Table 38) are shown.

N20-00442 Trials

N20-00442 is a *C. difficile* isolate from retail meats. It is a NAP11, ribotype 106, PF type 1191. The best 10 assemblies (Table 39) and 10 best sequence coverage trials (Table 40) are shown.

11ACD0075 Trials

11ACD0075 is a *C. difficile* isolate from a human clinical case. It is a NAP1, ribotype 027, PF type 1. The best 10 assemblies (Table 41) and 10 best sequence coverage trials (Table 42) are shown.

Robotic Extraction vs DNEasy Extraction Assembly

Assembly results of LuminUltra (LuminUltra, Canada) robotic extraction comparted to manual Qiagen's DNEasy Blood & Tissue Kit (Qiagen, Germany) extraction (Table 43). The *de novo* assemblies of the LuminUltra robotic extracted DNA were also compared to the de novo assemblies of Qiagen's DNEasy Blood & Tissue Kit extracted DNA (Table 44). Outliers were not removed.

Table 37. Best 10 Optimization Assemblies of N20-00231

Strain	Mean Contig Length	Number of Contigs	Number of Contigs in N50
N20-00231-E3	40049.93	102	10
N20-00231-I	38205.85	107	10
N20-00231-E2	36829.67	111	10
N20-00231-N1	31192.07	131	16
N20-00231-D1	30931.31	132	15
N20-00231-J	30127.75	135	14
N20-00231-C1	30057.62	136	16
N20-00231-C2	29187.3	140	17
N20-00231-E1	27796.46	147	17
N20-00231-G2	27592.16	148	13

Table 38. Best 10 Optimization Sequence Coverage Trials of N20-00231

Strain	Mean Coverage	>=50X
N20-00231-I	152.7X	87.95%
N20-00231-E3	152.1X	87.71%
N20-00231-F2	157.2X	86.50%
N20-00231-H	148.1X	86.08%
N20-00231-F3	158.9X	85.98%
N20-00231-B1	201.2X	85.34%
N20-00231-E2	134.4X	81.59%
N20-00231-E1	126.5X	80.92%
N20-00231-L	164.5X	78.46%
N20-00231-C1	121.0X	78.15%

Table 39. Best 10 Optimization Assemblies of N20-00442

Strain	Mean Contig Length	Number of Contigs	Number of Contigs in N50
N20-00442-D1	53361.28	78	11
N20-00442-E3	39972.84	104	17
N20-00442-B3	37104.51	112	16
N20-00442-I	37152.16	112	18
N20-00442-B2	35272.89	118	18
N20-00442-H	34946.37	119	16
N20-00442-B1	34077.02	122	20
N20-00442-F2	29871.06	139	18
N20-00442-G3	28826.93	144	25
N20-00442-D2	28188.65	147	26

Table 40. Best 10 Optimization Sequence Coverage Trials of N20-00442

Strain	Mean Coverage	>=50X
N20-00442-F2	258.0X	89.34%
N20-00442-H	139.1X	84.27%
N20-00442-I	127.7X	81.60%
N20-00442-N1	173.1X	80.99%
N20-00442-E3	133.7X	80.83%
N20-00442-D1	136.1X	80.09%
N20-00442-F1	131.1X	77.11%
N20-00442-A3	115.7X	75.04%
N20-00442-D3	124.3X	74.26%
N20-00442-B3	124.7X	74.07%

Table 41. Best 10 Optimization Assemblies of 11ACD0075

Strain	Mean Contig Length	Number of Contigs	Number of Contigs in N50
11ACD0075-I	47072.17	87	13
11ACD0075-E2	39369.26	104	16
11ACD0075-E3	38620.27	106	17
11ACD0075-N1	37072.49	110	17
11ACD0075-D3	34401.44	119	18
11ACD0075-B3	33241.62	123	18
11ACD0075-G3	31444.09	130	22
11ACD0075-B1	30769.74	133	20
11ACD0075-F3	30168.69	135	22
11ACD0075-A3	30073.39	136	19

Table 42. Best 10 Optimization Sequence Coverage Trials of 11ACD0075

Strain	Mean Coverage	>=50X
11ACD0075-I	150.2X	88.81%
11ACD0075-E3	137.2X	85.05%
11ACD0075-E2	134.2X	83.01%
11ACD0075-F3	141.7X	79.80%
11ACD0075-L	150.4X	78.88%
11ACD0075-B1	130.2X	78.20%
11ACD0075-E1	126.2X	77.55%
11ACD0075-D3	130.8X	75.81%
11ACD0075-A3	113.0X	75.75%
11ACD0075-C1	123.1X	74.68%

Table 43. Assembly Statistics of *C. difficile* DNA Extracted by LuminUltra Robotics

	LuminUltra Robotic	**DNEasy**
Average Contig Length	7917.17 95% CI [3103.5, 12730.83]	42923.58 95% CI [38475.59, 47371.56]
Average Number of Contigs	386.95 95% CI [274.4, 499.5]	101.86 95% CI [91.91, 111.82]
Average Number of Contigs in N50	197.72 95% CI [145.02, 250.43]	14.1 95% CI [12.84, 15.37]

Table 44. Robotic Extraction vs DNEasy Extraction Assembly

Strain	Average Contig Length		Average Number of Contigs		Average Number of Contigs in N50	
	LuminUltra	DNEasy	LuminUltra	DNEasy	LuminUltra	DNEasy
03A18040	4981.85	44856.34	786	90	145	14
03B18016	16412.28	55880.66	247	73	39	11
04A15056	2758.12	38423.85	1,370	107	251	16
04A18052	2541.58	48172.89	1,486	85	267	13
04A18086	9352.55	39766.54	432	103	71	16
04B15008	1917.98	32826.44	1,826	126	363	15
04B15013	1955.04	25512.39	1,855	164	370	20
04B18027	2675.42	32985.2	1,424	124	257	16
06D18031	4729.58	57249.63	842	71	145	11
08A18022	2854.4	46299.48	1,308	87	244	10
17A18003	4517.9	62453.72	871	65	148	9
17A18011	5040.36	60987.66	854	73	146	9
17A18026	6068.55	46179.27	653	88	116	12
17A18037	4036.94	60649.96	964	67	181	10
17A18041	997.1	71803.95	2,517	57	628	8
21A18004	1706.03	36336.15	1,959	113	401	18
21A18006	1890.23	29345.71	1,840	138	365	20
21A18026	15039.52	37224.05	266	108	41	16
21C18032	67355.16	35424.02	61	116	8	18
21C18041	11384.15	32385.96	354	125	53	15
23A18032	4460.03	33828.5	878	120	144	16
23A18035	3757.54	32977.36	1,028	123	184	18
23B18047	26117.1	43481.5	156	94	25	14
23B18053	2200.39	49467.48	1,655	82	313	12

23B18062	7453.89	46365.02	528	86	95	13
24A18027	3958.2	31404.35	1,014	132	175	15
24A18037	4001.57	43600	991	95	183	12
70A18038	6878.49	28482.01	579	141	102	18
70A18046	2555.85	40413.6	1,494	101	274	14

Appendix E – 300-1,000 vs 600-1,000 Base Pair Library Preparation

The use of a library gated between 600 and 1,000 base pairs is the normal protocol at the National Microbiology Lab for the sequencing of *C. difficile*. However, in an effort to decrease the number of contigs when sequenced genomic DNA is assembled, the use of a 300 to 1,000 base pair library was used to potentially capture smaller fragments of genomic DNA. Unfortunately, this did not improve the quality of the produced assemblies. While the 300 to 1,000 base pair lead to fewer failed sequences, it produced lower quality assemblies overall. The average number of contigs increased by almost 20% from 258.48 contigs per assembly to 308.14 contigs per assembly (Table 45, Table 46, and Table 47). Outliers were not removed.

Table 45. Assembly Statistics of *C. difficile* sequenced with a 300-1,000 Base Pair Library vs 600-1,000 Base Pair Library

	300-1,000 Base Pair Library	**600-1,000 Base Pair Library**
Successful Sequences	95	90
Failed Sequences	1	6
Average Contig Length	17609.89 95% CI [14929.14, 20290.64]	24890.62 95% CI [21173.25, 28608]
Average Number of Contigs	308.14 95% CI [264.63, 351.65]	258.48 95% CI [211.63, 305.33]
Average Number of Contigs in N50	62.28 95% CI [51.4, 73.17]	50.93 95% CI [40.31, 61.55]

Table 46. 300-1,000 Base Pair Library Preparation Sequence Assemblies

Strain	Mean Contig Length	Number of Contigs	Number of Contigs in N50
02A16015	50937.01	81	13
02B15017	3268.24	1,202	216
02B15022	8098.94	508	78
02B16024	34513.22	122	19
03A15035	7690.3	541	90
03A15042	6424.17	628	105
03A15049	5394.95	744	131
03A15050	62040.3	66	8
03A15056	29116.17	140	17
03A16035	37485.13	110	17
03A16049	15614.13	261	38
04A15069	4891.62	820	145
04A15078	10744	379	63
04A16042	11077.97	367	55
04A16058	13740.02	297	44
04A16068	5760.11	699	115
04A16086	44113.37	93	15
04A16090	27031.66	151	22
04A16096	21475.34	190	30
04A16106	13381.78	301	55
04A16108	18450.62	242	30
04A16123	23705.99	172	30
04A17063	3619.91	1,071	191
04B15019	31937.16	130	23
04B15028	33187.28	122	19
04B15030	26784.81	150	24

04B15038	10134.48	395	65
04B16031	17826.92	225	34
04B16034	14577.87	279	41
04B16057	21658.98	187	27
04B17014	6722.2	592	93
04B17020	12134.76	335	50
06B15012	12462.08	325	49
06C15032	23931.13	168	26
06C16027	23196.89	175	25
06C17023	12121.79	336	50
07B15037	43635.92	93	13
07B16058	8619.2	474	78
07B17034	6727.95	597	103
07B17046	18754.25	214	32
07B17052	16253.14	248	37
07B17056	12603.45	321	51
07B17058	8387.63	480	85
07C15025	15926.27	252	40
07C17017	6583.41	601	102
07C17022	16556.19	250	38
07C17027	19261.07	211	33
07C17028	25378.46	160	25
08A15023	13630.35	292	44
08A15038	15474.97	262	37
08A16032	14068.66	285	40
09A15003	10771.28	377	60
09A15037	4134.83	971	170
09A16006	11640.85	350	56

09A16015	4811.47	827	150
09A16035	10246.85	391	63
10A15026	10607.86	377	67
10A15028	6801.58	585	107
10A15038	8681.08	465	71
10A16023	3677	1,068	193
10A16046	20158.87	201	32
10A17044	10530.75	384	63
15B16048	16965.99	242	33
17A15009	12846.21	312	48
17A16006	5511.38	740	121
17A16009	6286.97	639	107
17A16023	3679.03	1,071	190
17A16034	21537.55	187	24
17A16036	17987.04	225	32
17A17005	9019.02	447	75
17A17013	2374.66	1,587	302
17A17023	75913.45	53	5
17A17028	32476.12	125	18
21A16003	5941.56	666	118
21A16027	37654.38	108	15
21A16028	10522.82	381	62
21A16043	49483.72	82	15
21C15055	32703.67	123	19
21C15078	25372.29	159	25
21C16046	17311.55	232	35
21D15031	20304.51	201	30
21D17009	4007.05	977	178

21D17031	12246.82	329	52
23A15033	18135.92	224	33
23B15065	21724.39	187	25
27A15012	18854.28	216	30
27A16010	31172.12	130	21
27A16016	15607.61	259	41
27A16017	21572.1	188	28
31A16012	15027.94	278	41
32A16032	10961.45	370	55
32A17030	25651.35	156	22
70A16038	11830.21	342	54
70A16043	6162.36	644	113

Table 47. 600-1,000 Base Pair Library Preparation Sequence Assemblies

Strain	Mean Contig Length	Number of Contigs	Number of Contigs in N50
02A16015	65520.48	63	9
02B15022	6133.5	664	100
02B16024	48390.13	87	15
03A15035	7958.95	522	82
03A15042	4601.44	860	146
03A15049	4147.31	956	170
03A15050	61078.16	67	10
03A15056	42410.9	96	14
03A16035	52167.67	79	13
03A16049	15354.27	265	39
04A15078	1,0004.15	405	64
04A16042	17555.44	232	32
04A16058	17967.52	227	34
04A16068	4621.56	859	142
04A16086	65154.92	63	10
04A16090	41707.86	98	15
04A16096	38528.8	106	17
04A16106	19075.46	211	32
04A16108	23846.77	187	25
04A16123	34558.04	118	18
04A17063	3255.36	1,156	208
04B15019	43295.69	96	13
04B15028	42148.42	96	17
04B15030	48428.12	83	10
04B15038	11118.29	360	56

04B16031	29466.2	136	22
04B16034	18732.76	217	33
04B16057	44979.44	90	14
04B17014	8015.84	495	78
04B17020	20992.26	194	27
06B15012	14410.81	281	44
06C15032	35906.97	112	16
06C16027	58107.5	70	12
06C17023	10061.66	400	67
07B15037	54128.41	75	12
07B16058	21484.49	191	29
07B17034	4896.77	810	141
07B17046	12602.37	316	48
07B17052	24084.86	167	24
07B17056	16449.67	246	38
07B17058	5703.56	685	118
07C15025	17816.14	225	33
07C17017	6191.76	636	102
07C17022	26156.59	158	23
07C17027	28204.6	144	21
07C17028	39435.39	103	16
08A15023	14710.13	270	42
08A15038	25013.56	162	23
08A16032	30633.56	131	18
09A15003	10053.75	400	68
09A16006	12590.44	323	48
09A16015	3207.49	1,203	223
09A16035	9189.84	431	70

10A15026	15299.05	262	39
10A15028	7103.49	560	90
10A15038	5068.45	784	139
10A16023	3743.39	1,040	185
10A16046	27019.39	150	22
10A17044	10054.98	398	63
15B16048	15970.32	256	42
17A15009	15668.53	256	36
17A16006	7563.58	539	85
17A16009	8699.58	461	75
17A16034	35573.94	113	20
17A16036	27905.98	145	22
17A17005	7556.83	526	86
17A17023	70568.79	57	8
17A17028	43072.73	94	19
21A16003	7207.12	551	87
21A16027	54939.2	74	11
21A16028	9168.22	434	64
21A16043	54856.03	74	9
21C15055	58315.48	69	9
21C15078	33942.49	119	18
21C16046	17285.66	232	33
21D15031	27813.55	147	20
21D17009	3429.94	1,104	190
21D17031	17132.77	235	36
23A15033	33296.37	122	20
23B15065	28573.47	142	23
27A15012	31599.92	129	15

27A16010	46561.61	87	14
27A16016	35482.01	114	17
27A16017	37605.53	108	13
31A16012	16682.56	250	36
32A16032	24666.45	165	22
32A17030	37414	107	16
70A16038	20586.68	197	30
70A16043	6153.39	645	109