PHENOTYPIC CHARACTERIZATION OF ENVIRONMENTAL CLOSTRIDIOIDES DIFFICILE ISOLATES FROM WASTEWATER AND SEAFOOD IN TAIWAN

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PHENOTYPIC CHARACTERIZATION OF ENVIRONMENTAL CLOSTRIDIOIDES DIFFICILE ISOLATES FROM WASTEWATER AND SEAFOOD IN TAIWAN

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Abstract: *Clostridioides difficile* (*C. difficile*) is a gram-positive, toxin-producing, spore-forming anaerobe, and leading cause of nosocomial antibiotic-associated diarrhea (AAD). *C. difficile* is present worldwide and the disease caused by this bacterium, *C. difficile* infection (CDI) affects individuals of all ages and health statuses. The spores formed by *C. difficile* are readily transmissible and can persist in animals, food, and environmental sources such as water and soil. The aim of this research was to complete phenotypic characterization of environmental *C. difficile* isolates to contribute to the first analysis of *C. difficile* environmental contamination in Taiwan. Assays were performed to evaluate the growth rate, colony morphology, sporulation efficiency, surface motility, and swimming motility. Notably, 078-lineage *C. difficile* isolates displayed significantly lower surface motility compared to toxigenic non-078 lineage isolates and non-toxigenic isolates. Also, toxigenic and non-toxigenic isolates of *C. difficile* demonstrated similar sporulation efficiency and swimming motility. The implications of *C. difficile* contamination in wastewater and seafood may suggest that more stringent wastewater treatment practices are needed and illustrate the importance of safe food handling practices. Moreover, having access to a complete profile for *C. difficile* isolates could help healthcare providers and researchers make better informed decisions in their respective lines of work.

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Abbreviations

° degree
µ microliter
% percent
AAD antibiotic-associated diarrhea
ANOVA Analysis of Variance
AP Anping
BHIS brain heart infusion supplemented
C. difficile Clostridioides difficile
c-di-GMP cylic-di-guanosine-5' monophosphate
CA-CDI community-associated CDI
CDAD *C. difficile*-associated disease/diarrhea
CDI *Clostridioides difficile* infection
CDT *C. difficile* binary toxin
CdtLoc binary toxin locus
CFU colony forming unit
cm centimeter
CPD cysteine protease domain
CROPs combined repeated oligopeptides
FMT fecal microbiota transplant
HW Huweiliao
g gram
L liter
mL milliliter
NCKUH National Cheng Kung University Hospital
OSU-CHS Oklahoma State University Center for Health Sciences
PaLoc pathogenicity locus
PCR polymerase chain reaction
RBD receptor binding domain
ROI region of interest
RT ribotype
SM(c) sporulation medium (cysteine)
SM sporulation medium
TcdA toxin A
TcdB toxin B
TFP type IV pili
WWTP wastewater treatment plant

CHAPTER I

INTRODUCTION

Clostridioides difficile (*C. difficile*) is an anaerobic, spore-forming, toxin-producing, gram positive bacterium [1]. It should be mentioned that *Clostridioides difficile* was formerly known as *Clostridium difficile* until 2016 when the *Clostridium* genus was limited to *Clostridium butyricum* plus related species [2]. *C. difficile* is the causative agent of the disease *Clostridioides difficile* infection (CDI), one of the most common causes of nosocomial antibiotic-associated diarrhea illness [1, 3]. There are both toxigenic and non-toxigenic strains of *C. difficile*, but only the toxigenic strains will promote CDI disease symptoms. TcdA (toxin A) and TcdB (toxin B) are the major toxins produced by *C. difficile* [3-5]. CDI is a gastrointestinal illness with diarrhea being the major symptom, plus serious complications including death can occur [5, 6]. *C. difficile* is spread by the spores it produces and these bacterial spores persist in numerous environments such as water and food, and animals may be colonized as well [7-9]. Typing of *C. difficile* by PCR (polymerase chain reaction) ribotyping is one way in which the bacteria can be classified [9].

C. difficile is present in Taiwan but most studies focus on CDI patients in healthcare settings [10-13]. One of the aims of our lab is to examine environmental sources for contamination with *C. difficile*. Previously, a member of our lab in Taiwan isolated *C. difficile* from seafood,

hospital wastewater, and from multiple points of the wastewater treatment process at several wastewater treatment plants (WWTPs). This work marked the first time that *C. difficile* had been isolated from seafood and wastewater in Taiwan. The genotypic and phenotypic characteristics of these environmental *C. difficile* isolates were subsequently analyzed [14]. Isolates of *C. difficile* belonging to different PCR ribotypes (RT) may exhibit distinct biological behaviors that can be relevant for *C. difficile* transmission and CDI progression in patients. Therefore, it is important to fully characterize all aspects of bacterial behavior by performing phenotypic analysis [15-22]. In this study, the growth rate, colony morphology, sporulation efficiency, surface motility, and swimming motility are characterized to provide a complete analysis of environmental *C. difficile* in Taiwan.

CHAPTER II

REVIEW OF LITERATURE

Epidemiology

Transmission of *C. difficile* and subsequently CDI, largely transpires via the fecal-oral route of infection with the spores produced by the bacterium. *C. difficile* spores are capable of surviving for several months in water, soil, food, and on environmental surfaces. Cases of CDI occur worldwide, though surveillance rates vary from country-to-country [9]. The most significant risk factor for developing CDI is recent antibiotic use [23]. Clindamycin, cephalosporins, penicillins, and fluoroquinolones are the classes of antibiotics associated with increased risk for developing CDI [6]. Individuals that are aged 65 and older, reside in a healthcare setting, have recently been hospitalized, have an underlying medical condition (i.e. compromised immune system, inflammatory bowel disease, etc.,), or have had one or more previous CDI cases are also at increased risk of infection [6, 23].

C. difficile was discovered in 1935, though its relevance as a human pathogen was not documented until the 1970s. In the 1970s, the relationship between *C. difficile* and diarrhea was identified and was referred to as *C. difficile*-associated disease/diarrhea (CDAD), now known as CDI [23]. The incidence of CDI surged in the 1990s before an even larger increase began in the 2000s. The increased CDI transmission rates, disease severity, and mortality observed at the

beginning of the twenty-first century coincided with the rise of epidemic/hypervirulent strains [9]. These rapid escalations in CDI incidence and mortality started to be seen in North America and parts of Europe after 2003, and in Australia, Asia, and Central America after 2008 [23]. While the definition of hypervirulent has yet to be formally agreed upon, it is often used when discussing *C. difficile* ribotypes that exhibit characteristics such as hyper-sporulation, toxin production, a frameshift deletion mutation in the *tcdC* gene, and reduced susceptibility to antibiotics [9, 15, 17, 24].

Classification of *C. difficile* by PCR ribotype is the preferred *C. difficile* typing method in Europe and Australia and is often used in North America as well [9]. PCR ribotyping is based off amplification of the 16S-23S rRNA intergenic spacer region by PCR [9, 23]. *C. difficile* BI/NAP1/027 (RT027) was first identified in 2002 and has been responsible for causing substantial epidemics of CDI. Much of the increased CDI morbidity and mortality during the early 2000s was a result of infections with ribotype 027 [9]. *C. difficile* ribotype 078 is another hypervirulent ribotype that has been the cause of CDI outbreaks, but its association to CDI mortality and incidence during the 2000s was less prevalent than RT027 [9, 23]. Recently, the hypervirulent RT 078 lineage has been dominant among *C. difficile* isolates from hospitalized patients in Taiwan [25, 26]. Ribotypes related to the hypervirulent 078 include RTs 126, 127, 237, 280, 281, and 598 [17, 27]. Nevertheless, it is important to mention that *C. difficile* epidemics are not restricted to just RT027 and RT078 as RTs 001, 002, and 014/020 are often responsible for CDI clusters in Europe and the United States [23].

Regarding *C. difficile* in the United States, a recent estimate from Mayo Clinic is that 200,000 people acquire CDI from a healthcare setting and 170,000 acquire CDI from a non-healthcare setting every year in the United States [6]. *C. difficile* has been a massive health problem during the 21st century as the incidence of CDI approximately doubled among adult hospital patients from 2001 to 2010 [23]. In 2017, according to a report from the CDC, approximately 223,900 people needed hospital care for CDI and at least 12,800 deaths occurred [28]. The

economic burden of *C. difficile* in the United States has been estimated to be $1 billion annually with longer healthcare stays and the cost of providing care to patients being the major expenses contributing to these high costs [23, 28].

C. difficile also affects children and an increase in CDI rates has been seen in both children with underlying conditions and children with no risk factors [29]. Lastly, it is important to note that community-associated CDI (CA-CDI) is on the rise and over 30% of these patients are absent of traditional risk factors - they are younger in age and have no history of recent antibiotic use or hospitalization [9, 23].

C. difficile and the microbiome

The human microbiome is comprised of the numerous microorganisms that live inside and on the human body. In particular, seventy percent of the microbiome is composed of gut microbiota and the largest numbers of microorganisms within the gut microbiota occupy the distal gastrointestinal tract [30]. The gut microbiota acts as a barrier against pathogens by providing colonization resistance, influencing the immune response, and regulating bile acid metabolism [30-32]. Through colonization resistance, commensals outcompete pathogens for nutrients and space within the host intestine thus limiting infections [32]. Primary bile acids are synthesized in the gallbladder and a small portion travel to the large intestine. Upon arriving in the large intestine, primary bile acids are transformed to secondary bile acids by members of the gut microbiota through deconjugation and dihydroxylation [30]. This is importance because primary bile acids (such as cholate) induce germination of *C. difficile* spores into vegetative cells which facilitates *C. difficile* growth while secondary bile acids (such as chenodeoxycholate) inhibit germination thus decreasing outgrowth of *C. difficile* vegetative cells. Germination of *C. difficile* spores into vegetative cells is what promotes overgrowth of *C. difficile* and onset of CDI [30-33].

Dysbiosis is the term used to describe the state in which the healthy microbiota has been disturbed and its diversity is diminished; it has been shown to be connected to CDI susceptibility

[32]. Antibiotics immensely change the host microbiota composition and treatment with antibiotics can induce dysbiosis [30, 32, 33]. Antibiotics reduce the number of commensals thus increasing primary bile acids that stimulate germination and decreasing secondary bile acids that hinder germination [32]. Members belonging to the phyla Bacteroidetes and Firmicutes are assumed to be crucial for bacterial metabolism as they comprise the largest biomass in the gut microbiota. Studies with animal models have shown that antibiotic-treated animals exhibited decreases in the phyla Bacteroidetes and Firmicutes and an increase in the phylum Proteobacteria. Moreover, patients with recurrent CDI patients exhibited increased Bacteroidetes and decreased Proteobacteria fecal microbiota populations after a successful fecal microbiota transplant (FMT) [30]. Ongoing research efforts are seeking to determine which microorganisms within the microbiota aid in resistance to *C. difficile*. So far, a negative correlation between *Clostridium scindens* and colonization of *C. difficile* has been observed [31].

C. difficile infection (CDI)

Diarrhea is the most common clinical presentation of CDI and overall, is the most widely recognized symptom of the disease [6, 23, 31]. There are a number of individuals who carry *C. difficile* in their large intestine (colon) while never exhibiting disease symptoms, but have been shown to be asymptomatic carriers through spore shedding [6, 23]. CDI symptoms normally arise 5-10 days after the beginning of treatment when the disease occurs because of antibiotic treatment. CDI patients with mild-to-moderate CDI normally experience at minimum three or more episodes of watery diarrhea per day and minor abdominal cramping may also occur. Manifestation of severe CDI symptoms may result in the patient requiring hospitalization. Symptoms of severe CDI include (but are not limited to) 10-15 watery diarrhea episodes per day, weight loss, rapid heart rate, and blood or pus in the feces [6]. Further complications of severe CDI include pseudomembranous colitis, toxic megacolon, sepsis, multiple organ dysfunction syndrome, and even death [23].

Antibiotics are usually the first treatment prescribed for CDI despite being the biggest risk

factor as certain antibiotics are crucial and very effective for treating most infections. Vancomycin and fidaxomicin are currently the two recommended antibiotics while a combination of metronidazole and vancomycin can be used for treating severe CDI cases [34]. Moreover, treatment with only antibiotics is no longer effective for a rising number of CDI patients and about 25% of patients treated for CDI experience recurrent disease [34, 35]. Antibody-based therapy and FMT are two treatment approaches for recurrent CDI [34]. Bezlotoxumab is an FDA-approved therapeutic that uses neutralizing antibodies against TcdB to inhibit toxin activity and can lower risk of recurrent CDI in high-risk patients [31, 34]. FMT consists of placing a stool sample from a donor into the patient's colon to replenish healthy bacteria in the gut microbiota [23, 34]. FMT is not FDA approved and while it is still considered experimental, FMT has an over 85% success rate [34].

Pathogenesis of CDI

Onset of *C. difficile* pathogenesis begins with spore ingestion [3]. *C. difficile* spores germinate into vegetative cells in the small intestine when CspC, the germinant receptor on the spore surface, is stimulated by bile salts (i.e., cholate and its derivatives) and a co-germinant (L-glycine) [3, 16]. Several bacterial components such as type iv pili, flagella, and cell wall proteins Cwp66, SlpA and its modifying protease Cwp84 aid in *C. difficile* adherence [36, 37]. Colonization ability is largely determined by the host microbiota. As mentioned earlier, antibiotic use (the biggest risk factor for CDI) vastly changes the healthy microbiota generating favorable conditions for *C. difficile* to overgrow [23, 32]. When the normal microbiota has been disrupted, *C. difficile* toxin production and colonization occurs in the colon [3]. The TcdA and TcdB toxins primarily facilitate CDI pathogenesis [3, 20, 35]. TcdA and TcdB are monoglucosyltransferases that irreversibly alter and inactivate the Rho family GTPases Rho, Rac, and Cdc42 which are essential for modulating many cellular processes [3, 20, 38]. Secretion of *C. difficile* toxins induces many effects that are detrimental to the host such as breakdown of actin cytoskeleton, deterioration of

epithelial barrier function (allowing fluid accumulation and intestinal damage), release of inflammatory cytokines from epithelial and mucosal immune cells, cell rounding, and cell death. Moreover, production of the binary toxin CDT may facilitate production of microtubule-based protrusions on epithelial cells which may increase *C. difficile* adherence and colonization during CDI [3, 20]. *C. difficile* then sporulates in the colon producing more spores that are emitted from the body through diarrheal shedding [3, 16]. The transmission of *C. difficile* will then continue to other people upon shedding of these spores [3].

Spore-forming abilities

The spores formed by *C. difficile* are essential for the bacterium's survival outside the host and function as the infectious vehicle for transmission of the bacterium. Since *C. difficile* is a strict anaerobic pathogen, formation of metabolically dormant spores enable the bacteria to withstand harsh environmental conditions and survive outside a host for an extended period of time [8]. *C. difficile* spores can be transmitted by the fecal-oral route, contaminated hands, or even airborne dispersal in high-risk environments such as hospitals and nursing homes. The exact number of *C. difficile* spores needed to trigger CDI symptoms is unknown, but it is estimated to be quite low-between 100 to 1000 spores [7]. While the number of spores per gram of feces is estimated to be between 1×10^4 and 1×10^7, up to 1×10^6 - 1×10^7 skin particles containing spores can be dispersed in the air in 24 hours [39, 40]. Furthermore, one study reported that *C. difficile* spores can be recovered from clinical surfaces (i.e., hospital surgical gown, vinyl hospital flooring) even after exposure to the disinfectant sodium dichloroisocyanurate (NaDCC) which illustrates the high likelihood of transmission of *C. difficile* in a healthcare environment [41].

The pathways/signals that initiate sporulation have yet to defined, but may be connected to quorum sensing, nutrient starvation, and other unknown stress factors [16]. Though, it has been well established that Spo0A is the transcriptional regulator of sporulation and gets activated upon phosphorylation [1, 8, 16, 36]. Five kinases (CD1352, CD 1492, CD1579, CD 1949, and CD 2492),

that phosphorylate Spo0A have been identified for *C. difficile* strain 630 and kinase CD1579, can directly phosphorylate Spo0A in vitro. Phosphorylated Spo0A activates sporulation-specific RNA polymerase sigma factors that regulate many different genes during sporulation [16, 36]. A positive feed forward loop has been identified between SigH and Spo0A that helps initiate sporulation. SigH stimulates *spo0A* gene transcription by increasing Spo0A associated kinase CD2492 transcription while Spo0A upregulates expression of the *sigH* gene [8]. The proteins CodY and CcpA are negative regulators of sporulation [1]. CodY represses transcription of two sporulation regulator genes, *sinI* and *sinR* [1, 36]. CcpA explicitly represses the *spo0A* gene [8, 36].

Dormant spores are extremely resistant to chemical and environmental stressors such as bleach-free disinfectants, ethanol, desiccation, oxygen exposure, and high temperatures [8, 16, 42]. One study demonstrated that *C. difficile* spores belonging to RT027, 012, and 078 have exhibited over 50% survival rate after exposure to high concentrations (85.5%) of ethanol with strain 5325 (RT078) exhibiting the highest, but non-statistically significant, survival rate. This study also showed that viable spores can still be detected even after 20-minute exposure to a range of temperatures from 65°C to 85°C with spores of R20291 (RT027) being more resistant at the 65°C to 75°C range. Household bleach was the only tested chemical in this study that brought down the viable spore concentration below the detection limit <10 colony forming unit/ml (CFU/ml) [42]. Chlorhexidine gluconate is the active ingredient in Hibiscrub® and is recommended for handwashing. *C. difficile* spores of RT012, 017, and 027 exhibited resistance to Hibiscrub® upon exposure with spores of RT027 exhibiting significantly higher resistance [39].

Spores are also naturally resilient to antibiotic treatment and the defense mechanisms elicited by the host immune system [16]. Furthermore, spore formation may contribute to *C. difficile* recurrence as spores might not be eliminated from the patients gut during antibiotic treatment so spores germinate back to their vegetative form to cause further disease [1].

Toxins

The two most important virulence factors of *C. difficile* are TcdA and TcdB as production of these toxins is adequate to facilitate all CDI symptoms and only toxin-producing (toxigenic) strains are associated with disease [3, 4, 20]. Both toxins have the same ABCD domain structure: the 'A' activity domain is located at the N-terminus and contains the glucosyltransferase domain (GTD) which is responsible for the toxins' enzymatic activity; the 'B' binding domain located at the C-terminal is made up of combined repeated oligopeptides (CROPs) which form the receptor binding domain (RBD) and is involved in receptor binding; the 'C' cutting domain has the cysteine protease domain (CPD) which facilitates auto-catalytic toxin cleavage; the 'D' delivery domain contributes to toxin translocation into the cytosol and toxin binding to host cells. Accessibility to certain nutrients, alterations in temperature, and changes in redox potential are some of the factors that dictate TcdA and TcdB expression [35].

The *tcdA* and *tcdB* genes that encode for TcdA and TcdB are located on the pathogenicity locus (PaLoc) along with *tcdC, tcdE*, and *tcdR* and the location of PaLoc is conserved across all strains that are toxigenic [20, 43]. The gene *tcdR* encodes an RNA polymerase sigma factor that is responsible for initiating TcdA and TcdB production. [20, 23]. The holin encoded by *tcdE* plays a role in extracellular toxin release [20]. TcdC has been proposed to be a negative regulator of TcdA and TcdB production as an inverse relationship between *tcdC* transcription and other PaLoc genes has been observed and strains belonging to hypervirulent lineages have a nonsense mutation in the *tcdC* gene implying that an inactive TcdC could be responsible for the increased virulence in hypervirulent lineages. However, different studies still report conflicting results regarding TcdC's role in toxin production [20, 23]. In *C. difficile* strains that are non-toxigenic, PaLoc is replaced with an extremely conserved 115/75-bp non-coding region [43].

The binary toxin (CDT) is another toxin produced by certain *C. difficile* strains [20, 23, 44]. CDT has two components: the CDTa component is an ADP-ribosyltransferase that alters the

actin cytoskeleton resulting in its destruction and cell death and CDTb is the binding component that brings CDTa into the host cytosol [23, 44]. The *cdtA* and *cdtB* genes plus *cdtR* are located on the binary toxin locus (CdtLoc) and the environmental signals responsible for CdtLoc gene regulation are unknown [23]. The relevance of CDT to virulence is not well understood as CDI cases caused by toxigenic strains that only produce CDT are uncommon and studies with animal models have yielded results that are difficult to interpret [20, 44]. Nevertheless, CDT production in strains belonging to the *C. difficile* hypervirulent ribotypes 027 and 078 that are often associated with severe CDI cases implies that CDT is important for CDI outcome, but the mechanism has yet to be determined [20, 23].

Biofilm formation

Biofilm formation is positively regulated by the second messenger cylic-di-guanosine-5' monophosphate (c-di-GMP) and biofilms shield bacteria by forming a matrix of extracellular polymeric substance (EPS) consisting of DNA, polysaccharides, and proteins [20, 45]. Previous studies have demonstrated that *C. difficile* strains mutant in any of the following Cwp84 (essential for maturation of S-layer proteins), flagellin (a key component of flagella), LuxS (mediates quorum sensing), and Spo0A (regulates sporulation) exhibit reduced or no biofilm formation. The following explanation was proposed by Dapa and Unnikrishnan as to how Cwp84, flagellin, LuxS, and Spo0A contribute to biofilm formation: an intact S-layer is essential during the initial stages of biofilm formation, hence explaining the cruciality of Cwp84; Spo0A controls early surface adhesion by upregulating adhesin expression during the initial step of biofilm formation; LuxS mediated quorum sensing is likely key in both early and late stages of biofilm production when cells are forming the biofilm matrix; flagella are also thought to play a role in biofilm maturation by directing bacterial cells to the correct sites of attachment [45].

Biofilm robustness varies among different strains [20, 45]. For example, it was shown that *C. difficile* strain R20291 formed more robust biofilm than *C. difficile* strain 630. The impermeable

layer that biofilms form protects *C. difficile* from defense mechanisms of both the innate and adaptive immune responses, makes antibiotic treatment for CDI more difficult or even unsuccessful, and makes *C. difficile* cells within biofilms more resistant to oxygen stress [45]. Lastly, biofilms may serve as a reservoir for recurring CDI as bacterial cells within biofilms are less susceptible to antibiotic treatment with vancomycin and metronidazole [18, 19].

Flagella

C. difficile may also produce flagella that enable swimming motility and attachment to host cells [37]. There are three operons that encode genes responsible for flagella construction. The F3 operon includes early-stage flagellar genes such as the FliA sigma factor (also known as SigD) while the F1 operon includes the late-stage flagellar genes that encode flagellin FliC and cap protein FliD. The FliA sigma factor is responsible for controlling *fliC* and *fliD* expression. Lastly, the purpose of the F2 regulon is related to posttranslational modifications [20]. Flagella expression is not uniform across different *C. difficile* ribotypes and is under complex regulation [37]. Moreover, some strains of *C. difficile* do not produce flagella such as those belonging to the 078-lineage as these strains are missing the F3 operon, which encodes the early-stage flagella genes [20]. The synthesis of both flagellum and toxins is phase variable and dictated by the orientation of an invertible DNA sequence known as the flagellar switch. Flagellar phase on (flg-on state) occurs when the orientation of the flagellar switch leads to flagellum production, toxin production, and swimming motility. On the contrary, flagellar phase off (flg-off state) occurs when orientation of the flagellar switch is in the opposite direction leading to lack of flagellum production, lower toxin production, and sessility [37]. The second messenger c-di-GMP represses both swimming motility and toxin production [46, 47]. Flagellar phase lock is when flagellar switch inversion does not occur [37].

Type IV pili (TFP)

Type IV pili (TFP) are hair-liking appendages composed of repeating subunits called pilin that extend from the surface of bacterial cells and have recently been shown to be essential for surface motility [48, 49]. TFP also have roles in mediating bacterial adherence to host tissues and stimulating initial biofilm formation as *pilA1* mutants exhibit reduced biofilm formation [21, 48, 49]. The *pilA1* gene encodes the major pilin subunit and genes encoding up to nine pilins are present in the *C. difficile* genome [21]. There are two proposed mechanisms for which TFP increases biofilm formation: TFP can bind to surface proteins which inhibits bacterial cells from moving freely (cell attachment model) or individual TFP are capable of interaction to form bundles and tie their cells together (pilus binding model) [48]. The formation of TFP is also under the regulation of secondary messenger c-di-GMP as high intracellular concentrations of c-di-GMP positively regulate expression of pilus genes [20, 49]. Lastly, the genes needed for TFP formation appear to be present in all *C. difficile* strains [20].

***C. difficile* and One Health**

The CDC's One Health approach, the notion that human health is intimately related to the health of animals and the environment, very much applies to *C. difficile* [50]. Carriage of *C. difficile* has been detected in nearly all mammals tested and *C. difficile* is commonly isolated from environmental sources such as soil and water [7, 51, 52]. Human contact with animals whether it be for work purposes (i.e., on a farm) or at the home (i.e., domestic pets) may place these individuals at an increased risk of zoonotic *C. difficile* transmission. *C. difficile* strains belonging to the hypervirulent RT078 have been detected in pigs and cattle in large numbers (predominant ribotype in these studies) and in dogs as well, but on a smaller scale [52-54]. Furthermore, cattle and dogs have been shown to carry the hypervirulent *C. difficile* RT027 while the toxigenic *C. difficile* RT014 has been identified in cats [52, 54]. The identification of *C. difficile* from swimming pools,

rivers, and lakes is indicative of more potential sources for environmental transmission as human contact with these bodies of water for recreational activities is common [51, 55]. While more detailed typing and toxin genotype studies are needed, toxigenic *C. difficile* belonging to ribotypes (RT014 and RT126), which are also found among human isolates, have been identified in rivers [56]. Previous studies have identified toxigenic strains belonging to *C. difficile* RT012 and RT014 in soil samples. Landscapers, soil scientists, or anyone else who has extended contact with soil may be at increased risk of environmental *C. difficile* transmission [51, 57]. It is important to study the different ways in which *C. difficile's* presence in the environment can impact human health.

Previous studies

Studies examining *C. difficile* presence in wastewater have reported high recovery rates. Even more important, recovery of toxigenic *C. difficile* from WWTPs could indicate a potential source of environment-to-human transmission placing humans at increased risk for developing CDI. Toxigenic *C. difficile* has been recovered in WWTP samples in Switzerland, Canada, New Zealand, and England [58-61]. One treated and untreated wastewater sample was collected from nine different WWTPs (so 18 samples in total) in Southern Switzerland and *C. difficile* was detected in all 18 samples. Furthermore, of the 55 *C. difficile* isolates obtained during this study, the majority (47/55 isolates) were toxigenic and 8/13 of the PCR ribotypes from WWTPs had also been isolated from *C. difficile* patients in the area. *C. difficile* PCR ribotype 078 made up 40% of the WWTP isolates [58]. In Southern Ontario, Canada, the presence of toxigenic *C. difficile* was not affected by seasonality as 61 out of the 77 recovered isolates that were toxigenic were recovered in the summer, and 90 out of the 96 recovered isolates in the winter from primary and digested sludge samples at one WWTP also were toxigenic. The frequency of RT078 in WWTPs was one of the focuses of this study and a total of 29 isolates belonging to RT078 were recovered in primary and digested sludge samples from two different WWTPs [59]. The study out of New Zealand recovered 10 toxigenic wastewater isolates from two WWTPs. Moreover, 4/8 PCR ribotypes recovered from

WWTP samples matched PCR ribotypes from human CDI cases in the country [60]. A study done in the East of England also reported high *C. difficile* recovery rates as the pathogen was detected at 18 of the 20 WWTPs in this study. Of the 186 wastewater isolates obtained, 126 were toxigenic [61]. On the contrary, low *C. difficile* recovery rates in Iran would suggest that *C. difficile* presence in WWTPs is not a major threat regarding environment-to-human transmission. One study done in Iran identified *C. difficile* in only 1/72 samples that had been collected from three different WWTPs and another Iran study reported a 5/95 *C. difficile* recovery rate in samples from two different WWTPs [62, 63]. Overall, previous research indicates a potential connection between CDI and *C. difficile* in wastewater; thus, more studies are needed to elucidate this relationship.

C. difficile has been isolated from a variety of food categories, including seafood and seafood related animals. One study in southern Italy reported that *C. difficile* was recovered from 36/925 edible bivalve mollusks samples. Notable ribotypes among the strains in this study were 078 and 126 which are similar to reported human CDI cases in Europe. Of the 36 isolates, 19 (52%) were toxigenic [64]. An earlier study conducted in the same city in Italy found that 26/53 edible bivalve mollusks samples contained *C. difficile* and once again many of these isolates' ribotypes matched those identified in clinical CDI cases in Europe [65]. A study with seafood samples from the North Adriatic Italian Sea found that of the 702 shellfish, 387 mussels and 315 clam samples collected, 118, 45, and 75 were contaminated with *C. difficile*, respectively. Ribotypes 014 and 078 were the two most common isolates in this particular study [66]. Regarding *C. difficile* in food in Canada, the 5 samples (out of 119) from Canadian grocery stores that were contaminated with *C. difficile* were from frozen scallop, fresh perch, frozen shrimp, fresh salmon, and cooked shrimp, respectively. Notably, 4 out of 5 of these isolates belonged to the hypervirulent ribotype 078 [67]. A study in the United States that examined *C. difficile* contamination in oysters reported that 9/19 oyster samples were contaminated [68]. Overall, the widespread contamination of *C. difficile* in seafood poses a health risk for humans as consumption of seafood may serve as a potential route of transmission.

CHAPTER III

METHODOLOGY

C. difficile isolates

The environmental *C. difficile* isolates characterized in this study were isolated from wastewater and seafood in Tainan, Taiwan (Table 1).

Table 1 *C. difficile* isolates analyzed in this study

PCR ribotype	Toxin genotype				Toxin classification	Source
	tcdA	*tcdB*	*cdtA*	*cdtB*		
RT002/2	+	+	-	-	Toxigenic	NCKUH
RT012	+	+	-	-	Toxigenic	laboratory strain
RT027	+	+	+	+	Toxigenic	laboratory strain
RT043	+	+	-	-	Toxigenic	NCKUH
RT060	-	-	-	-	Non-toxigenic	Oyster shell
RT106	+	+	-	-	Toxigenic	AP
RT126	+	+	+	+	078-lineage	NCKUH
RT127	+	+	+	+	078-lineage	NCKUH
RT235	+	+	-	-	Toxigenic	NCKUH
RT462	-	-	-	-	Non-toxigenic	NCKUH
RT592	-	-	-	-	Non-toxigenic	Oyster shell
RT596	-	-	-	-	Non-toxigenic	AP
RT598	+	+	+	+	078-lineage	HW
RT607	-	-	-	-	Non-toxigenic	Oyster shell
RT633	+	+	-	-	toxigenic	NCKUH
RT647	-	-	-	-	Non-toxigenic	NCKUH
RT713	-	-	-	-	Non-toxigenic	NCKUH
RT AI-60	-	-	-	-	Non-toxigenic	Oyster shell
RT AI-74	-	+	-	-	Toxigenic	Oyster shell
RT AI-83	-	+	+	+	Toxigenic	NCKUH

(+): *tcdA*, *tcdB*, *cdtA*, or *cdtB* positive; (-): *tcdA*, *tcdB*, *cdtA*, or *cdtB* negative; AP: Anping; HW: Huweiliao; NCKUH: National Cheng Kung University Hospital; PCR: polymerase chain reaction; RT: ribotype

The wastewater isolates were obtained from National Cheng Kung University Hospital (NCKUH) and from two different WWTPs - Anping (AP) and Huweiliao (HW). The seafood isolates were collected from oyster shells purchased at LiuKong Pier in Tainan, Taiwan. These isolates were shipped to Oklahoma State University Center for Health Sciences (OSU-CHS) for further analyses. One isolate of each *C. difficile* PCR ribotype represented in our lab's collection was chosen for the following experiments, 18 different *C. difficile* PCR ribotypes were analyzed in total. *C. difficile* strains R20291 (RT027) and 630 (RT012) were included as toxigenic control strains for all experiments.

C. difficile media and growth conditions

C. difficile was cultured in BHIS media containing 37 gram/Liter (g/L) brain heart infusion (OXOID) supplemented with 5 g/L yeast extract (Fisher BioReagents) and 1 g/L L- cysteine (Alfa Aesar), unless otherwise noted. 1.5 g/L agar or 1.0 g/L agar (Alfa Aesar or Thermo Scientific) was also supplemented when making solid media in petri plates. 70:30 medium was utilized to measure sporulation efficiency. The 70:30 medium contained 63 g/L Bacto™ peptone (Gibco), 3.5 g/L proteose peptone (Oxoid), 11.1 g/L BHI (Oxoid), 1.5 g/L yeast extract (Fisher Bioreagents), 1.06 g/L Tris base (Fisher Scientific), 0.7 g/L ammonium sulfate (Fisher Bioreagents), and 15 g/L agar (Thermo Scientific).

All media were pre-reduced for at least four hours prior to use inside a Coy vinyl anaerobic chamber (Coy Laboratory Products, Grass Lake, Michigan, United States) with an atmosphere of 5% H_2, 5% CO_2, and 90% N_2. *C. difficile* was always grown at 37°C inside the anaerobic chamber incubator. All work with *C. difficile* was performed in a LabGard Class II, Type A2 Biological Safety Cabinet (NuAire, Plymouth, Minnesota, United States).

Preparation of *C. difficile* glycerol stock

Glycerol frozen stock was made for each *C. difficile* isolate and stored in a -80°C freezer

(Thermo Scientific, TSX Series). In short, *C. difficile* isolates were grown in BHIS broth for approximately 2 days at 37°C in the anaerobic chamber incubator upon arrival at OSU-CHS. Next, a portion of *C. difficile* culture was inoculated onto 1% BHIS agar plates. Approximately 48 hours later, an overnight culture was made for each isolate by using a disposable inoculating loop to inoculate a single colony in BHIS broth. Finally, the overnight culture was used for making *C. difficile* frozen stock which contained a 1:1 ratio of 500µL *C. difficile* and 500µL 50% glycerol.

Growth curve

Each environmental *C. difficile* isolate plus control strains R20291 and 630 were struck from glycerol frozen stock onto 1.5% BHIS agar plates and grown anaerobically at 37° for 48-hours. An overnight culture was then made by inoculating a single colony into 10 mL BHIS broth. After overnight incubation, *C. difficile* cultures were refreshed 1:100 in BHIS broth and the optical density 600 (OD_{600}) was measured using a Spectronic 20D+ Digital Spectrophotometer (Thermo Scientific). OD_{600} was measured at hourly intervals for 8 hours. A growth curve was constructed using GraphPad Prism 9.3.1. (471).

Colony morphology

The following protocol was adapted from Camorlinga et al., though slight modifications were made [22]. In summary, *C. difficile* isolates were streaked from the -80°C glycerol stock onto 1.5% BHI agar plates and grown at 37°C in the anaerobic chamber incubator. After an approximate 48-hour incubation period, an overnight culture (18 hours) was prepared for each isolate by inoculating a single colony into BHI broth. The following day, 3µL of overnight culture was spot inoculated onto 1.5% BHI agar plates. The plates were wrapped in parafilm after the inoculum spots had dried and then placed inside the incubator. Seven days later, the morphology (i.e., size and shape) of each single colony was macroscopically examined and a photograph of each colony

was taken using an Apple iPhone XS. *C. difficile* strains R20291 and 630 were included as controls [22].

Sporulation efficiency

The experiment protocol was adapted from Edwards and McBride and several modifications were added [69]. Environmental *C. difficile* isolates along with control strains R20291 and 630 were streaked from glycerol stock onto 1.5% BHIS agar plates and incubated anaerobically at 37°C for approximately 45 hours. An overnight culture (18 hours) was then made for each *C. difficile* isolate by inoculating a single colony from the BHIS agar plates. Following overnight incubation, 200 µL culture was inoculated onto 70:30 sporulation medium plates. The 200 µL culture was spread using a glass spreader. *C. difficile* isolates were incubated on 70:30 sporulation medium at 37°C in the anaerobic chamber for 24-hours. In order to visually observe bacterial spores and cells under a microscope, a 200 µL pipette tip was used to scrape a small portion of cells from the 70:30 sporulation medium plate to resuspend in 100 µL BHIS broth. The cells were centrifuged for 30 seconds at 13,000 rpm then the supernatant was promptly discarded. The pellet was resuspended in 10 µL BHIS broth and 5 µL of the resuspended culture was added to a glass microscope slide containing 5 µL of a 0.7% agarose pad. The vegetative cells and spores were visualized under a 1000x magnification lens with oil immersion. Vegetative cells materialized as rod-shaped while free spores appeared circular, and the endospores still within their mother cells were at a terminal location. A photograph of three different microscope viewpoints was taken for each isolate using an Apple iPhone XS. The cell counter on ImageJ was utilized to directly count the spores and vegetative cells. Sporulation efficiency was calculated using the following formula: sporulation efficiency (%) = (spores)/(vegetative cells + spores) x 100 [69].

Surface motility

This experiment was performed as previously described by Purcell et al. with minor

modifications [49]. *C. difficile* isolates were streaked from glycerol stock onto 1.5% BHIS agar plates and incubated for approximately 48 hours at 37°C in the anaerobic chamber incubator. Overnight cultures were prepared for each isolate by growing a single colony of each isolate in BHIS-1% glucose broth. Following an 18-hour incubation period, 5µL of overnight culture was spot inoculated onto plates containing 2.1% agar BHIS-1% glucose in triplicates. Each plate was wrapped in parafilm once the inoculum spots dried and moved to the anaerobic chamber incubator. The migration diameter of each spot inoculum was measured at strict 24-hour intervals for up to 120 hours. Plates were kept inside the anaerobic chamber while the measurements were collected. After collecting the final migration diameter, plates were removed and photographed using an Apple iPhone XS. *C. difficile* strains R20291 and 630 were included in this experiment as toxigenic controls [49]. Statistical analysis was performed using GraphPad Prism 9.3.1. (471). An unpaired t-test was done to compare the migration diameter of toxigenic isolates vs. non-toxigenic isolates and an ordinary one-way Analysis of Variance (ANOVA) was used to compare the migration diameter of 078-lineage isolates vs non 078-lineage toxigenic isolates vs non-toxigenic isolates.

Swimming motility

The following experiment protocol was adapted from Karpinski et al. with some additional modifications [70]. A 1.5% BHIS agar streak plate for each *C. difficile* isolate was prepared from glycerol frozen stock and grown for approximately 48 hours at 37□ inside the anaerobic chamber incubator prior to inoculation. A metal inoculation needle was used to stab inoculate (6 centimeter (cm) above the bottom of the tube) a single colony of each isolate into test tubes containing 8 mL .175% BHI agar. This step was done in triplicates for each isolate. Test tubes were removed from the incubator after a 24-hour incubation period and the motility of each isolate was photographed using an Apple iPhone XS [70]. For statistical analysis, the software program ImageJ was used to quantify the image brightness of each isolate using a defined region of interest (ROI) to allow for semi-quantitative comparison of swimming motility between isolates with different toxin

genotypes. *C. difficile* strain R20291 was the motile control and RT126, RT127, and RT598 were the non-motile controls [20]. GraphPad Prism 9.3.1 (471) was utilized for statistical analysis to compare the motile toxigenic isolates and the motile non-toxigenic isolates.

Statistical analysis

GraphPad Prism 9.3.1 (471) was utilized for analyzing the growth curve, sporulation efficiency, surface motility, and swimming motility experimental results. $P < 0.05$ was chosen to represent statistically significant differences for the sporulation efficiency, surface motility, and swimming motility assays.

CHAPTER IV

FINDINGS

Growth curve analysis of environmental *C. difficile* isolates

A growth curve represents the bacterial cell population density in liquid culture over a defined period of time and may be obtained by measuring the optical density at 600 nm (OD_{600}) [71]. Most of the environmental *C. difficile* isolates exhibited similar growth rates as they followed an S-shaped growth curve. However, there were a few isolates that grew slower such as the isolates representing RT126, RT127, RT596, RT598, and *C. difficile* strain 630 (Figure 1).

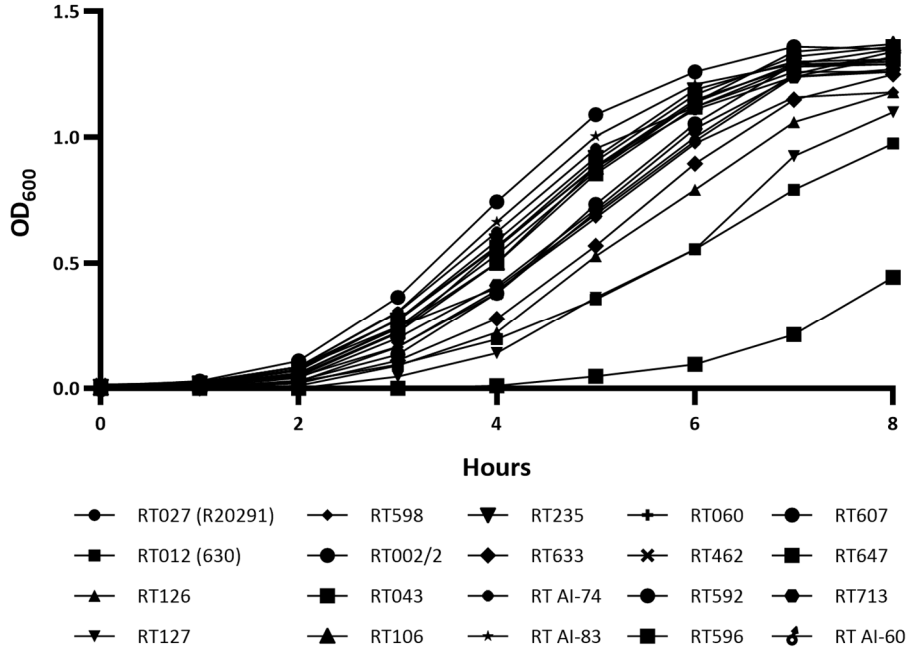


Figure 1 Growth curve

The growth rate of environmental *C. difficile* isolates plus control strains R20291 and 630 grown in BHIS was measured at OD_{600} at 1-hour intervals for up to 8 hours

The colony morphology of environmental *C. difficile* isolates

We began by examining the colony morphology of the environmental *C. difficile* isolates. Colony morphology is observed by growing bacteria on an agar plate and visually examining the colony characteristics [72]. Morphological characteristics may be used for characterization purposes and reveal biological differences between isolates [22, 72]. Overall, we observed diverse colony morphology in terms of size and shape between the different *C. difficile* ribotypes in our environmental isolate collection (Figure 2). A couple of things we noticed was that the larger sized colonies (such as RT647 and RT AI-74) tended to have more ruffled edges while the colonies of the isolates belonging to the 078-lineage (RT126, RT127, and RT598) each had moderately ruffled edges. Lastly, there did not appear to be any visible patterns between toxigenic isolates and non-toxigenic isolates.

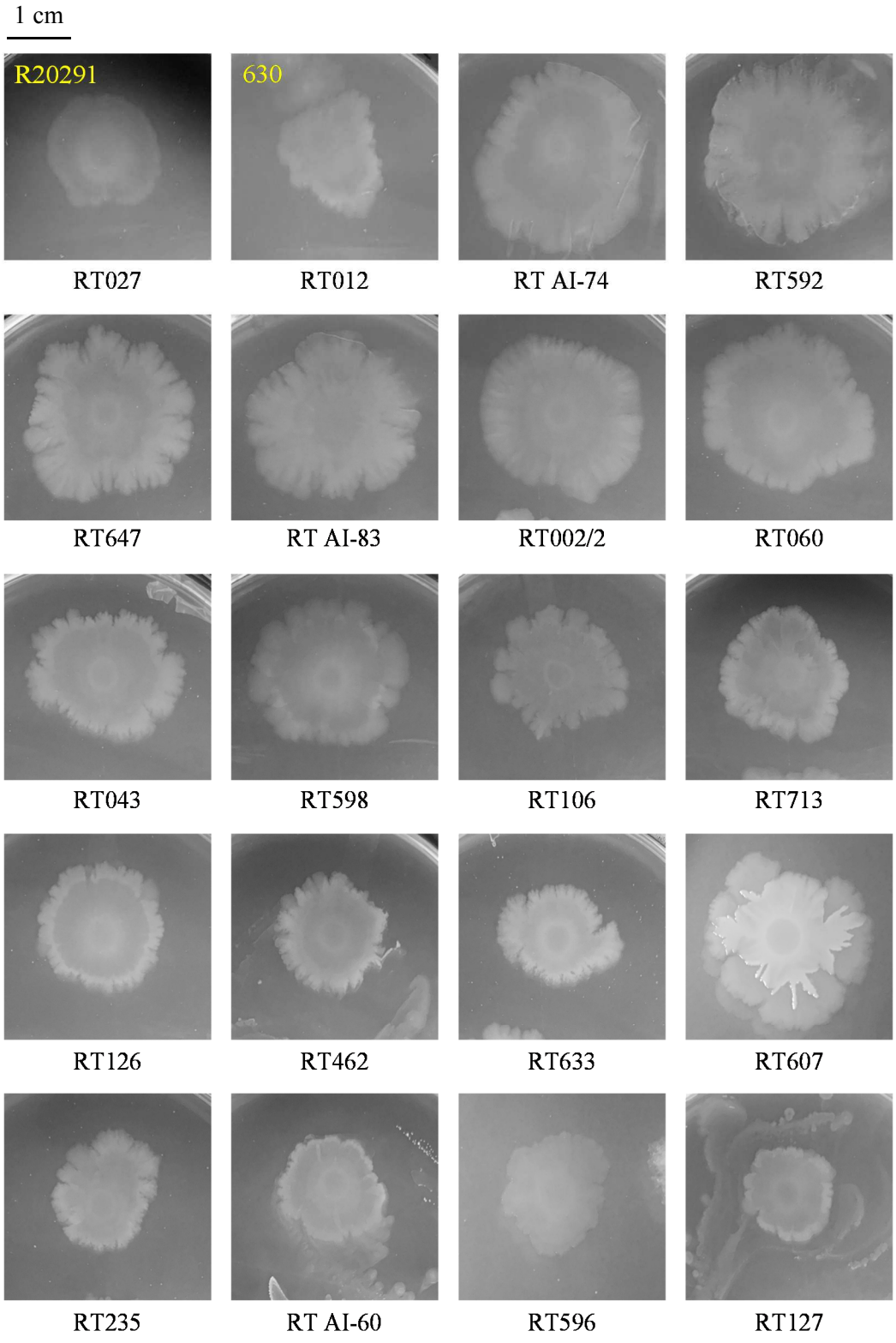
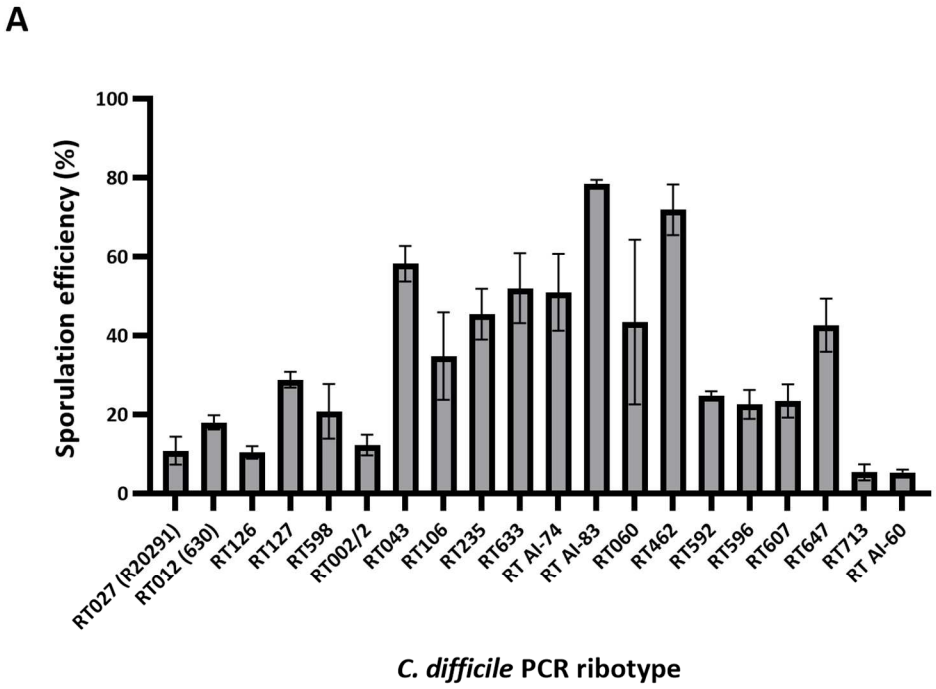


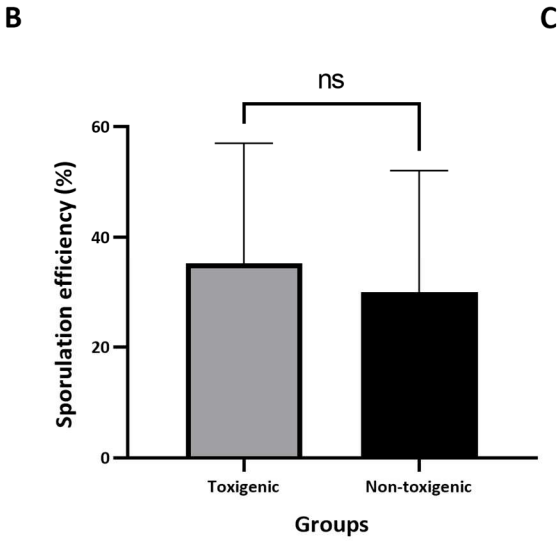
Figure 2 Colony morphology of *C. difficile*

The colony morphology of environmental *C. difficile* isolates along with control strains R20291 and 630 was visually observed after a 7-day incubation period on 1.5% BHI agar

Sporulation efficiency of environmental *C. difficile* isolates

C. difficile bacterial spores are a distinctive attribute that sets *C. difficile* apart from other healthcare-associated pathogens since they are resistant to multiple disinfection measures and the production of spores has been shown to differ between certain *C. difficile* PCR ribotypes. For example, *C. difficile* RT017 was shown to be a higher spore-former than RT012 and RT027 [39]. We compared the sporulation efficiency (percentage of spores among total number of vegetative cells and spores in culture) among the different PCR ribotypes in our environmental isolate collection [69] (Figure 3). We observed the sporulation efficiency after a 24-hour incubation period as there will be a mixture of vegetative cells and spores at this time point [69]. In this study, there were no significant differences in the sporulation efficiency based on toxin genotype (Figures 3B and C). Nonetheless, there was significant variation in the spore-forming abilities within the groups. Among the toxigenic *C. difficile* isolates RT AI-83 had a sporulation efficiency of 78.4% while RT126 had a sporulation efficiency of 10.38%. For the non-toxigenic *C. difficile* isolates, RT462 exhibited 71.89% sporulation efficiency while RTAI-60 and RT713 exhibited 5.32% and 5.34%, respectively (Figure 3A).


A
Sporulation efficiency (%)
100
80
60
40
20
0
RT027 (R20291)
RT012 (630)
RT126
RT127
RT598
RT002/2
RT043
RT106
RT235
RT633
RT AI-74
RT AI-83
RT060
RT462
RT592
RT596
RT607
RT647
RT713
RT AI-60
C. difficile PCR ribotype


B
ns
Sporulation efficiency (%)
60
40
20
0
Toxigenic
Non-toxigenic
Groups

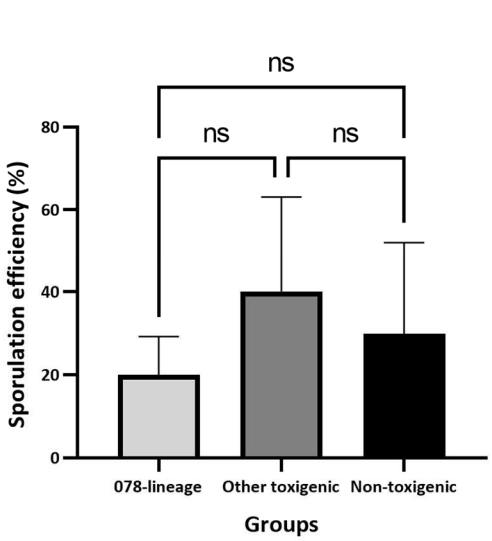

C
ns
ns
ns
Sporulation efficiency (%)
80
60
40
20
0
078-lineage
Other toxigenic
Non-toxigenic
Groups

Figure 3 Sporulation efficiency of *C. difficile*

A) Sporulation efficiency of environmental *C. difficile* isolates after 24-hour incubation on 70:30 medium. Three different microscope views were observed in order to determine sporulation efficiency. The toxigenic *C. difficile* PCR ribotypes are listed on the left side of the graph starting with control *C. difficile* strain R20291 and the non-toxigenic *C. difficile* PCR ribotypes are listed on the right side of the graph beginning with RT060. B) The sporulation efficiency of toxigenic (n=12, including control strains R20291 and 630) and non-toxigenic (n=8) environmental *C. difficile* isolates was compared using an unpaired t-test (ns=non-significant). C) The sporulation efficiency of 078-lineage toxigenic isolates (n=3; RT126, RT127, and RT598) was compared with other toxigenic isolates (n=9, including R20291 and 630) and non-toxigenic isolates (n=8) using a one-way ANOVA (ns=non-significant).

Surface motility of environmental *C. difficile* isolates

TFP-mediated surface motility is necessary for *C. difficile* to be capable of moving across host tissues to new adherence sites while the bacterium is simultaneously maintaining contact with the epithelium and its nutrients [49]. The mechanism for TFP movement consists of pilus extension, attaching to the surface, and then retracting the pilus which pulls *C. difficile* toward its desired attachment site [48]. We observed surface motility of the environmental isolates over a 5-day period and photographs documenting motility were taken on day 5 [49]. This experiment was done in triplicates and one colony image per isolate illustrating surface motility is shown in Figure 4. Notably, we found that *C. difficile* isolates belonging to the hypervirulent 078-lineage (RT126, RT127 and RT598) exhibited significantly lower surface motility compared to both other toxigenic isolates and non-toxigenic isolates (Figure 5C). There were no significant differences between toxigenic isolates and non-toxigenic isolates (Figure 5B).

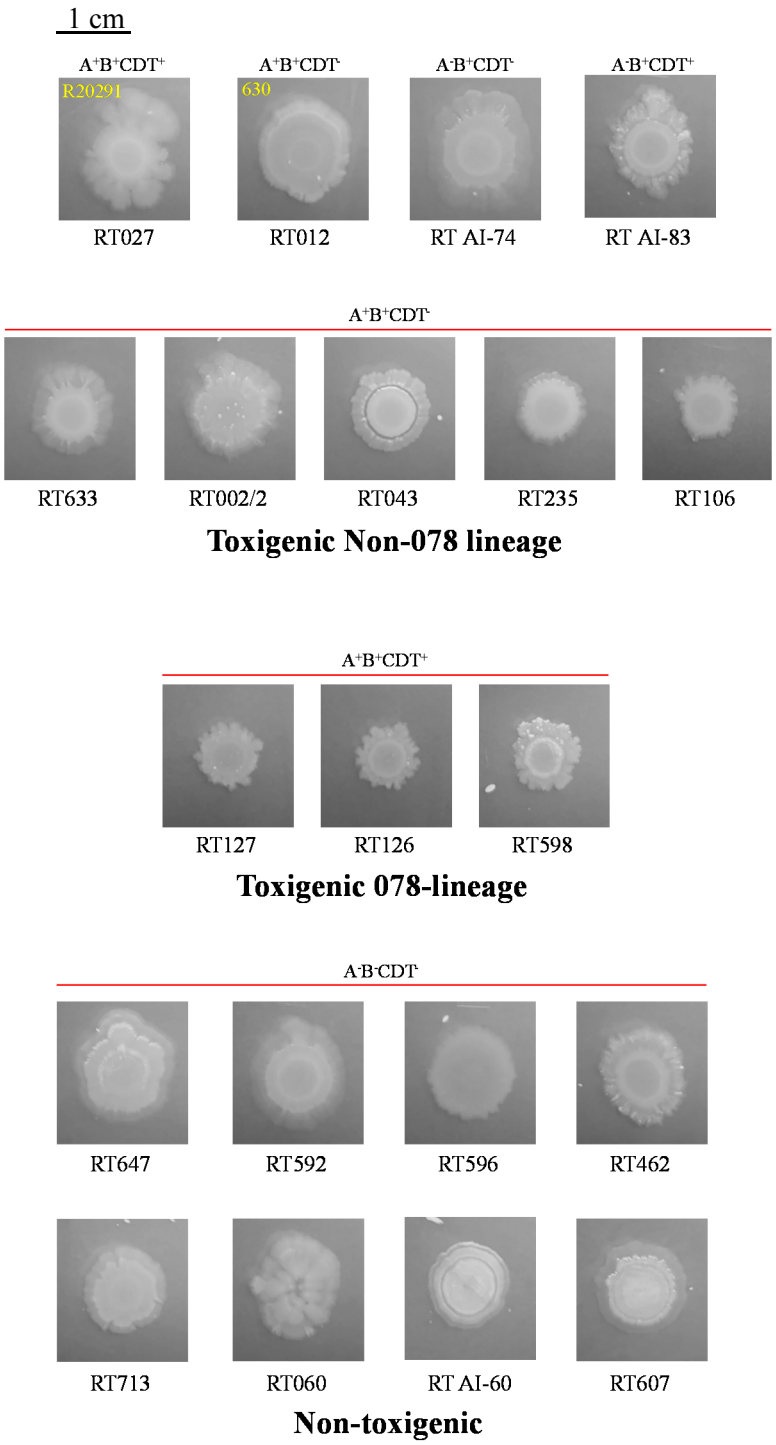
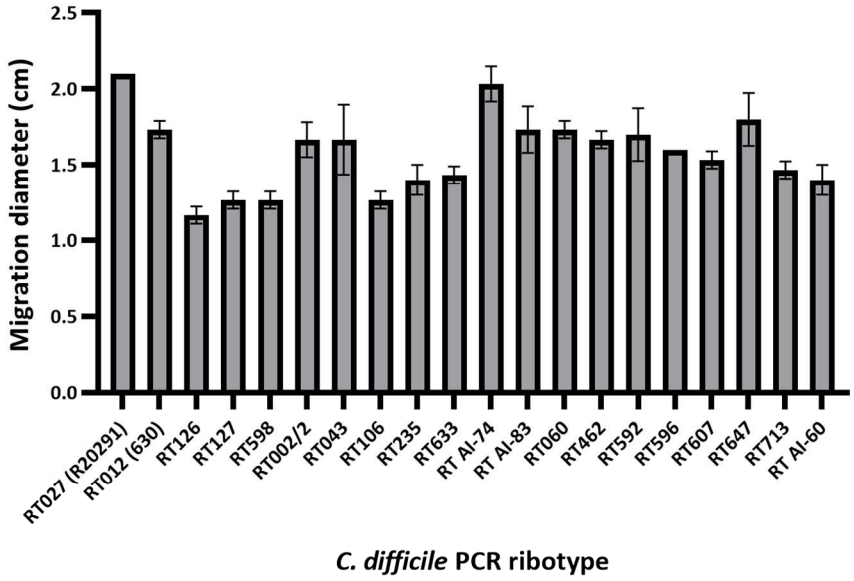


Figure 4 Surface motility images

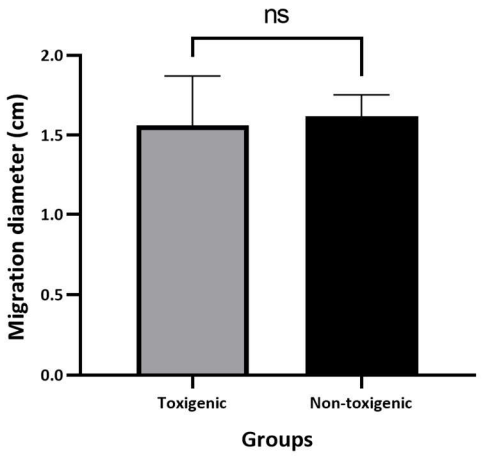
The surface motility of environmental *C. difficile* isolates and control strains R20291 and 630 was photographed after a 120-hour incubation period on 2.1% agar BHIS-1% glucose.

A: TcdA; B: TcdB; CDT; binary toxin; (+): positive; (-): negative

A


Migration diameter (cm)
2.5
2.0
1.5
1.0
0.5
0.0
RT027 (R20291)
RT012 (630)
RT126
RT127
RT598
RT002/2
RT043
RT106
RT235
RT633
RT AI-74
RT AI-83
RT060
RT462
RT592
RT596
RT607
RT647
RT713
RT AI-60
C. difficile PCR ribotype

B


ns
Migration diameter (cm)
2.0
1.5
1.0
0.5
0.0
Toxigenic
Non-toxigenic
Groups

C

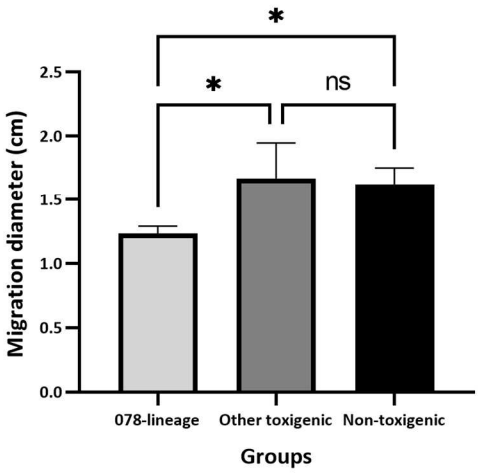

*
*
ns
Migration diameter (cm)
2.5
2.0
1.5
1.0
0.5
0.0
078-lineage
Other toxigenic
Non-toxigenic
Groups

Figure 5 Surface motility of *C. difficile*

A) Migration diameter (cm) represents the surface motility exhibited by environmental *C. difficile* isolates after 120 hours. Toxigenic *C. difficile* PCR ribotypes are shown on the left side of the graph beginning with control strain R20291 and non-toxigenic *C. difficile* PCR ribotypes are shown on the right side of the graph starting with RT060. B) The surface motility exhibited by toxigenic isolates (n=12; including control strains R20291 and 630) and non-toxigenic isolates (n=8) after 120 hours was compared using an unpaired t-test (ns=non-significant). C) The surface motility of 078-lineage isolates (n=3; RT126, RT127, and RT598) vs other toxigenic isolates (n=9; including control strains R20291 and 630) vs non-toxigenic isolates (n=8) were compared after 120-hours using an ordinary one-way ANOVA. *C. difficile* isolates of the 078-lineage displayed significantly lower surface motility than other toxigenic isolates and non-toxigenic isolates (* $P < 0.05$).

Swimming motility of environmental *C. difficile* isolates

In addition to analyzing surface motility, we also analyzed swimming motility which is the movement of *C. difficile* in a liquid environment mediated by flagella [37]. RT126, RT127, and RT598 were the non-motile controls for this experiment since it has been established in the literature that RT078-lineage isolates lack flagella and are non-motile [20]. This experiment was done in triplicates and the image brightness of each isolate was measured using ImageJ to determine which isolates were motile. RT127 exhibited the highest image brightness of the 078-lineage isolates and any isolate with a lower image brightness was deemed non-motile. In total, 10 of the environmental isolates were non-motile (Figure 6 and 7A). Moreover, the swimming motility differed by PCR ribotype as 8 environmental isolates and R20291 and 630 exhibited swimming motility-visible movement in tube relative to initial inoculum (Figure 6 and Figure 7A). For example, of the isolates with $A^+B^+CDT^-$ toxin genotype, RT106 was motile and clearly exhibited motility while RT 633 was non-motile (Figure 6). We sought to determine if there were any differences in swimming motility between motile toxigenic isolates and motile non-toxigenic isolates and the non-motile isolates were not included in the following analysis. We found that there were no significant differences in swimming motility between motile toxigenic isolates and motile non-toxigenic isolates (Figure 7B).

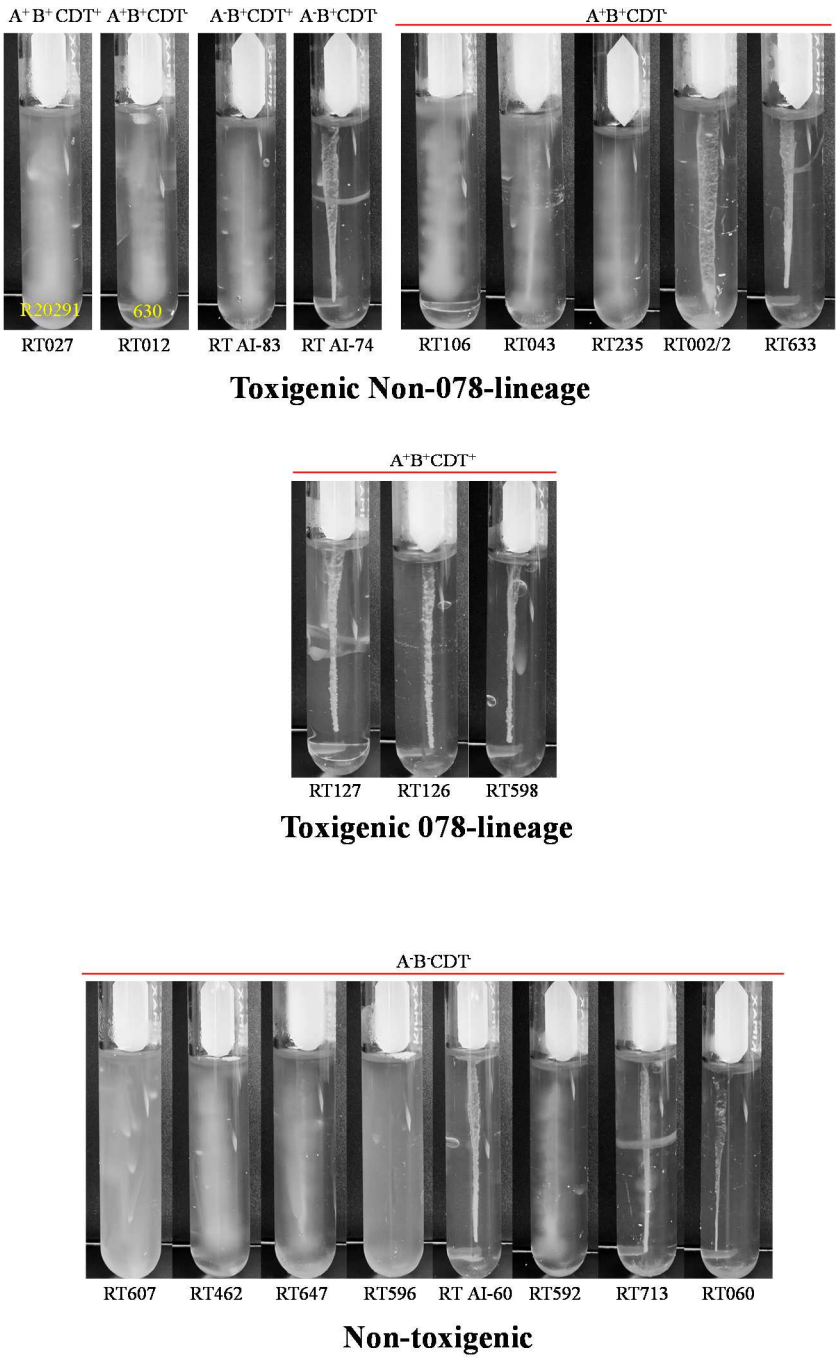


Figure 6 Swimming motility images

The swimming motility of environmental *C. difficile* isolates, motile control strains (R20291 and 630), and non-motile control isolates (078-lineage) was examined after a 24-hour incubation period in test tubes containing .175% BHI agar. One image per PCR ribotype is shown.

A: TcdA; B: TcdB; CDT: binary toxin; (+): positive; (-): negative

A

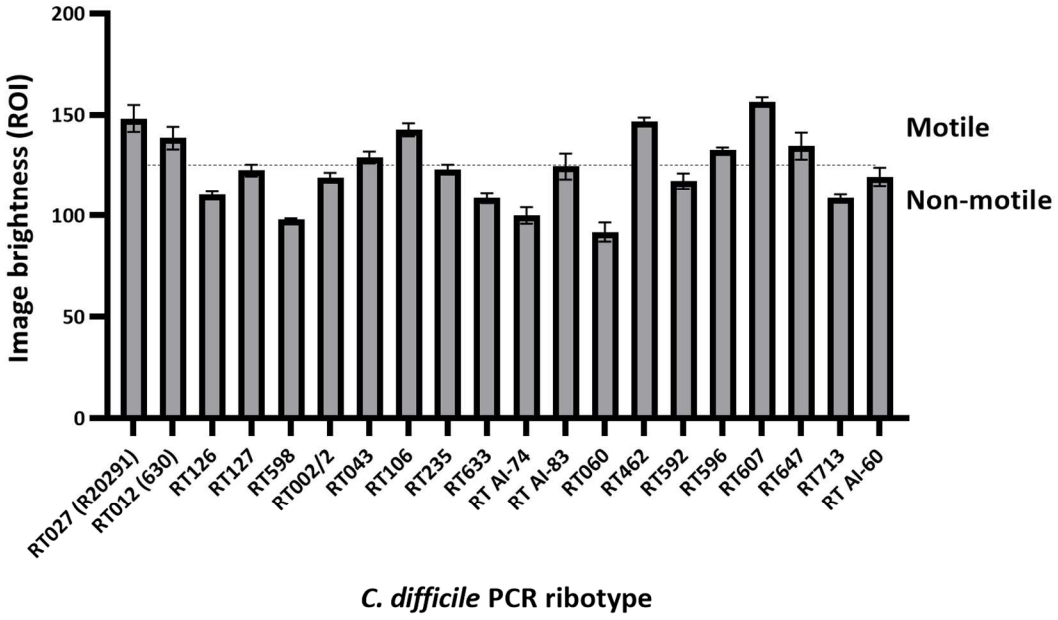


Image brightness (ROI)
200
150
100
50
0
Motile
Non-motile
RT027 (R20291)
RT012 (630)
RT126
RT127
RT598
RT002/2
RT043
RT106
RT235
RT633
RT AI-74
RT AI-83
RT060
RT462
RT592
RT596
RT607
RT647
RT713
RT AI-60
C. difficile PCR ribotype

B

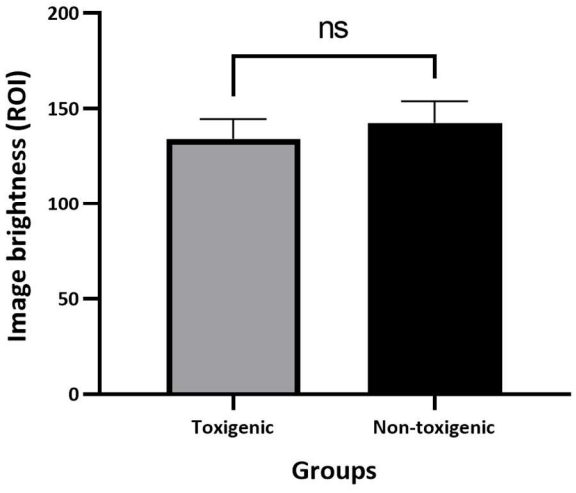


Image brightness (ROI)
200
150
100
50
0
ns
Toxigenic
Non-toxigenic
Groups

Figure 7 Swimming motility of *C. difficile*

A) Swimming motility of environmental *C. difficile* isolates and *C. difficile* strains R20291 and 630 after 24-hours. Image analysis was performed using ImageJ and the image brightness for a defined region of interest (ROI) was determined. Any isolate that had a lower image brightness than RT127 (of the 078-lineage) was defined as non-motile. Motile isolates (n=10) are listed above the horizontal line on the graph and non-motile isolates are below the line. RT235 is motile as its image brightness was 122.9183 and the image brightness for RT127 was 122.5643. The toxigenic *C. difficile* PCR ribotypes are listed on the left side of the graph starting with *C. difficile* strain R20291 and non-toxigenic *C. difficile* PCR ribotypes on the right side beginning with RT060. B) The image brightness of each motile toxigenic isolate (n=6, including R20291 and 630) was compared to the image brightness of each motile non-toxigenic isolate (n=4) and an unpaired t-test was performed to analyze differences in swimming motility (ns=non-significant).

CHAPTER V

CONCLUSION

The phenotypic characteristics of *C. difficile* are relevant for CDI patients, especially in regard to antibiotic treatment. As mentioned earlier, *C. difficile* produces spores that are responsible for enabling bacterial transmission and are naturally resistant to antibiotics [16]. CDI patients that are infected with strains of *C. difficile* that exhibit increased sporulation and consequently antibiotic resistance (such as those belonging to RT027) will have less success when taking antibiotics such as vancomycin or one of the fluoroquinolones [15, 17, 73]. *C. difficile* spores and vegetative cells hide within biofilms rendering treatment with commonly prescribed antibiotics to be less effective in patients that are infected with a strain that forms biofilms [18, 19, 45, 74]. Moreover, since sporulation rates differ among different *C. difficile* ribotypes, knowing that a hospitalized CDI patient is infected with a strain that exhibits hypersporulation could prompt healthcare providers to follow a stronger disinfection approach to limit the spread of *C. difficile* as these strains spread faster than others and success in killing spores varies based on chemical/method used [15, 39].

In short, knowing the sporulation and biofilm-forming abilities of the *C. difficile* strain the patient is colonized with could reduce the use of unnecessary antibiotics in situations where the patient is unlikely to benefit from antibiotics [21]. It is also known that flagella, which mediate swimming motility and adherence are active during CDI as anti-flagellin antibodies have been identified in

CDI patients and TFP are involved in promoting initial biofilm formation thus subsequently, antibiotic resistance [20, 21, 37].

Several studies have examined the prevalence of CDI in patients at National Cheng Kung University Hospital (NCKUH) in southern Taiwan [10, 11]. In fact, several of the isolates tested in this study came from this hospital's wastewater (Table 1). From January 2007 to March 2008, the incidence of CDI at NCKUH was 42.6 cases per 100,000 patient days with a higher prevalence being noted in patients in the ICU [10]. Another study from NCKUH specifically examined the incidence of CDI in ICU patients and reported 8.8 cases per 10,000 patient days from March 2013 to March 2014 [11]. Regarding CDI in northern Taiwan, 30 patients were found to have toxigenic CDI from 2000 to 2010 at National Taiwan University Hospital [12]. Lastly, in regard to CDI in central Taiwan, stool samples from 149 patients at six different hospitals were tested for *C. difficile* from April 2009 to October 2009. Patients from all six hospitals were found to have *C. difficile* colonization, but only patients at two of the six hospitals had CDI. PCR ribotyping was performed with the *C. difficile* isolates collected from patients and RT106 was one of the most common PCR ribotypes [13]. *C. difficile* PCR RT106 was analyzed in this study and was isolated from the AP WWTP (Table 1). *C. difficile* has also been detected in carcasses, raw pork and ready-to-eat pork at a slaughterhouse in Taiwan. RT126 was the predominant ribotype in this study and RT127 and RT014 were also present [75]. Future research should explore *C. difficile* in non-human sources to examine a potential relationship between environmental *C. difficile* and CDI in patients in Taiwan.

The environmental isolates exhibited a similar growth rate in BHIS medium except for RT596 (Figure 1). At this time we are unsure of why RT596 grows relatively slower than the rest as no further analyses were carried out. This phenomenon should be further examined.

The colony morphology of *C. difficile* has been analyzed by multiple research groups and we found several studies with interesting data to compare to our results. One such study was done in Mexico with non-toxigenic *C. difficile* isolates obtained from patients who were experiencing hospital-acquired diarrhea. The images published in this paper show both diversity in colony

size and appearance, same as what we observed with the environmental *C. difficile* isolates in our study. Moreover, we referenced the protocol in this paper for our methodology so comparing the colony morphology of isolates grown on the same medium (BHI agar) allows for a more direct comparison. Several differences were that this group observed that colonies of non-toxigenic *C. difficile* strains were larger compared to the control toxigenic *C. difficile* strains while there were no visually clear patterns in terms of toxin-producing status in our study. Camorlinga et al. also did not compare colony morphology among isolates belonging to different PCR ribotypes [22]. Gross et al. examined the phenotypic characteristics of three *C. difficile* isolates obtained from the same patient. However, this group used a different growth medium-*Clostridium difficile* (CLO) agar plates (bioMérieux, Nürtingen, Germany) so comparing the colony morphology images from this study to what we observed is not a direct comparison. In short, the colony morphology of both RT012 and RT027 noticeably differ in appearance on CLO agar plates compared to BHI agar plates, plus the incubation period followed in this study was only 2 days while ours was 7 days. The colonies of RT027 appear irregularly shaped and whitish in color while RT012 appears both white and cloudy in color on CLO agar plates [76]. On the contrary, RT012 and RT027 appear golden brown in color and are irregularly shaped on BHI agar (Figure 2). A study published in 2019 examined *C. difficile* colony morphology in context of the CmRST system. Similar to our study, they compared colony morphology of multiple ribotypes, but also demonstrated that some *C. difficile* strains yield both smooth and rough colonies. The methodology followed by this group differed as they specifically streaked for both smooth and rough colonies while we spot-inoculated overnight culture so only a single colony per isolate would form, so naturally we did not observe this phenomenon in our study. Moreover, the colony morphology images published in this study were taken under 2X magnification, but ultimately the colony morphology did differ between different *C. difficile* PCR ribotypes [77].

We hypothesized that sporulation efficiency would differ by toxin genotype with toxigenic isolates and 078-lineage isolates exhibiting significantly higher sporulation than non-toxigenic

isolates. Since only toxigenic isolates are associated with CDI disease symptoms and the 078-lineage isolates are in the same family as the hypervirulent RT078, we wondered if toxigenic and 078-lineage isolates of *C. difficile* were more efficient spore-formers than non-toxigenic isolates or if toxigenic *C. difficile* just has more opportunities to be spread as the bacteria is ultimately transmitted via spores [4, 17, 27, 39]. Surprisingly, we observed no significant differences in sporulation efficiency between groups (Figures 3B and C) and there was more variation in sporulation efficiency within the groups than expected (Figure 3A). However, differences in sporulation efficiency by toxin genotype may be observed under different study conditions. One possible reason why we did not see differences between groups could be because of the small number of samples per group. For instance, only three *C. difficile* isolates belonging to the 078-lineage were tested. Another limitation of this study is that sporulation efficiency was only measured after incubation on 70:30 medium due to time constraints. While 70:30 medium yields high sporulation for a broad range of *C. difficile* strains, certain strains may exhibit their maximum sporulation abilities on sporulation medium cysteine (SM(c)) [78]. 70:30 medium is actually a mixture of 70% SMC and 30% BHIS and was created for quantifying sporulation frequency [69]. In the 1980s, Wilson et al. originally showed that sporulation medium (SM) yields high spore counts and the ingredients for SM consisted of Trypticase peptone, Proteose peptone, ammonium sulfate, and Tris [79]. It is also important to note that we did not measure sporulation efficiency using the most common methodology which consists of alcohol shock or heat shock to kill vegetative cells and performing serial dilutions and plating these dilutions to calculate the CFU/ml of heat resistance spores and CFU/ml of total cells in order to determine the percentage of heat resistance spores [22, 39, 76, 80, 81]. We tried this approach, and it did not work in our lab. When quantifying CFU/ml, it is essential that *C. difficile* vegetative cell exposure to oxygen is minimized. Due to the large number of *C. difficile* isolates that we had to work with at the same time, it is possible that prolonged exposure to oxygen may have reduced the number of viable vegetative cells leading to an overestimation of sporulation efficiency. Nonetheless, there are other ways that *C.*

difficile sporulation efficiency can be determined, and several studies have been published that measured sporulation efficiency by directly counting the number of spores, spore-forming cells, and vegetative cells under a microscope (Fawley et al. and Underwood et al.) plus a study by Edwards and McBride validated this protocol [69, 82, 83]. For directly counting spores, the need to minimize oxygen exposure so the vegetative cells will stay viable is less urgent. However, it should be emphasized that on the day *C. difficile* spores were counted under the microscope, the 70:30 plates were out of the anaerobic environment for no more than 25 minutes and this amount of time will not affect the number of viable vegetative cells that can be seen under the microscope. When searching the literature for similar studies, we found one study that reported a similar spore count for *C. difficile* isolates belonging to RT012, RT027, and RT078 [76]. From our sporulation efficiency study, we found that RT027 (R20291), RT012 (630), and RT078 family members (RT126, RT127, and RT598) exhibited similar sporulation efficiency (Figure 3A). Though it must be mentioned that BHIS media was used for assessing sporulation in this study and BHIS media has been shown to yield lower sporulation than 70:30 media, plus this research group used the heat shock approach to kill vegetative cells and determined the CFUs to calculate sporulation efficiency while we visually counted sporulation efficiency, so this study was only vaguely similar to ours [76, 78]. We also found that Dawson et al. reported similar sporulation efficiency for RT012 and RT027 which we observed as well (Figure 3A) [39]. Though it should be mentioned that our methodology differed as this group calculated sporulation efficiency by using the heat-shock approach to eliminate vegetive cells and calculate CFUs [39].

To our knowledge, this study was the first to report that isolates belonging to the 078-lineage demonstrated significantly lower surface motility (Figure 5C). We speculate that the relationship between c-di-GMP concentration, toxin production and type iv pili formation may explain our results. Toxin production is one characteristic of the 078-lineage and toxin synthesis is inhibited by c-di-GMP while high levels of c-di-GMP promotes formation of TFP [20, 24, 47]. Though when searching the literature, we found that there have only been a few studies that have

analyzed the surface motility of *C. difficile*. In fact, the requirement of type iv pili for surface motility was only first published within the last seven years [49]. For our surface motility assay, we partially followed the methodology from the study that examined the role of TFP for mediating *C. difficile* surface behavior and reported the necessity of TFP in the surface motility of this bacteria. The focus of the Purcell et al. study was different than ours as this group did not compare factors such as surface motility in context of toxin genotype or motility among *C. difficile* strains with different ribotypes. Nevertheless, Purcell et al. found that the presence of TFP is essential in order for R20291 to exhibit surface motility and that TFP have a more important function in surface motility of strain R20291 compared to 630Δerm [49]. Two of the other studies that examined surface motility did so in the context of the CmrRST system and smooth/rough colonies. There is little to compare as both studies by Garrett et al. followed a shorter incubation period, used a lower agar concentration (1.8%) for BHIS-1% glucose plates, and did not compare toxigenic and non-toxigenic *C. difficile* strains or strains of different *C. difficile* PCR ribotypes [77, 84]. Despite the strong probability that surface behaviors of *C. difficile* are applicable during the course of CDI, little research has been devoted to this aspect of *C. difficile* biology. Future research efforts should examine TFP dependent surface motility and other surface behavioral characteristics of *C. difficile* [49].

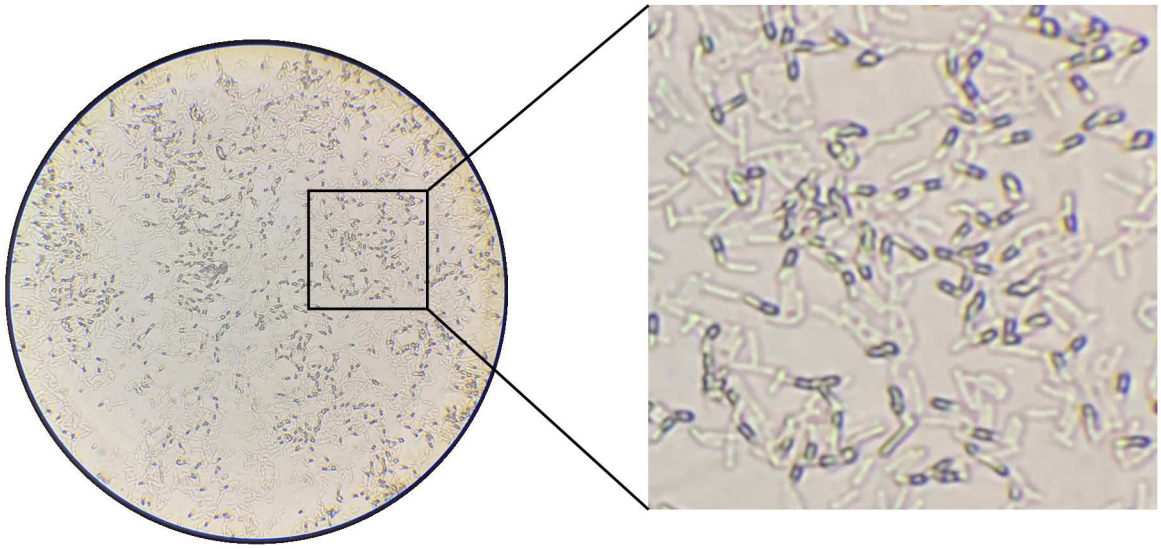
C. difficile swimming motility has been well studied. There are two ways in which swimming motility may be analyzed: one method consists of macroscopic examination of *C. difficile* isolates in test tubes containing BHI and a low concentration of agar (such as .175%) and the other method involves measuring the swimming diameter of *C. difficile* isolates on BHI or BHIS plates containing a low agar concentration (such as 0.3%), which is the preferred method as motility differences can be directly/quantitatively compared by statistical analysis [22, 70, 76, 80, 84, 85]. We analyzed *C. difficile* swimming motility using the test-tube approach after several unsuccessful attempts of measuring *C. difficile* swimming diameter on BHI plates with low agar concentration. When we used the plating method with *C. difficile* strains R20291 and 630, there

was minimum-to-no *C. difficile* growth beyond the initial inoculum stabbed underneath the BHI agar. However, we took analysis of *C. difficile* swimming motility one step further than other research groups that used the test-tube approach by using ImageJ to determine image brightness and comparing motile isolates by toxin genotype using these image brightness measurements (Figure 7B) [70, 76, 80, 85]. While this was only a semi-quantitative measurement of *C. difficile* swimming motility because motility was compared by image analysis as opposed to measuring swimming range, determining image brightness enabled us to discover which isolates were non-motile in addition to the three 078-lineage isolates (Figure 6 and Figure 7A). One potential drawback of our methodology for examining swimming motility was that not all *C. difficile* isolates were tested at the same time due to material constraints, so small differences, such as moisture amounts in the anaerobic chamber on that day, could slightly impact results. We found several studies that observed similar *C. difficile* swimming motility results compared to what we observed. Karpinski et al. macroscopically examined the swimming motility of isolates belonging to different *C. difficile* PCR ribotypes. Likewise, they observed a mixture of both motile and non-motile isolates across the different ribotypes after a 24-hour incubation period in .175% BHI agar growth medium (Figure 6) [70]. Gross et al. showed that isolates belonging to RT027 and RT012 are motile while isolates in the RT078 family are non-motile (Figure 6) [76]. One difference from our study was that Gross et al. observed motility 1-, 2- and 3- days post inoculation while we only observed motility 24-hour post inoculation illustrating that the protocol for swimming motility can be carried out in different manners and still yield consistent results [76]. Camorlinga et al. quantified the swimming motility of non-toxigenic *C. difficile* isolates on petri plates containing .15% BHI agar (so a different methodology than our study) by measuring the swimming range and observed that some isolates exhibited higher swimming motility than others. However, when the non-toxigenic *C. difficile* isolates were compared to toxigenic isolates, there was no difference in swimming motility ability based on toxin genotype [22].

In closing, *C. difficile* isolates were obtained from wastewater and seafood in Taiwan for

the first time [14]. The phenotypic characterization performed in this study will contribute to a better understanding of the characteristics of the *C. difficile* isolates responsible for contamination of wastewater and seafood in Tainan, Taiwan. We hope that this study shines light on the importance of extensively investigating environmental sources for pathogen contamination and the importance of following proper disinfection measures in a world with spore-forming bacteria.

SUPPLEMENT DATA



Supplementary Figure 1 Endospore staining

C. difficile strain R20291 endospores were visualized by staining with malachite green after a 48-hour incubation period on 70:30 medium. The endospore staining procedure was modified from Microbiologie Clinique and our methodology is as follows: a bacterial smear was prepared on a glass slide and air dried in the biosafety cabinet, the smear was heat fixed by passing over a flame three times then allowed to cool, the bacterial smear was covered with 1% malachite green stain for 5 minutes, the smear was heat fixed again by passing over a flame three times and allowed to cool, the glass slide was rinsed with ddH20 and a Kim wipe was used to blot the slide dry, and lastly, spores and spore-forming cells were observed under 1000X magnification with oil immersion and a photograph of the microscope view was taken using an Apple iPhone XS [86].

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VITA

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Completed the requirements for the Master of Science in Biomedical Sciences at Oklahoma State University Center for Health Sciences, Tulsa, Oklahoma in July 2022.

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Experience:
Poster Presentations:

Moulis G., Li Y.R., Tsai B.Y. …& Huang I.H. (2022, March 19). Isolation and Characterization of Environmental *Clostridioides difficile* Isolates from Wastewater Treatment Plants and Farmed Mollusks in Taiwan. Annual Meeting of the Missouri Valley Branch of the American Society for Microbiology, Stillwater, OK, United States.

Moulis G., Huang I.H. (2022, February 18). Analyzing the phenotypic characteristics of environmental *C. difficile* isolates. 13th Annual OSU-CHS Research Week, Tulsa, OK, United States.

Professional Memberships:

Served as the secretary for the Biomedical Sciences Graduate Student Association at OSU-CHS during the 2021-2022 school year.