John C. Vitucci, A picture of *C. difficile* epidemic and non-epidemic ribotypes: Is there a difference in virulence? Doctor of Philosophy (Cell Biology, Immunology, & Microbiology) November 2019, 114 pp., 17 illustrations, bibliography, 130 titles

The purpose of these studies was to determine if the epidemic ribotype of *C. difficile* (now *Clostridioides difficile)* was more virulent than non-epidemic ribotypes, to ascertain whether clinics contribute to community-acquired *C. difficile,* and to bridge gaps in understanding between *C. difficile* epidemiology and pathology. Virulence of the epidemic isolates was determined to be greater than non-epidemic isolates within LD_{50} studies utilizing a hamster model of *C. difficile* disease. In the epidemic isolates, increased production of toxins A and B, increased spore adherence ability, and increased production of spores when antibiotic treatment was administered were factors that are believed to play a role both in increased virulence and in the ability of the epidemic isolate to persist as epidemic.

Our results indicate that primary care clinics have higher frequency of contamination of *C. difficile* spores than hospitals. The study also revealed that of all the samples positive for *C. difficile,* approximately 90% contained the genes for toxins A, B or both. Thus, primary care clinics can be a source of *C. difficile* and contribute to community-acquired *C. difficile*. However, the epidemic ribotype of *C. difficile* was not isolated at significantly increased levels compared to non-epidemic ribotypes. This suggests that other factors may contribute to its increased frequency of *C. difficile* infection diagnosis.

Isolates of the epidemic ribotype were found to be more virulent than other non-epidemic isolates in both the hamster and mouse models of CDI. In particular, the epidemic ribotypes of *C. difficile* had lower LD_{50} values in hamsters than the non-epidemic isolates. The increased severity of disease was associated with higher levels of toxins A and B, but not the number of organisms recovered. The increased toxin production was observed in both the hamster and mouse models of CDI. In addition, it is believed that increased ability of epidemic isolate spores to adhere to the intestinal epithelium *in vitro*, and produce

more spores when treated with the antibiotic vancomycin in hamsters are also important contributors to the enhanced virulence and prevalence associated with epidemic isolates of *C. difficile.*

This revealed a possible link between *C. difficile*'s epidemiology and pathology, and suggested that this connection can potentially explain how epidemic ribotypes persist as epidemic. Though it is likely that the factors discussed throughout this dissertation play a significant role in the epidemic ribotype's ability to persist as epidemic, it is important to note that there may be other contributing factors, such as those found in the *in vivo* environment. These other factors should be accounted for during future studies of *C. difficile*'s virulence, as future ribotypes are characterized, and as novel treatments are developed to combat *C. difficile* infections. Still, it is strongly believed that the findings in this dissertation contribute significantly to understanding why the epidemic ribotype is epidemic, and to exposing virulence characteristics that are contributing to this.

A PICTURE OF *C. DIFFICILE* EPIDEMIC AND NON-EPIDEMIC RIBOTYPES: IS THERE A DIFFERENCE IN VIRULENCE?

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A PICTURE OF *C. DIFFICILE* EPIDEMIC AND NON-EPIDEMIC RIBOTYPES: IS THERE A DIFFERENCE IN VIRULENCE?

DISSERTATION

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LIST OF ABBREVIATIONS

ABSL-2 - Animal biosafety level 2

ANOVA – Analysis of Variance

CCFA – Cycloserine-Cefoxitin Fructose Agar

CDI - *C. difficile* infections

CFU – Colony forming units

DMEM - Dulbecco's Modified Eagle's Medium

ELISA – Enzyme-Linked Immunosorbent Assay

EMEM - Eagles Minimal Essential Medium

FBS - Fetal bovine serum

PBS – Phosphate-buffered saline

PCR - Polymerase chain reaction

SM - Sporulation medium

ST-80 - Surfactant tween 80

TGY-veg - Tryptone glucose yeast abstract vegetative

TSA – Tryptic soy agar

TSB - Tryptic soy broth

CHAPTER I

INTRODUCTION TO THE PROJECT

"To give anything less than your best is to waste the gift" – Steve Prefontaine

Between the years 2000-2007, *Clostridium difficile* (*C. difficile*) infections (CDI) increased by 400% in the United States [1]. This rise in the *C. difficile* infection rate was predominantly attributed to the increased prevalence of the epidemic NAP/BI/027 (epidemic-027) ribotype [2]. The characterization of this ribotype revealed its ability to produce increased quantities of toxin and spores [3, 4], factors which are key in enabling the organism to infect a host and sustain an infection [5]. In conjunction with these factors, an increase in cases that involved the epidemic-027 ribotype, and the difficulty associated with the treatment of this organism contributed to the organism being labeled as an "urgent threat" level pathogen in 2013 [6]. Currently, the question of whether the epidemic-027 ribotype does possess increased virulence when compared to non-epidemic ribotypes is still disputed [7]. Irrespective of the conflicting data, incidence of *C. difficile* infection continues to rise through present day. In 2015, *C. difficile* was responsible for half a million infections among medical patients and was additionally responsible for 100,000 infections in nursing home residents [8]. These infections were directly linked to 15,000 deaths and found to be a contributing factor in an additional 29,000 deaths [8]. Even with the development of a variety of *in vitro* assays and animal models for *in vivo* studies, as well as a plethora of scientific studies utilizing these resources, there is still a dearth of knowledge associated with *in vitro* virulence factor production and its relevance to *in vivo* disease presentation in relation to individual *C.*

difficile strains. It also remains unclear whether the epidemic-027 ribotype exhibits increased virulence in comparison to non-epidemic ribotypes. These gaps in knowledge, combined with other factors continue to be a significant reason that the infection and mortality rates associated with *C. difficile* infections are not decreasing.

C. difficile Physiology:

C. difficile is a Gram-positive, spore-forming, bacillus bacterium located in the class *Clostridia [5]*. It is an obligatory anaerobe. It is an obligatory anaerobe relaying primarily on fermentation and gluconeogenesis for energy and utilizes multiple sugars in these processes [9]. While the organism is not known to produce biofilms, it has been observed to exhibit quorum sensing using small molecular messengers. *C. difficile* is not considered to be an ubiquitous organism; however, the spores it produces can persist in most environments for extended periods of time [10]. The ability of *C. difficile* to sporulate increases the likelihood of encounter or translocation by intermediates or hosts. This translocation of spores to new areas increases the chance of the spores being ingested by potential hosts [11].

Potential hosts are typically people who are immune-compromised, who have been recently administered a broad-spectrum antibiotic, or people who have recently had a procedure or event that significantly altered their gut microbiological flora [12]. Though these individuals have a higher probability of contracting the disease, anyone has the potential to contract and exhibit the disease and its symptoms [12]. Specifically, one demographic that is at an increased risk of *C. difficile* infection is people ages 55+, especially if they reside in long-term living facilities [6]. This is thought to be due to the continuous movement of residents between hospitals and the long-term living facility [13]. In 2016, may individuals born in the later years of the “baby boomer” era, commonly considered to span from 1946-1964, will reach about 50 years of

age. Therefore, in association with the negative population growth within the United States, this places an increasing percentage of the population at risk for this *C. difficile* infection.

Ribotypes and Ribotyping:

C. difficile associated disease (CDAD) has been an issue for many years, but between 2000 and 2006, it rose in both prevalence and the severity [8]. The increases associated with these are attributed to the emergence of *C. difficile* ribotypes known as epidemic ribotypes [3, 14]. The epidemic ribotype(s) are defined as those found to be the most prevalent within a specific geographical region (i.e., North America) [14]. The ribotype of a specific *C. difficile* isolate is determined by the heterogeneity of the ribosomal intergenic spacer region as compared to previously identified sequences from other strains of *C. difficile [15]*.

Why specific ribotypes evolved into epidemic strains have been theorized for a variety of reasons. Two of the more prevalent theories suggests these ribotypes produce an increased number of spores, enabling them to more easily spread between hosts in the environment [16, 17]. The other prevalent theory suggests that these ribotypes produce an increased amount of toxins within the host, causing more damage and increased shedding of the spores due to symptoms associated with the disease [2, 3]. This allows the bacterium to cause a more severe disease, and in turn cause the host to shed more spores into the environment. This increases the likelihood of new hosts contacting the spores.

A recent publication in Nature provided new insight into why the 027 and 078 ribotypes emerged as the epidemic ribotypes within North America [18]. This paper denotes how ribotypes 027 and 078 have increased fitness from other ribotypes because they acquired the ability to utilize trehalose as an additional energy source [18]. Overall, it is suggested that the combination of these ribotypes' ability to utilize

trehalose, and the implementation of trehalose as a food additive shortly before the emergence of these ribotypes as epidemic has directly contributed to the rise of 027 and 078 ribotypes as epidemic[18].

Pathology:

C. difficile can be found as a small component of the colon's microbiome and is not believed to cause disease when the microbiome is undisturbed [5]. There are two primary reasons supporting this theory. The first reason is that some *C. difficile* strains, which can be found as components of the microbiome, are non-toxigenic strains [19]. It has been shown that animals carrying non-toxigenic strains show few to no signs of disease and the damage associated during infection with a toxigenic strain [20]. The second reason derives from evidence suggesting that the biosalt composition of the environment helps to control *C. difficile* spore germination and outgrowth of viable cells [21, 22]. Within the digestive tract, there are subsets of bacteria, such as *Clostridium scindens,* which transport unconjugated primary biosalts from the environment into their cytosol, and through a series of enzymatic reactions produce secondary biosalts [23]. These secondary biosalts are then excreted into the extracellular environment. This sequence of events primes the environment to be significantly less conducive for the outgrowth of viable *C. difficile* cells [21, 23]. If an event disrupts the microbiome and eliminates the bacteria that balance the level of secondary biosalts within the intestines, there will be a shift in the environment which favors an increased level of primary biosalts. One example of what causes disruption to the intestinal microbiome causing this sequence of events to occur is the administration of broad-spectrum antibiotics, such as clindamycin. Once the level of primary biosalts is increased, this supports the outgrowth of *C. difficile* from its spore form and its colonization of the colon [21-23]. Once outgrowth of the spores has occurred and disease has manifested, multiple factors make it difficult to treat the disease and also hinder the microbiome's ability to reestablish itself. One factor involves treatment with antibiotics such as metronidazole and vancomycin, which do not solely target *C. difficile* and thus make it challenging for natural flora to recover

and reestablish [13, 24]. Secondly, *C. difficile* occupies similar niches to the bacteria which produce secondary biosalts used to keep germination and outgrowth of *C. difficile*'s spores in check [23]. Lastly, the ability of *C. difficile* to produce spores when challenged with antibiotics used for treatment, as well as the ability of these spores to germinate and cause recurrence in an individual, additionally hamper disease treatment and microbiome recovery [25, 26].

When an individual is infected with a toxigenic strain, *C. difficile* can cause disease ranging in severity. Latent symptoms can mimic the flu, whereas less serious cases can cause the patient to have mild inflammation of the colon [27]. As the disease progresses and gastrointestinal epithelial cells are destroyed, cytoplasmic leakage occurs and fluid balance is unable to be maintained. This leads both to dehydration and *Clostridium difficile*-associated diarrhea [27, 28]. If the disease continues to go untreated, pseudo-membranous colitis occurs, followed a life-threatening inflammation of the colon known as toxic megacolon [12, 27]. This condition can lead to both sepsis and death if treatment is not rendered or is ineffective [12, 16, 29].

Adherence:

One factor that is essential to *C. difficile* colonization of a host is the ability of exogenous spores to bind to host intestinal epithelial cells [10]. Several studies suggest a correlation between adherence of the spores to the intestinal epithelial cells and the virulence of a strain *in vivo [30]*. Studies indicate that the ability of *C. difficile* to adhere to an environment can directly influence the severity of disease by delivering toxin to a target cell in an optimal fashion [31]. *C. difficile* is shown to adhere to multiple cultured cell lines such as Caco2, and Vero cells, and this adherence is mediated by proteinaceous components [32]. *C. difficile* is also shown to produce fimbriae and various cell-surface carbohydrates, which are known to mediate adherence in other bacteria, though their role in *C. difficile* colonization is

not well understood [11]. It is worth noting that it is thought *C. difficile*, like other bacteria, could have multiple adhesion factors. An early study suggests that *C. difficile* adhesion protein expressions were affected by various physical and chemical treatments [33]. Heat shock causes an increase in adhesion of the Wilkins strain (highly toxigenic) and FD strain (moderately toxigenic) to both Caco2 and HT29-MTX cells [34]. Another factor shown to influence the expression of adhesion proteins was the addition of blood to the culture media [34]. It was observed that two main protein bands, at 12K and 27K, disappeared from the electrophoretic profile of the surface-associated proteins when not cultured in the presence of blood [35]. The study concludes that two surface proteins are involved in adhesion when exposed to blood and both play a similar adhesion role [35, 36]. While, heat shock increases the association of the cells to cultured human intestinal cells, a more recent study identifies one adhesion protein named Cwp66 [26]. This adherence factor is a surface protein with repeated motifs. When *C. difficile* is stressed (i.e., such as by heat shock), this adherence factor becomes prevalent on the organism [26, 34]. When cells exhibiting Cwp66 are paired with competitive inhibitors Cwp66-C or N, the relative adherence to the control was 15-49% lower [26]. A third study outlined the finding of three different adhesive surface proteins with molecular masses of 70, 50, and 40kDa. It was observed that both the 50 and 40kDa proteins were expressed at various levels when the environment differed from a baseline, while the 70kDa was expressed at a constant level no matter the environmental conditions [35]. There is still more to be understood about the structure and total number of adherence proteins utilized by *C. difficile*, but this organism has multiple ways to adhere to intestinal epithelial cells in varying environment conditions.

Spore Production and Regulation:

As mentioned, the growth and colonization of *C. difficile* within a host does not always lead to the development of symptomatic disease. Disease development is mediated by specific virulence factors [4]. One of these factors is *C difficile*'s ability to produce both spores and exotoxins. Clostridial spores are

made up of a core layer, cortex layer, and coat [10]. The spore coat has distinctive laminations (i.e., lamellae) [37]. These are comparable to spores produced by *Bacillus subtilis*, though only 25% of the genes encoding the coat have homology between the two bacteria [30]. In *C. difficile*, the outermost layer of the spore is the exosporium [11, 37]. *C. difficile*'s exosporium layer has hair-like projections, but, unlike most other spores, lacks the gap between the spore coat layer that surrounds the cortex [11]. It also has been observed that different strains of *C. difficile* produce spores with different exosporium phenotypes [11]. It is hypothesized that this layer can be shed in the environment. Studies with strain R20291 produce spores with a defective exosporium layer, and these spores show increased binding to Caco2 cells [38]. The increased binding enhances the spores' ability to outgrow into colonies *in vitro*. Currently, the fraction of spores that have a firmly attached exosporium coat and the half-life of this layer in the environment is unknown [11, 36]. Clostridial spores are resistant to many kinds of physical and chemical treatments [11]. This allows *C. difficile* spores to evade techniques typically fatal to the bacteria in the environment and host. This is believed to be due to a combination of coat proteins found on the spore, and the interior environment of the spore. Recently, five coat proteins were identified to as playing a role in protecting the spore during environmental stress and attack by the host's immune system. These proteins are: CotA, CotB, CotCB, CotD, and CotE [11]. The proteins CotCB, CotD, and CotE have activity that correlate with catalase, peroxiredoxin and chitinase activity respectively [11]. CotCB and CotD also have been shown to be putative manganese catalases [11]. CotE, along with exhibiting chitinase activity at its carboxy terminus, is thought to be bifunctional and have peroxiredoxin activity at the amino terminus [11, 37]. In combination with macromolecule degradation, CotE is hypothesized to play a role in inflammation and could contribute to the gastrointestinal symptoms exhibited during infection [11]. The roles of CotA and CotB have yet to be identified.

The mechanisms of spore production and germination in *Bacillus* is well understood, and genes important for this process are currently identified in *Clostridium subtilis*; however, the genes that regulate spore production and germination in *C. difficile* are significantly less understood [39]. The stimuli that signal the beginning of sporulation, *in vitro* and *in vivo*, have yet to be identified but, it is thought that they could be related to nutrient starvation, quorum sensing, antibiotic exposure, and other environmental stress factors [30, 39, 40]. The genes for *C. difficile* sporulation are spread throughout multiple areas of the bacteria's genome, but it is understood that *C. difficile* utilizes up to five orphan histidine kinases to trigger spore production through the phosphorylation of the master transcriptional regulator, Spo0A [41, 42]. This regulator governs the Spo0 operon in *C. difficile*. Spo0A has proven pivotal for spore production in *C. difficile*. During a study where Spo0a was knocked out, it was found that in both riobotype-027 and 012 (a non-epidemic strain), Spo0A is essential for spore formation [41]. When this gene is knocked out, the result is an asporogeneous form of *C. difficile* in both epidemic and non-epidemic ribotypes [41]. Recently, a study took a genomics approach to understand more about the *C. difficile* spore production pathway by comparing homologs and regions conserved from *Bacillus subtilis* to regions in *C. difficile*'s bacterial chromosome. It was determined that within the Spo0 operon, there are multiple genes that can be transcribed based on which specific sigma factor is bound to RNA polymerase [42]. These sigma factors have been observed to be similar to the sigma factors utilized during *B. subtilis* sporulation [42]. In Saujet et. al., it has recently been shown there is more compartmentalization of each sigma factor during the development of *C. difficile* spores in comparison to *B. subtilis* spore formation [42]. The four sigma factors identified during analysis of *C. difficile* spore formation are: sF, sE, sG, and sK regulons. It is believed that approximately 225 genes are regulated by these four sigma factors while these same sigma factors regulate approximately double the number of genes in *B. subtilis [42]*. Many of these gene's specific functions have yet to be identified in *C. difficile.* Identification of each sigma factor's function

during spore development was completed by developing strains of *C. difficile* in which each sigma factor was knocked out individually. The *sigF* and *sigE* mutant's spore production is arrested at stage II of sporulation [42, 43]. While the *sigG* mutant's process was arrested after the completion of engulfment, and that the *sigK* mutant form spores that lack coat material [42, 43]. This was important in understanding that *sigF* and *sigG* activity is confined to the forespore, while *sigE* and *sigK* is restricted to the mother-cell [42, 43].

Toxins and Mechanisms of Action:

In conjunction with spores, *C. difficile's* toxins are key virulence factors for the development of CDAD. Due to toxin A and B's importance in disease development, they have been studied extensively. *C. difficile* has been observed to produce up to three different toxins [44, 45]. These toxins have been designated toxin A (TcdA), an enterotoxin, toxin B (TdcB), a cytotoxin, and a binary toxin (tdc) [44, 45]. Strains of *C. difficile* have been identified to be positive for all three toxins, positive for toxin A and B, positive for toxin A only, and positive for toxin B only. Currently, there has been no strain of *C. difficile* identified to be positive for only the binary toxin [46-48].

TcdA and *TcdB* are located within a pathogenicity locus. For comparison, *tcdA*'s reading frame is 8,133 nucleotides, and *tcdB*'s length is 7,098 nucleotides [48]. The sequences of these two toxins exhibit a 66% overall similarity. Due to the similarity between these two genes, it is hypothesized that they arose due to a gene-duplication event [48]. This theory is also supported by the genes' proximal locations, as well as the similarity exhibited in their mechanisms of action. This action uses the N-terminal domain, which is about 74% conserved, to modify identical substrates [48]. Most of the conserved regions between the two toxins occur within their receptor-binding and enzymatic regions [31, 48]. TcdA and TcdB also

have been shown to have homology and similar functions to other clostridial toxins, such as TcsH and TcsL found in *Clostridium sordellii [48]*.

In *C. difficile,* the pathogenicity locus containing the genes for TcdA and TcdB is approximately 19.6-kb [49]. These toxin genes are located next to one another separated by an intervening sequence thought to transcribe *tcdE,* whose function will be described later [50]. TcdA and TcdB are both transcribed in the same direction [47]. In conjunction with *tcdA'*s and *tcdB'*s reading frames, the pathogenicity locus contains three other open reading frames believed to contribute to one of two actions [48]. These actions are the regulation of toxin production or the release of toxin from the cell. Genes located within the pathogenicity island, downstream from the promoter region, are ordered *tcdD, tcdB, tcdE, tcdA,* and *tcdC [49]*. In this group, *tcdC* is notable because it is transcribed in the opposite direction from the other genes [50]. This gene also merits attention because it has been observed to have its transcription upregulated during the exponential phase of growth while its expression declines as the cells shift into stationary phase [50]. Once *tcdC* expression declines, an increase in t*cdA* and *tcdB* expression is observed [45]. This relationship suggests that TcdC functions as a negative regulator of toxin production [51]. Meanwhile, *tcdD* is expressed with *tcdA* and *tcdB [48]*. TcdD shows similar homology to other DNA-binding proteins and UViA, which regulates a bacteriocin gene from *Clostridium perfringens [48]*. In *C. perfringens,* this gene is a positive regulator of toxin production. Evidence also suggests that *tcdD* enhances the expression of promoter reporter fusions involving the TcdA and TcdB [4, 48]. Recently, the *tcdD* protein was observed acting as an alternative sigma factor for RNA polymerase in *C. difficile [48]*. The expression of *tcdD* is influenced by *C. difficile*'s stage of growth and environmental conditions [48]. TcdD is upregulated during the stationary phase, while its expression is downregulated in the presence of glucose [48]. Additionally, there is evidence that supports TcdD's ability to autoregulate its expression in response to environmental cues and the phases of *C. difficile*'s

lifecycle [48]. These functions for *tcdD* suggests that it can act as a positive regulator of *tcdA* and *tcdB*. Lastly, *tcdE* encodes for a protein which exhibits homology with holin proteins [47, 48]. These proteins are typically associated with assisting the release of molecules through the cell wall. Though the exact mechanism in which these genes interact is unknown, it is thought that *tcdA* and t*cdB* production rely on enhancement by *tcdD [48]*. This is followed by t*cdE*-mediated release from the cell, which occurs once *tcdC* is downregulated during the stationary phase [48].

Some studies have observed stark differences between toxin production of epidemic-027 strains and non-epidemic strains within the United States [3, 52, 53]. It is hypothesized that this increased toxin production correlates with increased virulence observed clinically and in studies involving epidemic-027 strains [3, 28, 53]. Increased toxin production is believed to be connected to one of two mutations within the pathogenicity locus [53]. Specifically, these mutations are observed in *tcdC* or *tcdE* genes [54, 55]. The mutation observed in the *tcdE* is thought to cause increased binding specificity of the sigma factor to the DNA [55]. This, in turn, causes *tcdA* and *tcdB* to be transcribed at a higher rate, leading to an increased production of TcdA and TcdB [48]. Strains with a mutation within *tcdC* have shown a downregulation in the gene's transcription rates [48, 54]. These mutations lead the epidemic-027 ribotype to produce toxin earlier in its life cycle, making it unable to downregulate both the toxin genes and their positive regulators throughout the cells lifespan [48].

While the environmental stimuli that influence both toxins' production are not well understood, the mechanisms in which toxins A and B function are well documented [31, 45, 48]. Both CdtA and CdtB primarily target intestinal epithelial cells, and receptor binding is an essential first step [4, 48]. On intestinal epithelial cells, the binding of TcdA and to some extent TcdB is thought to be mediated by nonproteinaceous structures showing the disaccharide GalB1-4GlcNac [48]. Although TcdB has affinity for this receptor, it is not thought to be the primary receptor for this toxin [46, 48]. The exact receptor for

TcdB has yet to be discovered, but in studies, TcdB has exhibited the ability to bind to multiple cell types [48]. *C. difficile* TcdA and TcdB target the Ras superfamily of GTPases for modification by glucosylation [56]. Specific targets within this family include Rho, Rac, and Cdc-42 [4, 48]. For the modification of these Ras family proteins, internalization of these toxins through receptor-mediated endocytosis and an acidified endosome are required for translocation across the cellular membrane [48, 57]. Once translocation into the cytosol is complete, TcdA and TcdB modification of Ras family proteins commences by glucosylation of a specific threonine residue [48]. The result of this action leads to the cell losing control of its actin cytoskeleton, which in turn triggers rounding of the cell, membrane blebbing, or apoptosis of the target cell.

C. difficile isolates also carry a gene for the production of a binary toxin (CDT) [58]. This two-component toxin is encoded by the genes *ctdA* and *ctdB* and the proteins transcribed by these genes are CDTa and CDTb [59, 60]. This toxin is distinct from TcdA and TcdB and is not required for symptomatic infection. Studies have found that 6.4 - 15% of *C. difficile* isolates tested positive for this binary toxin, while other studies have observed approximately 20% of isolates were positive for CDT in a hospital setting [44]. *C. difficile*'s binary toxin exhibits ADP-ribosyltransferase activity, which acts as a capping protein to prevent actin filament elongation [60]. The specific role of CDT in *C. difficile* disease progression is still unclear. However, the binary toxin has been associated with increased white blood cell count, and elevated risk of recurrence in recent clinical studies.

C. difficile's toxins are necessary for a specific strain to cause disease, as well as influence its severity [28, 31, 45]. Spores are also an important virulence factor for *C. difficile [4]*. Though encoded in separate locations on the bacterial genome, spore production and toxin production work together to produce disease [61]. This has been shown during a study of the bacterium's two-component signal transduction system used to produce spores [41]. When Spo0A is inactivated, it results in an asporogenous

phenotype. This Spo0A knockout was also observed to produce less than 10% of the total toxin A created by the parental strain [41]. This observed relationship supports the theory that the ability to produce spores through the Spo0 genes and a strain's ability to produce toxin is linked in some fashion [41, 61]. In a second study, the HK CD2942 knockout also showed a 40-50% reduction in total toxin A production from that of the parental strain [30]. These studies show a definitive link between spore production and toxin production, and suggest that the relationship between these two virulence factors can influence the severity of disease within the host [30, 41, 61].

Animal Models:

Multiple animal models are important to advance the understanding of *C. difficile*'s interactions and disease within hosts [62-65]. These have been developed to study various aspects of *C. difficile* disease, virulence, and treatment. These animal models cover a broad range of species, but the most commonly studied models are the mouse, hamster, and rat models [62, 64, 65]. The various aspects of CDI studied include: colonization, disease histology, toxin production, sporulation/shedding, recurrence, and pharmacological studies, as well as other aspects not mentioned here [62, 64]. Each *C. difficile*-associated animal model has advantages and disadvantages. Therefore, it is essential to consider multiple factors when determining which model is the best for a given study. It is also important to understand how the model is relevant to human disease and whether the disease pathology exhibited in the specific animal model relates to pathological features seen in humans.

One of the more integral models to understanding *C. difficile* pathogenesis is the hamster model. In the 1970s the hamster model was shown to be a viable animal model for use in studying *C. difficile* infections [64]. This model was shown to be most effective when the animal was treated with antibiotics prior to intercaecal administration of the bacteria [52, 64]. Once this model was established, it was

followed by the discovery of a link between toxins and *C. difficile* disease *in vivo [64]*. Since then, the model has continued to be developed, refined, and utilized for *C. difficile* pathogenesis studies [62, 64]. This is due to the pathology observed within this model mirroring many of the pathological aspects of human CDI [62, 64]. Once the bacteria are introduced to the animal and a dose of clindamycin, or other broad-spectrum antibiotic, is administered, hamsters develop hemorrhagic caecitis [62, 66]. This commonly presents as "wet tail" and diarrhea, but also can include symptoms such as ruffled fur, hunched posture, dehydration leading to weight loss, and death if left untreated [62, 64, 66]. Studies have shown cecal tissues taken from hamsters presenting with CDI show similar mucosal tissue damages typically associated with polymorphonuclear influx [67, 68]. This includes tissue hemorrhaging [64, 67]. One difference that is important to note between the CDI in hamster's versus humans the disease site. In hamsters, the disease establishes within the caecum, in contrast, CDI in humans occurs in the colon [62, 64]. Despite this difference, this model has many advantages and is used to study multiple facets of CDI. This includes studies observing disease pathology, colonization, drug efficacy and treatment and recurrence, as well as other facets of the disease [62, 64]. Due to the hamster's sensitivity to *C. difficile* toxins, one of the most useful roles of this model lies in comparative studies of virulence between different isolates of *C. difficile [62, 64]*. Although this model is used to study a multitude of CDI pathological aspects, one of the major limitations associated with it is the lack of immunological reagents available to study host response to the disease [64].

One way researchers worked around this lack of immunological reagents was the development of a CDI mouse model, which recently has become the predominant model [62-64]. This shift can be attributed to multiple factors, including improved methods of inducing the disease within mice, the larger number of immunological reagents for use with mice, the increased ability to genetically alter mice, and the cost efficiency of utilizing mice versus hamsters [62-64]. Improving the methods by which *C. difficile*

can colonize and establish disease within mice was important for the evolution of this model because mice are less susceptible to colonization by *C. difficile* than hamsters [62, 64]. Mice are relatively resistant to CDI. When treated with a broad-spectrum antibiotic such as clindamycin or cyproflaxin, the development of fatal infections does not normally occur [63]. It is believed that colonization resistance against *C. difficile* is provided by the microbiome composition within the intestinal tract. Typically, mice treated solely with one of the two antibiotics mentioned above become asymptomatic carriers and shed only low numbers of spores [62-64]. This phenomenon is similar to when hamsters are administered only *C. difficile* toxins, instead of the live organism or spores [62]. For example, if antibiotic treatment is not given to the hamsters after being administered the *C. difficile* toxins, inflammation of the colon and tissue damage is observed, but mortality is not observed [62]. Uses of the mouse CDI model advanced following the discovery of various antibiotic cocktails with the ability to disrupt the microbiome, thus allowing for symptomatic disease development and mortality [62, 63].

There are multiple mouse CDI models; some are used to study disease features that differ from those studied in the hamster model, while others enable study of disease aspects that can be explored in either model [62, 64]. One model utilizes germ-free mice. Another model uses a single antibiotic (as does the hamster CDI model) to induce disease. A third model utilizes an antibiotic cocktail to induce the mouse's susceptibility to infection by *C. difficile* [62-64]. The antibiotic cocktail mouse CDI model administers antibiotics to the mice prior to administration of *C. difficile* [63]. This model is important because it increased the reproducibility of the disease model, as well as reproducing key features of the disease as observed for CDI in humans [62, 63]. These aspects include: diarrhea, weight loss, colonic damage, and death [62, 63]. One of the most common ways the mouse CDI model is utilized is to determine the colonization of specific *C. difficile* strains. This is determined by enumerating the shedding associated with the viable cells and spores in the fecal matter [62, 64]. This shedding is also used to

elucidate the ability of a specific strain of *C. difficile* to colonize the host [62, 64]. Another common use for the mouse model, which most closely resembles the hamster model of CDI, is the antibiotic cocktail mouse model [62, 63]. This model was developed to allow for study of relapse associated with the treatment and clearance of the disease [62, 63]. In addition to characterization of the disease, this specific mouse model has also elucidated how microbiome composition plays a role in mice that develop severe disease compared to mice that only show signs of fecal shedding [62, 69]. Overall, the development of this model offers an avenue to study many aspects of CDI, but one advantage it has over other commonly used models (i.e., the hamster CDI model) is the number of reagents tailored to mouse studies, and the ability of those reagents to be utilized to more easily study the disease from an immunological standpoint [62].

Final Thoughts:

Further studies are needed to elucidate virulence phenotypes of different *C. difficile* ribotypes. This includes the current NAP/BI/027 ribotype, 078 ribotype, and other non-epidemic ribotypes. Conclusive studies also are needed to determine if the *in vitro* virulence phenotype of a strain or ribotype is indicative of its *in vivo* virulence. It is clear that there is varying virulence between different *C. difficile* ribotypes [4, 16, 28, 52, 53, 70, 71]. However, whether the epidemic ribotype's virulence can be classified as hyper-virulent when compared to other ribotypes is still debated [28, 52, 53, 70, 71]. Variation of disease severity, toxin production, and production of spores in the mouse model is already demonstrated [28, 51, 53]. Questions of whether this variation is seen between the clinical isolates utilized in these studies, and if whether the same variation is observed when a mouse model and a hamster model are infected with these same isolates remain to be answered. Understanding if *C. difficile*'s virulence varies *in vitro, in vivo,* and between different models may offer insight into factors that influence virulence and help to predict potential future epidemic ribotypes.

The work summarized in this dissertation clarifies how *C. difficile*'s virulence compares both *in vitro* and in multiple *in vivo* models, and additionally draws comparisons between the virulence factors of these ribotypes. The studies conducted demonstrate that a ribotype's *in vitro* virulence is not predictive of its virulence *in vivo*. This suggests that there is an *in vivo* component that has an effect on virulence which is not present *in vitro*. These studies also conclude that toxin production by the isolates in the hamster and mouse models of CDI was greater when compared with their *in vitro* toxin production. In our study comparing *C. difficile* isolate distribution within clinics and hospitals, surprisingly, the distribution of the epidemic-027 ribotype was less prevalent than multiple other ribotypes. This suggests that increased exposure in healthcare settings is not the sole factor causing the NAP/BI/027 ribotype to be classified as "epidemic" or "the most prevalent" in North America. This ability to persist as epidemic could be linked to the epidemic ribotype's ability to exhibit severe disease with a lower number of inoculant of spores. This characteristic, in conjunction with increased toxin production, spore adherence, and spore production, when treated with antibiotics, are likely to enhance its ability to flourish as the epidemic ribotype over other ribotypes. Overall, this work brings to light a novel understanding and comparison of not only multiple *C. difficile* ribotypes and isolates, but also an understanding of whether the behavior of these ribotypes is consistent between multiple models of study. Understanding this work will help link *C. difficile*'s epidemiology and pathology, and this link may partially explain why epidemic ribotypes' are able to persist as epidemic. This understanding could also influence research into alternative treatments for *C. difficile*, beyond traditional antibiotics.

CHAPTER II

CLOSTRIDIUM (NOW *CLOSTRIDIOIDES*) *DIFFICILE* SPORE FORMATION IS HIGHER IN EPIDEMIC ISOLATES WHEN TREATED WITH VANCOMYCIN *IN VIVO* AND *IN VITRO*

The purpose of the present study was to determine the impact of vancomycin treatment on multiple *Clostridium* (*Clostridioides) difficile* strains' spore production. Thus, using the hamster model of *C. difficile* animals were inoculated with *C. difficile* spores, disease progression was allowed to occur, and the animals were treated with vancomycin. It was discovered that all strains of *C. difficile*, both non-epidemic and epidemic produced greater numbers of spores *in vitro* than *in vivo*, but the epidemic isolates produced greater numbers of spores than the non-epidemic isolates both *in vitro* and *in vivo.* It was also observed that the epidemic isolates produced greater numbers of spores:CFUs recovered than the non-epidemic isolates. This work suggests that treatment with an antibiotic commonly used to treat *C. difficile* potentially confers an advantage to the epidemic isolate's ability to persist within the host and disseminate increased numbers of spores into the environment.

Background

Clostridium difficile (now *Clostridioides*), a spore forming bacillus, is the cause of *C. difficile*-associated disease. In the United States of America, the occurrence of *C. difficile* infections (CDI) increased by a factor of 400% between 2000-2007 [1]. *C. difficile* is estimated to cause 500,000 infections in the US each year that results in 29,000 deaths and associated annual healthcare costs of approximately

$3 billion [72, 73]. This increase in the frequency and severity of CDI in the United States is partially attributed to the emergence of the epidemic *C. difficile* clinical isolates, e.g. BI/NAP1/027 (ribotype-027) [18, 74]. Ribotype-027 is commonly associated with healthcare-associated CDI and is responsible for 19 to 22.5% of hospital-acquired CDI [75].

The increased frequency of diagnosis, associated with the epidemic ribotype-027, is potentially linked to increased numbers of virulence determinates compared to other ribotypes. One such virulence determinate are the ability to form spores [71]. Clostridial endospores are essential for the environmental transmittance of *C. difficile* in humans and are resistant to a broad variety of physical and chemical treatments [76, 77]. Within the host, *C. difficile* spores germinate into vegetative cells, which enables colonization of the intestinal tract, toxin production, and eventual disease [12, 27]. Stages of disease progression include intestinal inflammation, perforation, toxic megacolon, pseudo-membranous colitis, and death [27, 28]. Typically, the antibiotic vancomycin is utilized for the treatment of CDI, and when treated with an antibiotic *C. difficile* produces endospores [11]. These spores are not only essential for transmission of the bacteria, but they are also essential for episodes of recurrence to occur in the host.

There are multiple *in vitro* studies that characterize ribotype-027's spore production, but these studies have produced conflicting results. Some *in vitro* studies have concluded that ribotype-027 isolates produce a significantly greater number of spores compared to non-epidemic isolates, while other studies suggest this may not be the case [7, 16, 51, 53, 70]. There are also a smaller number of *in vivo* studies, and these have observed that the ribotype-027 does not produce significantly more spores that non-epidemic ribotypes. Thus, it is unclear whether epidemic ribotype's spore production could be a potential factor that contributes to the increase of CDI cases associated with ribotype-027.

To examine spore production, we determined the *in vivo* spore production of four *C. difficile* isolates [2 non-epidemic and 2 epidemic (ribotype-027)] in the hamster model of *C. difficile* when the

hamsters were untreated or treated with vancomycin. The hamster model is very sensitive to *C. difficile* and, though there are differences, closely parallels the characteristics of clinical *C. difficile* associated-disease [62]. By using this approach, there was no difference in the recovery of spores from untreated animals; however, higher numbers of spores were recovered from vancomycin-treated hamsters infected with epidemic isolates in comparison to the non-epidemic isolates. Thus, the epidemic isolates produced greater number of spores when animals were treated with vancomycin, and that this could be a potential factor contributing to the spread or relapse of disease associated with the epidemic ribotype of *C. difficile.*

Materials and Methods

Ethics statement

Animal studies were conducted in accordance with protocols 2016-0015 approved by the Institutional Animal Care and Use Committee (IACUC) at the University of North Texas Health Science Center (UNTHSC). IACUC established guidelines ensuring that approved protocols are in compliance with federal and state laws regarding animal care and use activity at UNTHSC. The UNTHSC animal program in USDA registered (74-R0081) and fully AAALAC accredited.

Bacterial strains and Ribotype Confirmation

All *C. difficile* isolates used in this study are listed in Table 1. *C. difficile* UNT 101-1, UNT-103-1, UNT 107-1, and UNT 108-1 were kindly provided by Dr. Curtis Donskey (Cleveland VA). The source of relevant characteristics of each isolate can be found in Table 1. Ribotypes were confirmed by running polymerase chain reaction (PCR) ribotyping with primers found in Bidet *et. al.* [15]. PCR fragments were analyzed in a Hitachi 3500xL genetic analyzer with a 36 cm capillary loaded with a POP4 gel (Applied Biosystems). The size of each peak was determined using Peak Scanner software (Applied Biosystems). A database was generated from the results of the capillary gel electrophoresis-based PCR ribotyping result

of each strain (http://webribo.ages.at). An error margin of ±4 bp was incorporated into the analysis algorithm of the database [78].

Media

Sporulation medium (SM) contained 90 g Trypticase Peptone, 5 g Proteose Peptone no. 3, 1 g Ammonium Sulfate, and 1.5 g of Tris in 1 liter of distilled water. The pH was adjusted to 7.4 at 37^{o} with 1 M NaOH. SM is a broth medium made according to what has been previously described [79].

TSA with 5% blood agar was made with 1L of distilled water (DI), 30 grams of TSB, and 15 grams of granulated agar with constant mixing over low heat. Once the granulated agar was dissolved, the mixture was autoclaved (20 minutes, 121 °C, 15 psi). Once cooled to approximately 50 °C, 50 mL of the medium was removed, and 50 mL of sterile defibrinated sheep blood (Remel, Lenexa, KS) was added and mixed into the medium. Approximately 12 mL of medium was then poured into petri dishes and cooled overnight to solidify and stored in a 4 °C refrigerator until used.

TGY-vegetative medium contained 5 g Tryptone, 5 g Yeast extract, 1 g Glucose, 1 g Potassium Phosphate, 15 g agar, and 1 liter of distilled water. This liquid-based medium was made according to what has been previously published [80].

CCFA and CCFA with Taurocholate contained 40g Proteose Peptone #2, 5g Sodium Phosphate, 1.0g of Potassium Phosphate, 2.0g Sodium Chloride, 0.1g Magnesium Sulfate, 6.0g Fructose, 3.0mL of 1% stock Natural Red solution, 15g of granulated agar, and 1.0g sodium taurocholate (Only in CCFA with Taurocholate). The media was heated until just before boiling and autoclaved (20 minutes, 121 C, 15 psi). The media was then cooled to 50 C before the volume was adjusted to 990 mL with autoclaved DI water, followed by the addition of heat sensitive components: 10 mL of a 50 mg/mL stock of cycloserine and 1.0 ml of a 15.5 mg/ml stock of cefoxitin. Plates were then poured, cooled overnight to solidify, and refrigerated at 4 C until used.

Preparation of C. difficile spore stocks

Spore stocks of each *C. difficile* strain were generated for use in the cellular adherence assay and the experimental animal models of CDI. These stocks were generated by growing each strain on 5% TSAb plates incubated at 37^{o}C in anaerobic conditions for 7 days. Plate growth was collected in a 1X PBS solution containing 1% (V/V) Tween-80 (ST-80), and suspensions were washed 3 times in equal volumes of ST-80. Suspensions were incubated for 1 hour at 65 ± 2°C, washed with ST-80, and re-suspended in 4 mL of sterile nanopore water. Suspensions were then stored overnight at 4°C in order to promote the maturation of endospores for each strain. Spores were separated from vegetative cells and residual debris by density gradient centrifugation (10 minutes at 4,500 x *g*) with a 25% (W/V) HistoDenz solution. Spore pellets were washed 3 times with ST-80 and suspended in sterile nanopore water to a final volume of 2 mL. Spore stocks for each strain were stored at -80°C until used in *in vitro* or *in vivo* studies

Minimum inhibitory concentrations (MIC) assay

In vitro susceptibility to metronidazole, vancomycin, rifampin, clindamycin, and moxifloxacin were performed using a broth dilution method. Wells with doubling concentrations of 0.03–64 μg/mL of clindamycin, 0.12–32 μg/mL of metronidazole, 0.03–16 μg/mL of moxifloxacin, 0.004–8 μg/mL of rifaximin, and 0.12–64 μg/mL of vancomycin was made and allowed to reduce for 3 hours prior to the assay. Frozen *C. difficile* isolates were subcultured on TGY-Veg plates twice prior to dilution in Brucella broth. The organism suspension was measured and diluted to an optical density of 1.00, the suspension was then diluted again 1:20. The organism suspension and the antimicrobial solution was then added to each well in a 1:1 equivalent into a new 96-well plate. Once inoculated, the plates were placed in an incubator at 37 °C for 48-hours. After 48-hours growth or lack of was checked for each antimicrobial and the MIC was determined.

Hamster C. difficile associated disease models

Male Golden Syrian hamsters that were 6 to 7 weeks old were purchased from Envigo RMS Inc., and individually housed in sterile cages. 30 hamsters were used in each study with 15 animals in each group that were orally inoculated with either UNT 101-1, 103-1, 107-1, 108-1. The animals were inoculated with 0.5mL of *C. difficile* spores from a spore preparation culture though oral gavage. The inoculation dose for all strains ranged from $5x10^5 – 1x10^6$ spores/mL, and the exact titers chosen for each strain were based on previously conducted studies. Clindamycin was administered subcutaneously to each animal at 10 mg/kg per body weight approximately 24 hours after infection. At day 3 of the infection, animals were treated with 20 mg/kg of Vancomycin and this treatment continued throughout the rest of the study. On days 3, 4, 7, and 11, three hamsters were culled to take cecum samples. Starting the day of infection, and each day after, approximately 0.1 to 0.2 g of feces was collected from each cage to determine *C. difficile* counts. Bedding was changed daily to ensure fresh feces were collected for analysis, and census of survivors were recorded daily for 11 days after infection. Sterile 1x PBS was added to the recovered feces, this solution was then homogenized and 1 mL was separated for each total CFU and spore recovery. Viable cell and spore counts were quantified as described in the Material and Methods. The homogenized solution separated for spore quantification was heated to 65 ± 2°C for 1 hour to facilitate the isolation of only spores.

In vitro growth of C. difficile vegetative cells and spore formation

Plate growth of each *C. difficile* isolate was transferred into TGY-veg broth and anaerobically incubated at 37°C for 24 hours. TGY-veg associated growth for each strain was adjusted to an optical density of 0.1 (600nm) in either SM or TGY-veg broth, which were anaerobically incubated at 37°C. Samples from each broth culture were collected in triplicate every 24 hours through 72 hours of total incubation, and these samples were 10-fold serially diluted and plated onto Columbia horse blood agar.

Additionally, a second sample from each culture were possessed for spore counts by incubating each sample in an equal volume of 200 proof ethanol for 30 minutes, and then incubating the samples at 65 ± 2ºC for 1 hour. The ethanol and heat-treated samples were centrifuged, washed with PBS, and the spore-containing pellets were suspended in a volume of PBS equal to the original volume of the sample. Ethanol and heat-treatment at 65 ± 2ºC were tested and sufficient to remove all viable vegetative cells during this stage. The spore suspension of each sample was 10-fold serially diluted and plated on Columbia horse blood agar supplemented with 0.1% sodium taurocholate. Both sets of plates were anaerobically incubated at 37°C for 48 hours and colony counts were used to calculate the vegetative CFU or spore counts per mL at each time point.

Statistical analyses

Data were evaluated by Two-way ANOVA with Tukey's post-hoc test. A p value ≤ 0.05 was considered statistically significant. Representation of survival rate against Log10 [daily dose]. Analyses were performed using Prism 8 software (Graphpad Software).

Results

Epidemic isolates of *C. difficile* produce greater numbers of spores and CFU when treated with vancomycin in the hamster model of CDI compared to non-epidemic isolates

A hamster model of CDI was used to compare the spore and CFU production of the non-epidemic and epidemic *C. difficile* isolates *in vivo* when vancomycin was administered. This is a commonly used model to study shedding and disease progression than closely mirrors the disease in humans. In this model, the intestinal microbiome was disrupted with clindamycin and then the hamsters were orally inoculated with approximately 5 x 10^4 *C. difficile* spores. On day 3, the remaining hamsters were then treated with

20mg/kg of vancomycin, and treatment was continued daily until the end of the study. Hamsters were culled on days 1, 2, 3, 4, 7, and 11 to obtain cecum samples, and feces were also collected on those days to determine CFU and spore counts. In previous studies, all animals would have died that were untreated with vancomycin, while most survived when treated with this dose of antibiotic.

The epidemic isolates (UNT 107-1 and UNT 108-1) produced greater amounts of CFU and spores recovered from hamsters after treatment with vancomycin than non-epidemic isolates (UNT 101-1 and UNT 103-1) (Fig 1). This was true for both the feces recovered and in the cecum samples that were taken. CFU numbers were higher on days 7 and 11 after infection (4 and 8 days after treatment began). Increased spore production in the epidemic isolates was also observed after vancomycin was administered to the animals. Interestingly, untreated animals prior to treatment produced similar amounts of CFU and spores regardless of the isolate they were infected with. The differences in spore numbers were present beginning one day after vancomycin treatment (day 4) with about 8- to 9-fold higher spore numbers from animals infected with epidemic isolates than non-epidemic isolates. When the spore numbers were normalized to the number of CFU recovered was compared between the isolates, the epidemic isolates produced spores in higher quantities compared to the non-epidemic isolates during treatment with vancomycin. On days 4 and 7 after infection (after 1 and 4 days of vancomycin treatment), epidemic isolates were found to produce 2.1 to 2.6 more spores per CFU recovered than non-epidemic isolates, indicating that there was higher spore formation relative to vegetative cells (CFU).

In vitro **growth and spore production are higher in epidemic isolates of** ***C. difficile*** **in the presence of vancomycin**

To confirm that there are no inherent differences in growth and spore production of the isolates, *in vitro* growth and spore formation of the four *C. difficile* isolates were determined over a 72-hour period,

and, it was found, that non-epidemic and epidemic isolates have similar *in vitro* growth patterns. Also, when places in sporulation medium, there was no difference over a 72-hour period between the epidemic and non-epidemic isolates in spore formation or the number of remaining vegetative cells.

In the presence of vancomycin, however, the epidemic isolates did produce higher numbers of spores *in vitro*, while the number of vegetative (CFU) cells dropped due to the antibiotic (Fig 2). By 48 hours in culture, the epidemic isolates produced 2.7- to 4.2-fold more spores than the epidemic isolates when exposed to vancomycin *in vitro*, indicating an inherent difference between the two groups of isolates.

To determine if there was a difference in resistance to vancomycin between the isolates, *in vitro* minimum inhibitory concentrations (MIC) were determined. All isolates were susceptible to vancomycin. The epidemic isolates, UNT 107-1 and UNT 108-1, both had MIC values of 2 mg/L vancomycin, while the non-epidemic isolates, UNT 101-1 and 103-1, had MIC values of 1 and 4 mg/L, respectively. Thus, differences in susceptibility to vancomycin does not account for the higher recovery of CFU and spores from animals infected with the epidemic isolates and treated with vancomycin.

Discussion

With the identification and labeling of the NAP/BI/027 ribotype as epidemic, there is an ongoing debate if this ribotype is epidemic due to virulence, or if the increased frequency in diagnosis is due to other factors [18, 70, 81]. One key virulence factor may be linked to the ability to produce spores [11, 82]. Some papers found the ribotype-027 isolates can produce greater amounts of spores *in vitro* that non-epidemic ribotypes [16, 53]. In contrast, other papers have stated there is little differences between the ribotype-027 and other non-027 ribotype's spore production *in vitro* [7, 70]. Therefore, we undertook a set of *in vitro* and *in vivo* studies of four clinically relevant *C. difficile* isolates (2 non-epidemic and 2 epidemic) isolates and compared their ability to grow and produce spores. To do this, the isolates were not only characterized *in vitro* and *in vivo* using a hamster model of CDI, but also used a unique approach

of characterizing the impact of the same isolates' *in vivo* growth and spore production during treatment with a clinically relevant antibiotic. With this approach, we were able to further our understanding about *C. difficile*'s growth and spore production for the epidemic ribotype in comparison to other non-epidemic ribotypes prior to and after antibiotic therapy.

In the hamster model of *C. difficile*, epidemic isolates produced greater amounts of spores than non-epidemic isolates when exposed to vancomycin. The hamster model is very sensitive to *C. difficile* and closely parallels the characteristics of clinical *C. difficile* associated disease [62]. Epidemic and non-epidemic isolates had similar *in vivo* growth patterns and numbers of spores recovered in hamsters prior to beginning treatment with vancomycin. However, once treatment with vancomycin started on day 3, there was a significant difference in spores and CFU recovered from hamsters infected with epidemic and non-epidemic isolates. Spore and CFU numbers from hamsters infected with the epidemic isolates and treated with vancomycin averaged approximately half a $\log_{10}$ greater than spores recovered from similarly treated hamsters infected with the non-epidemic isolates. During treatment, the epidemic isolates also had a significantly greater ratio of spores:total CFU recovered than non-epidemic isolates, indicating that after normalization of results to vegetative organisms there was a higher production of spores. The difference in spore numbers in response to the presence of vancomycin also occurred in *in vitro* cultures. These differences between the epidemic and non-epidemic isolates were consistent despite there being no difference to sensitivity to vancomycin *in vitro*. Thus, antibiotic treatment promoted higher levels of spores *in vivo* and *in vitro* than found in other non-epidemic ribotypes, suggesting this difference in response to clinically relevant antibiotics is a factor that contributes to the ribotype-027 being more frequently diagnosed in *C. difficile* cases.

Within the last decade, *C. difficile* has become an ever-increasing threat, and a major reason for this is the rise of the NAP/BI/027 ribotype [74, 83]. Previous studies debated whether the current epidemic

ribotype is able to produce greater amounts of spores, and if this could be a potential factor in its ability to persist as epidemic over this period of time [16, 51, 53, 70, 74]. The results presented here helps to bridge the gap between the organism's microbiology and the public health and disease outcome. Although there were no differences in spore production between the epidemic and non-epidemic isolates in the absence of antibiotic, the epidemic isolates produced more spores when exposed to vancomycin. This increased spore production could contribute to an increased environmental shedding of epidemic *C. difficile* spores, and therefore, increase exposure to epidemic ribotypes. Another possible outcome is that infection with the epidemic isolates may more frequently result in relapse disease after treatment with antibiotics. The higher numbers of vegetative cells due to infection epidemic isolates after treatment may contribute to more severe and prolonged disease. However, further studies are needed to determine the impact of this response to antibiotic therapy on the spread and disease outcome in patients. Thus, this characterization will contribute to our understanding of this pathogen and its role in healthcare associated infectious diseases.

Conclusion

Epidemic isolates of *C. difficile* produced greater number of spores during *in vivo* treatment or in the presence *in vitro* of vancomycin, a clinically relevant antibiotic. This increased spore production could be a potential factor contributing to the more frequent appearance and relapse of disease associated with the epidemic ribotype of *C. difficile*, as compared to the other non-epidemic ribotypes.

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Conflicts of interest

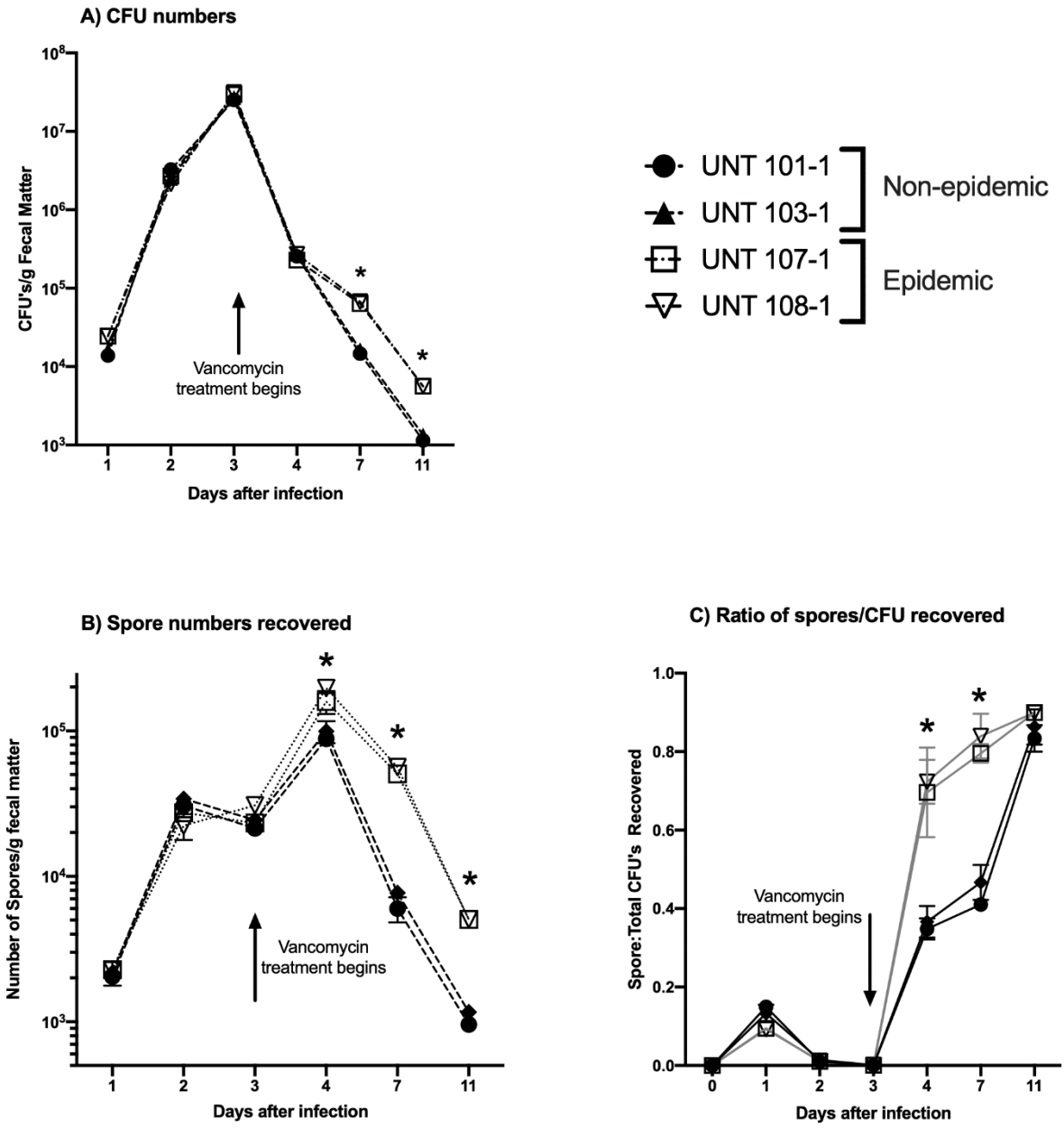
The authors declare that they have no conflicts of interest.

Tables and Figures

Clostridium difficile **Isolates and Sources**			
UNT Strain #	**Species**	**Source**	**Relevant Characteristics**
UNT 101-1	*Clostridium difficile*	Ohio VA Medical Center (Curtis Donskey)	Non-epidemic, Other Designation VA1
UNT 103-1	*Clostridium difficile*	Ohio VA Medical Center (Curtis Donskey)	REA J-type strain, binary toxin negative, non-epidemic, Other Designation VA 11
UNT 107-1	*Clostridium difficile*	Ohio VA Medical Center (Curtis Donskey)	(BI/NAP1, binary toxin positive, Ribotype 027; Epidemic), Other Designation VA17
UNT 108-1	*Clostridium difficile*	Ohio VA Medical Center (Curtis Donskey)	(BI/NAP1, binary toxin positive, Ribotype 027; Epidemic), Other Designation VA20

1. Table 1. *Clostridium difficile* Strain Designation, Sources and Characteristics

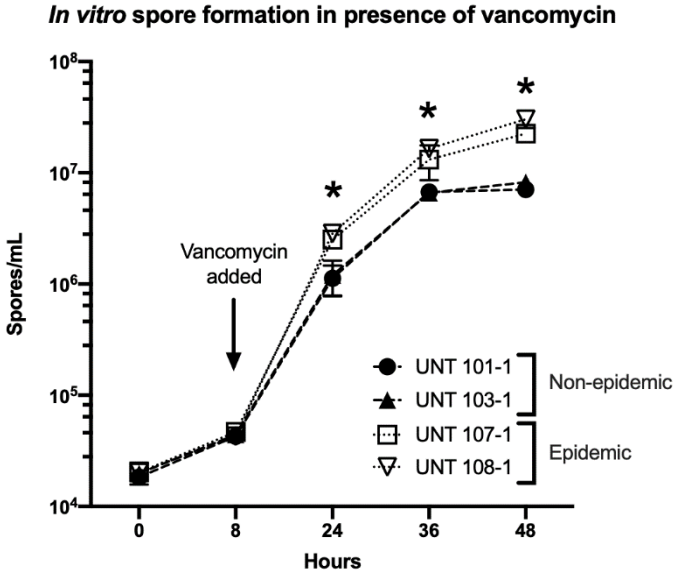
This table denotes the source of the individual isolates, other designations for each isolate, and some of the major characteristics associated with each of the isolates.



1. Figure 1. Epidemic isolate infected hamsters had significantly more fecal-associated spores than hamsters infected with non-epidemic isolates of *C. difficile* when treated with vancomycin

For each isolate, 15 hamsters were split into 5 groups, housed individually, and inoculated with approximately $1x10^5 – 5x10^5$ *C. difficile* spores. Fecal pellets were then collected, weighed, and processed to measure for total CFU's and spores recovered. **(A)** Total CFU's recovered per gram of feces that was

collected from epidemic or non-epidemic infected hamsters on days 0 to 11 of the studies. **(B)** Total spores recovered per gram of feces that was collected from epidemic or non-epidemic infected hamsters on days 0 to 11 of the studies. **(C)** Ratio of the total spores:total CFU's that were collected from epidemic or non-epidemic infected hamsters on days 0 to 11 of the studies. These data represent the average of three independent groups, and error bars indicate the standard errors of the means. An asterisk denotes significant difference at $p \leq 0.05$ (Two-way ANOVA with Tukey's post-hoc test).



2. Figure 2. Mean spore recovery from *in vitro* cultures differed between non-epidemic and epidemic isolates when exposed to vancomycin

The 4 isolates (2 non-epidemic and 2 epidemic) were incubated in TGY-veg broth over a 72-hour period, and vancomycin was administered at hour 8 of the studies. A representative sample was then taken from each culture and plated on an agar medium ± 0.1% taurocholate to measure the number of spores/mL at each timepoint. The non-epidemic isolates are represented by the black symbols, and the epidemic isolates are represented by the open symbols. This data represents the average of three independent experiments

and error bars indicate the standard errors of the means. An asterisk denotes significant difference at $p \leq 0.05$ (Two-way ANOVA with Tukey's post-hoc test)

CHAPTER III

EPIDEMIC RIBOTYPES OF *CLOSTRIDIUM (*NOW *CLOSTRIDIOIDES) difficile* ARE LIEKLY TO BE MORE VIRULENT THAN NON-EPIDEMIC RIBOTYPES IN ANIMAL MODELS

The prior chapter demonstrated that epidemic-027 ribotypes of *C. difficile* produce greater numbers of spores both *in vitro* and *in vivo* compared to non-epidemic ribotypes when treated with vancomycin. Though important to determine, this increased spore production does not answer a central question: Are the epidemic ribotypes of *C. difficile* more virulent than non-epidemic isolates, and, if so, why? Thus, using the hamster model of *C. difficile* an LD_{50} model was developed and utilized to compare the epidemic and non-epidemic isolates virulence. It was determined that the epidemic isolates were more virulent than the non-epidemic isolates, and these results were supported through survival results using the mouse-model of *C. difficile.* In both models, it was determined that toxins A and B production were greater in the epidemic isolates than the non-epidemic isolates. This increased toxin production was also observed *in vitro* at the 72-hour timepoint, but the levels were significantly lower than *in vivo.* This suggested that toxin production may play a central role in increased virulence, however, there may be *in vivo* factors that influence the production of toxin within certain ribotypes more than others. Another factor potentially contributing to the lower LD_{50} values observed was the increased spore adherence during *in vitro* assays associated with the epidemic isolates. These results suggest that the epidemic ribotypes of *C. difficile* are more virulent than the non-epidemic ribotypes, and that virulence and environment factors could be contributing to this phenomenon.

Introduction

Clostridium difficile (now *Clostridioides*), a spore forming bacillus, is the cause of *C. difficile*-associated disease. In the United States of America (US), the occurrence of *C. difficile* infections (CDI) increased by a factor of 400% between 2000-2007 [1]. *C. difficile* is estimated to cause 500,000 infections in the US each year that results in 29,000 deaths and associated annual healthcare costs of approximately $3 billion [72, 73]. Clostridial endospores are essential for the environmental transmittance of *C. difficile* in humans and are resistant to a broad variety of physical and chemical treatments [76, 77]. Within the host, *C. difficile* spores germinate into vegetative cells, which enables colonization of the intestinal tract, toxin production, and eventual disease [12, 27]. Stages of disease progression include intestinal inflammation, perforation, toxic megacolon, pseudo-membranous colitis, and death [27, 28]. Mortality associated with CDI is approximately 5% but has been as high as 20% during particular outbreaks [75]. *C. difficile* is capable of producing two different Rho glucosylating exotoxins, TcdA (toxin A) and TcdB (toxin B) [31, 70], which are responsible for the pathology typically associated with CDI [45, 48]. Toxin A and B both produce multiple cytopathic and cytotoxic effects on the targeted cells [31]. These can include disruption of Rho-dependent signaling, disruption of the actin cytoskeleton and of the tight adherence junctions, all causes of increased epithelial permeability which cause the diarrhea associated with *C. difficile* associated disease [31]. *C. difficile* isolates can produce another toxin, binary toxin, which can disrupt normal cytoskeletal function of cells [84]; however, studies have yet to show that binary toxin plays a significant role in disease severity or virulence [85, 86]. Therefore, both *C. difficile* spores and toxins play an important role in disease transmission and pathogenesis, and these virulence determinates have been shown to vary between different *C. difficile* ribotypes [31, 45, 53, 70].

The increase in the number and severity of CDI in the United States is largely attributed to the emergence of the epidemic *C. difficile* clinical isolates, e.g. BI/NAP1/027 (type 027) and ribotype 078

[18, 74]. Interestingly, ribotype 027 is common among healthcare-associated CDI cases, while the type 078 is more commonly associated with community-acquired CDI [74]. Ribotype 027 is responsible for 19 to 22.5% of hospital acquired CDI cases, and most of these cases are significantly associated with increased disease severity, recurrence, and mortality [3, 74, 87]. It was recently suggested that one possibility why ribotypes 027 and 078 have become epidemic strains was due to their ability to utilize low concentrations of the sugar trehalose [18]. The increased usage of trehalose as a food additive in both the US and Europe coincides with the emergence of both ribotype 027 and 078 outbreaks. Thus, the ability to utilize this sugar may provide a competitive advantage over other ribotypes, resulting in the increased frequency of infection within a complex host environment [18]. Still, this does not account for the increased frequency of disease associated with the epidemic ribotypes, as well as the increased severity of disease associated with them when compared to other non-epidemic ribotypes.

The apparent increased severity of disease due to the epidemic ribotypes of *C. difficile* suggests that these isolates may be more virulent than other ribotypes, and if so, this is likely linked to enhanced expression of virulence determinates, such as spores and toxins A and B [71]. There are limited studies examining *in vivo* virulence of multiple isolates of the epidemic ribotypes using animal models [63, 88]. However, there are multiple *in vitro* studies that characterize type 027's spore and toxin production, but these studies have produced conflicting results. Some *in vitro* studies indicate that ribotype 027 has increased spore and toxin production [51, 53, 71, 83]. Increased toxin production was highlighted in a study by Warny et al, which found a ribotype 027 isolate expressing 16 times more toxin A and 23 times more toxin B that other ribotype isolates [71]. In contrast, other *in vitro* studies found that spore production for other ribotype 027 isolates were not significantly different from other ribotypes, and toxin production by ribotype 027 is not as robust as shown in the study by Warny et. al [7, 89]. These studies, as well as other studies, have not definitively compared the *in vitro* profiles of various *C. difficile* isolates

with their ability to cause disease *in vivo,* leading others to speculate that clinical outcomes may be isolate dependent. Thus, it is unclear whether epidemic ribotypes are more virulent than other ribotypes, and whether the *in vitro* virulence phenotype of an isolate or ribotype is useful in predicting *in vivo* virulence of individual isolates.

To examine the virulence of epidemic isolates, we initially determined the *in vivo* virulence of thirteen *C. difficile* isolates (7 non-epidemic and 6 epidemic) in two different animal models of CDI. The first animal model that was used in these studies was the murine model of CDI. Being that mice are less susceptible to *C. difficile,* this model is an excellent shedding model and has been used, with some success, as a survival model [63, 64]. Also, due to this decreased sensitivity to *C. difficile*, the mouse model is better suited for determining subtle differences between isolates that pose an issue in more sensitive animal models, such as toxin production over extended periods of time [3]. The second animal model that was used in these studies is the hamster model of CDI. In contrast to mice, hamsters are very sensitive to *C. difficile* and, though there are differences, closely parallels the characteristics of clinical *C. difficile*-associated disease in humans [3]. This enhanced sensitivity makes the hamster model of CDI a strong choice for survival studies and the subsequent calculation of LD_{50} values for *C. difficile* strains [52, 64, 68]. By using this approach, we found collectively that the epidemic isolates had increased virulence in both experimental animal models when compared to non-epidemic isolates. In particular, the group of epidemic ribotypes of *C. difficile* had lower LD_{50} values in hamsters. Additionally, we also examined the *in vitro* production of toxins A and B, growth rates, spore formation and adherence of spores to intestinal epithelial cell lines, and although there was increase production of toxins and adherence for the group of epidemic isolates, the *in vitro* profiles of individual isolates were not predictive of their *in vivo* virulence. Overall, the group of epidemic ribotypes of *C. difficile* were more virulent *in vivo* despite individual isolates having similar phenotypes to the non-epidemic isolates *in vitro*.

Results

Isolates of the epidemic ribotypes of *C. difficile* are more virulent in the murine CDI model when compared to isolates of non-epidemic ribotypes

A mouse CDI model was used to compare the virulence of the non-epidemic and epidemic *C. difficile* isolates *in vivo*. This is a frequently used model to study colonization, shedding, disease progression, and, in some cases, survival [64]. For this model, the intestinal microbiome of the mice was disrupted with antibiotics and then they were orally inoculated with approximately 1 x 10^6 *C. difficile* spores. Survival was monitored for the entire study, and feces were sampled each day for 7 days post-infection and every other day thereafter, until the end of the study (Day 12). *C. difficile* CFU and toxin levels in fecal samples were determined.

The epidemic ribotype isolates caused greater mortality than those with non-epidemic ribotypes (Fig 1). The notable exception to this trend was non-epidemic ribotype isolate UNT 106-1. This isolate had a mortality rate that was equivalent to UNT 109-1 and greater than UNT 210-1 (both, epidemic, type 027 isolates). As a whole, mortality rates ranged from 15% - 30% for mice infected with epidemic ribotype isolates, while the mortality rates for nice infected with non-epidemic ribotype isolates ranged from 5 – 20%.

Despite the differences in survival, there were no significant differences between fecal *C. difficile* CFUs recovered from mice infected with epidemic and nonepidemic ribotype isolates (Fig 2). All isolates followed a similar pattern of growth, and growth for the isolates reached its apex between 1 x 10^7 and 1 x 10^8 CFU per gram of feces on days 2 and 3 of the studies. After this apex, there was a similar decline in the recovered fecal counts observed for each isolate.

Significant levels of Toxin A and B in fecal samples were found in mice infected with non-epidemic or epidemic ribotype *C. difficile* isolates (Fig 3). The data per gram of feces were similar to that

if normalized to CFU numbers recovered. Measurable concentrations of Toxin A for both the non-epidemic and epidemic ribotype isolates were initially detected 2 days after infection and were continued through day 10 of each study. Toxin A production for both sets of isolates peaked 4 days after infection, and there were significant differences observed between the non-epidemic and epidemic mean Toxins A levels associated with feces collected between days 3-8 ($p \leq 0.05$). During this time, the feces collected from mice with epidemic ribotype isolates had between 1.5-2.5x higher mean levels of Toxin A/gram than feces collected from mice infected with non-epidemic ribotypes. Similar trends were observed for fecal-associated Toxin B production titers determined for animals infected with epidemic and non-epidemic *C. difficile* ribotype isolates. During this time, between 3-4x higher levels of Toxin B was found in feces collected from epidemic ribotype infected mice than those infected with non-epidemic ribotypes ($p \leq 0.05$). When toxin levels were normalized with numbers of CFU recovered, Toxin A levels per CFU in feces from epidemic ribotype infected mice were 2-3x more ($p \leq 0.05$) than feces from mice infected with the non-epidemic ribotypes. In addition, there was approximately 3.3x higher levels of Toxin B per CFU in feces from epidemic ribotype infected mice than the non-epidemic ribotype infected mice. ($p \leq 0.05$).

Epidemic ribotype isolates of *C. difficile* are more virulent than non-epidemic ribotype isolates in the hamster model of CDI

The previous studies using the mouse model of CDI suggested the epidemic ribotype isolates were more virulent than the non-epidemic ribotype isolates. The virulence of the two sets of *C. difficile* isolates were further investigated using the hamster model of CDI. The hamster model is well established and shares some common features of *C. difficile* disease associated with the human clinical condition [62, 64]. Like humans, hamsters also exhibit increased susceptibility to *C. difficile* infection after administration of a broad spectrum antibiotic that often leads to consistent clinical disease outcomes in the experimental model [52, 62]. To perform these studies, groups of hamsters were inoculated with a range of spore titers

per isolate, and then treated with clindamycin to facilitate infection and subsequent disease establishment. After this, the condition of the hamsters was assessed multiple times a day, and fecal samples were collected daily until the conclusion of the study on day 7. Fecal samples were processed for CFU and assayed for Toxin A and B concentration via ELISA.

When LD_{50} values were compared between the isolates in the hamster CDI model, the epidemic isolates had a lower mean LD_{50} value than the non-epidemic isolates did in the model (Fig 4). The average LD_{50} value was 3.57 ± 0.025 log CFU for hamsters infected with epidemic strains, and hamsters infected with non-epidemic strains had a LD_{50} value of 3.94 ± 0.051 log CFU ($p \leq 0.05$). As a whole, the LD_{50} values ranged from 3.27 – 3.72 log CFU for the hamsters infected with epidemic ribotype strains, while the LD_{50} values for the hamsters infected with non-epidemic ribotype isolates ranged from 3.76 – 4.13 log CFU.

For this model, we chose not to compare fecal-associated CFU counts, because determining the LD_{50} values led to varying inoculation doses for each isolate. Due to differences observed between the isolate's toxin production in the mouse model, we chose to examine fecal-associated Toxin A and B concentrations to determine if this was similar in the hamster model. To do this, toxin levels/CFU was assayed from the fecal samples collected daily for 6 days after infection, and the results were separated into multiple groups for comparison purposes. Fecal-associated Toxin A and B were initially detected 2 days after infection for both the non-epidemic and the epidemic ribotype infected animals (Fig 5). When comparing non-epidemic and epidemic ribotype infected groups that survived, the epidemic isolate infected hamsters had approximately 2-3x more Toxin A/CFU in their feces than did non-epidemic isolate infected hamsters ($p \leq 0.05$), and the feces collected from epidemic ribotype infected animals had approximately 3-4x Toxin B/CFU higher levels than hamsters infected with isolates of the non-epidemic ribotype ($p \leq 0.05$).

In vitro growth and spore production are similar between non-epidemic and epidemic ribotype isolates of *C. difficile*

Epidemic isolates were shown to be more virulent than non-epidemic isolates *in vivo*, despite having no differences in recovered CFU. To confirm that there are no inherent differences in growth and spore production of the isolates, in vitro growth and spore formation of all the *C. difficile* isolates were determined over a 72-hour period, and, it was found that non-epidemic and epidemic strains exhibited similar *in vitro* growth patterns. Furthermore, when placed into sporulation medium, there was no difference over a 72-hour period between epidemic and non-epidemic isolates in spore formation or the numbers of remaining vegetative cells (Fig. 6).

In vitro Toxin A and B production is higher in epidemic ribotype isolates than non-epidemic ribotypes.

Infection of animals with epidemic ribotype isolates were shown to result in higher levels of Toxin A and Toxin B in fecal samples. Toxin A and Toxin B production is a major factor in intestinal epithelial damage and increased severity of disease [31, 48], and previous studies found variable levels of *in vitro* toxin production between non-epidemic and epidemic ribotypes [31, 45, 53]. Therefore, we performed sets of *in vitro* experiments to determine if the non-epidemic and epidemic *C. difficile* isolates produced similar amounts of Toxin A and Toxin B over a 72-hour period. These studies were performed in parallel with the sporulation studies, and spent medium from each time point was used to determine Toxin A and B titers by ELISA.

Mean Toxin A and B values were significantly different between the non-epidemic and epidemic ribotype groups at 72-hours (Fig 7) (Two-way ANOVA with Tukey's post-hoc test; $p<0.05$). Isolates with the epidemic ribotype produced approximately 1.4x Toxin A and 2x Toxin B than the non-epidemic

isolates in 72-hour culture. Although there was a significant difference between the groups, there was variability within the individual isolates within non-epidemic and epidemic ribotype groups. For example, the non-epidemic isolate UNT 101-1 produced Toxin A levels that were not significantly different than the levels produced by the epidemic isolates, while producing Toxin B levels significantly greater than two epidemic isolates (UNT 110-1 and UNT196-1; $p \leq 0.05$). Toxin B levels were more variable within the groups of isolates than Toxin A.

In vitro adherence of non-epidemic and epidemic ribotype *C. difficile* spores to Caco-2 and C2BBe1 cells are significantly different

Adherence to intestinal epithelial cells is integral for *C. difficile* colonization and subsequent infection. Therefore, *in vitro* studies comparing the ability of non-epidemic and epidemic spores to adhere to two different intestinal epithelial cell lines (i.e., Caco-2 and C2BBe1) were done. Caco-2 cells are traditionally used for studies involving intestinal epithelial cells, while C2BBe1 cells are a clone of Caco-2 cells [90]. The C2BBe1 cells are more homogenous than Caco-2 cells in regards to brush border expression and are morphologically similar to the human colon [91]. To perform these studies, wells containing confluent intestinal epithelial cells were infected with *C. difficile* spores and incubated for 3-hours. Selection of this timepoint was chosen based on preliminary studies, where adhesion was found to plateau at 3-hours. Non-adherent spores were removed by washing plates, and intestinal cells were collected and pleated to determine percent adherence.

Overall, the mean percentages of adhered epidemic *C. difficile* spores to both intestinal epithelial cells were significantly higher than the mean percentages determined for adherent non-epidemic spores. Spores from epidemic isolates adhered at a 5% higher level to Caco-2 cells than non-epidemic isolates (Fig 9, Supplemental Table 2) ($p \leq 0.05$). When comparing the non-epidemic and epidemic spore's adherence to C2BBe1 cells, there was also a 5% difference between the groups ($p \leq 0.05$).

Discussion

With the identification of the epidemic NAP/BI/027 ribotype, there has been an ongoing debate if this genetic cluster of *C. difficile* is more virulent than non-epidemic ribotypes [3, 16, 28, 51, 70, 71, 74, 92]. This debate is supported by papers which have stated the type 027 is more virulent because it hyper-produces toxins and spores *in vitro* [51, 53, 74, 88]. Whereas, other papers have stated there is little differences between the 027 ribotype and other non-027 ribotypes *in vitro* [28, 29, 70]. However, there is also a question whether *in vitro* characterizations accurately predict the *in vivo* virulence of individual *C. difficile* isolate or a group of isolates of the same ribotype. Therefore, we undertook a comprehensive set of *in vitro* and *in vivo* studies of thirteen *C. difficile* isolates (7 of non-epidemic ribotypes and 6 of epidemic ribotypes) to examine whether isolates of the epidemic ribotype are more virulent than non-epidemic isolates *in vivo*. To do this, we not only characterized the isolates *in vitro,* but also used a unique approach of characterizing the same isolates' *in vivo* virulence within two different animal models of *C. difficile* infection. With this approach, we were able to answer questions about *C. difficile*'s epidemic ribotype in comparison to other non-epidemic ribotypes. Such as, is an isolate's *in vitro* virulence phenotype predictive of its *in vivo* virulence and, is there truly a difference between non-epidemic and epidemic isolate's *in vivo* virulence?

As a group, isolates of an epidemic ribotype were more virulent than those from non-epidemic ribotypes, although there was variability within each group of ribotypes. Difference in *in vivo* virulence was found using two animal models, murine and hamster. The mouse model is an excellent shedding model and has been used, with some success, as a survival model [63, 64]. In mice, there were differences in survival after infection with epidemic isolates or non-epidemic isolates. Between 4-8 days after infection the average mortality of the mice infected with epidemic isolates of 22.5% while the mice infected with non-epidemic isolates averaged 10.7% mortality. In the hamster model *C. difficile* infection,

we confirmed the results observed in the mouse CDI model in that epidemic isolates have increased virulence when compared to the non-epidemic isolates. Hamsters are more sensitive to *C. difficile* toxin, and this sensitivity makes it a strong choice as a survival model and determining the median lethal dose or LD_{50} value [62, 64]. Epidemic isolates had significantly lower mean LD_{50} values in the hamster model than the non-epidemic isolates. Thus, the *C. difficile* strains of the epidemic ribotype were more virulent than non-epidemic isolates *in vivo*.

The differences in survival in mice infected with epidemic and non-epidemic isolates occurred even though the numbers of *C. difficile* recovered from the animals were the same, suggesting a factor other than growth are responsible for the difference in virulence. Consistent with the *in vivo* results, there were no differences in the *in vitro* growth or spore formation between epidemic and non-epidemic isolates. Previous *in vitro* studies found that epidemic ribotype 027 isolates produced more spores and higher levels of toxin than nonepidemic isolates [16, 53]. Although we did not show a difference in spore formation, there was a significant difference in toxin production between the epidemic isolates and the non-epidemic isolates in the animal models of *C. difficile* infection. In both mice and hamsters, there were two to three times higher levels of both toxins after infection with the epidemic isolates. Consistent with the previous published studies {[53, 71]}, higher levels of toxin production, by epidemic isolates, was also found during *in vitro* culture, but was only significant at 72-hours in culture. Approximately two times more toxin production was associated with the epidemic isolates in *in vitro* cultures when compared to the non-epidemic isolates. It is worth noting increased toxin production for some ribotype 027 isolates is associated with genetic mutations within its pathogenicity island, this could also play a role in the epidemic isolates' increased virulence *in vivo* [50, 51, 54]. Thus, the increased virulence of the epidemic isolates was linked to the higher production of Toxin A and Toxin B.

Although toxin levels may be the most critical factor involved in increased disease severity, there may be other factors. One factor that may contribute to *C. difficile* virulence is an isolate's ability to adhere to intestinal epithelium. Adherence of *C. difficile* spores to epithelium is dependent on the characteristics of exosporium, and the composition of this outmost layer can vary between strains [11, 36, 82]. Recently, two cysteine-rich proteins, *cdeC* and *cdeM*, were shown to influence the ability of *C. difficile* spores to adhere to intestinal epithelium [82]. In the mouse model of infection, spores lacking the CdeC protein had increased colonization rates, recurrence rate, and were correlated with higher toxin titers during disease [82]. These results suggest that adherence mediated factors could play a role in the increased virulence associated with the epidemic isolates. In the current studies, the ability of *C. difficile* spores to adhere to two sets of human epithelial cells, Caco-2 and C2BBe1, *in vitro* was investigated, and the epidemic isolates had about 5% greater adherence to both cell lines than non-epidemic isolates. The ability of the epidemic strains to better bind to the epithelium suggests that these strains will more easily reach the inoculation threshold needed for the establishment of disease. In support, lower doses of epidemic ribotype isolates are needed to cause disease, e.g. $LD_{50,}$ in the hamster, but further studies are needed.

In vitro virulence phenotypes of individual *C. difficile* isolates were not predictive of their *in vivo* virulence. Although the group of epidemic isolates had higher levels of toxin production *in vitro*, the level of toxin production *in vitro* did not predict *in vivo* virulence for each individual isolate. For example, UNT 101-1, a non-epidemic isolate, expressed Toxin A and Toxin B at levels similar to those of the epidemic isolates in *in vitro* cultures. In contrast, *in vitro* characterizations showed that UNT 110-1 and 210-1, two epidemic isolates, had toxin levels that were approximately equal with non-epidemic isolates. However, UNT 101-1, though producing high levels of toxin *in vitro*, was one of the least virulent isolates *in vivo*, while UNT 110-1 and 210-1 were equal to other epidemic isolates' observed virulence in the mouse and hamster CDI models. Not only does this suggest that the evaluation of an individual isolate's virulence

should be done using an *in vivo* model, but it is a strong possibility that factors in the *in vivo* environment influence an isolate's toxin production and virulence [21, 69, 82]. In fact, previous studies demonstrate that *C. difficile* epidemic ribotype isolates can have increased *in vivo* fitness compared to non-epidemic isolates [18, 88]. They are capable of interacting more efficiently with metabolites produced by the host's GI microbiome and have the ability to utilize additional nutrients that other ribotypes are unable to use. In addition, other factors may contribute to the *in vivo* virulence of *C. difficile*. For example, although the role of binary toxin in virulence is unclear [85, 86], a study suggests that binary toxin may suppress host immune responses which results in enhanced virulence of epidemic ribotype 027 strains in a mouse model [93]. Most likely complex combinations of factors of *C. difficile* influences the outcome of infection, and to further complicate the ability to assess virulence solely using in vitro studies, the level and types of factors may be differentially expressed in the *in vivo* environment. Thus, *in vitro* characterization of virulence factors produced by *C. difficile* alone is not reliable approach to assess the potential to cause disease by individual isolates, but this approach may still be useful in comparing the potential of different groups, e.g. ribotypes, of organisms to cause disease.

Overall, these studies demonstrated that epidemic ribotypes of *C. difficile* are likely to be more virulent than non-epidemic ribotypes. Within the last 10 years, *C. difficile* has become an ever-increasing threat, even being designated an urgent threat level organism in 2013 by the Centers for Disease Control, and the major reason for this is linked to the rise of the epidemic NAP/BI/027 ribotype, along with other "hyper-virulent" ribotypes [74, 83]. Results described in these studies provide a comprehensive examination of virulence between different *C. difficile* isolates through multiple methods and provides an important contribution in further understanding what causes the NAP/BI/027 ribotype to be labelled as, epidemic, hyper-virulent, and such a prevalent threat to healthcare. Previous studies debated whether the current epidemic ribotypes are more virulent than the non-epidemic ribotypes [16, 51, 53, 70, 74]. This

appears to be the first study to compare the abilities of isolates of epidemic and non-epidemic ribotypes to cause disease in both the mice and hamster models of CDI. Although all *C. difficile* isolates examined were able to cause disease in both hamsters and mice, the group of isolates with epidemic ribotype caused more severe disease than the non-epidemic group of isolates, providing a compelling case that the epidemic ribotype is indeed more virulent. Additionally, the *in vivo* and *in vitro* data supports the idea that the levels of toxins A and B production are likely to contribute to the increased virulence of the epidemic isolates. Other factors, such as the ability to adhere to epithelial cells, may also play a role. However, there was variability in disease severity between individual isolates within the group of epidemic and non-epidemic ribotypes, with one non-epidemic isolate caused disease as severe as one of the epidemic strains. Furthermore, *in vitro* expression of virulence factors, such as toxin production and adherence to epithelial cells, corresponded with disease potential of the ribotype groups, but was not a reliable approach to assess the potential to cause disease by individual isolates. These results suggest a link between the ability to cause disease and the likelihood of a ribotype's ability to be epidemic and more easily transmissible between hosts. However, further studies are needed to directly link the ribotype with increased virulence and spread of infection.

Materials and Methods

Bacterial strains and Ribotype Confirmation

All *C. difficile* isolates used in this study are listed in Table 1. *C. difficile* UNT 101-1 to UNT-110-1 were kindly provided by Dr. Curtis Donskey (Cleveland VA); UNT 008-1, UNT 210-1, and UNT 196-1 were obtained from the American Type Culture Collection (ATCC). The source of relevant characteristics of each isolate can be found in Table 1. Ribotypes were confirmed by running polymerase chain reaction (PCR) ribotyping with primers found in Bidet *et. al.* [15]. PCR fragments were analyzed in a Hitachi 3500xL genetic analyzer with a 36 cm capillary loaded with a POP4 gel (Applied Biosystems).

The size of each peak was determined using Peak Scanner software (Applied Biosystems). A database was generated from the results of the capillary gel electrophoresis-based PCR ribotyping result of each strain (http://webribo.ages.at). An error margin of ±4 bp was incorporated into the analysis algorithm of the database [78].

Media

Sporulation medium (SM) contained 90 g Trypticase Peptone, 5 g Proteose Peptone no. 3, 1 g Ammonium Sulfate, and 1.5 g of Tris in 1 liter of distilled water. The pH was adjusted to 7.4 at 37^{o} with 1 M NaOH. SM is a broth medium made according to what has been previously described [79].

TSA with 5% blood agar was made with 1L of distilled water (DI), 30 grams of TSB, and 15 grams of granulated agar with constant mixing over low heat. Once the granulated agar was dissolved, the mixture was autoclaved (20 minutes, 121 °C, 15 psi). Once cooled to approximately 50 °C, 50 mL of the medium was removed, and 50 mL of sterile defibrinated sheep blood (Remel, Lenexa, KS) was added and mixed into the medium. Approximately 12 mL of medium was then poured into petri dishes and cooled overnight to solidify and stored in a 4 °C refrigerator until used.

TGY-vegetative medium contained 5 g Tryptone, 5 g Yeast extract, 1 g Glucose, 1 g Potassium Phosphate, 15 g agar, and 1 liter of distilled water. This liquid-based medium was made according to what has been previously published [80].

Columbia horse blood agar with 0.1% sodium taurocholate was made by adding 869 mL of distilled water, in combination with 35 g of Columbia broth (Remel), and 15 g of Difco Agar, granulated (BD). The mixture was autoclaved (20 minutes, 121 °C, 15 psi). Once cooled, 70 mL of horse blood and 50 mL of a 20 mg/mL stock of sodium taurocholate, 10 mL of a 50 mg/Ml stock of cycloserine and 1 mL of a 15.5 mg/mL stock of cefoxitin were also added.

Preparation of *C. difficile* spore stocks

Spore stocks of each *C. difficile* strain were generated for use in the cellular adherence assay and the experimental animal models of CDI. These stocks were generated by growing each strain on 5% TSAb plates incubated at 37^{o}C in anaerobic conditions for 7 days. Plate growth was collected in a 1X PBS solution containing 1% (V/V) Tween-80 (ST-80), and suspensions were washed 3 times in equal volumes of ST-80. Suspensions were incubated for 1 hour at 65 ± 2°C, washed with ST-80, and re-suspended in 4 mL of sterile nanopore water. Suspensions were then stored overnight at 4°C in order to promote the maturation of endospores for each strain. Spores were separated from vegetative cells and residual debris by density gradient centrifugation (10 minutes at 4,500 x *g*) with a 25% (W/V) HistoDenz solution. Spore pellets were washed 3 times with ST-80 and suspended in sterile nanopore water to a final volume of 2 mL. Spore stocks for each strain were stored at -80°C until used in *in vitro* or *in vivo* studies

Mouse *C. difficile* associated disease model

Female C57 BL/6 mice that were 7 to 8 weeks old were obtained from Charles River Laboratory and housed in sterile caging for the in-life portion of each study. Animals were randomly organized into groups of 20 and placed on drinking water supplemented with a cocktail of antibiotics immediately upon arrival. These antibiotics and their concentrations were: Kanamycin (0.4 mg/mL), Colistin (850 units/mL), Gentamicin (0.035 mg/mL), Metronidazole (.215 mg/mL), Vancomycin (0.045 mg/mL). Animals were left on the antibiotic supplemented water for 5 days, and then switched to normal water for 24 hours. Mice were orally inoculated with 1 x 10^6 *C. difficile* spores, and clindamycin was administered subcutaneously at 10 mg/kg of body weight. Starting the day of infection, and each day after, approximately 0.1 – 0.2 g of feces was collected from cages to determine *C. difficile* counts and associated amounts of toxin A and B. Bedding was changed daily to ensure fresh feces were collected for analysis,

and census of survivors were recorded daily for 14 days after infection. Sterile 1x PBS was added to the recovered feces, this solution was then homogenized, and 1 mL was separated for each total CFU recovery, spore recovery, and toxin A and B expression. Viable cell counts, spore counts, and toxin expression were quantified as described in the Material and Methods. The homogenized solution separated for spore quantification was heated to 65 ± 2^{o}C for 1 hour to facilitate the isolation of only spores, while the fecal matter separated for toxin expression was diluted approximately 100x - 500x for quantification. This allowed it to fall within detection range of the ELISA used to determine toxin concentration.

Hamster LD-50/Survival *C. difficile* associated disease models

Male Golden Syrian hamsters that were 6 to 7 weeks old were purchased from Envigo RMS Inc., and individually housed in sterile cages. Up to 30 hamsters were used in each study with 5 animals in each group that were orally inoculated with a designated spore titer of each strain. The animals were inoculated with 0.5mL of *C. difficile* spores from a spore preparation culture though oral gavage. The inoculation dose for all strains ranged from 800 – 30,000 spores/mL, and the exact titers chosen for each strain were based on previously conducted studies and observation of higher titers with non-epidemic and epidemic strains. Clindamycin was administered subcutaneously to each animal at 10 mg/kg per body weight approximately 24 hours after infection. Starting the day of infection, and each day after, approximately 0.1 to 0.2 g of feces was collected individually from each cage to determine *C. difficile* counts and associated amounts of toxin A and B. Bedding was changed daily to ensure fresh feces were collected for analysis, and census of survivors were recorded daily for 7 days after infection. Cecal fluid was collected from deceased hamsters for *C. difficile* enumeration and toxin A and B quantification Sterile 1x PBS was added to the recovered feces, this solution was then homogenized and 1 mL was separated for each total CFU recovery, spore recovery, and toxin A and B expression. Viable cell counts, spore counts, and toxin expression were quantified as described in the Material and Methods. The homogenized

solution separated for spore quantification was heated to 65 ± 2ºC for 1 hour to facilitate the isolation of only spores, and the fecal matter separated for toxin expression was diluted approximately 100x - 500x for quantification. Cecal fluid was processed identically to the fecal samples, with the exception that they were not homogenized. This allowed it to fall within detection range of the ELISA used to determine toxin concentration.

In vitro growth of *C. difficile* vegetative cells and spore formation

Plate growth of each *C. difficile* isolate was transferred into TGY-veg broth and anaerobically incubated at 37°C for 24 hours. TGY-veg associated growth for each strain was adjusted to an optical density of 0.1 (600nm) in either SM or TGY-veg broth, which were anaerobically incubated at 37°C. Samples from each broth culture were collected in triplicate every 24 hours through 72 hours of total incubation, and these samples were 10-fold serially diluted and plated onto Columbia horse blood agar. Additionally, a second sample from each culture were possessed for spore counts by incubating each sample in an equal volume of 200 proof ethanol for 30 minutes, and then incubating the samples at 65 ± 2ºC for 1 hour. The ethanol and heat-treated samples were centrifuged, washed with PBS, and the spore-containing pellets were suspended in a volume of PBS equal to the original volume of the sample. Ethanol and heat-treatment at 65 ± 2ºC were tested and sufficient to remove all viable vegetative cells during this stage. The spore suspension of each sample was 10-fold serially diluted and plated on Columbia horse blood agar supplemented with 0.1% sodium taurocholate. Both sets of plates were anaerobically incubated at 37°C for 48 hours and colony counts were used to calculate the vegetative CFU or spore counts per mL at each time point.

In addition to determining spore counts associated with each culture by counting the colonies recovered on agar media, the Schaeffer-Fulton endospore staining method was used to visually enumerate

spores associated in 72-hour cultures of each *C. difficile* isolate. This was done by generating heat-fixed smears of samples taken from each culture every 24 hours on glass slides and staining with 0.5% (W/V) malachite green as each slide was being steamed for 5 minutes. Slides were counterstained with Gram's safranin for 2 minutes in order to contrast vegetative cells from endospores and spores in each sample. The number of endospores and free spores were visually counted among 100 non-sporulating vegetative cells with a bright-field microscope at 1,000x total magnification, and the percentage of cells that had undergone sporulation was calculated for each *C. difficile* strain in triplicate at each 24-hour time point.

At the time of the viable cell quantification, 1.0 mL from the same sample vials were pipetted into 1.5 mL centrifuge tubes and centrifuged at 10,000 x g for 5 minutes. The supernatant was pipetted into a new 1.5 mL centrifuge tube and stored at -80°C until the quantification was performed.

Quantification of toxins

The levels of toxins A (TcdA) and B (TcdB) in fecal and culture samples were determined using an enzyme-linked immunosorbent assay kit purchased from tgcBIOMICS (Bingen, Germany). Samples were centrifuged at 10,000 *x g* for 5 minutes, and the recovered supernatants were diluted in kit supplied sample buffer. Toxin A and B concentration values for each sample were interpolated from standard curves generated for each toxin by non-linear regression analysis.

In vitro C. difficile adhesion assay

The Caco-2 cell line (ATCC HTB-37) and the C2BBe1 cell line were purchased from the ATCC. The Caco-2 cells were cultured in Eagles Minimal Essential Medium (EMEM) supplemented with 20% (V/V) fetal bovine serum (FBS), which was heat-inactivated, and 2 mM L-glutamine. The C2BBe1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 0.01 mg/mL human transferrin and 10% (V/V) FBS. Other than the use of different growth media, the cell lines were grown

and treated the same during the studies. The cells were grown at 37ºC in an atmosphere of 5% CO_2/95% O_2**,** and spent media was replaced every other day until the cells reached 80-90% confluency. Caco-2 or C2BBe monolayers were removed from the growth flask with trypsin and transferred into 12-well tissue culture plates, which were placed into an incubator for 2 days, 37ºC in 5% CO_2/95% O_2, to allow the cells to adhere to the wells.

To prepare for the assay, four aliquots of prepared *C. difficile* spore suspension of were washed twice by centrifugation and resuspended in PBS. For the adhesion assay, non-supplemented EMEM or DMEM replaced the medium currently in the wells containing the Caco-2 and C2BBe1 cells at least 1 hour prior to the assay, and *C. difficile* spores were seeded at a concentration of roughly 5 x 10^3 spores per well in triplicate. A negative control with PBS containing no bacteria was also added to additional wells in triplicate. Plates were incubated at 37ºC in 5% CO_2/95% O_2 for 3 hours. Plates were removed from the incubator and the wells were washed twice with 1x PBS then the Caco-2 cell monolayer was detached from each well by adding a 1% (W/V) trypsin solution and anaerobically incubating the plates for 5 minutes at 37ºC. The wells were, again, washed with 1x PBS, and the effluent was centrifuged at 8,000 x *g* for 5 minutes. Supernatants were discarded and each pellet suspended in 1mL of 1x PBS that was ten-fold serially diluted and plated onto Columbia horse blood agar. To enumerate spores the solution was plated on Columbia horse blood agar containing 0.1% sodium taurocholate.

Statistical analyses

Data were evaluated by One- or Two-way ANOVA with Tukey's post-hoc test or unpaired Student's t test. A p value ≤ 0.05 was considered statistically significant. Representation of survival rate against Log10 [daily dose]. LD50 values were calculated with the variable slope model ($Y=100/ (1+10^{((LogEC50 - x) * HillSlope))}$) (Curve fitting, Prism 8, Graphpad Software, La Jolla, CA) and were compared for

statistical significance using the extra sum-of-squares F test ($p \leq 0.05$). Analyses were performed using Prism 8 software (Graphpad Software).

Declaration

Ethics statement

Animal studies were conducted in accordance with protocols 2016-0015 and 2017-0002 approved by the Institutional Animal Care and Use Committee (IACUC) at the University of North Texas Health Science Center (UNTHSC). IACUC established guidelines ensuring that approved protocols are in compliance with federal and state laws regarding animal care and use activity at UNTHSC. The UNTHSC animal program in USDA registered (74-R0081) and fully AAALAC accredited.

Acknowledgments

We would like to thank Kiahrae Carter, David Valtierra and Phung Nguyen for all the technical support and advice they provided.

***Clostridium difficile* Isolates and Sources**

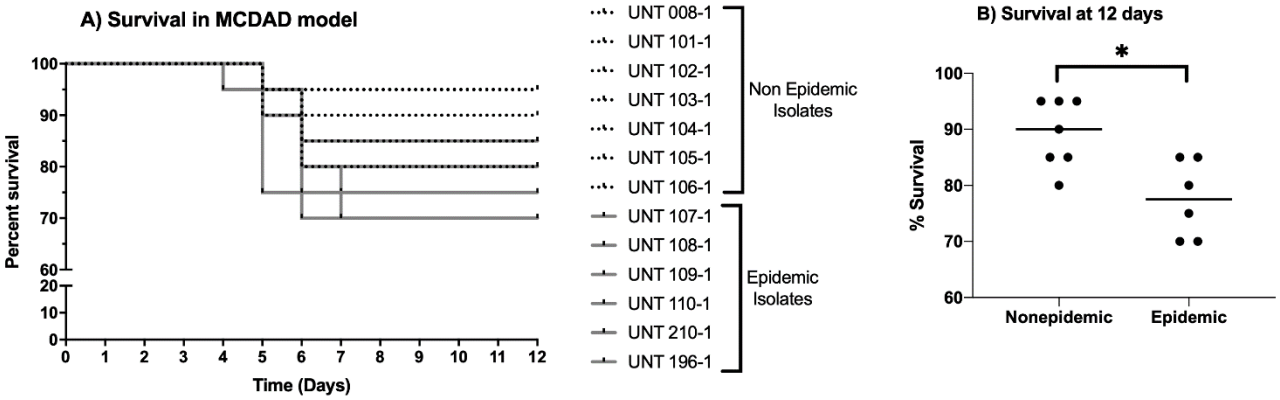
UNT Strain #	Species	Source	Relevant Characteristics
UNT 101-1	*Clostridium difficile*	Ohio VA Medical Center (Curtis Donskey)	Non-epidemic, Other Designation VA1
UNT 102-1	*Clostridium difficile*	Ohio VA Medical Center (Curtis Donskey)	Non-epidemic, Other Designation VA10
UNT 103-1	*Clostridium difficile*	Ohio VA Medical Center (Curtis Donskey)	REA J-type strain, binary toxin negative, non-epidemic, Other Designation VA 11
UNT 104-1	*Clostridium difficile*	Ohio VA Medical Center (Curtis Donskey)	Non-epidemic, Other Designation UH15
UNT 105-1	*Clostridium difficile*	Ohio VA Medical Center (Curtis Donskey)	Non-epidemic, Other Designation UH18
UNT 106-1	*Clostridium difficile*	Ohio VA Medical Center (Curtis Donskey)	(BI/NAP1, binary toxin positive, Ribotype 027; Epidemic), Other Designation VA5
UNT 107-1	*Clostridium difficile*	Ohio VA Medical Center (Curtis Donskey)	(BI/NAP1, binary toxin positive, Ribotype 027; Epidemic), Other Designation VA17
UNT 108-1	*Clostridium difficile*	Ohio VA Medical Center (Curtis Donskey)	(BI/NAP1, binary toxin positive, Ribotype 027; Epidemic), Other Designation VA20
UNT 109-1	*Clostridium difficile*	Ohio VA Medical Center (Curtis Donskey)	(BI/NAP1, binary toxin positive, Ribotype 027; Epidemic), Other Designation CC20
UNT 110-1	*Clostridium difficile*	Ohio VA Medical Center (Curtis Donskey)	NAP-1, Epidemic, Other Designation L32
UNT 196-1	*Clostridium difficile*	ATCC	BAA-1875 (Other Designation: 5325) Ribotype 078, Binary toxin positive, Toxinotype V PFGE tye NAP7, REA type BI 8
UNT 210-1	*Clostridium difficile*	ATCC	BAA-1870; Ribotype 027, Binary toxin positive, Toxinotype IIIb PFGE tye NAP1, REA type BI 8
UNT 008-1	*Clostridium difficile*	ATCC	Non-epidemic

2. Table 2. *Clostridium difficile* Strain Designation, Sources, and Characteristics

This table denotes the source of the individual isolates, other designations for each isolate, and some of the major characteristics associated with each of the isolates.

Figure legends

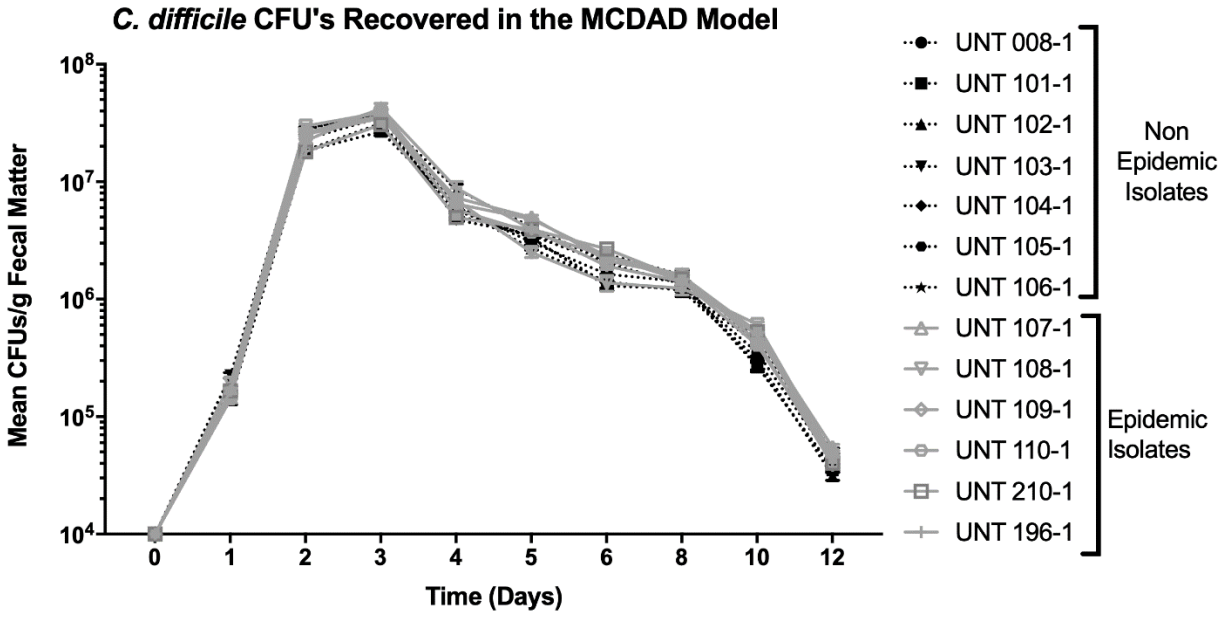
Fig 1



3. Figure 1. Mice infected with epidemic ribotype isolates had lower survival than mice infected with non-epidemic ribotype isolates

For each isolate, groups (n=20) were housed 5 to a cage and inoculated with approximately $1x10^6$ *C. difficile* spores. A) The non-epidemic ribotype isolates are denoted by black survival curves, and the epidemic ribotypes are denoted by gray. Survival was monitored for 12 days, and there were no additional deaths for any isolate after day 7. B) Percent survival at 12 days after infection. An asterisk denotes significant difference at $p \leq 0.05$ (Student's unpaired t test).

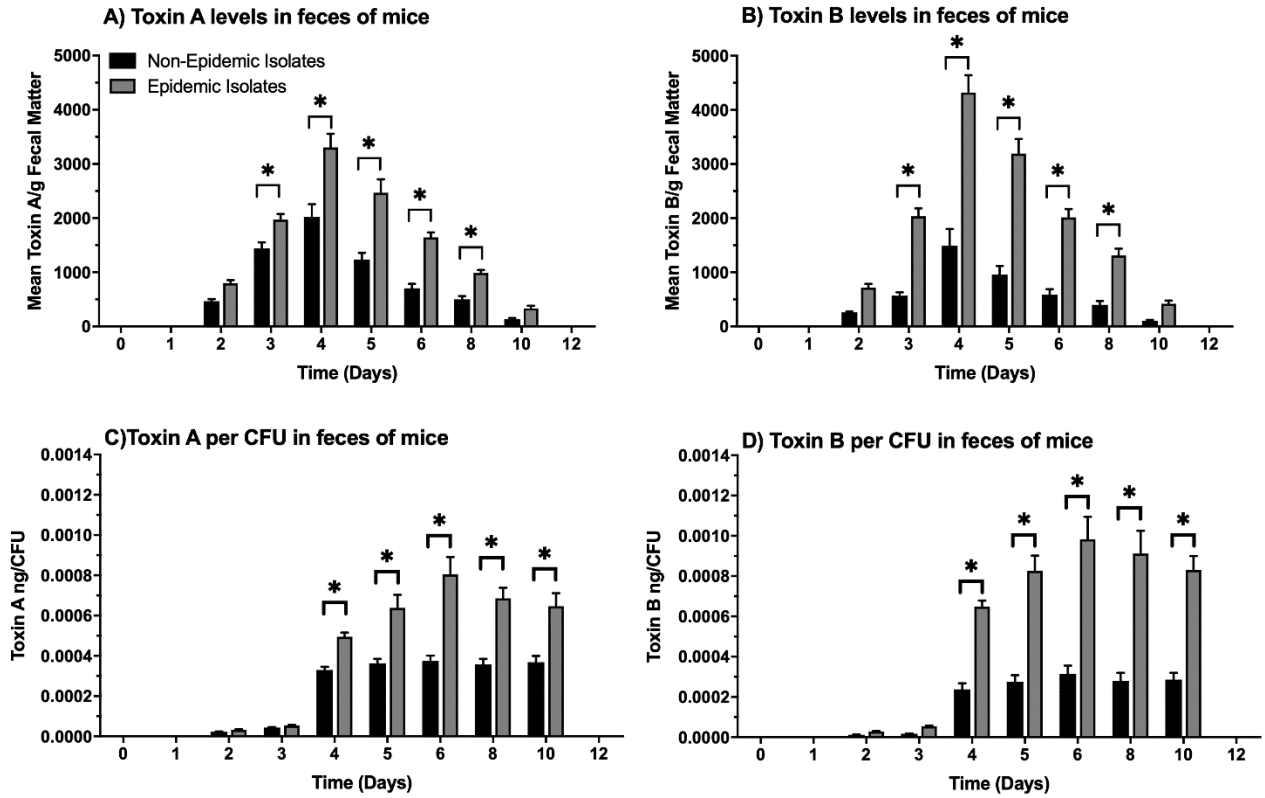
Fig. 2



4. Figure 2. *In vivo* fecal-associated CFU counts were not different between isolates

For each isolate, groups (n=20) were housed 5 to a cage and inoculated with approximately $1x10^6$ *C. difficile* spores. Fecal pellets were then collected, weighed, and processed to measure CFU counts throughout the study. Mean fecal counts were not significantly different between the non-epidemic and epidemic ribotypes, and CFU counts peaked 3 days after infection which declined until the end of the study. These data represent the average of four independent groups, and error bars indicate the standard errors of the means.

Fig 3

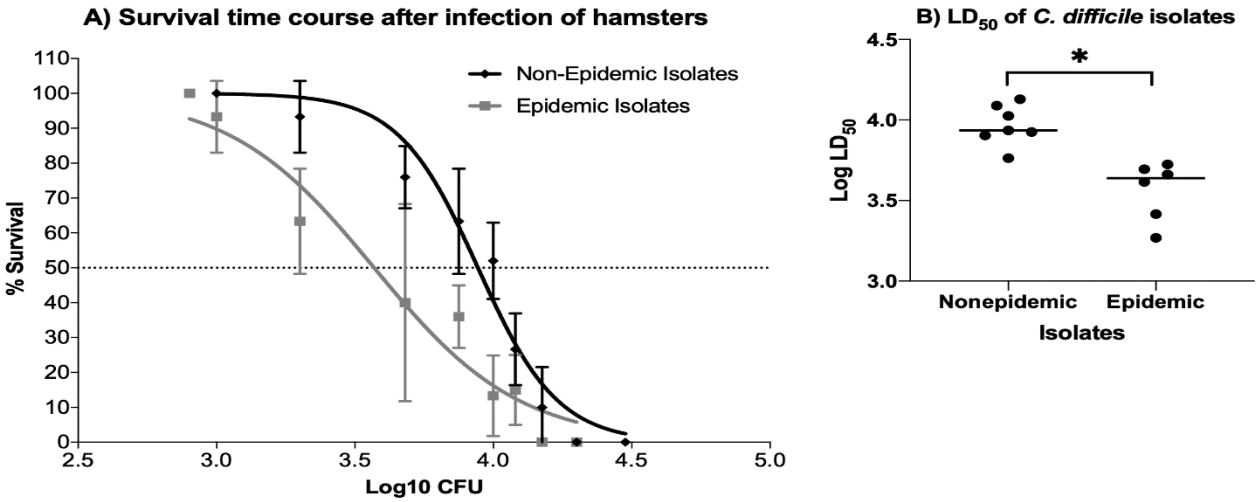


5. Figure 3. Epidemic ribotype infected mice had significantly more fecal-associated Toxin A and B than mice infected with non-epidemic ribotype isolates of *C. difficile*

For each isolate, groups (n=20) were housed 5 to a cage and inoculated with approximately $1x10^6$ *C. difficile* spores. Fecal pellets were then collected, weighed, and processed to measure Toxin A and B concentrations via ELISA. **(A)** Mean Toxin A titers per gram of feces that was collected from epidemic or non-epidemic ribotype infected mice on days 0 to 12 of the studies. **(B)** Mean Toxin B titers per gram of feces that was collected from epidemic or non-epidemic ribotype infected mice on days 0 to 12 of the studies. **(C)** Normalized mean Toxin A titers per CFU that was collected from epidemic or non-epidemic ribotype infected mice on days 0 to 12 of the studies. **(D)** Normalized mean Toxin B titers per CFU that

was collected from epidemic or non-epidemic ribotype infected mice on days 0 to 12 of the studies. These data represent the average of four independent groups, and error bars indicate the standard errors of the means. An asterisk denotes significant difference at $p \leq 0.05$ (Two-way ANOVA with Tukey's post-hoc test).

Fig 4

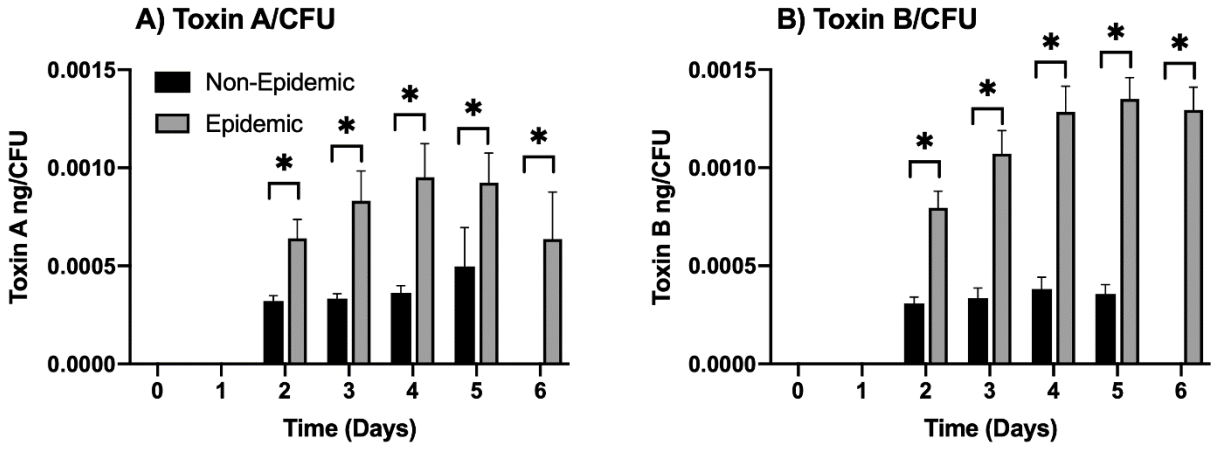


6. Figure 4. Epidemic ribotype isolates of *C. difficile* are more virulent than non-epidemic isolates in the hamster model of CDI

For each isolate, groups (n=5) were orally inoculated with a titration range of *C. difficile* spores as needed to define the LD_{50}. A) The graph compares the mean survival of each group inoculated with either non-epidemic or epidemic strains at specific $\log_{10}$ spore titers. Error bars represent the standard deviation of

mean survival percentages at specific spore titers, and average LD_{50} values were calculated for each group with the variable slope model (Y=100/ (1+10^(($LogEC_{50}$ – x) * HillSlope))) and were determined to be significantly different using the extra sum-of-squares F test ($p < 0.05$). B) The individual LD_{50} values for epidemic and nonepidemic ribotype isolates are shown. An asterisk denotes significant difference at $p \leq 0.05$ (Student's unpaired t test).

Fig 5

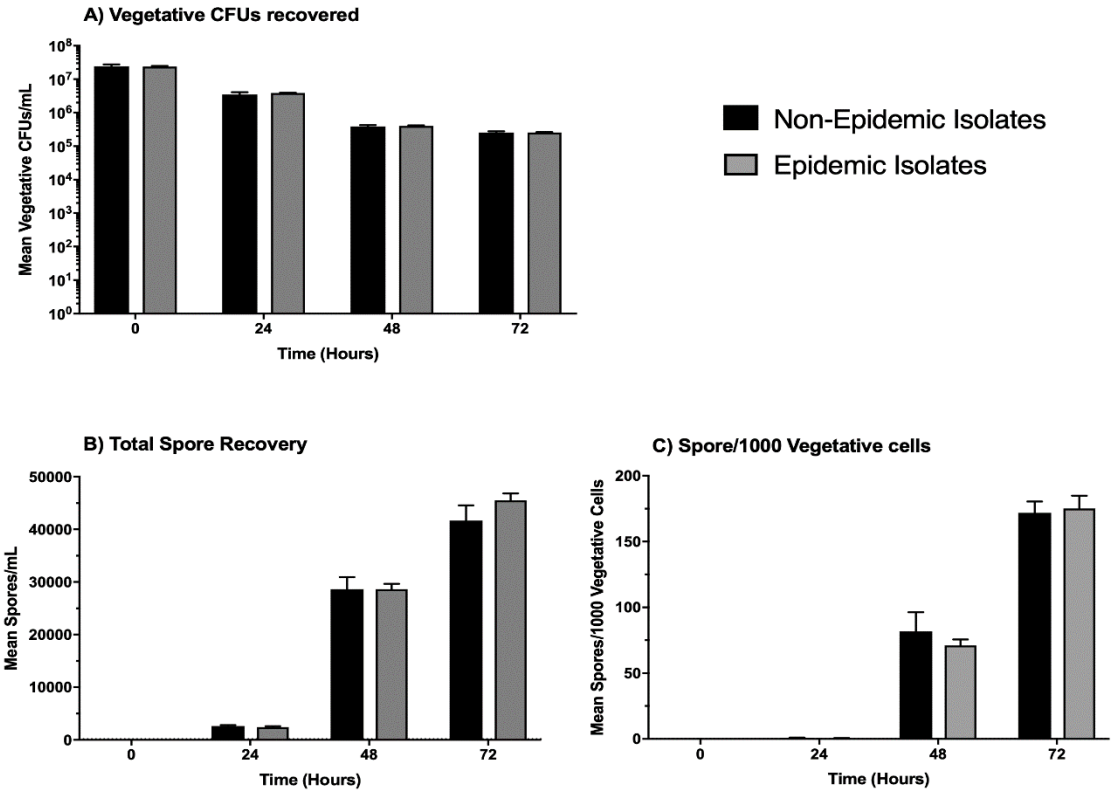


7. Figure 5. Fecal-associated Toxin A and B was significantly higher in hamsters infected with epidemic ribotype of *C. difficile* in the hamster CDI model

For each isolate, hamsters were split into groups of 5, housed individually, and orally inoculated with a specific titer of spores. Fecal pellets were collected every 24 hours, then weighed and processed for detection of Toxin A and B by an ELISA. Toxin levels were normalized to the numbers of CFU recovered. **(A)** Toxin A and **(B)** Toxin B levels were higher in hamsters infected with epidemic isolates. These data represent the average of 5 independent data points, and error bars indicated the standard error of the means.

Asterisks denote significant differences between toxin values at $p < 0.05$ (Two-way ANOVA with Tukey's post-hoc test; $p<0.05$).

Fig 6

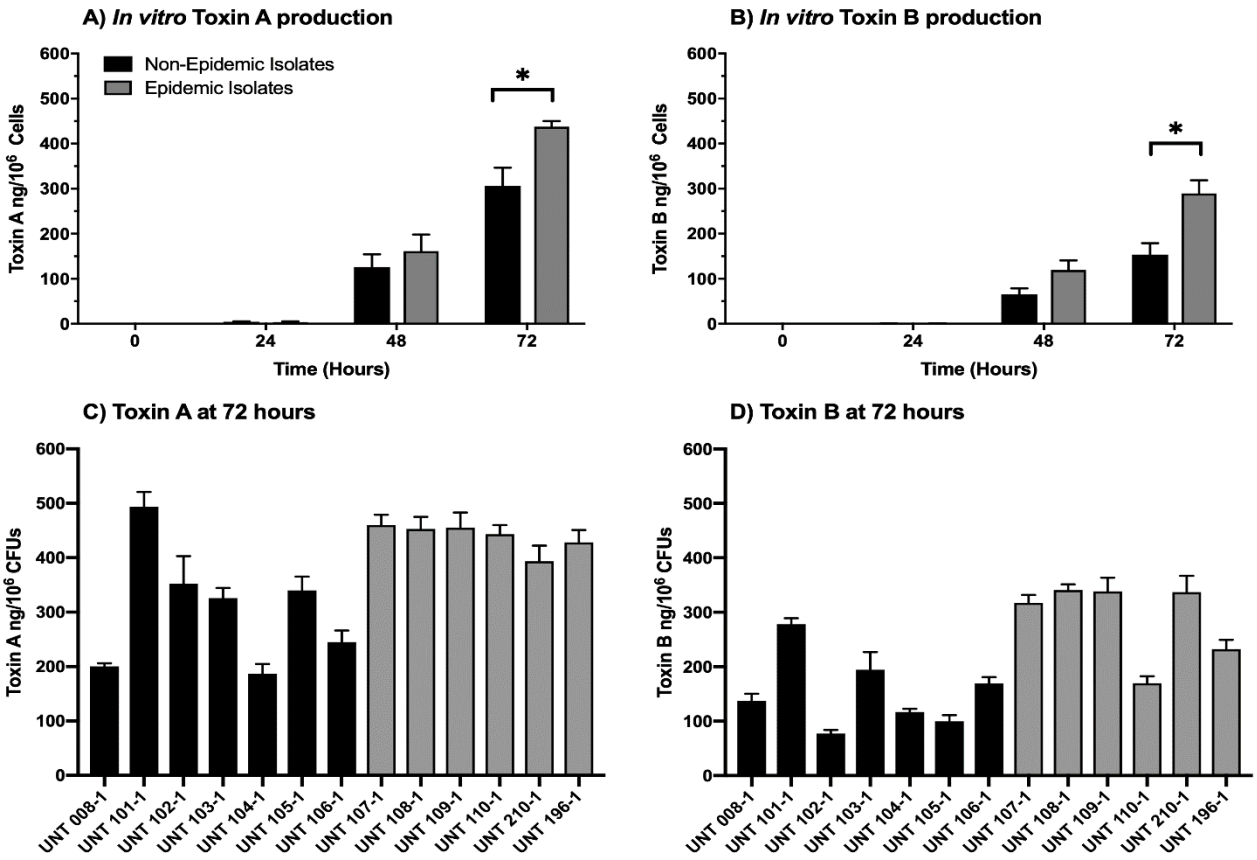


8. Figure 6. Mean vegetative CFUs and spore recovery between non-epidemic and epidemic ribotype isolates did not differ over 72-hours

The 13 isolates (7 non-epidemic and 6 epidemic) were incubated in SM broth over a 72-hour period. A representative sample was then taken from each culture and plated on an agar medium ± 0.1% taurocholate. The non-epidemic isolates are represented by the black bars, and the epidemic isolates are represented by the gray bars. This data represents the average of three independent experiments and error bars indicate the standard errors of the means. **A)** Mean vegetative CFU's recovered from 72-hour SM broth cultures. **B)** Mean spores/mL recovered from 72-hour SM broth culture. **C)** Mean number of spores

recovered from SM broth cultures normalized per 1,000 vegetative cells recovered at the corresponding time point.

Fig. 7

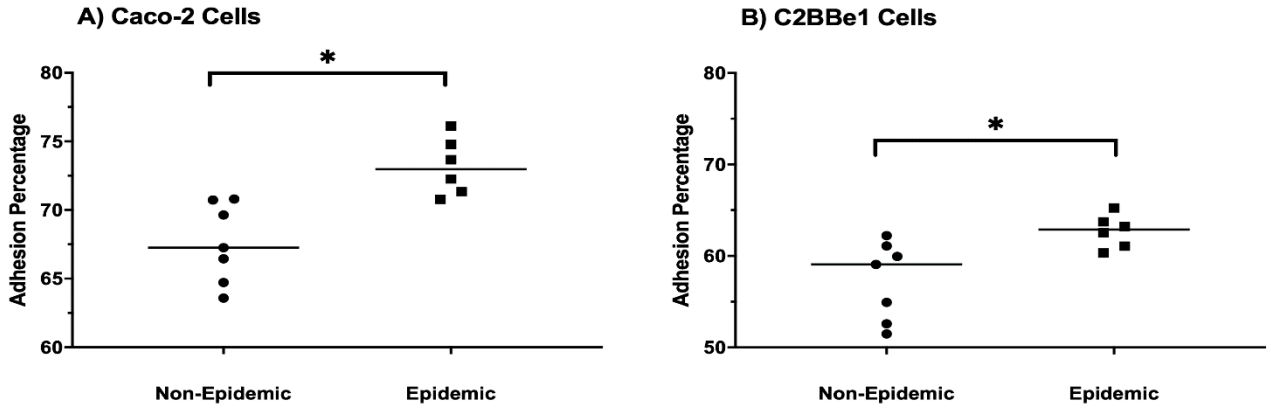


9. Figure 7. Normalized *in vitro* Toxin A and B production differs between non-epidemic and epidemic ribotype isolates at 72-hours.

The 13 isolates (7 non-epidemic and 6 epidemic) were cultured in SM broth over a 72-hour period. (A) Toxin A and (B) Toxin B production was determined from spent medium by ELISA and normalized per 10^6 vegetative cells recovered. (C) Toxin A and (D) levels at 72 hours in culture for each of the individual isolates are shown. Mean toxin titers for non-epidemic isolates are represented by the black bars, and mean toxin titers for epidemic isolates are represented by the gray bars. These data represent the average of

three independent experiments, and error bars indicate the standard errors of the means. An asterisk denotes significant difference at p <0.05 (Two-way ANOVA with Tukey's post-hoc test; $p<0.05$).

Fig. 8



10. Figure 8. Spores of epidemic ribotype adhere significantly different than those from the non-epidemic ribotype *in vitro* to Caco-2 and C2BBe1 Cells

C. difficile isolates (7 non-epidemic and 6 epidemic) were incubated with either Caco-2 or C2BBe1 cells for 3-hours, washed, plated and counted to determine the adhesion for each isolate. The non-epidemic isolates are denoted by the black symbols and the epidemic isolates by the gray symbols. **(A)** The isolates were incubated with Caco-2 cells and the mean adhesion percentages were determined as the percentage of spores bound after washing as compared to the original inoculum dose. **(B)** The isolates were incubated with C2BBe1 cells and the mean adhesion percentages were determined as the percentage of the spores bound after washing as compared to the original inoculum dose. These data represent the average of three independent experiments and error bars indicate the standard errors of the means, and a statistically

significant difference between each group at p <0.05 (One-way ANOVA with Tukey's post-hoc test; $p<0.05$).

CHAPTER IV

PRIMARY CARE CLINICS CAN BE A SIGNIFICANT SOURCE OF EXPOSURE TO VIRULENT *CLOSTRIDIUM difficile*: AN EVVIRONMENTAL SCREENING STUDY OF HOSPITALS AND CLINICS IN THE DALLAS-FORT WORTH REGION

The prior chapters demonstrated that epidemic ribotypes of *C. difficile* are more virulent than non-epidemic ribotypes, and this could be attributed to increased *in vivo* production of toxins A and B. It was also previously observed that epidemic ribotypes produced greater numbers of spores both *in vitro* and *in vivo* compared to non-epidemic ribotypes when treated with vancomycin. Spores are integral components for *C. difficile*'s ability to persist in oxygenated environments, and spread between hosts. Historically, hospitals have been associated with the greatest number of *C. difficile* cases, known as hospital-acquired *C. difficile.* Recently, cases contracted outside of hospitals have become an issue. These cases are known as community-acquired *C. difficile.* The location that CACD is contracted is still unknown, but it has been hypothesized that clinics may be a source contributing to CACD. The present study examined the contamination of different hospitals and clinics around Dallas-Fort Worth for *C. difficile* spores before and after education. It was found that both hospitals and clinics were equally contaminated, but after education the clinics lowered their frequency of contamination in comparison to hospitals. The ribotypes of the samples isolates from hospitals and clinics was determined to better understand if ribotypes can potentially be shared between the locations. It was determined that both hospitals and clinics had ribotypes that were unique to those locations, but there were also ribotypes shared between the locations as well. It

was also determined that approximately 90% of those isolates were capable of causing disease. These results suggest that clinics could be a source for community-acquired *C. difficile.* Interestingly, it was worth noting that during this study, epidemic ribotypes of *C. difficile* were not found as a large percentage of samples. This suggests that other factors studied and discussed in this dissertation may play a contributing role to why the epidemic ribotypes are epidemic within a geographical area.

Background

Clostridium difficile-associated disease (CDAD) is a leading cause of gastroenteritis associated deaths [94, 95] and becoming the most common cause of health-care associated diseases in the United States [95-97]. Health care costs due to *C. difficile* infections are estimated to be about $5 billion annually [1]. *C. difficile* is an endospore-forming pathogen that can affect the gastrointestinal system primarily in at-risk patients [2]. Both asymptomatic and symptomatic patients can shed *C. difficile* spores into the environment, which are resistant to desiccation, heat, and various disinfectants [98, 99]. The spores can reside in the gut of healthy individuals and not germinate while in the presence of an intact and healthy gut microflora. However, disruption of the gut microflora after antibiotic treatment can result in a permissive environment and germination of spores to vegetative cells. Production of exotoxins by vegetative *C. difficile* results in disruption of normal epithelial function and potentially severe diarrhea, leading to hospitalization and mortality, especially in elderly or immunocompromised patients.

Environmental contamination of health-care facilities with *C. difficile* spores is a major concern in the transmission of this pathogen. Hospitals are recognized as sites for exposure to this pathogen and subsequent development of disease [99-101]. However, community-acquired *C. difficile* disease occurs in patients who have not been recently admitted to health care facilities (i.e. within 90 days) and is becoming

recognized as a significant problem, despite an unclear source of acquisition [102, 103]. It has been suggested that outpatient care clinics are a significant source of community exposure to this pathogen [104], but further studies are needed to determine if primary care clinics have a similar potential for exposure of patients as found in the hospital environment.

Environmental and clinical isolates of *C. difficile* can vary in their potential to infect and cause disease. Virulence of *C. difficile* is linked to the expression of toxin genes [105], as well as known ribotypes used to identify epidemic/hypervirulent isolates [106-108]. The development of *C. difficile* disease is dependent upon the expression of toxins A (TcdA) and B (TcdB) [105, 109, 110]. These glycosylating toxins damage the intestinal epithelium, leading to inflammation and diarrhea. A third toxin, binary toxin (CDT), has also been identified in 17% to 23% of *C. difficile* strains, but it is currently unclear whether CDT plays a significant role in disease pathogenesis [86, 93, 111]. Additionally, there are multiple ribotypes of *C. difficile*, which are often identified in epidemiological studies for the assessment of environmental transmission and potential virulence. In North America, the most prevalent epidemic strain of *C. difficile* is BI/NAP1/027 or ribotype 027 [106, 108], but other ribotypes are also found as a cause of disease, including ribotype 078 [74, 112-115]. Importantly, there may be differences in virulence associated with different ribotypes of *C. difficile* [116]. In addition to clinical evidence, ongoing studies in our lab have shown that epidemic ribotypes, like 027, are more virulent in animal models than non-epidemic isolates (Manuscript in preparation). Thus, ribotyping in addition to the presence of toxin genes can be used to assess the potential virulence and epidemiology of *C. difficile* isolates.

To address the potential for primary care clinics as a source of environmental exposure to virulent *C. difficile*, we measured the frequency of environmental contamination with spores in clinic examination rooms and hospital rooms in Dallas-Fort Worth (DFW) area of Texas. Furthermore, the ribotypes and presence of toxin genes from a portion of the environmental isolates were determined and compared. In

addition, procedures to disinfect hospital rooms from environmental contamination with *C. difficile* spores can be inadequate [117], and it is possible that cleaning procedures can be improved in primary care clinics. Thus, we examined whether disclosure of the presence of spores impacted subsequent attempts to detect spores in the environment. In clinics, we not only provided this information but also included an educational discussion to emphasize the importance of this infection and methods of infection control to prevent transmittance of environmental *C. difficile*. Our results indicate that indeed primary care clinics have higher frequencies of contamination than that found in hospitals, but after notification and education, environmental contamination in clinics was reduced to that found in hospitals. Thus, primary care clinics can be a significant source of exposure to virulent *C. difficile,* and recognition of this possibility can result in improved infection control, potentially reducing community-acquired *C. difficile* infections and subsequent disease.

Materials and methods

Health care facilities

A total of 33 healthcare facilities in Texas were recruited to determine if *C. difficile* is detected through environmental sampling of patient/examination rooms. Three hospitals chose to withdraw from the study after the first sampling day because of their participation in a similar study. Out of the 30 remaining facilities, the 19 hospitals and 11 Family Medicine clinics were sampled (Table 1). Of the 11 clinics, 5 were located in rural areas making up 18.2% of the total healthcare facilities. Most of the sampling was done in the inner city of an urban area (36.4%).

Environmental samples of each facility were collected over two rounds, and within each round, each facility was visited three times with approximately one week between each visit. The hospital rooms were all on the general care floors and did not include intensive care units. For each visit, seven sites (exam

table/bed rails, doorknob, keyboards, light switches, restroom sink handles and faucet, toilet handles/pushbutton, and window blind wands/curtain) within a single patient/examination room were sampled. After the first round, each facility was provided the results of the environmental screening. For clinics, we also included an informational/educational session to increase awareness of the impact and problem with *C. difficile* infections after the first round of sampling. The hospitals elected to not participate in the additional education session. All facilities maintained their standard cleaning processes. The first round of sampling began on June 25th, 2014 and ended on August 22nd, 2014. The second round of sampling began on September 29th, 2014 and ended on November 24th, 2014.

Survey

A 16-question survey was completed by personnel at each site during the first sample collection (Supplemental material). The survey included questions about site specific cleaning practices/policies and patient demographic information. Specifically, sites were asked if they had guidelines for *C. difficile* infection prevention and what their cleaning practices were for each of the seven sites that were sampled for surface-associated contamination. An infection control nurse generally completed the survey for the hospitals; while, an administrative director generally completed the survey for the primary care clinics. Survey questions were developed by the research team.

Environmental sampling

Wet wiping with sterile cloths (Swiffer TM, Proctor and Gamble) were used to collect environmental samples in each of the healthcare facilities. Within a patient/examination room at a healthcare facility, there were seven sites sampled during each visit. These sites included: light switches, doorknobs, window blind wands/curtains, toilet handles/pushbutton, restroom sink handles and faucet, keyboards, and exam table/bed rails. When the rooms did not have restrooms located within the room, the nearest restroom was

used for sampling. The same procedure was used for keyboards if one was not located in the room. If there were no window blind wands/curtains in the room, then the opthalmoscope/otoscope handles or the chair in the room was sampled. In addition to these seven sites, a negative control was used to ensure that the sampler was not carrying *C. difficile* on their hands. Sterile gloves were worn during sampling and were changed between each sampling site.

Initial isolation and identification of environmental *C. difficile*.

Samples were processed similarly to a previous study [118, 119]. The bags containing the sampling cloths were transferred into a Don Whitley A35 or DG250 anaerobic workstation (Microbiology International, Fredrick, MD), and 30 mL of reduced brain heart infusion broth supplemented with 0.5% taurocholate (BHI-TA) was added into each bag containing the sampling cloth. After 5 days anaerobic incubation in the workstation at 37°C, 2 mL of the culture was removed and transferred to a sterile 2-mL Eppendorf tube. The sample was centrifuged for 5 minutes at 8,000x*g*, and the pellet was re-suspended in 2 mL of 70% ethyl alcohol for 1 hour and centrifuged again for 5 minutes at 8,000x*g*. The pellet was re-suspended in 0.2 mL of reduced BHI-TA and spread onto reduced cycloserine-cefoxitin-fructose agar plates (CCFA) containing 0.1% (w:v) sodium taurocholate, which is selective for growth of *C. difficile.* The plates were anaerobically incubated for 4 days at 37°C. As a positive control was included with each set of environmental samples, which included transferring 0.2 mL of prepared spores from *C. difficile* BAA-1875 to a sterile sample bag containing a PBS saturated Swiffer cloth, and the control sample was processed identically to all of the other samples. After each plate has been incubated for 4 days, they were inspected for *C. difficile* growth. Positive *C. difficile* growth was identified as colonies with spreading morphologies, irregular margins, and a yellowing of the medium that results from acid being produced with the fermentation of fructose by *C. difficile*. Colonies initially identified as *C. difficile* were expanded onto CCFA agar and tryptic soy agar (TSA) containing 5% sheep's blood and anaerobically incubated for

48 hours at 37°C. CCFA agar growth was evaluated for yellow-green fluorescence under long-wave UV light, and growth on blood agar plates was used in a *C. difficile* simple latex agglutination assay (Oxoid Ltd, UK). Based on the phenotypic results of these tests, all presumed isolates of *C. difficile* were assigned a strain number and stored in cryogenic cultures at -80°C.

C. difficile informational/educational session

C. difficile education session took place after the first round of sampling. An infection preventionist attended meetings with clinic staff either in person or by phone and used a PowerPoint® (Microsoft®) presentation to provide background information and the definition of *C. difficile* (Supplemental material). Signs and symptoms of disease were reviewed in the presentation along with general methods to avoid *C. difficile* such as washing hands with soap and water, placing patients in isolation, cleaning with bleach, and antibiotic stewardship programs. The education provided to the participating locations was not specifically aimed at cleaning for *C. difficile* but did reference established cleaning guidelines for each facility. All participants were given brochures and posters to distribute and display to help increase the public's awareness.

Ribotyping of isolates

The ribotype of the 39 *C. difficile* isolates, collected from various hospitals and clinics around Dallas-Fort Worth in the 2014 environmental study, was performed by PCR analysis [78]. Control strains for the *C. difficile* ribotypes 027 and 078, previously obtained from American Type Culture Collection (ATCC) and characterized in the Simecka lab, were also included in the study.

Primers 16S (5'-carboxyfluorescein (Fam) Dye-GTGCGGCTGGATCACCTCCT-3') and 23S (5′-CCCTGCACCCTTAATAACTTGACC-3′) (Thermo-Fischer Scientific) were used in capillary electrophoresis polymerase chain reaction (PCR) ribotyping. These primers were described by Bidet *et.*

al. [120]. DNA was extracted from cultures to a final volume of 20 µl using the High Pure Product DNA kit (Roche) according to manufacturer's instructions. Amplification reactions contained 5 µl of Buffer II, 1 µl DNTP's, 1 µl of forward and reverse primers as previous described, 31.75 µl water, 0.25 µl of Taq Polymerase, and 10 µl of sample DNA. Samples were amplified in a commercial PCR thermocycler running a 95 °C initial step for enzyme activation followed by 35 cycles of 1 min at 95 °C for denaturation, 1 min at 57 °C for annealing and 1 min at 72 °C for elongation, plus a 5 min 72 °C final elongation step.

PCR fragments were analyzed in a Hitachi 3500xL genetic analyzer with a 36 cm capillary loaded with a POP4 gel (Applied Biosystems). The size of each peak was determined using Peak Scanner software (Applied Biosystems).

Peaks in Bioanalyzer were counted as bands when they showed at least 5% of the height of the highest peak of the individual run. Double peaks were counted only if they were separated by more than 1.5 base pairs (bp). A web-based database (http://webribo.ages.at) was crafted for capillary gel electrophoresis-based PCR ribotyping results. An error margin of ±4 bp was incorporated into the analysis algorithm of the database. Using this web-based database, all users are able to enter their own data and receive a ribotype identification for each submitted isolate.

Detection of toxin A and B genes

PCR analysis was used to detect the presence of the toxin A (*tcdA*) and toxin B (*tcdB*) genes. For the toxin A gene, a set of oligonucleotides were utilized to amplify different regions of the toxin A gene found in *C. difficile*. Primers YT-28 (5′-GCATGATAAGGCAACTTCAGTGG-3′) and YT-29 (5′-GAGTAAGTTCCTCCTGCTCCATCAA-3′) were designed by Y.J. Jang et. al. (1998) to amplify a region of the toxin A gene. For the toxin B gene, primers NK-104 (5′-GTGTAGCAATGAAAGTCCAAGTTTACGC-3′) and NK-105 (5′-

CACTTAGCTCTTTGATTGCTGCACC-3′), as described by H. Kato et. al. (1998), were used to amplify a non-repeating portion of the toxin B gene. The DNA samples from the 39 isolates described above were amplified in 2.5 µl of Buffer II, 0.5 µl DNTP's, 0.5 µl of forward and reverse toxin specific primer pair, 15.5 µl water, 0.5 µl of Taq Polymerase, and 5 µl of sample DNA. Samples were amplified in a commercial PCR thermocycler running a 95 °C initial step for 2 minutes followed by 35 cycles of 45 seconds at 95 °C for denaturation, 30 min at 55 °C for annealing and 45 seconds at 70 °C for elongation. The PCR results were analyzed by Experion 1K DNA chips read on an Experion Bioanalyzer (Bio-Rad). The chips were set-up with the included reagents (DNA stain and DNA 1K gel, ladder, and loading buffer) and then ran according to included manufacturer's instructions. Isolates or control strains positive for the toxin A gene showed amplification at a size between 630-640 bp. Whereas, those that were positive for the toxin B gene showed amplification at a size between 230-240 bp.

Statistical analyses

Data were evaluated by Fisher's exact test or unpaired t test. A p value ≤ 0.05 was considered statistically significant. Two-tailed tests were used to determine whether there were differences between groups, while one-tail tests were used to determine if there was a reduced frequency of *C. difficile* after notification/educational information was given to the facilities. Analyses were performed using JMP 10 (SAS Institute, Cary, NC) or Prism software (Graphpad Software, La Jolla, CA).

Results

Infection control survey of clinics and hospitals prior to sampling for environmental *C. difficile*

Data were collected for 19 hospitals and 11 Family Medicine clinics. Of the clinics, 5 were located in rural Texas with 2 of those clinics serving a community with less than 2,500 people. Five (45.5%) of the 11 clinics were solo practices. Six (54.5%) clinics and 19 (100.0%) hospitals reported having a policy on

infection control; while, 5 (45.5%) clinics and 19 (100.0%) hospitals reported that they facilitate training/education to staff on infection control. Only 1 (9.1%) clinic said they have specific guidelines for *C. difficile* infection prevention compared to all 19 (100.0%) of the hospitals.

Responses to the question "How often does your clinic/hospital clean (disinfect) these surfaces" are provided in Table 1. Respondents reported that most hospital sites were cleaned every day or every week. The exceptions included 2 hospitals for which keyboards were cleaned every month and 1 hospital for which window blind wands/curtains were cleaned every 6 months. More variation in cleaning practices was reported for clinics. Exam tables/bedrails were never cleaned for 3 clinics, and doorknobs were never cleaned for 1 clinic. Additionally, light switches, window blind wands/curtains, and keyboards were never cleaned for 2 clinics.

Environmental contamination with *C. difficile* was common in clinics

Although hospital-acquired *C. difficile* infections are well recognized [100, 121], recent studies suggest that the presence of *C. difficile* spores within the environment of clinics is a source of community-acquired *C. difficile* infections [81, 121]. In our study, *C. difficile* environmental isolates were recovered more frequently from clinics than hospitals (Table 2). Environmental samples from 10 out of the 11 clinics tested had at least one positive sample, while 5 out of the 19 hospitals tested had *C. difficile* recovered from environmental samples. However, clinic and hospital facilities, where environmental *C. difficile* were recovered, had a similar percentage of positive samples (7.6%). Thus, clinics were a frequent source of potential exposure to *C. difficile*, even more commonly contaminated than hospital rooms.

In both clinics and hospitals, the primary sites where *C. difficile* was recovered included bed rails or examination bed and doorknobs (Table 3). In hospitals, restroom sink handles and faucets were also commonly contaminated; whereas in clinics, keyboards were a major site for *C. difficile* recovery. Thus,

it appears that locations in rooms that are often touched by hands of patients and staff are more often potential sources of transmission.

Increased education reduced the frequency of spore recovery in clinics

After the first round of environmental sampling, each of the healthcare facilities was provided the results of the testing. The facilities were offered an additional educational seminar for the staff that emphasized the impact of *C. difficile* (Supplemental information) on healthcare and the importance of infection control. The hospitals did not accept the offer for additional training; whereas, all of the clinics did. After the first round of sampling, notification of results, and educational seminar (if given), a second round of environmental sampling was performed. As in the first round, each of the facilities were sampled on three different dates and 7 sites within an empty patient or examination room were sampled during each visit, as done in the first round (Table 4).

There was a difference in the frequency of *C. difficile* positive rooms in clinics, but not hospitals after notification and/or education environmental contamination in these facilities. Prior to education and notification of culture results, *C. difficile* was found in 10 out of 11 clinics, but afterwards, only 5 out of the 11 clinics were positive. Thus, there was a significant reduction ($p \leq 0.05$) in the frequency of environmental contamination in the clinics. In contrast, there was no overall difference between the frequency of hospitals that were positive before (5 out of 19) and after (9 out of 19) notification of culture results, although there may have been an effect on specific hospitals. Interestingly, there was no difference in the frequency of environmental contamination between clinics and hospitals after the education of clinic staff.

Ribotypes and presence of toxin genes were similar in isolates from hospitals and clinics

Environmental isolates of *C. difficile* can vary in their potential to infect and cause disease. To further compare the isolates from clinic and hospitals, 20 different clinic isolates and 19 hospital isolates were characterized for their ribotype and presence of toxin genes. Both ribotyping and presence of toxin genes can be used to assess the potential virulence of *C. difficile* isolates, and by comparing those results among the environmental isolates, the likelihood of environmental contamination of clinics being a source of community-acquired disease can be evaluated.

Six out of 10 clinics had isolates with similar *C. difficile* ribotypes to that found in hospitals, indicating that a similar profile of isolates can be found in both hospitals and clinics (Table 5). Interestingly, ribotype 078 isolates were found in 3 clinic or hospital facilities. Importantly, *C. difficile* ribotype 078 was most frequently found in animals, but the 078 ribotype has been characterized as hypervirulent and increasingly found as a cause of CDAD in humans [18, 108, 122]. In contrast, 027 ribotype is an epidemic strain found in North America [73, 108], and only one hospital isolate, and none of the clinical isolates, was found to be this ribotype.

About 90% of the isolates from clinics and hospitals had the genes encoding either toxin A and toxin B. In one clinic (C016), two isolates (ribotype AI58) had the toxin B gene but not the toxin A gene, but other isolates from that clinic had both toxin genes. There were some isolates that did not have either of the toxin genes (1/20 clinic isolates; 2/19 hospital isolates). Two isolates (ribotype 413) from a hospital (H008) had neither toxin A nor toxin B genes, but other isolates from that hospital had both genes. Additionally, a single isolate (ribotype 413) from one clinic (C022) did not have either toxin gene as well. Thus, environmental isolates from clinics were similar to those found in hospitals, indicating a similar virulence potential. This suggests that clinics could be a potential source of exposure and subsequent development of community-acquired *C. difficile* infections in susceptible individuals.

Discussion

C. difficile is becoming the most common cause of health-care associated disease in the United States [95-97]. *C. difficile* is an endospore-forming pathogen that can cause severe gastrointestinal disease in some patients. Both healthy and symptomatic patients can shed *C. difficile* spores into the environment [123], which can survive for long periods, being resistant to desiccation, heat, etc. In healthcare facilities, environmental contamination with *C. difficile* is a major concern as this is a potential source of exposure to this pathogen and risk of disease in susceptible patients [124]. Although hospital-acquired infection is recognized [74, 81], community-acquired CDAD, i.e. absence of recent hospital admission, is also a potential health problem. Primary care clinics may be a significant source of exposure to this pathogen [74, 81], but there are limited data about presence of environmental *C. difficile* within clinics. To examine this possibility, the current study compared the presence of *C. difficile* in clinic examination rooms and hospital rooms in Dallas/Fort Worth area in Texas.

C. difficile was recovered from both clinic examination rooms and hospital rooms. During the first round of sampling, *C. difficile* was recovered more frequently from clinics than from hospitals. However, there was no difference in the percentage of samples between a "contaminated" clinic and hospital, indicating a similar level of environmental contamination. *C. difficile* was reported on the hands of nurses after handling a patient with *C. difficile* in previous research. In both clinics and hospitals sampled in the current study, the primary sites contamination included locations often touched by the hands of patients and/or staff, indicating a potential source of exposure to and spread of *C. difficile* [124]. Analysis of the ribotype distribution among the environmental isolates from clinics and hospitals show a commonality between the two types of health care facilities. As hospitals are associated with the spread of hospital-acquired *C. difficile* infections [100, 121], it is highly likely that clinics are a source of exposure to similar ribotypes of the pathogen. Different ribotypes may be more virulent than others [83] (Vittuci, J., Pulse, M. and

Simecka, J.W. Manuscript in preparation). Furthermore, the presence of toxin A and B genes in most of the isolates from either clinic or hospital environments is supportive that the isolates were of similar virulence potential. Based on these results and recognition that environmental exposure can lead to hospital-acquired infections [121, 124], there is clearly the potential for clinics to be a significant source of community exposure to *C. difficile* and subsequent development of disease from infection, especially in susceptible patients. Our study found that environmental isolates were more commonly recovered in clinics than in hospitals, further supporting the fact that clinics are probably significant sites of community exposure to *C. difficile.*

Most likely, infection control and cleaning procedures in clinics need to be reemphasized. The frequency of sites where *C. difficile* was recovered was compared prior to and after disclosure of the results from the first round of sampling of clinic and hospital rooms. In all of the clinic sites, an educational presentation emphasizing the importance of CDAD and infection control was given. Although hospitals were given this option, none accepted. As described above, primary care clinics had higher frequencies of contamination than that found in hospitals, but after the educational presentation, the frequency of *C. difficile* recovery in clinics was reduced to that found in hospitals. These results suggest that notification of environmental contamination and impact of CDAD influenced the efficiency of infection control procedures in clinics. However, *C. difficile* spores are resistant to heat, dehydration and many detergents [11, 76], and thus, in addition to improved hygiene practices by staff and clinicians, better cleaning procedures that inactivate *C. difficile* spores may reduce potential exposure to patients in clinics and hospitals.

Overall, clinics are likely a significant source of community-acquired *C. difficile* infection and subsequent disease. Clinics were a frequent source of potential environmental contaminants with *C. difficile*, even more often than in hospital rooms. The majority of the clinical isolates did have genes encoding the toxins

responsible for the pathology of *C. difficile* disease, supporting their potential to cause disease. Furthermore, many of the isolates were similar ribotypes to those found in hospitals, including known highly virulent ribotypes. There still may be differences in antibiograms of isolates obtained from hospitals and clinics, which would be consistent with the profiles of isolates from patients with hospital-acquired and community-acquired infections [125]. The current study indicates that better cleaning procedures should reduce environmental contamination of clinics, and that improved personal hygiene of healthcare workers would likely reduce the spread and contamination within health care facilities, including primary care clinics. Future studies examining the possible association of patients with *C. difficile* disease and visits to primary care clinics would provide further support about their role in impacting community exposure to *C. difficile*; however, the current study does provide compelling evidence that environmental contamination of virulent *C. difficile* are found in these clinics.

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3. Table 1. Results from survey question, "How often does your clinic/hospital clean (disinfect) these surfaces?"

Hospitals	**every day**	**every week**	**every month**	**every 6 months**	**never**	**missing**
light switches	15 (78.9%)	2 (10.5%)	0	0	0	2 (10.5%)
door knobs	15 (78.9%)	2 (10.5%)	0	0	0	2 (10.5%)
window blind wands / curtains	7 (36.8%)	6 (31.6%)	0	1 (5.3%)	0	5 (26.3%)
restroom commodes	18 (94.7%)	1 (5.3%)	0	0	0	0
sink handles	18 (94.7%)	1 (5.3%)	0	0	0	0
keyboards	15 (78.9)	1 (5.3%)	2 (10.5%)	0	0	1 (5.3%)
bedrails	17 (89.5%)	0	0	0	0	2 (10.5%)

Clinics

	every day	every week	every month	every 6 months	never	missing
light switches	3 (27.3%)	6 (54.5%)	0	0	2 (18.2%)	0
door knobs	3 (27.3%)	7 (63.6%)	0	0	1 (9.1%)	0
window blind wands / curtains	1 (9.1%)	3 (27.3%)	3 (27.3%)	0	2 (18.2%)	2 (182%)
restroom commodes	9 (81.1%)	2 (18.2%)	0	0	0	0
sink handles	9 (81.1%)	1 (9.1%)	0	0	0	1 (9.1%)
keyboards	3 (27.3%)	4 (36.4%)	1 (9.1%)	0	2 (18.2%)	1 (9.1%)
bedrails	3 (27.3%)	1 (9.1%)	0	0	3 (27.3%)	4 (36.4%)

4. Table 2. Prevalence of *C. difficile* in samples obtained from participating health care facilities prior to information sessions

Clinics			**Hospitals**		
Facility	**No. Positive Samples**	**Percentage (Out of 21 total samples)**	**Facility**	**No. Positive Samples**	**Percentage (Out of 21 total samples)**
C012	**1**	**4.76**	**H001**	**1**	**4.76**
C013	**4**	**19.05**	**H002**	**0**	**0.00**
C014	**1**	**4.76**	**H003**	**1**	**4.76**
C015	**3**	**14.29**	**H004**	**0**	**0.00**
C016	**1**	**4.76**	**H005**	**0**	**0.00**
C017	**1**	**4.76**	**H006**	**0**	**0.00**
C022	**1**	**4.76**	**H007**	**1**	**4.76**
C023	**2**	**9.52**	**H008**	**2**	**9.52**
C024	**1**	**4.76**	**H009**	**0**	**0.00**
C025	**0**	**0.00**	**H010**	**0**	**0.00**

C032	**1**	**4.76**	**H011**	**0**	**0.00**
			H021	**0**	**0.00**
			H026	**0**	**0.00**
			H027	**0**	**0.00**
			H028	**0**	**0.00**
			H029	**0**	**0.00**
			H030	**0**	**0.00**
			H031	**0**	**0.00**
			H033	**3**	**14.29**
	Frequency of Positive Clinics	**% positive samples from positive facilities[a]**		**Frequency of Positive Hospitals**	**% positive samples from positive facilities**
Summary	**10 out of 11 facilities***	**7.6 (5.1)**		**5 out of 19 facilities**	**7.6 (4.2)**

[a]Mean (± SD) of number of samples from individual facilities where *C. difficile* was recovered.

*There was a higher frequency of clinics where *C. difficile* was recovered than hospitals ($p \leq 0.05$, Two tailed Fischer's exact test).

5. Table 3. *C. difficile* prevalence by type of health care facilities and sampling site

	Type of health care facility		
	Clinics		
Sampling site	No. of samples tested[a]	No. positive samples	%
Exam table	33	6	18.18
Doorknob	33	2	6.06
Keyboards	33	5	15.15
Light switches	33	0	0.00
Restroom sink handles & faucet	33	1	3.03
Toilet handles/ pushbutton	33	1	3.03
Window blind wands/curtain	33	1	3.03
Subtotal	231	16	6.93
	Hospitals		
Sampling site	No. of samples tested	No. positive samples	%
Bed rails	57	0	0.00
Doorknob	57	2	3.51
Keyboards	57	1	1.75
Light switches	57	0	0.00
Restroom sink handles & faucet	57	3	5.26

Toilet handles/ pushbutton	**57**	**1**	**1.75**
Window blind wands/curtain	**57**	**1**	**1.75**
Subtotal	**399**	**8**	**2.01**

[a]Clinics had 3 samples per site per facility (11 clinics); hospitals had 3 samples per site per facility (19 hospitals).

6. Table 4. Prevalence of *C. difficile* in samples obtained from participating health care facilities after the information sessions and/or notification of results from first round of sampling.

Clinics			Hospitals		
Facility	**No. Positive Samples**	**Percentage (Out of 21 total samples)**	**Facility**	**No. Positive Samples**	**Percentage (Out of 21 total samples)**
C012	**0**	**0.00**	**H001**	**0**	**0.00**
C013	**1**	**4.76**	**H002**	**3**	**14.29**
C014	**0**	**0.00**	**H003**	**1**	**4.76**
C015	**1**	**4.76**	**H004**	**2**	**9.52**
C016	**4**	**19.05**	**H005**	**1**	**4.76**
C017	**0**	**0.00**	**H006**	**0**	**0.00**
C022	**7**	**33.33**	**H007**	**0**	**0.00**
C023	**0**	**0.00**	**H008**	**2**	**9.52**
C024	**0**	**0.00**	**H009**	**0**	**0.00**
C025	**0**	**0.00**	**H010**	**0**	**0.00**
C032	**1**	**4.76**	**H011**	**3**	**14.29**
			H021	**0**	**0.00**
			H026	**2**	**9.52**
			H027	**5**	**23.81**
			H028	**0**	**0.00**
			H029	**3**	**14.29**
			H030	**0**	**0.00**
			H031	**0**	**0.00**
			H033	**0**	**0.00**

	Frequency of Positive Clinics	% positive samples from positive facilities[a]		Frequency of Positive Hospitals	% positive samples from positive facilities
Summary After information sessions/notification	**5 out of 11 facilities***	**13.3 (12.8)**		**9 out of 19 facilities**	**11.6 (5.9)**
Summary (Table 1) Before information sessions/notification	**10 out of 11 facilities***	**7.6 (5.1)**		**5 out of 19 facilities**	**7.6 (4.2)**

[a]Mean (± SD) of number of samples from individual facilities where *C. difficile* was recovered.

*There was a lower frequency of clinics where *C. difficile* was recovered after the information session, than prior to these sessions ($p \leq 0.05$, One tailed Fischer's exact test). There was no difference found in the frequency of *C. difficile* recovery in hospitals due to notification of results.

7. Table 5. Ribotypes of environmental *C. difficile* isolates collected from clinics and hospitals

Type of facility	Facility	Common ribotype*	Unique ribotype
Clinics	C012		707
	C013	078	066
	C014		241
	C015	078	039
	C016	078, AI83	AI58
	C017		699
	C022	413	
	C023	063	441
	C024		AI60
	C032	552	
Hospitals	H001	552	
	H003	078, AI83	
	H004	AI83	027
	H005		626
	H007		001 ecdc
	H008	413	582
	H011	078	218
	H026	AI83	693
	H029	063	
	H033	078	

*Common ribotypes refer to ribotypes found in both clinics and hospitals, while unique ribotypes are those found only in one site.

CHAPTER V

DISCUSSION

The objectives of these studies were to: 1) offer insight into whether clinics are a potential source of community-acquired *C. difficile*; 2) determine if epidemic ribotypes are more virulent than non-epidemic ribotypes; and 3) help bridge the gap between *C.* difficile's microbiology and epidemiology to better understand whether prevalence or virulence is a more likely the reason for the persistence of the current epidemic ribotypes. These important findings appear to differentiate the *in vivo* behavior of current epidemic ribotypes from non-epidemic ribotypes, further understanding of what makes specific ribotypes more virulent *in vivo*, and help form a phenotypic foundation from which to predict and identify which ribotypes pose a threat of becoming epidemic isolates in the future.

Recent studies have shown that community-acquired *C. difficile* cases are being diagnosed at an equal or even slightly greater rate than cases of hospital-acquired *C. difficile* [81, 102]. It is still unknown where community-acquired *C. difficile* is contracted, but it has been suggested that clinics may be a contributor to community-acquired *C. difficile*. In our study, clinics tested positive for *C. difficile* at 10 of 11 locations, whereas only 5 out of 19 hospitals tested positive. The discrepancy between clinics and hospitals that tested positive for *C. difficile* was surprising, as hospitals have been thought to be the primary sites from which *C. difficile* was disseminated into the population. These results suggest clinics are a potential site contributing to community-acquired *C. difficile*, and that they are a potentially greater source of *C. difficile* exposure for the community than hospitals. In addition to sampling positive for *C.*

difficile, approximately 90% of isolates from clinics and hospitals contained genes for toxin A, B or both. This also supports the theory that not only do clinics harbor *C. difficile,* but the majority of *C. difficile* isolates from these facilities are potentially capable of causing disease. To further understand the contribution of clinics to community-acquired *C. difficile* over time, it would be beneficial to work with clinics in a specific area to sample for contamination with *C. difficile,* while also censusing how many of their regular patients have been diagnosed with *C. difficile* disease. After an initial time period, the implementation of techniques to improve cleanliness and sterilization of *C. difficile* spores should be taught and followed. Once this has occurred, comparing future contaminations of the facilities to the number of regular patients who are diagnosed with *C. difficile* disease can once again be performed. This would allow for a comparative study on the effects of contamination on the rate of patients who contract and present with *C. difficile*. This would not preclude the chance they were exposed to spores elsewhere, but it would be a good start in quantifying an impact that clinics may have on the growing number of community-acquired *C. difficile* cases.

Within our study, hospitals and clinics were observed to have unique ribotypes associated with one set of locations or another; however, it was unexpected that there was an approximately 50:50 split of shared to unique ribotypes between the clinics and hospitals. When compared as epidemic and non-epidemic groups, these isolates compromised approximately 15% of the total samples. Interestingly, it also was found that the number of epidemic ribotypes was not disproportionately larger than other non-epidemic ribotypes when considered on a ribotype-by-ribotype basis. Further studies are needed to compare the potential virulence of the environmental isolates found between hospitals and clinics. The hamster model of *C. difficile,* as utilized in the studies presented here, would be a potentially beneficial approach for assessment of disease capacity. Still, the small representation of epidemic ribotypes within the sampling suggests that increased prevalence in healthcare settings is not an adequate explanation for

the greater frequency of diagnosis in *C. difficile* cases associated with these ribotypes. Based on the studies presented here, there are two pathological reasons that could explain why epidemic ribotypes are so prevalent in *C. difficile* disease cases.

The first possibility is that the epidemic ribotypes have a lower inoculation threshold that is needed to cause disease within the host. The potential virulence of a *C. difficile* isolate or ribotype is often characterized *in vitro*, although there have been some *in vivo* studies characterizing virulence [7, 16, 28, 53, 70, 88]. Differing methods and results presented throughout i*n vitro* studies, *in vivo* studies, and a limited number of healthcare studies has led to debate over whether the current epidemic ribotypes are truly more virulent than other isolates, or if they are epidemic due to a larger frequency of diagnosis in *C. difficile* cases [7, 51, 53, 71]. To approach this question in a novel way, our lab utilized an LD_{50} hamster model of *C. difficile.* Using this model enables for a more translatable measurement of an isolate's virulence, provides a better overall picture of differences in virulence between isolates within an individual ribotype, and allows for grouped comparisons between epidemic and non-epidemic ribotypes. When compared, epidemic isolates had significantly lower mean LD_{50} values than the non-epidemic isolates. Thus, the epidemic isolates were significantly more virulent than non-epidemic isolates. Not only was this important in confirming that clinical isolates of epidemic ribotypes are more virulent than clinical isolates of non-epidemic ribotypes, but also shed light on the requirement that a host be inoculated with fewer spores from epidemic isolates in order for severe disease to be established. Additional ID_{50} studies are needed to further support the theory that a lower inoculation threshold is needed to cause disease, regardless of severity.

The second possibility is that the increased virulence that has been associated with the epidemic ribotype helps promote increased disease severity, including damage to the intestinal epithelium; this in turn increases the possibility of spores shed into the environment. It is worth noting that this model did

not elucidate a definitive reason why virulence was increased for the epidemic group. However, it was observed that this group produced increased levels of toxin A and B when compared to the non-epidemic group. This increase in toxin production was also observed within the mouse model, and was correlated with decreased survival in mice infected with the epidemic isolates. To further study if increased toxin production is a definitive reason for a rise in mortality within the mouse model, future survival studies detailing histological assessments of the intestines and weight loss throughout the course of infection would be important. If increased toxin production is associated with increased damage to the intestinal epithelium, this does support the theory that there could be increased shedding of spores into the environment due to increased symptoms associated with the disease. If this is found to be the case, understanding potential mechanisms underlining the increased toxin production could be important to help predict other ribotypes that could be candidates for increased virulence and potential future epidemic ribotypes.

It is known that toxin production is an integral factor for the establishment and persistence of *C. difficile* disease, and toxins A and B are the major cause of damage to intestinal epithelium [31, 48, 93]. A potential explanation and future avenue of study for understanding the increased toxin production observed here is a mutation known to occur in the *tcdC* gene. Studies have shown *in vitro* toxin levels increase within isolates that carry this mutation in the *tcdC* gene, a negative regulator of toxin A and B production [50, 54]. This mutation renders the isolate unable to downregulate toxin production as the strain moves into the stationary phase or it faces environmental stress. However, it has been shown that not all epidemic isolates carry this genetic mutation. Those that do not are still able to produce greater quantities of toxin *in vivo* compared other non-epidemic ribotypes [53]. Currently, we have the sequenced genome data for 12 of the 13 isolates used in this study, and analysis of this genome data could be useful in determining the prevalence of this mutation or revealing other differences in the pathogenicity locus

between the isolates. Another use for the genomic data would be to identify whether any of the isolates studied here carry the gene for the binary toxin, TCD. Though not sampled for in our studies, this binary toxin is known to be produced by some strains of the epidemic ribotypes [60, 93]. Though the role of the binary toxin is not fully understood, it is not essential for disease pathogenesis. However, a recent paper has shown that, in mice, the binary toxin has the potential to suppress an immune response at the location of infection, potentially causing additional damage to the epithelium though an immune-mediated response [93].

In our studies, it also was observed that *in vivo* toxin production was higher than *in vitro* toxin production. This lends credence to the hypothesis that metabolites exist in the *in vivo* environment that are not found *in vitro*. One example of this is provided by metabolites normally utilized during quorum sensing and observed to have effects on toxin production within *C. difficile* [126, 127]. Other studies have shown that readily available glucose or a plethora of proline or cysteine within an environment also can have a regulatory role on toxin A and B production [128]. Therefore, when postulating why the epidemic isolate presents with greater virulence and increased production of some virulence factors, it is important to consider the interactions of the bacterium and the *in vivo* environment.

Lastly, it is plausible that the epidemic ribotype is able to produce more spores during disease, leading to more spores within contaminated locations. Though *C. difficile* disease is driven by toxin production, spores arguably perform some of the most integral roles throughout the life cycle of *C. difficile. In vitro* production of spores between epidemic and non-epidemic ribotypes did not significantly differ during our studies, and CFUs recovered during studies using the mouse models also showed no significant differences in recovery. However, in other previously conducted studies, we observed that isolates from the epidemic ribotype produced increased amounts of spores compared to non-epidemic isolates *in vivo* after hamsters were treated with the antibiotic vancomycin (chapter II, fig 1). This could

have direct impact within healthcare settings, as *C. difficile* disease is treated with the antibiotics metronidazole, vancomycin, or fidaxomicin. This could lead to larger subsets of spores from epidemic isolates being released into the environment from the host, increasing the likelihood of exposure and inoculation threshold needed to cause disease. Therefore, this could be a potential reason why there is a higher frequency of cases associated with the epidemic ribotypes, while recovery of isolates associated with the epidemic ribotypes from healthcare facilities were not a majority.

Still, as only a small subset of epidemic isolates was tested, this profile may not be representative for all epidemic isolates. Therefore, there may be other factors influencing why epidemic ribotypes are diagnosed in a greater frequency of cases. Our *in vitro* studies, as well as others, have identified a potential reason. It was observed that epidemic isolate spore adherence is greater than non-epidemic ribotype spore adherence to different subsets of colonic epithelial cells. This underscores the importance of developing and utilizing novel models, such as the LD_{50} model, to better understand how different virulence factors could impact *C. difficile* epidemiology. For example, when this is taken into account with the lower LD_{50} values of the same isolates, it reduces the importance of sheer spore production by an isolate and suggests the importance of other factors influencing *C. difficile* virulence. Greater adherence percentage and lower LD_{50} values synergize to produce a greater chance of the epidemic isolates reaching the threshold needed to allow for the development of CDAD, compared to a non-epidemic isolate, when a host is inoculated with "x" number of spores. This could also be a plausible explanation why the epidemic isolates are associated with greater numbers of *C. difficile* cases, even though they were seen to be only a partial percentage of the total number of ribotypes isolated from clinics and hospitals.

Though our study did not elucidate a reason for this increased adherence, a recent study has begun to identify how a difference in spore coat proteins contributes to adherence, infectivity, disease potential and severity. Due to the difficulty in genetically manipulating *C. difficile*, studies to understand how

different genes interact and influence different aspects of spores have been primarily completed by studying *Bacillus subtilis* [30, 129, 130]. However, recent studies have begun to work with *C. difficile* and identify important genes in spore production and important components that influence adherence and function [129, 130]. One such study demonstrated that cystine-rich proteins expressed in the exosporium are important in spore assembly and functionality. Recently, CdeC and CdeM have been identified as exosporium cystine-rich proteins for *C. difficile* [82]. When either *cdeC* or *CdeM* were knocked out, spore composition, functionality, or pathology were affected. Specifically, for *cdeC* defective spore coats, the ability to persist in harsh environments was compromised and adherence was lower than wild-type spores [82]. However, infection, fitness, and persistence were greater than in the wild-type *C. difficile* strain [82]. Therefore, this study, in conjunction with our adherence study, suggests that the composition of the exosporium potentially contributes to why the epidemic ribotype is more virulent than non-epidemic ribotypes, and why the epidemic ribotype is able to better adhere than other ribotypes during initial establishment of spores in the colon.

Overall, this work concludes that there is a likely link between *C. difficile*'s epidemiology and pathology, and that this link may partially explain how epidemic ribotypes are able to persist as epidemic. Factors such as the epidemic ribotype's ability to produce severe disease with a lower inoculation dose, increased toxin production, increased ability for spores to adhere, and increased spore production when treated with antibiotics, are likely to enhance its ability to persist as the epidemic ribotype over other ribotypes. Though it is probable that these factors play a major role in this persistence, it is important to note that other factors in the *in vivo* environment also are likely to contribute to this behavior. This should be considered as the epidemic ribotypes continue to be studied, as future hypervirulent ribotypes are characterized, and as novel treatments are developed to combat *C. difficile*. However, it is strongly believed that the findings presented here are imperative to understanding why the current epidemic

ribotype is epidemic, and what characteristics potential future epidemic and hypervirulent ribotypes are likely to exhibit.

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