OPTIMIZING DETECTION AND CONTROL OF *CLOSTRIDIUM DIFFICILE* AND ITS TOXINS

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by

Michael P. Shilling

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Dissertation written by
Michael P. Shilling
B.S., Kent State University, 1981
Ph.D., Kent State University, August 2013

Approved by
_______________________________, Chair, Doctoral Dissertation Committee
Christopher J. Woolverton

_______________________________, Members, Doctoral Dissertation Committee
John Johnson, Ph.D.

_______________________________
Mary Russell, Ph.D.

_______________________________
Kenneth Rosenthal, Ph.D.

_______________________________
Roger Gregory, Ph.D.

Accepted by
_______________________________, Chair, Department of Biomedical Sciences
Eric M. Mintz

_______________________________, Associate Dean, College of Arts and Sciences
Raymond Craig
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Chapter I

General Introduction to Clostridium difficile

Dissertation Abstract

Clostridium difficile infection (CDI) is a bacterial disease affecting the lower gastrointestinal tract of patients whose normal colonic microbiota are altered, generally by administration of antibiotic therapies. C. difficile produces toxins that cause severe diarrhea, with potentially fatal complications in the immunocompromised. CDI has spread unabated despite the best prevention efforts of clinical practitioners. This dissertation is a broad-based study of several factors of importance in prevention and control CDI.

In clinical environments, transport media are used to maintain the integrity of clinical samples for later laboratory testing. A transport medium for enteric bacteria was assessed as a preservative in outpatient fecal samples submitted for CDI testing. The transport medium used preserved C. difficile toxin out to five days. The possible effect of fecal pH and trypsin content on toxin stability was investigated. Fecal pH was ruled out as a factor due to CDI selected samples tending toward neutral pH. Trypsin degraded toxin in controlled experiments, although results varied using clinical samples.
Oils and fatty acids, including virgin coconut oil (VCO), have antimicrobial effects on a variety of human pathogens. Use of natural products like VCO may reduce or prevent CDI by killing *C. difficile* while preserving the protective bowel flora. Virgin coconut oil (VCO) and three of its constituent fatty acids were evaluated for their toxic effect on *C. difficile* and were found to have bactericidal activity, the most potent of which was lauric acid.

The outer surface of *C. difficile* spores is thought to contain proteins that are critical for attachment of the spores to surfaces, including human hands. This strong attachment assists in transmission of infectious spores; however, the source of this strong attachment in the spore remains unknown. Transmission electron micrography shows that *C. difficile* lacks a true exosporium, rather, they are coated in the remains of the mother cell. Results from subsequent fluorescence microscopy and ELISA with antibodies raised against spore coat proteins confirm that this residue is not part of the spore coat and can be removed using dithiothreitol, hydrogen peroxide and proteinase k, increasing anti-coat antibodies binding to the spore coat.

*Clostridium difficile*

*Clostridium difficile* is a member of the family *Clostridiaceae* and the genus *Clostridium*. The genus is described as obligate anaerobes: spore forming, Gram positive bacilli. The genus contains over one hundred fifty species, with several considered to be pathogenic to animals and humans (Woo, Lau et al. 2005). These pathogenic species
have been well characterized and are noted for their production of exotoxins which are some of the most potent known to man (Johnson 1999).

Vegetative cells of *C. difficile* are typically 2 to 8 µm in length and 0.5 µm in width when grown on blood agar plates at 37°C (Stubbe, Berdoz et al. 2000). Most strains of *C. difficile* are motile by peritrichous flagella which, in addition, are thought to aid in adherence to colonic mucosal cells (Twine, Reid et al. 2009). Subterminal, non-bulging spores are formed by the vegetative cells in response to declining nutrients or stress, and measure 2.0 µm in length and 1.1 µm in width (Panessa-Warren, Tortora et al. 1997). The spores of *C. difficile* are highly resistant to most sanitizing agents and environmental cleansers (Vonberg, Kuijper et al. 2008). These resistant spores are the key to the spread of *C. difficile* infection (CDI) from patient to patient (Vonberg, Kuijper et al. 2008).

Colonies of *C. difficile* are typically large, gray, flat, and spready, with a “ground glass” appearance when cultured anaerobically at 37°C. The colonies also display fluorescence under ultraviolet light; yellow when growing on cycloserine, cefoxitin, fructose, and egg yolk agar (CFFA), and chartreuse when growing on blood agar plates after 48 hours of incubation (Stubbe, Berdoz et al. 2000). *C. difficile* also produces a characteristic odor resembling fresh horse manure due to its production of iso-valeric acid, iso-caproic acid and p-cresol (Levett 1984). Diarrheal samples from patients infected with *C. difficile* have been noted to have a similar horse manure smell (Bomers, van Agtmael et al. 2012).

**History of *C. difficile***

In 1935 *C. difficile* was first described when studying the normal fecal biota of newborn infants. Originally it was named *B. difficilis*, due to the fact that it was very
difficult to grow outside of anaerobic broth cultures. \textit{C. difficile} was isolated with such frequency from newborn feces that it was considered part of the infant’s microbiota. Crude cell-free supernatants of \textit{C. difficile} cultures were injected into laboratory animals proving that \textit{C. difficile} produced toxins that would affect animal models. These toxins produced by \textit{C. difficile} were considered non-pathogenic to humans, however, as newborns had no pathology associated with carriage of these toxin producing bacteria (Hall and O’Toole 1935). Not until 1978 did the discovery linking \textit{C. difficile} toxin to pseudomembranous colitis prove that \textit{C. difficile} was a human pathogen, capable of producing diarrhea, colitis and death (Bartlett, Chang et al. 1978).

Pseudomembranous colitis (PMC) was first described by Finney in 1893 (Bartlett, Chang et al. 1978) as a post-operative complication, and described in autopsies from 40 cases of enterocolitis by Penner and Bernheim in 1939 (Anand and Glatt 1993). PMC is recognized as a pseudomembrane (multiple yellow discrete patches) developing on the mucosal surface of the colon (Nelson, Auerbach et al. 1994), which microscopically reveals partially disrupted mucosal layer glands covered with fibrin and inflammatory polymorphonuclear leukocytes (Nelson, Auerbach et al. 1994). (Figure 1) The remnants of colonic glands may become necrotic and inflamed. The submucosa also becomes inflamed with increased vasculature and inflammatory cells responsible for water loss and subsequent diarrhea (Nelson, Auerbach et al. 1994). The pseudomembrane is considered a scab that forms over and replaces the normal colonic mucosa in response to bacterial toxins (Bartlett, Chang et al. 1978).
PMC was considered rare until the increased use of antimicrobial agents in the 1950s, when PMC became a common complication with high mortality rates (Bartlett, Chang et al. 1978). Originally, *Staphylococcus aureus* was implicated to cause PMC by the Gram staining of plaques. The stains revealed many Gram positive coccis in clusters (Bartlett, Chang et al. 1978). This was also supported by the knowledge of staphylococcal enterotoxins produced by *S. aureus* caused enteric disease, and clinical improvement with vancomycin treatment in patients suffering from PMC (Bartlett, Chang et al. 1978). Attempts using animal models infected with *S. aureus* failed to reproduce the affects that were occurring in humans, so other potential explanations of the cause of PMC were pursued (Bartlett, Chang et al. 1978). The true cause of these
Figure 1. Pseudomembranous colitis (A) Colonoscopy reveals multiple yellow pseudomembranes composed of fibrin, necrotic tissue and white blood cells. (B) Histology of colon biopsy stained with hematoxylin and eosin demonstrating neutrophils infiltrating the lamina propria along with erupting purulent exudates onto the surface epithelium. Complements of New England Journal of Medicine.
infections remained unknown, and would not be tied to *C. difficile* until two decades later when the link between *C. difficile* toxins A and B and PMC was discovered (Bartlett, Chang et al. 1978).

Anaerobic bacteria were linked to the formation of abscesses in the 1960s, and new antibiotics were developed to target them. Clindamycin was especially well suited for intestinal infections caused by anaerobes due to clindamycin’s excretion in the bile (Bignardi 1998). A common side effect of clindamycin treatment was diarrhea, which in some patients was quite severe and often progressed into PMC (Bignardi 1998). The association between diarrhea and clindamycin usage was strong enough to name it “clindamycin colitis.” This antibiotic-associated diarrhea was seen with other broad-spectrum antimicrobials, so the name was replaced with antibiotic associated diarrhea (AAD) (Bignardi 1998). Once *C. difficile* toxins were identified in patients suffering from colitis the connection between *C. difficile*, antimicrobial use and PMC was established (Bartlett, Chang et al. 1978).

When the connection linking antimicrobial use, PMC and *C. difficile* became known, researchers worked to identify a single toxin responsible for the diarrhea associated with *C. difficile* infection (CDI). First, toxin B was identified by neutralizing the toxin’s affect using *Clostridium sordelli* antitoxin. As researchers attempted to purify the toxin, a second toxin became apparent. Purification and animal testing of the individual toxins followed showing that toxin B was lethal, caused severe hemorrhage, disrupted villae, and caused mucosal edema in the cecum of laboratory animals when
injected intracecally. An equal amount of toxin A injected by the same route was not lethal in animal models, and resulted only in focal hemorrhage (Taylor, Thorne et al. 1981). Further studies of both toxins revealed that toxin A was responsible for the pathology of CDI. Purified toxin A, given intragastrically, resulted in a disease pathology similar to CDI, while purified toxin B given in the same manner to test animals had no effect. Toxin B at this time was thought to require the presence of toxin A to have an effect on test animals (Lyerly, Saum et al. 1985; Mitchell, Ketley et al. 1986). However, a toxin A negative, toxin B positive strain was implicated in a CDI outbreak in Canada in 1998, and these strains have been isolated with increasing frequency (Al-Barrak, Embil et al. 1999; Voth and Ballard 2005). Both toxins are now considered responsible for the pathology, although toxin A positive, toxin B negative strains have not been found to be naturally occurring. In 2009, a laboratory created toxin-A positive, toxin B-negative mutant strains showed reduced pathology in a hamster model, where pathology in hamsters caused by toxin A-negative, toxin B positive mutants were unaffected in severity. This evidence demonstrates that toxin B is essential for *C. difficile* disease, and synergy with toxin A may not be necessary (Lyra, O’Connor et al. 2009).

*Clostridium Difficile* introduction into the host

Any organism that wants to colonize an animal host must evade or penetrate the protective barriers of the host. *C. difficile* achieves this first as the spore form when it is ingested and traverses through the stomach, and is not adversely affected by the host’s stomach acid. In patients receiving proton pump inhibitors that increase the pH of the
stomach, spores and some vegetative forms may survive to reach the small intestine (Cunningham, Dale et al. 2003). Upon reaching the duodenum, spores are stimulated to germinate by the presence of bile salts and the amino acid glycine (Sorg and Sonenshein 2008). Once germinated and transformed into the vegetative forms, *C. difficile* uses various cell associated proteins to adhere to the intestinal mucosa. Without adherence, the vegetative forms would simply be excreted with the host’s feces. Strains known to possess flagella are more likely to penetrate the protective mucous coating, allowing them to adhere to the enterocytes and colonocytes in the intestines (Delmée, Avesani et al. 1990; Tasteyre, Barc et al. 2000). Surface layer proteins produced by *C. difficile* have also been reported to aid in adherence by providing strong adherence to mucosal cells and the underlying lamina propria (Calabi, Calabi et al. 2002). Once *C. difficile* has colonized an individual it can begin the process of producing toxins if conditions are appropriate (Voth and Ballard 2005).

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

*Clostridium difficile* infection is primarily attributed to the two large exotoxins produced in the colon: toxin A, an enterotoxin that disrupts mucosal epithelium, and toxin B, a cytotoxin that induces apoptosis in a wide variety of mammalian cells. Production of these toxins is linked to the stress from antibiotics and nutrient availability to *C. difficile* vegetative cells (Onderdonk, Lowe et al. 1979; Honda, Hernadez et al. 1983; Dupuy, Govind et al. 2008). When nutrients are plentiful, toxin production is inhibited; but if essential nutrients are lacking, particularly biotin, toxin production will
cause intestinal epithelial cells to rupture, releasing nutrients (Yamakawa, Karasawa et al. 1996). Limiting biotin to 0.05 nM in a defined medium was shown to increase toxin A production by 35 fold and toxin B by 64 fold compared to strains grown in biotin-rich environments (Yamakawa, Karasawa et al. 1996; Voth and Ballard 2005).

Some strains of *C. difficile* lack the ability to produce toxins, and are not considered pathogenic (Kelly, Pothoulakis et al. 1994; Hammond and Johnson 1995). Both toxins are high molecular weight toxins; toxin A is 308 kDa and toxin B is 279 kDa (Barroso, Wang et al. 1990; Dove, Wang et al. 1990; Borriello 1998). Surprisingly, both toxins are nearly 45% identical, have 63% similar amino acid homology, and are thought to have been created by a gene duplication event from a single toxin gene (Eichel-Streiber, Laufenberg-Feldmann et al. 1992). Both A and B toxins are comprised of four domains: a receptor binding domain; an enzymatic domain; an auto-proteolytic cleavage during toxin-processing domain; and a hydrophobic translocation domain responsible for transferring the enzymatic domain into the cytosol (Figures 3, 4) (Belyi and Aktories 2010). The catalytic domains of toxin A and B are nearly identical (Voth and Ballard 2005). The catalytic toxin domains act as glucosyltransferases interfering with actin polymerization in mammalian cells resulting in cell rounding and death (Bignardi 1998). The ability of both toxins to cause pathology in the gut was demonstrated in 2001 and 2003, when patients infected with strains of *C. difficile* that were negative for toxin A, but positive for toxin B, developed diarrhea and colitis (Pothoulakis and Lamont 2001; Savidge, Pan et al. 2003). The C terminal receptor binding domain differs between the two toxins, and allows the toxins to attach to different intestinal epithelial cell receptors:
toxin A to the apical surface receptors, and toxin B to the basolateral surface receptors (Jank, Giesemann et al. 2007). The genes that encode toxin A and toxin B are part of 5 genes that make up an area termed the Pathogenicity Locus, or PathLoc, described by Eichel Streiber’s group in 1996 (Braun, Hundsberger et al. 1996). Toxin A and B are encoded for by the genes tcdA and tcdB, respectively. Additionally, other genes are present to regulate toxin production: tcdR, (formally tcdD) a positive regulator, and tcdC, an inhibitory negative regulator (Figure 2). CodY represses toxin production as it is available when GTP and other essential nutrients are plentiful (Figure 4). Limited transcription produces small amounts of positive promoter TcdR. TcdC, a membrane associated protein, is produced which inhibits the transcription of tcdB, tcdE, and tcdA by sequestering TcdR at the cell membrane. When nutrients are scarce, during stationary phase of growth or in times of stress, CodY is limited, and TcdR promotes transcription of tcdB, tcdE, and tcdA, allowing for toxin production and release. The role TcdE plays in toxin activity is not completely understood. It is presumed TcdE serves as a holin function due to its homology to phage holin proteins, which allows for the release of toxins though C. difficile’s cell membrane and into the environment before the cell dies from lysis (Tan, Wee et al. 2001).

Both toxins are produced when nutrients are depleted or in response to antibiotic stress. The toxins enter cells by binding to their specific receptors (Figure 5 and 6). Toxin A binds to the disaccharide Galβ1-4GlcNac found on I, X and Y blood antigens expressed on several cell types, including the apical surfaces of enterocytes and colonocytes (Tucker and Wilkins 1991). The receptor for toxin B is currently unknown,
Figure 2. PathLoc diagram indicating transcription areas for the production of A and B toxins. Pathloc components: *tcdR* is the positive regulator for toxin production, *tcdB* codes for toxin B, *tcdA* for toxin A, *tcdE* presumptively codes for holing function, and *tcdC* codes for a negative regulator inhibiting toxin production. Adapted from Voth *et al.* 2005
Figure 3. **ABCD model of toxin A and B domains.** B represents the cell receptor binding domain. The hydrophobic D domain allows for penetration of the endosome membrane once inside the cell. The autocatalytic domain C frees the glucosyltransferase A domain into the cytoplasm. Adapted from Belyi 2010.
Figure 4. Regulation of toxin production during exponential (A) and stationary (B) phases of growth. A. CodY represses toxin production as it is available when GTP and other essential nutrients are plentiful. Limited transcription produces small amounts of positive promoter TcdR. TcdC, a membrane associated protein, is produced which inhibits the transcription of tcdB, tcdE, and tcdA by sequestering TcdR at the cell membrane. B. When nutrients are scarce or in times of stress, CodY is limited and TcdR promotes transcription of tcdB, tcdE, and tcdA, allowing for toxin production and release. Adapted from O’Conner 2009.
Toxin processing and activity on eukaryotic cells. Toxin A and/or toxin B bind to specific cell surface receptors and are taken in via clathrin mediated endocytosis. Once inside, lysosomes fuse resulting in acidic conditions within the endosome. The shift in pH allows for hydrophobic D domain to insert itself within the membrane. The A and C domains protrude into the cytosol where cellular InsP6 activates cysteine protease C domain to cut and releases the glucosyltransferase A domain. Cytosolic Rho GTPases are glucosylated by A domain where they become inactive allowing for the disruption of actin cytoskeleton. Inset. Rho proteins are bound to GTP by guanine nucleotide exchange factors (GEFs) where they facilitate actin polymerization. GTP hydrolysis by GTPase activating proteins converts Rho-GTP to Rho-GDP which is inactive. Reprinted with permission from Belyi and Aktories 2010.
although it has been located to the basolateral sides of intestinal epithelial cells and many other eukaryotic cells (Stubbe, Berdoz et al. 2000; Belyi and Aktories 2010). This may lead to a synergistic relationship between the two toxins: as toxin A disrupts the actin cytoskeleton, opening up the tight lateral junctions of colonocytes, and allowing toxin B access to the cells lateral receptors (Stubbe, Berdoz et al. 2000). Once bound to their specific receptors, the toxins are taken into the cell by clathrin-mediated endocytosis (Figure 5). The endosome then fuses with lysosomes resulting in acidic conditions within the endosome. The shift in pH allows for hydrophobic D domain to insert itself within the membrane. The A and C domains protrude into the cytosol where cellular InsP6 activates cysteine protease C domain to cut and releases the glucosyltransferase A domain (Henriques, Florin et al. 1987; Belyi and Aktories 2010). The internalized and released toxin affects the Rho and Ras superfamilies, especially the Rho, Rac, Ras and Cdc42 of proteins responsible for regulation of the cytoskeleton, formation of tight junctions between cells, and parts of the cell cycle (Voth and Ballard 2005). GTPases are glycosylated by transferring the glucose moiety of UDP-glucose to the threonine residue switch region on the GTPase, permanently transforming them into their inactive form. This change interferes with Rho, Rac, Ras and Cdc42 protein’s regulatory activity within the cell and has a significant effect on the cell’s cytoskeleton (Figure 6). The Rho family members play a key role in the regulation of the actin cytoskeleton, and the loss of its activity results in cell rounding. Tight junctions between mucosal epithelial cells are disrupted by the cell rounding in addition to the loss of Rho protein regulation (Nusrat, Giry et al. 1995; Voth and Ballard 2005). The loss of the tight junction barrier results in
increased fluid loss and diarrhea for the host. Inactivation of GTPases also is responsible for triggering apoptosis of the affected cells (Brito, Fujji et al. 2002; Voth and Ballard 2005).

*C. difficile* toxins have also been found to cause systemic effects in severe cases of CDI (Dobson, Hickey et al. 2003). Patients have been documented with cardiopulmonary arrest, acute respiratory distress syndrome (Jacob, Sebastian et al. 2004), multiple organ failure (Dobson, Hickey et al. 2003), renal failure (Cunney, Magee et al. 1998) and liver damage (Johnson, Samore et al. 1999). Hamm *et al.* studied the effect toxin B had on zebrafish embryos and found the toxin localized in the heart, where it damaged cardiac tissue and induced apoptosis (Hamm, Voth et al. 2006).

The *C. difficile* toxins go beyond affecting the GTPases of cells. The toxins can also induce damage to mitochondria, resulting in apoptosis as demonstrated by affected Chinese hamster ovary cells (He, Hagen et al. 2000; Pothoulakis and Lamont 2001; Brito, Fujji et al. 2002). Enteric neurons located in the submucosal and myenteric plexuses have been shown to release substance P after exposure to toxin A. Substance P promotes inflammatory response in the intestinal mucosa, including vasodilation, leading to fluid loss; activating the innate immune cells; and degranulation of mast cells (Mantyh, Pappas et al. 1996; Galli, Maurer et al. 1999).
*C. difficile* transferase a Clostridial binary actin ADP-ribosylating toxin

*C. difficile* transferase (CDT) is a third toxin (aka, binary toxin) that acts as an actin-specific ADP-ribosyltransferase; it was first described by Popoff *et al.* among a few *C. difficile* strains in 1988 (Popoff, Rubin *et al.* 1988; Perelle, Gibert *et al.* 1997; Stubbs, Rupnik *et al.* 2006). Other similar toxins are produced by other species: iota toxin produced by *Clostridium perfringens* (Stiles and Wilkins 1986), *C. botulinum* ADP-ribosyltransferase C3 (Aktories, Weller *et al.* 1987), and *Clostridium spiroforme* toxin (CST) (Simpson, Stiles *et al.* 1989). The CDT toxin structure is composed of two separate proteins encoded by genes *cdtA* and *cdtB*; where A is the enzyme component and B is the receptor binding and translocation component. The receptor binding portion oligomerizes into a heptamer which binds to a surface lipoprotein receptor. The enzymatic portion then binds to form the functional toxin which is then taken into the cell by receptor mediated endocytosis (Perelle, Gibert *et al.* 1997). Lysosomal acidification causes membrane fusion of the binding portion, forming a pore in the endosomal membrane, allowing the enzymatic portion to enter the cytosol. Translocation into the cytosol is aided by Hsp90. Following release, the enzyme causes ribosylation of monomeric actin, preventing actin polymerization, and resulting in the destruction of the actin cytoskeleton. Cells exposed to CDT toxin display depolymerization of F-actin, leading to cell rounding (*Figure 7*). In addition, formation of surface microtubules occurs in these cells that have been shown to increase adherence of *C. difficile* bacteria. This increased adherence to cell surfaces may facilitate colonization, adding to the virulence of these CDT producing strains (Schwan, Stecher *et al.* 2009). *C. difficile* strains that
Figure 6. **Toxins effect on mucosa.** As *C. difficile* vegetative cells grow in the gut, toxins A and B are released concurrently with spore production. Toxin A attaches to specific receptors on the apical surface of mucosal cells and is endocytosed into the cells interior. Endosome containing toxin fuses with lysosome, acidifying the interior, leading to release of the catalytic enzyme. The catalytic enzyme disrupts the function of Rho, Rac, and Cdc42 resulting in the depolymerization of the actin cytoskeleton and destruction of tight junctions. Toxin B now can access the cell’s basolateral receptors and other cells deeper into the lamina propria. Mucosal cells swell, release cytokines and die by apoptosis. White blood cells migrate to the damaged mucosa to become part of the pseudomembrane. Neurons release substance P, causing mast cells to degranulate, increasing permeability and fluid loss. Endospores stick to the mucosal lining and wait to germinate (adapted from Voth 2005).
Figure 7. Clostridium difficile transferase activity on eukaryotic cells. CDT the components are secreted by the bacterial cell. The receptor binding portion oligomerizes and binds to a surface lipoprotein receptor. The enzymatic portion then binds and is taken into the cell by receptor mediated endocytosis. Lysosomal acidification causes membrane fusion of the binding portion, forming a pore in the endosomal membrane, allowing the enzymatic portion to enter the cytosol. Translocation into the cytosol is aided by Hsp90.

The active enzyme causes ribosylation of monomeric actin, preventing actin polymerization, and resulting in the destruction of the actin cytoskeleton and induces the migration of basal bodies to the cell surface. Basal bodies form long microtubules projecting from the cell surface, increasing the adherence of C. difficile bacteria (adapted from Aktories 2012).
produce CDT toxin in addition to toxins A and B have been shown to be more virulent, especially the hypervirulent PCR ribotype 027 and 078 strains. Some *C. difficile* strains only produce CDT toxin without toxins A and B, so its presence does not necessarily indicate a hypervirulent strain (Stubbs, Rupnik et al. 2006).

**Other virulence factors**

*C. difficile* also produces additional virulence factors, including hydrolytic and proteolytic enzymes, fimbriae, and a capsule (Seddon and Borriello 1992; Borriello 1998; Janoir, Péchiné et al. 2007). Hydrolytic and proteolytic enzymes are thought to play a role in the breakdown of host tissues to aid in adherence and colonization of the colon. These enzymes may also be necessary to release essential nutrients for bacterial growth within the gut (Seddon, Hemingway et al. 1990; Janoir, Péchiné et al. 2007) Fimbriae have been demonstrated in about a third of *C. difficile* isolates to aid in adherence (Borriello, Davies et al. 1990). *C. difficile’s* polysaccharide capsule prevents opsonization by neutrophils, increasing its virulence in the host. In order for white blood cells to phagocytize these encapsulated organisms, complement or antibodies produced by the host must be attached to the bacterium (Dailey, Kaiser et al. 1987; Davies and Borriello 1990).

**Sporulation of *C. difficile***

*C. difficile’s* ability to produce infectious endospores aids not only its survival outside of its host, but also inside the host. Sporulation is induced when reproduction of vegetative cells fails from nutrient deprivation or undesirable conditions. The spores,
once produced, are metabolically inactive, making them resistant to antimicrobial
treatments, heat, radiation, desiccation and chemical treatments. Spores are resistant to
the majority of cleaning products used in healthcare facilities. Sporicidal agents must be
utilized to effectively eliminate spores from contaminated environments (Gerding, Muto
et al. 2008). The spore form of *C. difficile* allows for fecal oral transmission in healthcare
facilities, either directly from patient to patient, by fomite transmission, or by
transmission from the hands of health care workers (Sorg and Sonenshein 2008; Lawley,
Croucher et al. 2009; Underwood, Guan et al. 2009). Airborne transmission of spores has
also been studied, and may in the future require additional respiratory contact
precautions, similar to those used to prevent the transmission of airborne pathogens like
*Mycobacterium tuberculosis*. Researchers found spores to be aerosolized by flushing
toilets or changing bed sheets contaminated with feces from *C. difficile* patients (Roberts,
Smith et al. 2008; Best, Fawley et al. 2010; Donskey 2010).

Spores are produced by the millions in patients suffering from CDI, and
sporulation is increased in response to antimicrobial therapy. Diarrheal samples have
been documented to carry approximately $10^5$ spores per gram of feces (Jump, Pultz et al.
2007). *C. difficile* can survive in its dormant spore state for months on contaminated
surfaces; when it arrives in a new host with a suitable growth environment it will be
stimulated to germinate. The persistence of spores in the gut has been documented to
range from days to weeks following resolution of diarrhea. The spores are thought to
adhere to mucosal epithelial cells until they are shed (Sethi, Al-Nassir et al. 2010).
Spores present in the patient’s environment have been thought to be related to the high
levels of relapse and reinfection in successfully treated patients (McFarland, Elmer et al. 2002; Tang-Feldman, Mayo et al. 2003).

Prokaryote sporulation is an evolutionary advantage ensuring species survival. Spore formation allows cells to wait until environmental conditions improve where they can switch back to their vegetative form and continue growing (de Hoon, Eichenberger et al. 2010). The process of sporulation has been thoroughly studied in *Bacillus subtilis*; its process can be applied to *C. difficile*. Sporulation can be divided into seven distinct stages (Figure 8). The process begins with stage I, where the vegetative cell grows in size and replicates its DNA followed normally by cell division resulting in stage 0. Unlike normal binary cell division, during the sporulation process the cell becomes divided into two unequal parts called stage II. The larger compartment is considered the mother cell, and the smaller compartment the forespore. In stage III the forespore then becomes engulfed by the mother cell, continuing the process of producing a mature spore. In stage IV, peptidoglycan forms the cortex layer separating the two cells. Calcium dipicolinate synthesized by the mother cell is concentrated in the spore core, stabilizing the DNA. Diplocolinic acid and calcium contribute to spore resistance to heat and oxidizing agents (Setlow 2007). During stage V the outer protein coat is formed surrounding the outer membrane. The spore finishes maturation in stage VI, completing coat synthesis, dehydration, and lysis of the mother cell wall by the action of lytic proteins releasing the dormant spore (Paredes, Alsaker et al. 2005; de Hoon, Eichenberger et al. 2010).
Figure 8. Stages of Sporulation. Cartoon of idealized mode of sporulation taken from *B. subtilis* demonstrates the vegetative and sporulation cycles. Vegetative cells divide by binary fission under good growth conditions. Sporulation is induced by stressful conditions. Stages of sporulation: **Stage 0**, normal cell growth; **Stage I**, duplication of DNA; **Stage II**, asymmetric septation; **Stage III**, engulfment of forespore; **Stage IV**, cortex synthesis; **Stage V**, coat and exosporium synthesis; **Stage VI**, spore finishes maturation followed by mother cell lysis; and **Stage VII**, dormant free spore (adapted from de Hoon 2010).
The structure of *C. difficile* endospores differs from its vegetative cells by the presence of additional layers (Figure 9). The outermost layer is the exosporium, consisting of hydrophobic glycoproteins that have been shown to aid in adherence to mammalian cells in cell culture (Paredes-Sabja and Sarker 2012). Deep to the exosporium is the highly proteinaceous spore coat, protecting the spore from chemical and enzymatic attack. The outer membrane separates the spore coat from the cortex. The cortex facilitates water removal from the core, enabling the dehydrated core to resist high temperatures. Beneath the cortex is the germ cell wall, composed of peptidoglycan, which becomes the vegetative cell wall following germination. The inner membrane separates the germ cell wall from the core, and acts as a permeability layer, also protecting the core from chemical attack. Furthermore, the inner membrane contains germination receptors that signal to the core when conditions are appropriate to resume vegetative growth (Setlow 2007). At the center of the spore lies the dehydrated core composed of DNA, ribosomes, and a large amount of dipicolonic acid which keeps the spore dormant. Small acid soluble proteins are also present in the core, providing resistance to UV radiation (Setlow 2007).
**Figure 9. C. difficile Endospore multiple layers.** A. Cartoon: The core contains DNA and is enclosed by the inner membrane. The germ cell wall which is enclosed by the cortex. The outer membrane separates the cortex from spore coat. The exosporium is the outermost layer which aids in adherence to cell surfaces. Adapted from Setlow 2007. Note: layers are not drawn to scale. B. TEM of *C. difficile* 630 spore displaying multiple layers. Complements Lawley 2009. Scale bar 100 nm.
Germination of *C. difficile* spores

Spores of *C. difficile* are transmitted naturally via the fecal-oral route. The acid-resistant spores easily withstand transit through the stomach. Upon arriving in the duodenum, exposure to germinants; taurocholate, one of the bile salts, and glycine, an amino acid, trigger germination (Sorg and Sonenshein 2008; Paredes-Sabja, Setlow et al. 2011). Interestingly, if normal fecal microbiota are present in the gut, they will hydrolyze taurocholate to secondary bile salts. The secondary bile salts chenodeoxycholate and deoxycholate inhibit germination and vegetative growth of *C. difficile*. The removal of normal gut flora may be one of the key determinates for the colonization of *C. difficile* (Sorg and Sonenshein 2009). The vegetative form now can release its toxins and virulence factors to cause disease in the patient.

Hypervirulent *C. difficile* Strains

A change occurred in a *C. difficile* strain that converted CDI from isolated annoyances in health care to epidemic, and potentially fatal infections (Loo, Poirier et al. 2005; McDonald, Killgore et al. 2005; Redelings, Sorvillo et al. 2007). Different molecular typing methods are used to identify the strains that have been involved in outbreaks. Multi-locus sequence typing (MLST) compares gene location on the DNA strains (Griffiths, Fawley et al. 2010). Toxinotyping compares the genes on the PathLoc for variations; hypervirulent strains are designated as toxinotype type Roman numeral, such as III or V (Rupnik, Avesani et al. 1998). Pulse-field gel electrophoresis (PFGE) and polymerase chain reaction ribotyping designate hypervirulent strains, while restriction
nuclease analysis (REA) designate them as specific groups; for example BI (Janezic and Rupnik 2010). Acceptable typing methods differ by country; in the U.S. PFGE is common, in Europe ribotyping is the preferred method. Some variance in laboratory methods causes confusion when trying to compare outbreak strains in different countries. MLST, REA and PFGE are labor intensive, with subjective interpretation making interlaboratory comparisons difficult, whereas PCR ribotyping requires little labor and offers excellent comparisons between laboratories (Killgore, Thompson et al. 2008).

The strain identified as toxinotype III, North American pulsed-field type 1(NAP1) ribotype 027, and group BI by restriction endonuclease analysis, has been deemed hypervirulent (Cookson 2007). Its genome had been altered by a genetic mutation, leading to an 18bp deletion in the tcdC portion of the pathogenicity location. This truncated TcdC protein, normally responsible for negatively regulating the production of toxins A and B during the exponential growth phase of the NAP1 strain, is no longer functional. Researchers found that this change allows for excessive amounts of toxins to be produced. Compared to other toxin producing strains, NAP1/027 isolates produce 16 times the amount of toxin A and 23 times the amount of toxin B (Warny, Pepin et al. 2005). These high levels of toxin production have caused the morbidity and mortality associated with CDI to drastically rise (Loo, Poirier et al. 2005). This strain also possesses a binary toxin also known as C. difficile transferase (CDT), which has been shown to cause higher levels of colonization in the gut by increasing adherence to mucosal epithelial cells (Deneve, Janoir et al. 2009). In addition to releasing more toxins, NAP1 strains have been found to produce higher levels of proteolytic and hydrolytic
enzymes, further increasing the likelihood of gut colonization (Deneve, Janoir et al. 2009). Adding to the spread of this hypervirulent strain is an increase in sporulation (Akerlund, Persson et al. 2008). During an outbreak associated with the hypervirulent NAP1 strain that occurred in Montreal, Canada between 2002-2003, there was a 400% increase in patients reported with CDI, along with 244 deaths attributed to infection (Loo, Poirier et al. 2005).

A second hypervirulent strain of *C. difficile*, designated NAP8/078, is the predominate strain found in pigs and calves (Goorhuis, Bakker et al. 2008). Between 2005 and 2008, a fourfold increase of CDI was seen in human cases. Differing from the NAP1/027 strain, the affected patient group was younger and the disease thought to be community acquired (Goorhuis, Bakker et al. 2008). It is possible that the NAP8/078 strain was foodborne, as it has been isolated from a variety of food products intended for human consumption (Goorhuis, Bakker et al. 2008; Gould and Limbago 2010). The NAP8/078 strain has a different mutation identified as a 39bp deletion in the *tcdC* portion of the pathogenicity location, and most isolates also produce binary toxin (Jhung, Thompson et al. 2008). In addition all strains display variable resistance to clindamycin, erythromycin, and fluoroquinolones. This strain has also shown to cause more severe diarrhea, higher mortality, and greater relapse rates when compared to patients infected with common animal strains (Dawson, Valiente et al. 2009). Recently the number of human cases of CDI attributed to NAP8/078 has surpassed the amount NAP1/027 strain cases in the Netherlands demonstrating that these hypervirulent strains are continuing to evolve. (Hensgens, Goorhuis et al. 2009; Gould and Limbago 2010).
Epidemiology

*C. difficile* infection remains the number one cause of antibiotic-associated diarrhea, accounting for 10-25% of all cases (Bartlett 1994; Barbut and Petit 2001; Bartlett 2002). Carriage and colonization rates vary widely between patient groups. In the normal adult population, *C. difficile* asymptomatic carriage is estimated at 2-3%, but much higher in those exposed to healthcare environments (Barbut and Petit 2001). Starting at birth, newborns are highly susceptible to acquiring *C. difficile* as they have no protective normal gut flora to inhibit colonization. Acquisition of *C. difficile* in newborns is thought to be caused by the child’s mother flora or the newborn nursery environment (Tabaqchali, O'Farrell et al. 1984). Carriage rates in newborns born in hospitals have been demonstrated to be as high as 70% (Tabaqchali, O'Farrell et al. 1984; Bartlett 1994; Kato, Kato et al. 1994). Carriage rates are also higher in healthcare providers and high-risk patients that have been hospitalized or received antibiotics (Barbut and Petit 2001; Giannasca and Warny 2004). Approximately one-third of the long-term care facility residents asymptotically carry *C. difficile* (Simor, Bradley et al. 2002).

*C. difficile* in Animals

Human CDI is of great concern to health care professionals, but animal CDI is also a concern to animal owners and farmers, particularly with food animals which are often given antibiotics to prevent infections (Songer and Anderson 2006; Jhung, Thompson et al. 2008). Piglets have been shown to carry the 078 and 027 strains that are
identical to strains isolated from humans suffering from CDI (Songer and Anderson 2006; Debast, van Leengoed et al. 2009). Not surprisingly, cultures of raw meat contain C. difficile contamination rates as high as 62% in pork braunschweiger, and 50% in ground beef (Songer, Trinh et al. 2009).

Visitation dogs contacting patients in healthcare settings have been shown to carry C. difficile and may be involved in transmission (Lefebvre, Reid-Smith et al. 2009). Hypervirulent NAP1 strains were isolated from 5% of dogs living in CDI patient homes in Ontario (Weese, Finley et al. 2010), where close contact between animals and humans may be a mode of interspecies transmission (Arroyo, Kruth et al. 2005).

**C. difficile in Packaged Ready to Eat Salads**

In 2008, 40 prepackaged ready to eat salads from Glasgow, Scotland from 7 different manufacturers were assayed for C. difficile contamination; PCR and enriched culture methods were employed. Three samples (7.5%) were positive for C. difficile; one identified as PCR type 001, toxin A and B positive, and the other two as PCR type 017, which is toxin A negative, toxin B positive. Both strains are common in the United Kingdom, but the salads were not prepared there. Possible sources of contamination were from manure used as fertilizer, field workers, food processing equipment, and workers packaging the salads (Bakri, Brown et al. 2009).

**C. difficile in the Environment in Wales**

The presence C. difficile prior to the wide use of antibiotics would suggest that it has been present in the environment for a very long time. A large study sampling many
different locations found that *C. difficile* is ubiquitous in nature. Culture results
determined river water had the highest rate of contamination, with 87.5% of the samples
being positive for *C. difficile*. Culture results on swimming pool water were also high,
with 50% testing positive. Hospital environments were found only to be 20% positive for
*C. difficile*. Feces from dogs and cats also tested positive for *C. difficile*, with 10% and
2% being positive, respectively. Even 2.4% of 300 raw vegetables tested positive for *C.
difficile* contamination. Although transmission of *C. difficile* to humans from
environmental sources has not been documented, it has to be considered a possibility (Al
Saif and Brazier 1996).

**Risk Factors Associated with CDI**

**Antibiotic Exposure**

The key risk factor associated with development of CDI is treatment with
antibiotics. Greater than 90% of all CDI occur during or following treatment with
antibiotics (Barbut and Petit 2001). With the exception of aminoglycosides, almost every
other antibiotic class has some risk of progression from asymptomatic colonization to
CDI (Sunenshine and McDonald 2006). Exposure to antibiotics may quickly progress to
CDI, but infection may be delayed for up to 8 weeks after antibiotics are given (Kelly,
Pothoulakis et al. 1994; Johnson and Gerding 1998). However, most CDI cases involved
the use of clindamycin, fluoroquinolones, or third generation cephalosporins (Gerding
2004; Pépin, Saheb et al. 2005). Studies demonstrated that restricting the use of these
antibiotics could significantly decrease the healthcare acquired infections (HAI) due to *C.
difficile (Carling, Fung et al. 2003; Valiquette, Cossette et al. 2007). It is important for individual healthcare institutions to be aware of the sensitivity and resistance patterns of *C. difficile* isolates that are endemic to their environment and patient population. This is part of antibiotic stewardship program that has been encouraged to limit HAIs and the increasing resistance of bacterial pathogens (Dellit, Owens et al. 2007). If a facility’s isolates are resistant to clindamycin, then the use of clindamycin should be highly restricted (Pear, Williamson et al. 1994; Owens, Donskey et al. 2008). If one’s *C. difficile* organisms are routinely sensitive to a particular antimicrobial, then use of that antibiotic should be encouraged (Donskey 2010; McGowan 2012).

The use of broad spectrum antibiotics often will eliminate the pathogen causing disease in the patient, but collateral damage to susceptible protective fecal microbiota will also occur. This opens up the colonic mucosa for colonization by opportunistic bacteria such as *C. difficile*. Not only does antibiotic therapy remove beneficial microbiota, but it also stresses *C. difficile* into production of toxins and spores (Honda, Hernandez et al. 1983; Pultz and Donskey 2005; Lawley, Clare et al. 2009). In addition, fluoroquinolones have been shown to induce *C. difficile* spore germination (Saxton, Baines et al. 2009); where colonization gene factors were up-regulated in vegetative forms when exposed to ampicillin and clindamycin (Denève, Deloménie et al. 2008).

**Age**

Patients older than 65 years of age or with severe underlying illness are at very high risk of CDI (Bignardi 1998; Barbut and Petit 2001; Pépin, Saheb et al. 2005). This
may also be associated with more frequency of hospitalization, decreasing immune function, and an increased likelihood to be treated with antibiotics. Patients living in long-term healthcare facilities are generally older, exposed to increased antibiotics, and routinely given antacids (Sunenshine and McDonald 2006). The emergence of hypervirulent strains has shifted susceptibility of infection from primarily the elderly to all age groups (Kim, Smathers et al. 2008; Rouphael, O'Donnell et al. 2008).

**Immune Response**

The disease progression can vary in patients, depending on their immune response to CDI. Those patients that produce high antibody titers to toxin A usually only develop diarrhea, which resolves without reoccurrence; patients failing to produce adequate antibodies to toxins are at increased risk for complications and recurrent infections (Kelly 1996; Kyne, Warny et al. 2001). Asymptomatic carriers normally have high antibody titers to toxin A, and are unlikely to develop severe symptoms when exposed to antibiotics (Salcedo, Keates et al. 1997). Antibodies to toxin A and toxin B interfere with toxin binding to cell surface receptors, preventing toxin endocytosis and disease (Kelly, Pothoulakis et al. 1992). This antibody protection has led to passive immunotherapy, where a treatment of antibodies to toxins A and B are given intravenously to patients in addition to metronidazole or vancomycin. Studies have found that this therapy effectively reduces symptoms of CDI (Salcedo, Keates et al. 1997).


**Pregnancy**

Prior to the emergence of hypervirulent strains of *C. difficile*, CDI was considered an unusual occurrence for pregnant women, with minor symptoms not involving hospitalization. Now an increased frequency and severity of disease is associated with CDI in peripartum women. More severe outcomes included increased colectomies, 3 stillbirths and 3 maternal deaths from CDI (Rouphael, O'Donnell et al. 2008). A review of discharge diagnosis of CDI in U.S. hospitals from 1998 to 2006 found that 1,706 peripartum women were discharged with diagnosis. The increase in CDI discharge diagnosis nearly doubled from 2004 to 2006 (Kuntz, Yang et al. 2010).

**Proton Pump Inhibitors**

Proton pump inhibitors (PPIs) are used commonly in hospitalized patients suffering from gastric ulcers and acid reflux. The use of PPIs reduces gastric acid secretion, raising the pH in the stomach. This is hypothesized to allow *C. difficile* vegetative forms, in addition to spores, to pass unharmed into the intestine (Cunningham, Dale et al. 2003; Dial, Alrasadi et al. 2004; Jump, Pultz et al. 2007; Dalton, Lye-Maccannell et al. 2009). However, two independent studies of patients receiving gastric acid suppression between 1995 and 1996, and 2003 to 2004, found no correlation between reduced stomach acid levels and increased incidence of CDI (Shah, Lewis et al. 2000; Pépin, Saheb et al. 2005).
Contact with Healthcare

Patients admitted to hospitals have increased rates of asymptomatic colonization regardless of progress to infection. Cultures on a variety of surfaces in healthcare facilities have documented high levels of *C. difficile* contamination (McFarland, Mulligan et al. 1989; Samore, Venkataraman et al. 1996). Screenings of patients upon admission has found carriage rates between 5.9 to 11%, which is higher than the normal distribution in adults of 2 to 3%. Acquisition rates vary amongst patient groups depending on other risks factors, but overall the rate is 4 to 21% in a non-outbreak environment. A study in one hospital demonstrated an acquisition rate of 13% for patients hospitalized for 1-2 weeks; the rate for patients requiring hospitalization for greater that a month was 50% (Clabots, Johnson et al. 1992). Approximately 63% of these patients will go on to be asymptomatic carriers. Studies of outbreaks have found acquisition rates can be as high as 32%, especially when highly susceptible patients are exposed (Barbut and Petit 2001).

Kyne *et al.*, found that the additional time needed to treat patients who developed CDI while being treated for another issue increased their extra length of stay in hospital by 3.6 days, at an estimated cost of over 1 billion dollars in the U.S., per year (Kyne, Hamel et al. 2002).

Other Risk factors

Severe underlying illness and any disruption of normal barriers, such as use of nasogastric tubes and enemas, place patients at higher risk for CDI (Barbut and Petit 2001). Chemotherapy with antineoplastic drugs, especially methotrexate, put patients at
higher risk to develop disease (Anand and Glatt 1993), also individuals suffering from AIDS also have more severe CDI symptoms (Barbut, Meynard et al. 1997).

**Asymptomatic Carriers**

Asymptomatic carriage of toxin producing and non-toxin producing strains of *C. difficile* is quite common. Starting at birth, greater than 50% of all neonates born in hospitals can be colonized with *C. difficile* (Viscidi, Willey et al. 1981; Larson, Barclay et al. 1982; Tabaqchali, O'Farrell et al. 1984; Barbut and Petit 2001). The rate of colonization drops dramatically to approximately 6% by age two (Mahony, Clow et al. 1991). Acquisition and asymptomatic carriage of *C. difficile* in the hospital setting has been shown to be as high as 20% in adult patients who have been hospitalized for at least 2 weeks (Shim, Johnson et al. 1998). Asymptomatic carriers can act as a reservoir for *C. difficile*, infecting others by spreading spores in their feces and on their skin. Higher levels of skin contamination have been identified in these asymptomatic carriers, and they may be placed unknowing in cohort rooms with susceptible patients. (Clabots, Johnson et al. 1992; Riggs, Sethi et al. 2007). The asymptomatic carriage of *C. difficile* has been shown to have a protective effect in patients as they produce antibodies to the toxins and don’t develop severe CDI, whereas patients who do not harbor *C. difficile* may lack these antibodies and be more susceptible to CDI (Shim, Johnson et al. 1998).

**Diagnosis of CDI**

Symptoms of mild CDI include diarrhea, *i.e.*, the passage of three or more loose-to-watery foul-smelling stools per day with some abdominal tenderness (Sunenshine and
McDonald 2006). Quick diagnosis is essential so that CDI does not progress to more severe CDI (Bartlett and Perl 2005). The symptoms of *C. difficile* colitis (severe CDI) include profuse watery diarrhea, abdominal cramping, fever, dehydration and nausea; blood in stool is rarely present (Sunenshine and McDonald 2006; Bartlett and Gerdin 2008). Sigmoid scope examination of the distal colon mucosal membrane reveals yellow patches characteristic of PMC. If left untreated, additional symptoms of paralytic ileus (obstruction and distention) and toxic megacolon (extreme dilation of colon) may develop. Systemic symptoms of fever and tachycardia can also be present. Once toxic megacolon develops, surgical intervention is required to prevent colon perforation, septic shock and death (Morris, Zollinger et al. 1990; Sunenshine and McDonald 2006). The mortality rate for patients following colectomy is high, with 32-50% succumbing to CDI (McFarland 2005).

**Antibiotic Treatment for CDI**

The first step in treatment of CDI is to stop the offending antibiotic, if possible, or switch to an antibiotic with a narrower spectrum. Nearly 25% of diarrhea from mild CDI will resolve with no further complication (Barbut, Richard et al. 2000). Additionally, supportive therapy to rehydrate and replace electrolytes is important, especially in patients with severe diarrhea. Antiperistaltic drug therapy should be avoided to limit patient exposure to *C. difficile* toxins, which may encourage the development of toxic megacolon (McFarland 2005). Unfortunately, with the rise of hypervirulent strains, some physicians may treat empirically with antibiotics until CDI has been ruled out by laboratory testing (Bartlett and Gerding 2008).
**Metronidazole**

Metronidazole remains the first line of antimicrobial treatment for mild CDI. Metronidazole, 250 or 500 mg can be given orally 4 times a day (Schroeder 2005), or given intravenously for those patients with ileus (Gerding, Johnson et al. 1995). The treatment normally takes 10 to 14 days to resolve CDI (McFarland 2005; Sunenshine and McDonald 2006). Metronidazole is more selective in its antimicrobial spectrum than vancomycin, having activity primarily on anaerobic bacteria. Oral metronidazole also is completely absorbed by the small intestine before reaching the colon in patients not suffering from diarrhea, but high levels are found in the colon in diarrheal patients (Teasley, Olson et al. 1983). The cost per day is much less for 500 mg capsule of metronidazole ($0.72/dose) than 125 mg capsule of vancomycin ($32/dose), being $3 and $128 per day, and $7.20, and $320 for a 10 day treatment, respectively (Lancaster 2012).

Reports of decreased susceptibility to metronidazole have been reported as early as 1999, when Wong et al. found one in 100 isolates resistant (Wong, Woo et al. 1999). With the emergence of NAP1/027 strains, reports of treatment failures with metronidazole increased to a point that it could only be used to treat mild CDI (Musher, Aslam et al. 2005; Baines, O’Connor et al. 2008).

**Vancomycin**

Oral vancomycin has excellent activity against *C. difficile* because it remains at high levels in the gut; it is not easily transported across mucosal membranes. This requires that vancomycin be taken orally (125 or 500 mg four times daily), but also leads
to concentrations as high as 3 mg/mL in the colon a thousand times more than the minimal inhibition concentration for *C. difficile* (Tedesco, Gurwith et al. 1978).

Unfortunately, these extremely high levels of vancomycin will be inhibitory to not only Gram-positive, but also Gram-negative bacteria, interfering with reestablishment of the normal protective bowel flora, which are necessary to prevent relapse or reinfection (Walters, Roberts et al. 1983). Use of vancomycin may also induce antibiotic resistant organisms found in gut, *i.e.*, vancomycin resistant enterococci (VRE).

Changing daily dosing of vancomycin to dosing every third day, or weaning vancomycin while extending the treatment over 25 days, have shown to reduce recurrence rates in patients suffering repeatedly from CDI. This change allows the normal protective bowel flora to recover before terminating the treatment (McFarland 2005).

**Fidaxomicin**

Fidaxomicin, also known Dificid, has been approved for treatment of CDI. Phase III clinical trials have shown Fidaxomicin to be as equally effective as vancomycin in treatment of CDI; the reoccurrence rates of CDI following treatment with Fidaxomicin were also significantly lower (Crook, Walker et al. 2012). Fidaxomicin has a narrower antibacterial spectrum, allowing for improved recolonization by protective gut flora (Louie, Miller et al. 2011). Unfortunately, Fidaxomicin is very expensive, costing $135 per tablet. Dosed twice a day, for 10 days, cost $2,700 per treatment (Lancaster 2012).
Other Antimicrobials

Several other antimicrobials have been tested to inhibit *C. difficile*. Linezolid has been tested *in vitro* where it effectively inhibits *C. difficile*. Linezolid is expensive and its use is reserved for treating vancomycin resistant bacteria (Peláez, Alonso et al. 2002). Fusidic acid has good activity *in vitro* and *in vivo*, but resistant isolates of *C. difficile* were recovered from patients after therapy; concerns of creating resistant organisms has limited its use (Norén, Wullt et al. 2006). Tigecycline is a newer broad spectrum antibiotic that has excellent activity *in vitro* against *C. difficile* (Hecht, Galang et al. 2007). A study involving four patients with refractory CDI showed clinical improvement in all four patients treated intravenously with tigecycline. Tigecycline may be useful as follow up therapy for patients with recurrent CDI, as the cost per day is three times that of vancomycin (Stein and Craig 2006; Herpers, Vlaminckx et al. 2009).

Alternative Therapies

Therapies that do not require the use of antibiotics are being pursued with the ultimate goal of reestablishing the protective normal gut flora. With continued use, resistance to metronidazole and vancomycin may be encouraged in *C. difficile*, and antibiotics have no effect on spores that may germinate after the antibiotic treatment has ended (McFarland 2005).

Probiotics containing one or several living beneficial microbes have been a popular addition to antibiotic therapy or as a replacement for it for many years. The benefits of probiotics are: multiple mechanisms of acting on pathogens, benefits to host immune system, survival to host colon, no drug interaction, and low risk to the patient.
The drawbacks to probiotics are: poor quality control, poor standardization, few clinical trials, possible infections with immunocompromised patients, and sometimes adverse reactions in patients (McFarland 2009).

*Saccharomyces boulardii* has been evaluated in several studies and found to support vancomycin therapy (McFarland 2005). The mechanism by which *S. boulardii* inhibits toxin binding has been determined to be the production of a protease, which digests the toxin A receptor in gut mucosal cells (Castagliuolo, LaMont et al. 1996). Studies showed significant improvement in patients treated with a combination of *S. boulardii* and high-dose vancomycin over vancomycin and placebo (Surawicz, McFarland et al. 2000).

Testing preparations containing *Lactobacillus* sp. in combination with *Streptococcus thermophiles* were used in different clinical trials as an adjunct to antimicrobial therapy with mixed results (Pochapin 2000). Hickson et al. in 2007 gave a probiotic fermented milkshake containing multiple *Lactobacillus* sp. and *St. thermophiles* to hospitalized patients over the age of 50 concurrently with antibiotics, then monitored the patients for 2 months for the development of antibiotic associated disease (AAD). The probiotic group developed significantly less AAD than the group given the milkshake placebo with their antibiotics (Hickson, D'Souza et al. 2007). A similar study was performed in 2003 using yogurt with the same live bacteria and achieved similar results (Beniwal, Arena et al. 2003). However, Thomas et al. gave *lactobacillus* sp. without milk products concurrent with antibiotics to hospitalized patients and found no improvement
in the subsequent development of AAD compared to the placebo group (Thomas, Litin et al. 2001).

**Fecal Bacteriotherapy**

Bacteriotherapy is the process of quickly replacing bowel flora so that the colon can be recolonized with bacteria that provide colonization resistance to *C. difficile*. Feces from a healthy family member is made into a slurry with normal saline and instilled into the patient’s upper GI tract through a nasoduodenal catheter or into the colon using a colonoscope or enema catheter (Bakken 2009). The reported success rate is 90-100%, depending on the study (Bakken 2009; Yoon and Brandt 2010). Instead of feces, a mixed culture of healthy bowel bacteria can be used to recolonize the colon (Tvede and Rasmussen-Madsen 1989). Instructions for home fecal bacteriotherapy are given to patients who chronically suffer from recurrent CDI (Silverman, Davis et al. 2010).

**Immune Therapy**

Toxoids of *C. difficile* toxins A and B are formalin inactivated and used to stimulate the production of serum immunoglobulins to toxin A and toxin B (Kotloff, Wasserman et al. 2001). A phase 2 clinical trial (ClinicalTrials.gov number, NCT00772343) has recently been completed in the United Kingdom using a vaccine containing toxoid A and toxoid B on patients suffering from their first episode of CDI. The goal of the trial is to see if the vaccine can induce high titers antibodies to toxin A and toxin B to prevent recurrence of CDI. Results of the clinical trial are currently unavailable.
Passive immunity can be used to help treat patients where antimicrobial therapy fails to resolve CDI symptoms. Patients who fail to produce sufficient antibodies to *C. difficile* toxins will continue to suffer from CDI and have more recurrences. Patients treated with antibodies from pooled human plasma quickly resolve from CDI symptoms and have fewer subsequent infections (Salcedo, Keates et al. 1997; Giannasca and Warny 2004). However, Juang *et al.* reviewed the use of passive immunotherapy on patients suffering from severe CDI in conjunction with antimicrobial therapy and found no significant reduction in the number of colectomies between patients receiving immunotherapy and those that did not (Juang, Skledar et al. 2007).

**Preventing Transmission of *C. difficile***

Infection control programs are responsible for limiting the spread of infectious organisms in hospitals and long-term care facilities. With the rise of hypervirulent *C. difficile* strains, increased pressure is being placed on these programs to prevent CDI outbreaks in their facilities. The Society for Healthcare Epidemiology and the Infectious Diseases Society of America have made recommendations to limit the spread of *C. difficile* (Dellit, Owens et al. 2007; Cohen, Gerding et al. 2010; Donskey 2010).

First, facilities need to monitor and be aware of their CDI rates. This will determine if the rate is acceptable compared to other facilities, and should additional resources be used to reduce it (Gerding, Muto et al. 2008). Laboratory reports for positive
toxin assays, detection of PMC by sigmoid scope, colectomies and autopsy reports can all be used to determine the rate of CDI in facilities (Valiquette, Cossette et al. 2007).

Antibiotic stewardship is perhaps the most challenging to implement, but also the most effective in reducing CDI rates in facilities. Reducing the use of antimicrobials associated with CDI has been shown to reduce healthcare acquired infections more effectively than hand washing and environmental cleaning (Valiquette, Cossette et al. 2007). Providing physicians with proper guidance to select antimicrobials, dose antimicrobials, and avoid antimicrobials that can cause problems by generating resistant organisms not only benefits the patient, but saves costs for the facilities also (Kyne, Hamel et al. 2002; Valiquette, Cossette et al. 2007).

On admission, patients need to be evaluated for symptoms of CDI. Suspect patients should be placed in private rooms, and if private rooms are unavailable, they should be cohorted with other CDI patients until CDI has been ruled out by the laboratory. Strict contact precautions must be implemented, including: gowns and gloves, dedicated equipment such as electronic rectal thermometers and blood pressure cuffs, and hand washing with soap and water for everyone before leaving the room. Gloves should be worn the entire time a person is in the room and removed when exiting (Vonberg, Kuijper et al. 2008). Hand washing with soap and water has been shown to be superior to alcohol based hand cleaners, but neither method will remove all spores from hands (Jabbar, Leischner et al. 2010). Rooms need to be cleaned with sporacidal agents, paying particular attention to high touch surfaces. Bleach freshly diluted 1:10 with water has
been found to effectively kill spores (Mayfield, Leet et al. 2000; Wilcox, Fawley et al. 2003; Loo, Poirier et al. 2005; Riggs, Sethi et al. 2007). Spores have been documented to be present in feces following cessation of CDI symptoms, so isolation is recommended to be continued for several days following cessation of diarrhea (Gerding, Muto et al. 2008).

When laboratories confirm the presence of *C. difficile* toxin in patient’s fecal samples, the result should be called immediately to the attending healthcare provider. Environmental services should be called next to ensure *C. difficile* decontaminating procedures are initiated (Cohen, Gerding et al. 2010).

**Current Statement Regarding C. difficile.**

*C. difficile* infection (CDI) continues to spread unabated by the best efforts of health care professionals, infection control practices, and environmental cleaning methods (Boyce 2007; O’Brien, Lahue et al. 2007). Epidemics caused by hypervirulent *C. difficile* strains are numerous (Pépin, Saheb et al. 2005; Redelings, Sorvillo et al. 2007; Rupnik, Wilcox et al. 2009). These epidemic strains are found to be endemic to health care facilities due primarily to the resistant spores that are passed in feces from infected patients and asymptomatic carriers (Viscidi, Willey et al. 1981; Riggs, Sethi et al. 2007). Laboratory testing is relied upon by health care professionals to identify infected patients quickly, to limit the spread of highly contagious spores, and most importantly prevent disease progression in untreated patients (Johnson and Gerding 1998; Wilkins and Lyerly 2003; Novak-Weekley, Marlowe et al. 2010). Laboratory testing for *C. difficile* needs to be highly sensitive and specific to avoid missing positive
patients, or causing undue treatment and isolation of those patients falsely identified (Ticehurst, Aird et al. 2006). The cost of treating patients with CDI has risen to over 3 billion dollars yearly in the United States alone (McGlone, Bailey et al. 2012), and CDI has surpassed methicillin resistant *Staphylococcus aureus* (MRSA) as the most numerous health-care-associated infection (MillerMD, ChenMD et al. 2011).

Approaches to identify and implement better test methods for the conformation of CDI are needed (Novak-Weekley, Marlowe et al. 2010; Wilcox, Planche et al. 2010). Laboratories will need to make decisions on how new and what tests will be made available in their facilities, along with educating doctors and nurses on the interpretation of laboratory reports. Also everyone needs to be aware of specimen transport methods to maintain the integrity of the fecal samples for testing.

Alternative therapies may help deter colonization of patients with *C. difficile*, that don’t rely on antibiotics that *C. difficile* may become resistant to. Natural products have previously been shown to inhibit bacterial infections such as cranberry juice with *Escherichia coli* urinary tract infections (Zafiriri, Ofek et al. 1989), or the use of silver coated urine catheters (Haynes and Mansour 1989) to prevent bladder infections. Yogurt supplemented with live bacterial strains have demonstrated reduced antibiotic associated diarrhea (Beniwal, Arena et al. 2003).

Interrupting the transmission of *C. difficile* spores spread by the hands of health care workers, would also be beneficial for patients who may be susceptible to CDI.
Treatments that would improve removal of spores from skin might help reduce transmission in health care facilities (Edmonds, Zapka et al. 2013).

Dissertation Rationale and Hypotheses

1. Use of Enteric Transport Medium for Detection *C. difficile* toxins and GlutamateDehydrogenase

   Hypothesis 1: Testing fecal samples by multiple methods will show that Enteric Transport Medium can preserve *C. difficile* antigens and toxins while being maintained at room temperature.

   Sub hypothesis 1.1: Acidic stools will test falsely negative for *C. difficile* toxin using enzyme immunoassay due to toxin degradation.

   Sub hypothesis 1.2: Fecal samples positive for *C. difficile* will also contain high levels of fecal proteases including trypsin due to disruption of normal fecal microbiota. Increased fecal trypsin will degrade toxin decreasing its detection.

2. Antimicrobial Effects of Virgin Coconut Oil and its Medium Chain Fatty Acids on *Clostridium difficile*.

   Hypothesis 2: As an alternative treatment, virgin coconut oil and its medium chain fatty acids will have a toxic effect of *C. difficile in vitro*. 
3. Visualization and Chemical Treatment of *C. difficile* 630 Strain Spore Surface

Hypothesis 3: *C. difficile* spores are partially coated with mother cell residues on the spore surface.

The first hypothesis is based on personal experience; the testing of patient fecal samples for *C. difficile* toxin has resulted in inconsistent test results when multiple samples from the same patient are submitted. These inconsistencies have resulted in the necessity of multiple patient fecal samples being collected. Physicians also question the laboratory results when one sample is positive and the following two samples are negative when nothing has changed in the patient’s treatment. A review of the literature has demonstrated these testing inconsistencies are attributed to poor sensitivity and specificity of common laboratory tests used to identify the presence of *C. difficile* toxin in patient fecal samples. The toxin becomes undetectable in patient fecal samples stored at room temperature in as little as two hours (Pelleschi 2008). Sample transport guidelines have been implemented by clinical microbiology laboratories requiring fecal samples to be delivered to the microbiology laboratory within an hour of collection. If this is not possible, the samples must be refrigerated until delivered.

Of major concern are the outpatient fecal samples collected at the patient homes. Here the sample must be refrigerated until delivered to the laboratory. Even under best conditions, patients may hesitate to store foul smelling diarrheal feces in their refrigerator, beside their food. To improve the transport of outpatient fecal samples, an
enteric transport medium (ETM) has been made available that claims to maintain *C. difficile* toxin for 5 days at room temperature. No independent studies have been published in peer-reviewed journals using this ETM transport medium.

The ability of ETM to preserve *C. difficile* antigens and toxins in feces was evaluated by analyzing 100 fecal samples submitted to the clinical microbiology laboratory with the possible diagnosis of *C. difficile* infection. These samples were stored frozen at -70°C until tested by multiple methods. Evaluation of toxin stability in feces, and prevention of its degradation are also evaluated.

The 2nd hypothesis deals with alternative therapies to prevent *C. difficile* colonization in animals and humans. Virgin coconut oil (VCO) and/or its constituent medium chain fatty acids (MCFA) have previously been reported to inhibit a wide variety of other microbes including; viruses, bacteria, and protozoa, but has not been tested for their ability to inhibit the growth of *C. difficile*. VCO and its MCFA were evaluated to determine if *C. difficile* growth inhibition is affected using the American Type and Culture Collection (ATCC) 9689 strain.

The third hypothesis uses different microscopic techniques to visualize *C. difficile*’s spore surface. Proteins on the spore surface have been implicated in increasing disease transmission by specifically adhering to surfaces including human skin. Previous studies have shown that *C. difficile* spores are harder to remove from the hands of volunteers than other bacterial spores (Edmonds, Zapka et al. 2013). Different hand washing methods failed to remove 100% of spores during controlled studies (Oughton,
We hypothesize that *C. difficile* spore surface proteins adhere to specific skin receptors similar to those for *Staphylococcus aureus* (Bibel, Aly et al. 1987; Roche, Meehan et al. 2003). Antibodies specific for spore surface proteins were used to visualize the localization of these proteins on the spore surface. Additionally chemical treatments were used to alter or remove the spore surface as determined by changes in the fluorescent images. An enzyme immunoassay was created to quantify the treatment effects on the binding of these spore antibodies to the spore surface. Chemical treatments that can remove or alter the spore surface proteins as determined by increases or decreases in antibody attachment, may prove valuable in designing cleaning or hand washing products to block the spore attachment to surfaces including human skin.

The following chapters will address the above hypotheses and conclude with a final synopsis.
References


Donskey, C. J. (2010). "Preventing transmission of Clostridium difficile: is the answer blowing in the wind?" Clinical Infectious Diseases 50(11): 1458-1461.


CHAPTER II

Use of Enteric Transport Medium for Detection *C. difficile* toxins and Glutamate Dehydrogenase

Abstract

Transportation of fecal samples for rapid and accurate detection of *Clostridium difficile* infection by clinical microbiology laboratories is an ongoing problem. Environmental conditions may have adverse effects on the detection of *C. difficile* toxins and antigens. Variation in test specificity and sensitivity also leads to repeat testing and empiric therapy decisions that increase the cost and reduce the accuracy of patient treatment. Fecal samples collected from one hundred from patients with the differential diagnosis of *C. difficile* infection were diluted in Enteric Transport Media (ETM) to establish its ability to preserve toxin antigens and glutamate dehydrogenase for detection by EIA and other test methodologies. Following preservation in room temperature ETM, fourteen percent of the one hundred samples submitted tested positive for toxin by EIA, with seven percent of the positive samples failing to have detectable toxin after 4 hours incubation at room temperature. *C. difficile* culture positive fecal samples preserved in ETM also demonstrated false negative glutamate dehydrogenase results in 12% of the
samples tested. Acidic feces was found not to be detrimental to toxin detection, but high fecal trypsin levels in patient samples had an effect on test sensitivity.

**Introduction**

In 1978 *Clostridium difficile* was acknowledged as one of the possible etiologic agents of pseudomembranous colitis along with the most probable cause of antibiotic associated diarrhea (Bartlett, Chang et al. 1978). There was a need for the clinical laboratories to confirm diagnosis of *Clostridium difficile* infection (CDI). Many labs relied on anaerobic cultures using selective agars such as cycloserine-cefoxitin-fructose agar (CCFA) (George, Sutter et al. 1979). *C. difficile* could be presumptively identified on agar by their characteristic swarming, ground glass appearance, fresh horse manure odor, and fluorescence. Gram staining of suspicious colonies would reveal Gram-positive rods with subterminal spores. Culture results would be reported within 48 to 72 hours, unfortunately it was soon discovered that not all *C. difficile* isolates produced toxins, and also, some patients may asymptotically carry toxigenic strains without any diarrheal symptoms. It was now clear that testing for toxin production from *C. difficile* isolates was essential for the diagnosis. A quicker solution to the problem would be to simply test diarrheal feces, from suspect patients, for the *C. difficile* toxins. However, the clinical picture is even more complex when considering certain populations, such as elderly, long-term patients in nursing facilities, who often have diarrhea, who carry *C. difficile* which may not responsible for their diarrheal symptoms. Studies have also found that asymptomatic carriage of toxigenic *C. difficile* by neonates born in hospitals ranges
from 2% to over 50% without any apparent diarrheal symptoms (Larson, Barclay et al. 1982; Humphries 2012). Toxigenic culture identification of *C. difficile* toxins from feces involves growing *C. difficile* isolates in broth cultures, and subsequently testing broth filtrates for toxin using the cell cytotoxin neutralization assay or the toxin A/B ELISA. Toxigenic culture is currently considered to be the new “gold standard” to which all other tests are compared.

*C. difficile* toxin was determined to be an exotoxin, as it was shown to be filterable, caused pathology and evoked an antibody response in laboratory animals, and was heat labile (Snyder 1937). The first assays to detect bacterial toxins were performed by either injecting bacterial cultures or culture filtrates directly into animals. The clostridial toxins caused edema in subcutaneous tissues of rabbits. Guinea pigs also suffered from toxin induced edema, but additionally suffered convulsions and death due to respiratory failure (HALL and O'TOOLE 1935; Snyder 1937). Antibodies to the toxins were prepared by carefully injecting sub-lethal doses of culture filtrates into laboratory animals. Animals could be protected from the *C. difficile* toxin by adequately injecting them with antisera (Snyder 1937).

By the late 1970s the connection between pseudomembranous colitis, antibiotic treatment and carriage of *C. difficile* was clear. Clinical laboratories were now being asked to confirm the diagnosis of CDI by either isolating *C. difficile* by anaerobic culture, or detecting its toxin using a human cell culture model; cell monolayers treated with stool filtrate paired wells pretreated with the antitoxin-treated stool filtrate were used (Wilkins
and Lyerly 2003). Both of the methods were time consuming, and required 48 to 72 hours for results. Isolating *C. difficile* with culture did not entirely confirm the diagnosis of CDI, as non-toxin producing strains could also be recovered. Confirmation of CDI required the production of cytopathic effect (CPE), *i.e.* toxin induced cellular deformity from *C. difficile*’s toxin B. A positive test was noted when cells within the human cell monolayers exposed to stool or culture filtrates from patients rounded while being incubated at 37°C. The test included paired wells where the filtrate was pretreated with a toxin neutralizing *Clostridium sordellii* antitoxin. Thus, a complete and positive test would display CPE within cell monolayers in the untreated filtrate wells, and normal cell morphology within monolayers pretreated with antitoxin (Wilkins and Lyerly 2003). The problems with using the cell cytotoxin neutralization assay (CCNA) were numerous; controlling which cell line was optimal for toxin detection, what percentage of cells needed to be affected, and which type of CPE was needed for confirmation? There were also laboratory differences in defining a positive result; CCNAs lacked standardization. Furthermore, labs had to have special rooms for tissue culture and specifically trained personnel. Finally, toxin testing needed to be performed in a more timely manner, on a larger scale, and with greater standardization (Wilkins and Lyerly 2003).

Marion Scientific introduced a latex agglutination test in the mid-1980s that could detect *C. difficile* toxin A from patient feces, in less than an hour, by the clinical microbiology laboratory. This provided a huge cost advantage over performing anaerobic culture or CCNA tests. However, this test was later shown not to detect toxin A, but instead detected glutamate dehydrogenase (GDH), an enzyme produced by all *C. difficile*
isolates regardless of toxin expression (Lyerly and Wilkins 1986). This test is now available in various test formats, and is often referred to as the “common antigen test”. GDH tests have a very high negative predictive factor for detecting the presence of \textit{C. difficile} bacterium, indicating that there are very few false negative results. Positive GDH samples would need to have a follow up toxin assay performed to ensure presence of toxin producing \textit{C. difficile} (Zheng, Keller et al. 2004).

Enzyme-linked immunoassays for the detection of toxin A quickly became available for use in the clinical laboratories (Aronsson, Granstr et al. 1985). These tests detected toxin in several hours, as compared to several days for anaerobic culture and cell cytotoxin assays. Originally designed to detect toxin A only, the tests were changed to detect either toxin A or toxin B, once the discovery of \textit{C. difficile} strains that exclusively expressed toxin B were identified (Laughon, Viscidi et al. 1984; Kato, Kato et al. 1998). Recently, the sensitivity of the Toxin A/B ELISA kits by numerous manufacturers, has been reported to be dramatically decreased compared to culture and CCNA assays (Bartlett and Gerditt 2008). Ticehurst reported an ELISA toxin A/B sensitivity of only 38% when compared to CCNA, in one study performed on samples collected in 2004 (Ticehurst, Aird et al. 2006). According to the College of American Pathologists the majority of clinical laboratories still perform a single ELISA test for detection of CDI (Peterson and Robicsek 2009).

The newest and rapidly-growing laboratory test for the identification of CDI is the nucleic acid amplification test (NAAT) which uses real time PCR, to both amplify and
quantitate the targeted DNA molecule, in about an hour. The majority of these tests target the tcdB gene of the pathogenicity locus (Novak-Weekley, Marlowe et al. 2010; Tenover, Novak-Weekley et al. 2010; Knetsch, Bakker et al. 2011). An assay from Meridian Bioscience differs from the others by amplifying the tcdA gene (Lalande, Barrault et al. 2011; Doing and Hintz 2012). The sensitivities of these assays to detect toxigenic strains are quite high, 90-100% have been reported in various studies (Novak-Weekley, Marlowe et al. 2010; Tenover, Novak-Weekley et al. 2010; Knetsch, Bakker et al. 2011; Lalande, Barrault et al. 2011; Doing and Hintz 2012). However, no test is perfect, and false negatives have been reported in fecal samples contaminated with blood. Blood contamination, however, is rarely found in CDI (Cohen, Gerding et al. 2010). The major disadvantage to NAATs is that they do not detect biologically active toxin in the feces, just the presence of the organism. When testing populations where asymptomatic carriage is high (children under age one or patients who access health care frequently), there is the possibility of misidentifying people as having CDI (Kufelnicka and Kirn 2011). An additional benefit of the Xpert C. difficile Epi assay (Xpert; Cepheid, Sunnyvale, CA) is that the test also presumptively identifies the newly-emerged NAP1 strains that over-express toxins A and B. The assay detects these NAP1 strains by targeting a base pair deletion in the tcdC gene (Kok, Wang et al. 2011). This additional information can be used for early intervention to prevent NAP1 outbreaks.

Environmental conditions have adverse effects on C. difficile and its toxins. Transportation of fecal samples is an ongoing problem for rapid and accurate detection of C. difficile infection by clinical microbiology laboratories. The toxins produced by the C.
*difficile* bacteria are very unstable at room temperature in stools with an acid pH, or having increased fecal proteases that will cause deterioration within hours if left at room temperature (Gumerlock, Tang et al. 1991). Fecal proteolytic activity is derived from two sources; trypsin, chymotrypsin, and pancreatic elastase are produced by the pancreas, whereas other proteases are produced by the microbiota residing in the colon (Macfarlane, Cummings et al. 1986).

Trypsin, one of the mammalian fecal proteases, is typically 30 µg of trypsin per gram of feces (Smith, Ediss et al. 1971). However, it can increase 100-fold in patients receiving antimicrobial therapy, where the activity of the normal fecal microbiota (*Bacteroides* spp.) breaking down trypsin is disrupted (Bohe, Borgström et al. 1983; Macfarlane and Macfarlane 1991). Trypsin is an endoprotease that cleaves after arginine or lysine, except when followed by proline. Trypsin can break down *C. difficile* proteins (toxins A and B, and glutamate dehydrogenase) when samples are not properly preserved (Bartlett, Chang et al. 1979). Patient fecal samples exposed to high levels of trypsin may test negative for *C. difficile* infection leading to false negative reports. Inaccurate test results may lead to inappropriate therapeutic decisions by physicians. Patients left untreated for *C. difficile* infection may go on to develop pseudomembranous colitis or possible toxic megacolon with life threatening complications.

The Society for Healthcare Epidemiology of America (SHEA) now recommends a two-step approach to streamline the detection of *C. difficile* disease (Ticehurst, Aird et al. 2006; Rüssmann, Panthel et al. 2007). Step one is to test the stool specimen for *C.
\textit{difficile} GDH (Novak-Weekley and Hollingsworth 2008). This test has been shown to have increased sensitivity and specificity over \textit{C. difficile} toxin ELISA assays, but does not specifically detect toxins A or B (Poultér, Doymaz et al. 2003; Zheng, Keller et al. 2004; Ticehurst, Aird et al. 2006; Reller, Lema et al. 2007; Fenner, Widmer et al. 2008; Gilligan 2008). All fecal samples that are negative for GDH are then to be reported as negative for \textit{C. difficile} and no further testing is performed, saving cost and technician time. The rapid GDH test may also assist in a differential diagnosis to permit patient isolation and treatment decisions. In step two of the SHEA recommendation, stool specimens testing positive for \textit{C. difficile} GDH are further tested using an enzyme-immuno assay toxin test, molecular test, or a cell cytotoxicity assay, for the specific detection of the \textit{C. difficile} toxins.

Enteric Transport Medium (ETM™ Alpha-Tec Systems, Vancouver, Washington) is a stool preservative, formulated to improve the detection of the glutamate dehydrogenase and toxins of \textit{C. difficile}. ETM is essentially a modified Cary-Blair medium that is used for preservation of feces for routine stool culture (Cary and Blair 1964). This transport medium is buffered to neutralize acidic stools. The ETM-preserved sample may be used for the recovery of \textit{C. difficile} bacteria, its GDH, and its toxins, when stored at 4°C. However, the manufacturer specifies room temperature transport until the sample is tested for other fecal pathogens (ETM product insert, Alpha-Tec Systems). Here we present the results of \textit{C. difficile} culture, antigen detection, and toxin recovery from room-temperature, ETM-preserved fecal specimens.
Methods

Samples.

Over a two month period, fresh fecal samples with soft or loose consistency, from 100 patients whose differential diagnosis included *C. difficile* infection (CDI), were obtained from a local hospital laboratory. The anonymous fecal samples were considered to be medical waste, and thus exempt from patient consent, as determined by Kent State Internal Review board under federal guideline (45 CFR 46, Subpart A, §46.101). The samples were aliquoted, on receipt, by laboratory workers and frozen at -70°C until evaluated together. Samples were prepared for testing by either diluting 400 µl of feces into 1600 µl ETM media with thorough mixing (ETM product insert, Alpha-Tec Systems) or testing the sample *de novo*.

*Clostridium difficile* Toxin A/B Enzyme Immunoassay.

Toxin A/B testing was performed by using ProSpec T® *C. difficile* Toxin A/B microplate assay (EIAPro;Remel, Lenexa, KS) following the manufacturer’s procedure for Cary-Blair preserved samples. Briefly, well-mixed feces in ETM were added to microtiter wells, coated with immobilized antibodies to the individual toxins, and incubated at room temperature for 60 minutes. These wells were manually washed and toxin detected by adding horseradish peroxidase conjugated secondary antibodies, washing, and evaluated by the addition of a chromogenic substrate. After 10 minutes incubation at room temperature, an inhibitory stop solution of 1.0 N sulfuric acid was added resulting in a color change. The test reactions were evaluated at 450/630 nm. A positive result was indicated by an O.D. 450/630 nm of ≥ 0.08; a negative result was
indicated by O.D. 450/630nm <0.08. EIA was not performed on fresh, unpreserved samples due to the limited amount of feces received.

EIA toxin testing was performed using three day old brain heart infusion broth (BHIB) with *C. difficile* isolates to determine if the isolates were toxigenic. To test the effect of altering the broth pH before testing, toxigenic *C. difficile* ATCC 9689 control strain was grow in anaerobic BHIB at 36°C for three days. The BHIB was sterile filtered, and the pH adjusted using concentrated hydrochloric acid or 10M sodium hydroxide. Following testing, broths with significant decrease in toxin detection were adjusted to neutral pH and retested.

**C. Diff Quik Chek.**

The C. Diff Quik Chek (TECHLAB Blacksburg, VA) assay was used to determine the presence of *C. difficile* in stool by detecting the presence of glutamate dehydrogenase (GDH), following the manufacturer’s procedure for Cary-Blair preserved samples. Briefly, one hundred microliters of ETM preserved sample was diluted in a conjugate consisting of antibodies to GDH coupled to horseradish peroxidase. This mixture was applied to a membrane with immobilized antibodies specific for *C. difficile* GDH. Antibody-bound GDH was visualized by the addition substrate (tetramethylbenzidine) resulting in a colored product.
**Fecal pH.**

Fecal pH was tested by adding equal amounts of fresh feces to sterile distilled water (pH 7.0). The samples were thoroughly mixed and the pH measured using an Accumet pH meter (Fisher Scientific, Pittsburgh, PA).

**Clostridium difficile Cell Cytotoxicity Neutralization Assay.**

Cell cytotoxicity assay (CCTA) was performed using the Diagnostic Hybrids *C. difficile* Toxin kit. (Athens, OH). Cary-Blair preserved samples were not acceptable for toxin testing by this manufacturer’s method, so only unpreserved specimens were tested per manufacturer’s instructions. Briefly, fecal samples were diluted into a buffer solution (pH 7.5) and centrifuged to form a solid precipitate. The liquid layer was removed and filtered through a 0.45 micron filter (VWR International, West Chester, PA.) The filtrate was incubated with an equal amount of either *Clostridium sordellii* antitoxin or sterile buffer for 30 minutes. After incubation, 50 µL of each suspension was added to separate microtiter wells containing human foreskin fibroblast cells (less than 10 passages), The final sample dilution was equal to 1:40. The wells were incubated in an environment of 5% CO$_2$ at 37°C, and observed after 4, 24, 48, and 72 hours. Confirmation of toxin B activity was by cell rounding indicating cytopathic effect (CPE) and no CPE with cells pretreated with *C. sordellii* antitoxin (Chang, Gorbach et al. 1978). Samples displaying CPE in the test well and also in the antitoxin well were further diluted to an additional 1:10 and 1:100 of the original 1:40 dilution, and retested using the paired well method as above.
Bacterial Culture Identification and Spore Isolation

Following the GDH, EIA, and cytotoxin testing, the remaining refrigerated stool samples along with the ETM preserved samples, were tested for *C. difficile* by anaerobic culture, using the alcohol shock method (Clabots, Gerding et al. 1989). Briefly, spore selection was performed by mixing equal amount of the refrigerated stool specimen (approximately 500 µL) with an equal amount of 95% ethanol. The room temperature ETM preserved samples were centrifuged for 15 minutes at 3,000 x g. The pellet was treated by the same method as unpreserved refrigerated stool samples; the suspension was incubated at room temperature for 30 minutes, centrifuged for 15 minutes at 3,000 g and decanted. The pellet was inoculated onto anaerobically-prerduced Remel *Clostridium difficile* agar (CDA, Remel, Lenexa, KS), and prerduced Columbia Sheep Blood Agar (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.), and incubated in an anaerobic chamber (Bactron, Sheldon Manufacturing, Cornelius, Oregon) at 36°C for 72 hours. Non-hemolytic colonies displaying Gram-positive rods that were L-proline-aminopeptidase (Pro disk, Remel, Lanexa, KS) positive were further tested with the RapID-ANA II System (Innovative Diagnostics Systems, Inc., Atlanta, Ga.), to identify them as *C. difficile*. Once identified, *C. difficile* isolates were additionally screened for Moxifloxacin resistance using 5 µg disc (Remel, Lanexa, KS) on Columbia Sheep Blood Agar plates. Testing for toxin and GDH production was performed using isolates confirmed to be *C. difficile*. The isolates were inoculated into BHI Broth (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.) and incubated for six days at 36°C. The broth cultures were subsequently centrifuged for 10 minutes at 3,000 RPM and the
supernatant diluted in sample diluent and tested for toxin by ProSpec T® *C. difficile* Toxin A/B microplate assay (EIAPro; Remel, Lenexa, KS) (Merz, Kramer et al. 1994). The supernatant was also tested for GDH production using C. Diff Quik Chek test kit (TECHLAB Blacksburg, VA).

**Broth and Agar Culturing using Variable pH.**

Brain heart infusion broth (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.), was prepared according to manufacturer’s instructions. The pH was adjusted using concentrated hydrochloric acid and 10M sodium hydroxide to a range from 4.5 to 9.8, using the Accumet pH meter, and allowed to acclimate overnight in the Bactron II anaerobic chamber. Overnight broth culture (100 microliters) of *C. difficile* ATCC 43595 was added to 10 mL of pH adjusted BHIB and incubated at 36°C overnight before testing for optical density. Aliquots of 1 mL were taken from each BHIB, placed into UV-transparent quartz cuvettes, and measured using a Genesys 10uv Thermospectronic spectrophotometer (Thermo Scientific, Pittsburgh, PA) at an optical density of 600 nm (OD$_{600}$).

Brain heart infusion agar (BHIA) plates (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.) were prepared according to manufacturer’s directions. The pH was adjusted using concentrated hydrochloric acid. An overnight culture of *C. difficile* ATCC 43595 grown on BHIA pH 7.3 was transfer using sterile loops to BHIA with adjusted pH. The culture plates were incubated anaerobically overnight at 36°C and checked visually for growth.
Fecal Proteases.

GDH-positive fecal samples (n=10) were assayed for total protease activity using azocasein as substrate, following a modified method by Macfarlane et al. (Macfarlane, Cummings et al. 1986). Briefly, well mixed fecal samples were diluted 10% (w/v) in sodium bicarbonate buffer [0.50% (w/v) NaHCO3 in deionized water, pH adjusted to 8.3 at 37°C with 1 M HCl], to make a fecal slurry. The fecal slurry was then centrifuged (IEC Centra MP4R, Needham Heights, MA) at 4,000 x g for 10 minutes at 4°C. Aliquots (1 mL) of the resulting supernatant were placed in 2 mL centrifuge tubes (Fisher Scientific Co., Pittsburgh, PA) and held on wet ice until tested. Aliquots were arranged into three groups: group A (total fecal protease assay), group B to be pretreated with soybean trypsin inhibitor (Sigma Aldrich, St. Louis, MO), and group C (azocasein added after incubation to act as spectrophotometric blank). Azocasein (300 µL) substrate (50 mg/mL in 0.50% sodium bicarbonate buffer) and 200 µL of 0.50% sodium bicarbonate buffer were added to group A aliquots. Group B aliquots received 200µL of 0.50% sodium bicarbonate buffer with soybean antitrypsin diluted 10 mg/mL and were allowed to incubate for 30 minutes at room temperature. After incubation, aliquots were returned to wet ice and 300 µL of azocasein buffer was added. Group C aliquots received only 200 µL of 0.50% sodium bicarbonate buffer. Trypsin standards were prepared by adding 10 mg of 202 µ/mg P trypsin powder (Worthington, Lakewood, NJ) to 10 mL of iced sodium bicarbonate buffer pH 8.3. The diluted trypsin was then diluted further to create standards ranging from 10 mg/mL to 0.001 mg/mL trypsin. Trypsin standards (1 mL) were then pipetted into 2 mL centrifuge tubes. Azocasein (300 µL) and 200 µL of sodium
bicarbonate buffer were added to the tubes. All tubes were vortexed for 5 seconds before being placed in a 37°C water bath. After 2 hours of incubation, the reactions were stopped by pipetting the entire content of the tube into 15 mL centrifuge tubes containing 1 mL of 5% trichloroacetic acid (Sigma Aldrich, St. Louis, MO). Azocasein (300 μL) was then added to group C. Mixed and incubated at room temperature for 30 minutes. All tubes were centrifuged at 4°C for 20 minutes 4,000 x g. Aliquots (1 mL) of supernatant were pipetted into 2 mL tubes containing 1mL of 1M NaOH. The suspensions were mixed and measured at 450 nm (BIO-TEK Instruments, Inc., Winooski, VT).

**Testing Fecal Samples Supplemented with Antitrypsin for Toxin by EIA**

To distinguish the effect of fecal trypsin on toxin from the effect of other fecal proteases, residual toxin activity was evaluated in samples treated or untreated with antitrypsin. Trypsin degradation of toxin activity after treatment with antitrypsin can be attributed to other fecal proteases. The fecal samples were inoculated as one part feces in 4 parts PBS pH 7.4, and similarly in PBS modified with soybean antitrypsin (Sigma Aldrich, St. Louis, MO) resulting in a 20 mg/mL concentration of antitrypsin. Soybean antitrypsin will inhibit the activity of trypsin, with a lesser effect on chymotrypsin. The samples were incubated in a 25°C water bath until tested for toxin by the EIA method described previously. The PBS samples were tested for toxin at 0, 4, 24, 48, 72 and 96 hours to see if antitrypsin treatment improved toxin detection.
Testing ETM Samples Supplemented with Antitrypsin or Trypsin-Chymotrypsin Inhibitor

Fecal samples (n=6) from the ten additional samples previously tested for fecal proteases that were positive for *C. difficile* toxin by EIA, were diluted in ETM following Alpha-Tec Systems’ directions. The samples were thoroughly mixed and divided into three aliquots. The first aliquot was left unchanged, while the second was supplemented with 20 mg/mL soybean antitrypsin (AT-ETM), and the third supplemented with 3 mg/mL soybean trypsin-chymotrypsin inhibitor which inhibits chymotrypsin to a greater extent (AC-ETM, Sigma Aldrich, St. Louis, MO). The ETM preserved samples were placed in a 25°C water bath until tested for toxin using the toxin EIA method described previously. The ETM samples were tested for toxin at 0, 24, 48, 72, and 96 hours, to determine the effect of inhibiting trypsin and chymotrypsin in ETM preserved samples.

Molecular Methods.

Evaluation of *Clostridium* DNA from *C. difficile* culture isolates was performed using the QIAampDNA Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer’s procedure with modifications for *C. difficile*. Toxin A gene DNA was amplified by PCR following the method of Kato *et al.* (1991) using the modified forward primer for toxin A, NK2 5’ GTA ATC CCC AAT AGA AGA TTC AATATTAGGCTT 3’, and the reverse primer NK3 5’ GGA AGA AAA GAA CTT CTG GCTCACTC 3’. Toxin B was detected by amplification of DNA with the forward primer, NK104- 5’ GTGTAGCAATGAAAGTCCAAGTTTAC 3’, and the reverse primer, NK105- 5’
CACTTAGCTCTTTGATTGCTGCACCT 3’. PCR products were evaluated by agarose gel electrophoresis using 1.5% (w/v) agarose gel containing 0.5 μg/mL ethidium bromide to detect genes for toxins A and B (Lou, Chong et al. 1997).

Isolates were typed by PCR ribotyping according to the method described by O’Neill et al. (1996). Primers 5′-CTGGGGTGAACTCGTAACAAGG-3’ 16S rRNA gene and 5′-GCGCCCTTTGATAGCTTGACC-3’ 23S rRNA gene complementary to the 3′ end of the 16S rRNA gene, and the 5′ end of the 23S rRNA gene, were used to amplify the variable-length intergenic spacer region. The *C. difficile* NAP1/027 (ATCC® BAA-1805-FZ) strain was used a control.

Amplification of the *tcdC* gene by PCR was performed following the method described by Spigaglia and Mastrantonio (2002) using the forward primer Tim2 5’ GCACCTCATCACCATCTTCAA 3’ and the reverse primer Struppi2 5’ TGAAGACCATGAGGGTGTCAT 3’. PCR products were evaluated by agarose gel electrophoresis to detect *tcdC* gene products.

**Interpretation of Toxin and Glutamate Dehydrogenase Results**

A true positive for *C. difficile* bacterial antigen (glutamate dehydrogenase), would be defined as a sample with a positive bacterial antigen test that also had a culture positive for the *C. difficile* bacterium. A false positive *C. difficile* bacterial antigen test is defined as glutamate dehydrogenase positive, but negative by anaerobic cultures for *C. difficile*. A true positive for the *C. difficile* toxin test would be a sample positive by EIA for toxin A or B, and the cell cytotoxin being positive either directly or with *C. difficile* bacterial isolates grown in BHI broth and the broth tested for toxin. A false positive EIA
result is defined as positive for toxin by EIA on feces and negative by cell cytotoxin assay, and negative EIA test from broth cultures with *C. difficile* isolates if present in those samples.

**Results**

**Glutamate dehydrogenase (GDH).** Of the 100 samples preserved in ETM, 17 tested positive for glutamate dehydrogenase as measured by the C. Diff Quik Chek. False positive GDH results were found in two of these 17 samples, which were negative by all other methods. False negative results were reported in two of the samples, where samples tested positive by culture, indicating a false negative result (Table 1).

**Toxin A/B Detection by EIA.** Of the 100 ETM-preserved samples initially tested, 15 were positive for *C. difficile* toxin A/B by EIA (Table 2). There were 12 low positive samples (absorbance ranging 0.08 to 0.68 OD 450/650) as determined by EIA, and negative by all other testing methods. Repeat toxin testing of these 12 samples by EIA resulted in absorbance less than 0.08 OD 450/650 and may have been caused by well to well contamination. All of the true EIA toxin A/B positive samples were strongly positive, with the lowest absorbance of OD 450/650 being 0.68, with the strongest absorbance being too high to measure spectrophotometrically >3.80 (Table 3). A single false sample (#31), was positive by the EIA method (negative by all other methods), was also strongly positive and the EIA reaction could not be read spectrophotometrically, even after being held at room temperature in ETM for 120 hours.

**Clostridium difficile culture.** There were nineteen culture positive isolates of *C. difficile* from the original 100 fecal samples tested. Paired cultures using either ETM
preservation at room temperature, or refrigerated samples were equivalent when tested by this method. Toxin negative isolates (n=3) were additionally negative for toxin when grown in anaerobic BHI broth and the toxin assay performed by EIA. The other 16 of the 19 C. difficile isolates were toxin positive by EIA when the supernatant was tested for toxin by EIA (Table 3).

**Cell Cytotoxin Neutralization Assay.**

Of the 100 samples tested, 17 were positive for toxin B, as determined by cytopathic effect (CPE). Cells were evaluated at 4, 24, 48 and 72 hours for evidence of CPE. Anti-toxin neutralization failed for six samples, three of these six samples (#47, 19, and 89) were neutralized by the anti-toxin at the additional 1:10 dilution. An additional 1:100 dilution was required to neutralize two (#28 and 36) of the 6 samples (Table 3). Of the six samples requiring additional dilutions, one sample (#16) was not neutralized at the additional 1:10 or 1:100 dilutions, and the CPE was not considered to be Clostridium difficile toxin related; it was also negative by culture, GDH and toxin A/B EIA methods.

**Toxin A and B DNA.**

Of the 19 culture-positive isolates, 16 had toxin A and toxin B genes detected by PCR amplification and gel electrophoresis (Figure 10). Non-toxigenic isolates 3, 50 and 55 were negative for toxin A and B genes.
Table 1. Stool samples were tested for *C. difficile* anaerobic culture and glutamate dehydrogenase

<table>
<thead>
<tr>
<th>Clostridium difficile Anaerobic Culture vs Glutamate Dehydrogenase (GDH)</th>
<th>Glutamate Dehydrogenase Positive (n=19)</th>
<th>Glutamate Dehydrogenase Negative (n=81)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture Positive (n=19)</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>Culture Negative (n=81)</td>
<td>2</td>
<td>79</td>
</tr>
</tbody>
</table>

Table 2. Results of cell cytotoxin assay (CCTA) versus EIA toxin A/B assay on 100 stool samples

<table>
<thead>
<tr>
<th>Clostridium difficile CCTA vs Toxin EIA</th>
<th>EIA Zero hrs.</th>
<th>EIA 4 hrs.</th>
<th>EIA 24 hrs.</th>
<th>EIA 48-120 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ (n=18)</td>
<td>- (n=82)</td>
<td>+ (n=16)</td>
<td>- (n=84)</td>
</tr>
<tr>
<td>CCTA Positive * (n=17)</td>
<td>15</td>
<td>2</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>CCTA Negative (n=93)</td>
<td>3</td>
<td>80</td>
<td>2</td>
<td>81</td>
</tr>
</tbody>
</table>

*One sample was positive by CCTA and Toxin EIA but failed to grow in anaerobic culture*
Table 3. Summary of positive culture isolates for *Clostridium difficile* compared with test methods.

<table>
<thead>
<tr>
<th>Sample#</th>
<th>pH</th>
<th>EIA</th>
<th>GDH</th>
<th>CCTA</th>
<th>1:10</th>
<th>1:100</th>
<th>Moxiflox</th>
<th>Proline</th>
<th>BT</th>
</tr>
</thead>
<tbody>
<tr>
<td>2*</td>
<td>7.1</td>
<td>1.04 P</td>
<td>Neg</td>
<td>25/N</td>
<td>NA</td>
<td>NA</td>
<td>R(0mm)</td>
<td>Pos</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>6.8</td>
<td>0.07 N</td>
<td>mod</td>
<td>Neg</td>
<td>NA</td>
<td>NA</td>
<td>S(19mm)</td>
<td>Pos</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>6.6</td>
<td>0.06 N</td>
<td>weak</td>
<td>100/N</td>
<td>NA</td>
<td>NA</td>
<td>S(19mm)</td>
<td>Pos</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>7.9</td>
<td>0.98 P</td>
<td>strong</td>
<td>100/U</td>
<td>100/N</td>
<td>NA</td>
<td>S(21mm)</td>
<td>Pos</td>
<td>-</td>
</tr>
<tr>
<td>28</td>
<td>7.2</td>
<td>2.48 P</td>
<td>strong</td>
<td>100/U</td>
<td>100/U</td>
<td>100/N</td>
<td>R(0mm)</td>
<td>Pos</td>
<td>+</td>
</tr>
<tr>
<td>36</td>
<td>8.2</td>
<td>&gt;4.0 P</td>
<td>strong</td>
<td>100/U</td>
<td>100/U</td>
<td>100/N</td>
<td>S(20mm)</td>
<td>Pos</td>
<td>+</td>
</tr>
<tr>
<td>45</td>
<td>7.8</td>
<td>2.87 P</td>
<td>strong</td>
<td>100/N</td>
<td>NA</td>
<td>NA</td>
<td>S(20mm)</td>
<td>Pos</td>
<td>-</td>
</tr>
<tr>
<td>47</td>
<td>7.1</td>
<td>3.16 P</td>
<td>strong</td>
<td>100/N</td>
<td>NA</td>
<td>NA</td>
<td>R(14mm)</td>
<td>Pos</td>
<td>+</td>
</tr>
<tr>
<td>49</td>
<td>7.7</td>
<td>0.07 N</td>
<td>Neg</td>
<td>100/N</td>
<td>NA</td>
<td>NA</td>
<td>S(20mm)</td>
<td>Pos</td>
<td>+</td>
</tr>
<tr>
<td>50</td>
<td>6.5</td>
<td>0.08 N</td>
<td>strong</td>
<td>Neg</td>
<td>NA</td>
<td>NA</td>
<td>S(20mm)</td>
<td>Pos</td>
<td>-</td>
</tr>
<tr>
<td>55</td>
<td>7.1</td>
<td>0.06 N</td>
<td>strong</td>
<td>Neg</td>
<td>NA</td>
<td>NA</td>
<td>S(19mm)</td>
<td>Pos</td>
<td>-</td>
</tr>
<tr>
<td>57</td>
<td>8.4</td>
<td>0.68 P</td>
<td>mod</td>
<td>100/N</td>
<td>NA</td>
<td>NA</td>
<td>S(20mm)</td>
<td>Pos</td>
<td>-</td>
</tr>
<tr>
<td>59</td>
<td>6.4</td>
<td>1.85 P</td>
<td>strong</td>
<td>100/N</td>
<td>NA</td>
<td>NA</td>
<td>S(25mm)</td>
<td>Pos</td>
<td>-</td>
</tr>
<tr>
<td>67</td>
<td>7.9</td>
<td>3.79 P</td>
<td>strong</td>
<td>100/N</td>
<td>NA</td>
<td>NA</td>
<td>R(0mm)</td>
<td>Pos</td>
<td>-</td>
</tr>
<tr>
<td>69</td>
<td>7.4</td>
<td>0.93 P</td>
<td>mod</td>
<td>100/N</td>
<td>NA</td>
<td>NA</td>
<td>S(20mm)</td>
<td>Pos</td>
<td>+</td>
</tr>
<tr>
<td>82</td>
<td>8.7</td>
<td>3.71 P</td>
<td>mod</td>
<td>100/N</td>
<td>NA</td>
<td>NA</td>
<td>S(25mm)</td>
<td>Pos</td>
<td>+</td>
</tr>
<tr>
<td>89</td>
<td>7.2</td>
<td>3.59 P</td>
<td>strong</td>
<td>100/U</td>
<td>100/N</td>
<td>NA</td>
<td>R(0mm)</td>
<td>Pos</td>
<td>-</td>
</tr>
<tr>
<td>91</td>
<td>7.8</td>
<td>2.89 P</td>
<td>mod</td>
<td>100/N</td>
<td>NA</td>
<td>NA</td>
<td>R(0mm)</td>
<td>Pos</td>
<td>+</td>
</tr>
<tr>
<td>98</td>
<td>6.8</td>
<td>2.98 P</td>
<td>strong</td>
<td>100/N</td>
<td>NA</td>
<td>NA</td>
<td>S(20mm)</td>
<td>Pos</td>
<td>-</td>
</tr>
</tbody>
</table>

*Sample #2 was negative for toxin by EIA after 4 hours in ETM. EIA = Enzyme immunoassay, P= positive EIA, N= negative EIA, GDH= glutamate dehydrogenase, CCTA= Cell cytotoxin assay, Moxifox = Moxifloxacin, R = no zone, S= zone. BT = binary toxin. Moxifloxacin resistance and binary toxin are both highly linked to NAP1 epidemic strain.
Figure 10. Agarose gel electrophoresis identifying toxin A and B DNA amplified by PCR from *C. difficile* isolates. Isolates negative for toxin A and B (white arrows).
Deletion in tcdC Gene.

DNA from six of the toxigenic isolates (#91, 89, 82, 67, 45, and 36) displayed electrophoretic migration in agarose identical to the NAP1 strain (Figure 11). Deletions were detected by PCR amplification using primers specific for the deletion area. Deletions larger than the 18 bp were found in two toxigenic isolates (#47 and 49) demonstrated by increased migration as compared to the NAP1 control strain, indicating a smaller PCR product. The toxigenic strains (n=8, #98, 69, 59, 57, 28, 19, 6, and 2) had migration patterns indicating that there was no deletion in the tcdC gene; similar to the ATCC 9689 control strain.

PCR Ribotyping, Binary Toxin detection.

Ribotyping was performed on 19 culture isolates using primers specific to the intergenic spacer region between the 16S-23S rRNA. NAP1 strains display a specific triple doublet to which other strains can be compared. Isolates #2, 28, 36, and 82 had similar banding as compared to the NAP1 strain, but no isolates were a match to the NAP1 strain (Figure 12).

Binary toxin testing was performed using specific PCR primers. Isolates #2, 28, 36, 47, 49, 69, 82, and 91 were positive for the presence of binary toxin (Figure 13).
Figure 11. Deletion in tcdC gene PCR agarose gel electrophoresis. White bar identifies migration equal to NAP1 control strain (N1). ATCC strain 9689 (AT) identifies the wild type without tcdC deletion.
Figure 12. PCR Ribotyping of 19 clinical isolates. NAP1 isolates display unique triple doublet (red box).
Figure 13. Binary toxin PCR gel electrophoresis. NAP1 strain (N) used as a positive control.
**pH and the Presence of *C. difficile***.

Freshly thawed fecal samples were tested for fecal pH using a pH meter. The pH range of the 100 samples was found to range from a low of 4.7, and a high of 8.9, with an average pH being 7.2 (Figure 14). *C. difficile* was cultured from 19 fecal samples with the lowest fecal pH being 6.4, the highest fecal pH being 8.7, and the average fecal pH being 7.4.

pH-adjusted BHIB media was used to determine *C. difficile* growth restrictions. *C. difficile* growth was significantly inhibited at and below pH 5.9, or above pH 9.5 (Figure 15). Bacteria were also evaluated for growth on BHI agar which was altered to vary pH. *C. difficile* displayed no growth at pH 4.5 and reduced growth at pH 5.0 as compared to the normal BHIA pH of 7.3 (Figure 16).

**Effect of Altering BHIB pH on EIA toxin testing**

*C. difficile* ATCC 9689 grown in BHIB in which the pH was adjusted prior to testing displayed a significant drop in toxin detection, when the bacteria were incubated for one or two hours at room temperature (Figure 17). Following the EIA test, the pH 4 sample was alkalized using 10M NaOH to pH 8 with no improvement in toxin detection indicating a permanent change in the structure of the toxin.

**Sensitivity and Specificity of tests.** Results of GDH testing were compared to those of *C. difficile* culture. EIA toxin testing for toxins A and B tested at various time points were compared to cell cytotoxin assay for toxin B. Sensitivities, specificities, positive predictive values, and negative predictive values were calculated for these tests using Stat pages.net (http://statpages.org/ctab2x2.html) (Table 4).
Figure 14. Histogram of pH for 100 patient fecal samples, and *C. difficile* culture positive samples.
Figure 15. C. difficile growth measured at 600nm in overnight BHIB with altered pH.

Growth of non-toxigenic C. difficile ATCC 43593 was measured at different pHs.

Cultures were measured following overnight incubation. * (p<0.0001)
Figure 16. *C. difficile* ATCC 43593 strain growth inhibition on BHI agar with altered pH.
Figure 17. Effect of altering BHIB pH on EIA Toxin Detection. BHIB pH was adjusted so as to evaluate the effect on *Clostridium difficile* toxin EIA detection using sterile filtered BHIB cultures of toxigenic ATCC 9689 strain. Reducing the pH to 4.0 had a very significant effect on the amount of toxin detected * (p<0.0001).
**Table 4.** Performance characteristics for GDH and Toxin EIA compared to Cell Cytotoxin Neutralization Assay and Culture.

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity%</th>
<th>Specificity%</th>
<th>PPV%</th>
<th>NPV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDH</td>
<td>89.5(71.6-97.3)</td>
<td>97.5(93.3-99.4)</td>
<td>89.5(71.6-97.3)</td>
<td>97.5(93.3-99.4)</td>
</tr>
<tr>
<td>EIA 0 hrs</td>
<td>88.2 (67.9-97.6)</td>
<td>96.4 (92.2-98.3)</td>
<td>83.3(64.1-92.2)</td>
<td>97.6(93.3-99.5)</td>
</tr>
<tr>
<td>EIA 4 hrs</td>
<td>82.4 (62.2-91.7)</td>
<td>97.6 (93.5-95.5)</td>
<td>87.5 (66.1-97.4)</td>
<td>96.4(92.3-98.3)</td>
</tr>
<tr>
<td>EIA 24 -120 hrs*</td>
<td>82.4(63.1-87.9)</td>
<td>98.8(94.9-99.9)</td>
<td>93.3(71.5-99.6)</td>
<td>96.5(92.6-97.6)</td>
</tr>
</tbody>
</table>

PPV (positive predictive value) NPV (negative predictive value)

**Table 5.** Determination of total fecal proteases and fecal trypsin using azocasein substrate.

<table>
<thead>
<tr>
<th>Patient feces (+) for <em>C. difficile</em></th>
<th>Total Protease Absorbance mean</th>
<th>Trypsin Blocked Absorbance mean</th>
<th>Calculated Trypsin Absorbance</th>
<th>Trypsin μg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.22</td>
<td>0.63</td>
<td>1.59</td>
<td>2116</td>
</tr>
<tr>
<td>2</td>
<td>2.61</td>
<td>1.06</td>
<td>1.55</td>
<td>2357</td>
</tr>
<tr>
<td>3</td>
<td>0.71</td>
<td>0.22</td>
<td>0.49</td>
<td>56</td>
</tr>
<tr>
<td>4</td>
<td>0.20</td>
<td>0.13</td>
<td>0.07</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>2.29</td>
<td>0.91</td>
<td>1.38</td>
<td>1122</td>
</tr>
<tr>
<td>6</td>
<td>2.1 (4.2*)</td>
<td>2.38</td>
<td>1.82</td>
<td>4447</td>
</tr>
<tr>
<td>7</td>
<td>2.02 (4.04*)</td>
<td>2.09</td>
<td>1.94</td>
<td>5917</td>
</tr>
<tr>
<td>8</td>
<td>2.23</td>
<td>1.11</td>
<td>1.12</td>
<td>453</td>
</tr>
<tr>
<td>9</td>
<td>1.79 (3.58*)</td>
<td>2.08</td>
<td>1.5</td>
<td>1610</td>
</tr>
<tr>
<td>10</td>
<td>2.47 (4.94*)</td>
<td>1.64 (3.28*)</td>
<td>1.66</td>
<td>2063</td>
</tr>
<tr>
<td>Normal feces</td>
<td>0.89</td>
<td>0.68</td>
<td>0.21</td>
<td>26</td>
</tr>
</tbody>
</table>

*Samples diluted 1:1 in 1M NaOH and calculated absorbance determined.*
Fecal Proteases

Additional GDH positive fecal samples (n=10) were tested to measure total fecal protease activity, using azocasein as the substrate (Table 5). Samples were also tested by blocking trypsin activity using soybean antitrypsin. Trypsin activity was determined by subtracting trypsin-blocked activity from total protease activity. Trypsin concentration was calculated by comparing patient trypsin OD 450 nm to OD 450 nm of known trypsin standards. The highest patient trypsin value was 5,947 μg/g feces, and the lowest was 17 μg/g feces. Normal values for random fecal trypsin are approximately 30 μg/g feces.

The effect of inhibiting the degradation of toxin by supplementing PBS with soybean antitrypsin resulted in higher levels of toxin being detected at the initial measurement, and measurements at 48 hours to 96 hours (Figure 18). Of the three samples tested, one sample with very high fecal trypsin (Figure 18c) had significantly higher toxin detection at every time point tested. Another sample with lower than normal fecal trypsin (Figure 18a), had significant improvement in toxin detection with the addition of antitrypsin, but to a lesser degree. The third patient sample tested had increased fecal trypsin level, however displayed little improvement in toxin detection upon supplementing the sample with antitrypsin (Figure 18b).

Following the determination of fecal trypsin concentrations on the ten additional samples, the samples were tested for *C. difficile* toxins using the EIA method. Of the ten samples tested, six were positive for *C. difficile* after being preserved in ETM (Table 6). Supplementing fecal samples preserved in ETM with either soybean antitrypsin, or soybean trypsin-chymotrypsin inhibitor at room temperature did not increase the amount
of toxin detected as seen after supplementing PBS samples. A single ETM sample (4) had improved toxin preservation after supplementation with protease inhibitors.

Following the 96 hour toxin EIA analysis on the six ETM preserved samples, the samples were retested by the GDH assay. All six samples contained detectable GDH.
Table 6. Decrease in EIA Absorbance in ETM preserved samples, with and without supplementation with protease inhibitors over time.

<table>
<thead>
<tr>
<th>Sample # and Treatment</th>
<th>Fecal Trypsin ug/g</th>
<th>EIA Zero hr Absorbance</th>
<th>EIA 24 hr Absorbance</th>
<th>EIA 48 hr Absorbance</th>
<th>EIA 72 hr Absorbance</th>
<th>EIA 96 hr Absorbance</th>
<th>% Change in Absorbance 0-96 hrs *</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Plain ETM</td>
<td>2357</td>
<td>2.57 (*5.14)</td>
<td>2.00 (*5.2)</td>
<td>2.55 (*5.1)</td>
<td>2.42 (*4.84)</td>
<td>2.18 (*4.36)</td>
<td>15%</td>
</tr>
<tr>
<td>AT – ETM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Plain ETM</td>
<td>46</td>
<td>2.41 (*4.82)</td>
<td>2.04 (*4.08)</td>
<td>2.59 (*5.18)</td>
<td>2.53 (*5.06)</td>
<td>2.08 (*4.16)</td>
<td>14%</td>
</tr>
<tr>
<td>AT – ETM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Plain ETM</td>
<td>17</td>
<td>2.90 (*5.98)</td>
<td>2.04 (*4.08)</td>
<td>2.90 (*5.80)</td>
<td>2.45 (*4.91)</td>
<td>1.96 (*3.92)</td>
<td>35%</td>
</tr>
<tr>
<td>AC – ETM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 Plain ETM</td>
<td>17</td>
<td>2.90 (*5.96)</td>
<td>2.20 (*4.20)</td>
<td>2.87 (*5.74)</td>
<td>2.35 (*5.70)</td>
<td>2.43 (*4.86)</td>
<td>19%</td>
</tr>
<tr>
<td>AT – ETM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 Plain ETM</td>
<td>4447</td>
<td>0.64</td>
<td>0.77</td>
<td>0.59</td>
<td>0.57</td>
<td>0.55</td>
<td>14%</td>
</tr>
<tr>
<td>AT – ETM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 Plain ETM</td>
<td>5917</td>
<td>0.71</td>
<td>0.72</td>
<td>0.76</td>
<td>0.7</td>
<td>0.68</td>
<td>4%</td>
</tr>
<tr>
<td>AC – ETM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 Plain ETM</td>
<td>17</td>
<td>0.69</td>
<td>0.72</td>
<td>0.62</td>
<td>0.51</td>
<td>0.65</td>
<td>14%</td>
</tr>
<tr>
<td>AT – ETM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 Plain ETM</td>
<td>1610</td>
<td>1.71 (*3.42)</td>
<td>1.90 (*3.80)</td>
<td>1.85 (*3.70)</td>
<td>1.82 (*3.64)</td>
<td>1.49 (*2.98)</td>
<td>13%</td>
</tr>
<tr>
<td>AT – ETM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 Plain ETM</td>
<td>228</td>
<td>1.75</td>
<td>1.75</td>
<td>1.75</td>
<td>1.75</td>
<td>1.75</td>
<td>14%</td>
</tr>
<tr>
<td>AT – ETM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 Plain ETM</td>
<td>2.28</td>
<td>1.83</td>
<td>1.83</td>
<td>1.83</td>
<td>1.83</td>
<td>1.83</td>
<td>14%</td>
</tr>
<tr>
<td>AC – ETM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(1) Decrease in absorbance at 96 hours was compared to absorbance at zero hours to determine percent decrease in absorbance when preserved.

Note: *Samples diluted 1:1 in 0.6 M H₂SO₄ and calculated absorbance determined.
Figure 18. Patient samples tested for toxin degradation in PBS, and PBS supplement with 20 mg/mL antitrypsin (AT) at 25°C (n = 3). A. Patient sample with low fecal trypsin (17 µg/g) B. Patient sample with high fecal trypsin (1,610 µg/g) C. Patient sample with very high fecal trypsin (5,917 µg/g) Statistically significant using paired t test **(p<0.001)* (p<0.0001)
Discussion

Testing for *C. difficile* disease is problematic due to the unstable nature of its toxins and specific antigens present in non-preserved or refrigerated feces. Transport of fecal samples at room temperature often results in the degradation of these antigens resulting in false negative laboratory tests. This is significant as patients left untreated for *C. difficile* disease can have increased morbidity and mortality. We believe that testing for the presence of toxin will continue in spite of molecular diagnostic testing for *tcdB* which is reported to have superior sensitivity and sensitivity when compared to the toxigenic culture method. Molecular testing’s downfall is related to the high number of asymptomatic carriers who test positive for the *C. difficile* toxin-positive organisms in their feces, but do not develop disease from the presence of toxin in their feces.

Of the 100 initial stool specimens evaluated in this study, fifteen samples initially tested positive for toxins A and B by EIA. A single sample that was initially a strong positive was negative after 4 hours of storage at room temperature when preserved in ETM. The 14 other samples remained positive after 5 days (120 hours) of storage at room temperature in ETM. Of these 14 samples, one was falsely positive even after 5 days; it was not positive by any other method. This result was most likely caused by non-specific antibody binding to a contaminating substance in the fecal sample.

Culturing the ETM preserved samples after 7 days at room temperature detected nineteen samples positive for *C. difficile*. These same samples were positive for growth when stored unpreserved at 2-8° C for 7 days before culturing. There was 100% correlation for the positive and negative cultures for *C. difficile* between the ETM
preserved samples at room temperature and the unpreserved refrigerated samples this indicated no loss of viability of the organism in ETM being held at room temperature for 7 days as compared to feces refrigerated at 2-8°C for 7 days.

Fecal pH did not affect toxin detection unless the pH was decreased to pH 4 (Figure 17). None of the 100 samples had a pH of 4 (Figure 14). The lowest fecal pH was 4.7, and it is likely that growth of *C. difficile* was inhibited at that pH, as the lowest fecal sample pH to harbor *C. difficile* was pH 6.4 (Table 3). Results from the BHIB and BHIA cultures that had been acidified resulted in significant growth inhibition when the pH was less than pH 5.9 (p <0.0001) for broth cultures, and pH 5.0 for solid agar cultures (Figure 16). *C. difficile* growth was also inhibited by alkaline pH in broth cultures at and above pH 9.5 (p <0.0001), although the highest fecal pH in the 100 samples was 8.9. The highest patient fecal pH to grow *C. difficile* was 8.7, which indicates that growth inhibition may occur in patient samples with high fecal pH. These facts may lead to alternative therapies where patient diets may be supplemented with food additives to acidify or alkalinize the intestinal environment to inhibit the colonization and growth of *C. difficile*. The acidification of feces using acidogenetic bacteria and oligosaccharides has previously been shown to inhibit the growth of *C. difficile* and other bacterial intestinal pathogens (May, Mackie et al. 1994; Campbell, Fahey et al. 1997).

Testing of an additional ten GDH fecal samples resulted in elevated total fecal proteases and fecal trypsin in 8 of 10 of patient samples tested, with the highest trypsin level being 5,917 μg/g of feces (Table 5). This is significantly higher than the normal patient spot fecal trypsin range of 30 μg/g of feces. Testing samples in PBS, and PBS
supplemented with antitrypsin, resulted in significantly greater levels of toxin detected by EIA in one sample at every time point (Figure 18c). This result did not hold true for the other sample with high fecal trypsin levels, where only a few of the time points saw improvement in detectable toxin (Figure 18b). The fecal sample with low fecal trypsin (17 μg/g) also displayed better toxin detection, at several time points that was statistically significant (Figure 18a). This may be due in part to testing only centrifuged supernatant for fecal proteases. Bacterial proteases are found attached to the solids in fecal samples, and these were removed by centrifugation during the stool processing before assaying the sample proteolytic activity (Macfarlane, Cummings et al. 1986). When testing patient feces for toxin preservation in PBS and ETM, whole feces was used in sample preparation and bacterial proteases may have played a role in toxin degradation.

No improvement in toxin detection was seen with supplementing ETM with protease inhibitors (soybean trypsin or soybean trypsin-chymotrypsin inhibitor) in five of the 6 additional toxin positive samples, indicating that fecal proteases did not degrade toxin while preserved in ETM (Table 6). Surprisingly ETM alone supported better toxin detection than did ETM with either of the supplements. This may be due to the protease inhibitors interfering with the components of ETM (formulation is proprietary). Improved toxin stability was seen in one fecal ETM preserved sample that was supplemented with protease inhibitors. This single sample (#4) had low total fecal proteases and a fecal trypsin of 17 μg/g. This result again suggests that there are proteases (bacterial) other than trypsin responsible for toxin degradation. ETM preservation of fecal samples had a
smaller decrease in detectable toxin (average 12%) as compared to the samples tested in PBS (50%) or PBS supplemented with antitrypsin (25-50%).

Molecular evaluation of *C. difficile* isolates for the identification of NAP1 strains was difficult to interpret. PCR ribotyping indicated that none of the strains recovered exactly matched the banding pattern from the control strain (Figure 12). Isolates #2, 28, 36, 82, and 91 had banding similar to the NAP1 control, but not identical. Binary toxin which is found in the all reported NAP1 strains was detected in isolates #2, 28, 36, 47, 49, 69, 82, and 91 (Figure 13). Evidence of the 18 base pair deletion was found in isolates #91, 89, 82, 67, 45 and 36, while isolates #47 and 49 had evidence of even a larger deletion in the *tcdC* gene (Figure 11). Moxifloxacin resistance is also common to NAP1 isolates, and this was found in isolates #2, 28, 47, 67, 89, and 91 (Table 3). From this evidence it could be said that isolate #91 is most similar to the NAP1 strain. Interestingly there were 8 isolates with deletions in the *tcdC* indicating they could be hyper-toxin producer strains. This deletion is usually associated with greater toxin production, although only 4 of these strains required additional dilutions to neutralize toxin activity in the cell cytotoxin assay (Table 3). DNA from isolates #47 and 49, which migrated faster than the DNA from the NAP1 strain, may actually be the newer NAP8 strain, which has a 39 base pair deletion in the *tcdC* gene, carries binary toxin, and produces increased levels of toxin. Direct comparison with a known NAP8 strain would need to be performed to confirm this.

Sample number 2 of the original 100 samples was initially very strongly EIA-positive, yet negative after four hours of room temperature incubation in ETM (Table 3).
Additionally, the cell cytotoxin result was weakly positive for sample #2, GDH assay was negative, and the *C. difficile* culture was positive. Repeat toxin EIA and GDH assays performed on a broth culture of this isolate were both positive. The aberrant results may have been caused by nonspecific fecal proteases in the original fecal specimen degrading *C. difficile* toxins and glutamate dehydrogenase. Testing for fecal proteases was not performed on this sample. The pH of this fecal sample was 7.2, so antigen degradation due to fecal acids was also unlikely.

Sample number 16 had a pH of 6.0 and was negative in all tests. The cells in the cell cytotoxin assay showed cytopathic effect even with the additional 1:100 dilution of the original 1:40 sample dilution. However, CPE was not considered to be due to *C. difficile* toxin since it was not neutralized by the anti-toxin, and the sample tested negative for *C. difficile* by all other methods. Other fecal samples with a pH of less than 6.0 did not cause nonspecific cytopathic effect on the cells in culture, so pH is unlikely to be the cause of CPE.

Sample number 31 was strongly positive by EIA for the entire 5-day room temperature incubation in ETM. All other tests were negative for *C. difficile*. This result was considered to be false positive for *Clostridium difficile* toxins A/B by the EIA method.

Glutamate dehydrogenase (GDH) is recommended as a screening method prior to toxin testing as a way to save resources and technologist time while improving test reliability (Ticehurst, Aird et al. 2006; Gilligan 2008). This test was found to have a high negative predictive value (97.5%). Our data indicates that if GDH-positive samples were
subsequently tested for EIA toxin, as recommended, the number of false positive EIA results would be reduced by two. The number of true EIA toxin-positive samples missed would then be one. The number of cell cytotoxin positive results missed would have been three. Importantly, three GDH-positive, but EIA toxin A and B, and cell cytotoxin negative isolates were found, thus making toxin testing essential for these samples (These fecal samples harbored non-toxigenic *C. difficile* bacteria).

GDH antigens were also maintained when preserved in ETM when held at room temperature. The six additional samples maintained their GDH antigens even when fecal proteases were at high levels. This ability to stabilize antigens is beneficial for transporting fecal samples to the laboratory for analysis when refrigeration is not available.

In conclusion, Enteric Transport Medium (ETM) is a reliable material for transporting fecal samples for multiple laboratory tests, including *C. difficile* toxins and glutamate dehydrogenase. In rare cases, non-specific fecal proteases may degrade the target epitopes affecting the *C. difficile* test results. Of the original 100 samples, 14 of the 15 EIA toxin positive samples were maintained for 120 hours at room temperature, and all six additional isolates maintained toxin with only small amount of toxin degradation; therefore, we conclude that room temperature preservation in ETM is a valuable tool for testing feces for *C. difficile* when refrigeration is not an option. Refrigeration of ETM preserved samples may reduce the activity of fecal proteases and provide better stability for antigens and toxins of *C. difficile*. 
References


Merz, C. S., C. Kramer, et al. (1994). "Comparison of four commercially available rapid enzyme immunoassays with cytotoxin assay for detection of Clostridium difficile"
toxin(s) from stool specimens." Journal of Clinical Microbiology 32(5): 1142-1147.


CHAPTER III

Antimicrobial Effects of Virgin Coconut Oil and its Medium Chain Fatty Acids on 

*Clostridium difficile*

Abstract

*Clostridium difficile* is the leading cause of hospital-acquired, antibiotic-associated diarrhea worldwide; in addition, the proliferation of antibiotic-resistant *C. difficile* is becoming a significant problem. Virgin coconut oil (VCO) has been shown to have antimicrobial activity. This study evaluates the lipid components of virgin coconut oil for the control of *C. difficile* in vitro. Lauric acid (C\textsubscript{12}) was the most bactericidal (*p* < 0.0001), as determined by a reduction in colony-forming units per milliliter. Capric acid (C\textsubscript{10}), and caprylic acid (C\textsubscript{8}) were also bactericidal, but to a lesser degree. Undigested virgin coconut oil did not inhibit growth of *C. difficile*; however, antimicrobial activity was present when bacterial cells were exposed to 0.15%-1.2% lipolyzed coconut oil. Transmission electron micrographs (TEM) showed disruption of both the cell membrane and the cytoplasm of cells exposed to 2 mg/mL of lauric acid. Changes in bacterial cell membrane integrity were additionally confirmed for VCO and select fatty acids using Live/Dead staining using the BacLight kit. This study demonstrates the antimicrobial properties mediated by medium chain fatty acids derived from virgin coconut oil on *C. difficile.*
Introduction

Risk of *Clostridium difficile* infection (CDI) continues to expand worldwide reaching nearly every continent (Warny, Pepin et al.; Gerding, Muto et al. 2008). CDI currently is the number one healthcare acquired infection (HAI) surpassing Methicillin resistant *Staphylococcus aureus* (MRSA) (May, Mackie et al. 1994). Of particular concern are the hypervirulent BI/NAP1/027 strains resistant to fluoroquinalone and cephalosporin antibiotics associated with outbreaks in the U.S. and Canada that have not only dramatically increased patient morbidity and mortality rates as well as the cost of treating these patients, estimated at 3.2 billion dollars yearly in the U.S. alone (O’Brien, Lahue et al. 2007; Dubberke and Wertheimer 2009). Low-risk, nontraditional patient groups including children and peripartum females have been found to be susceptible to this new strain (Kuijper, Coignard et al. 2006; Freeman, Bauer et al. 2010). This NAP1 strain has shown decreased sensitivity to metronidazole, and increased reoccurrence in patients treated with metronidazole and vancomycin (McFarland 2005; Pepin, Valiquette et al. 2007; Schwan, Stecher et al. 2009).

The traditional treatment for CDI has relied on the use of antibiotics that may further compromise the normal microbiota of the gut. Metronidazole has been used as the first line of treatment, with vancomycin reserved for more severe cases or relapses (Sunenshine and McDonald 2006; Zar, Bakkanagari et al. 2007). Introduced in 2011, fidaxomicin is a newer treatment option that matches the activity of vancomycin, but has been shown to have fewer relapses following treatment (Tannock, Munro et al. 2010; Louie, Miller et al. 2011; Mullane, Miller et al. 2011). However, antibiotics still have no
effect on residual spores that can cause recurrence after successful treatment. Exposing gut flora to antibiotics like vancomycin encourages the colonization of antibiotic-resistant organisms such as vancomycin-resistant enterococci (Louie, Miller et al. 2011).

Alternative therapies to treat CDI that would not involve disrupting protective normal fecal microbiota have been investigated. Antibiotics only target vegetative bacteria and not spores that may germinate once the treatment has ended. These non-antibiotic treatments include: fecal transplant, immunotherapy, probiotics, bacteriotherapy, and adsorbents as outlined in the introduction (McFarland 2005; Brandt, Aroniadis et al. 2012).

Oils containing medium chain fatty acids (MCFA) have been shown to have antimicrobial effects on a variety of microbes. High levels of MCFA are produced by kern oil plants, particularly coconut and palm kernel plants that are found in tropical countries. The MCFA offer protection to the plants by inhibiting the growth of viral, fungal and bacterial pathogens. The plant oils containing MCFA can be extracted by steam heating and pressure (Babayan 1968). Coconut oil can be extracted from the dried coconut meat using high temperature or a naturally drying process using sunlight that inactivates the biologically active components vitamin E and some of the polyphenols (Nevin and Rajamohan 2009). Virgin Coconut oil (VCO), however, is extracted by a wet process directly from coconut milk using low heat allowing for the preservation of the vitamins, phytosterols and polyphenols (Nevin and Rajamohan 2006).
VCO contains three biologically active MCFA; caprylic (C8), capric (C10) and lauric (C12). When consumed, VCO is lipolyzed into its constituent MCFA that are directly absorbed by the gut into the portal blood system and transported to the liver, where MCFA are absorbed and processed by cells in the liver (Guillot, Vaugelade et al. 1993). Researchers have shown MCFA do not have adverse effects on skin and mucous membranes of animals (Thormar, Bergsson et al. 1999).

Research on VCO has shown MCFA to contain antimicrobial active components that can inhibit the growth of various microbes including viruses, parasites, fungi and bacteria (Kabara, Swieczkowski et al. 1972; Kabara 2000; Bergsson, Arnfinnsson et al. 2001; Enig 2002; Preuss, Echard et al. 2005). MCFA inhibit the growth of various Gram-positive bacteria including: Streptococcus pyogenes, Staphylococcus aureus, and Clostridium perfringens (Bergsson, Arnfinnsson et al. 2001; Skrivanová, Marounek et al. 2005). MCFA have a lesser effect on Gram-negative bacteria (Preuss, Echard et al. 2005). Candida albicans, yeast, which is often involved as a secondary infection following antimicrobial therapy, is also susceptible to MCFA found in coconut oil (Bergsson, Arnfinnsson et al. 2001; Ogbolu, Oni et al. 2007).

VCO manufactured by "The Garden of Life" is an organic product comprised of greater than 99% triglycerides, with free fatty acids making up less than 0.2% (Marina, Che Man et al. 2009). Saturated fats account for 89% of this VCO, with polyunsaturated and monounsaturated fats being 7% and 4% respectively, as noted by the manufacturer. Lauric acid makes up approximately 48% of the saturated fat found in coconut oil, with
caprylic and capric accounting for 7 and 6% respectively (Laureles, Rodriguez et al. 2002). VCO triglycerides can be lipolyzed with lipase and water to form monoglycerides, diglycerides, glycerol, and free fatty acids (Mattson and Volpenhein 1961). Monoglycerides and free fatty acids have been reported to exhibit antimicrobial activity (Thormar, Isaacs et al. 1987; Bergsson, Steingrimsson et al. 1999; Bergsson, Arnfinnsson et al. 2001).

The mechanism by which MCFA exerts its antimicrobial effect is not completely understood. MCFA are believed to disrupt the lipid membranes of pathogens leading to lysis of microbes or loss of their ability to bind to cell receptors (Thormar, Isaacs et al. 1987; Preuss, Echard et al. 2005). Electron micrographs have shown membrane disruption in yeast and bacterial cells exposed to MCFA leading to cell death (Bergsson, Arnfinnsson et al. 2001; Bergsson, Arnfinnsson et al. 2001; Skrivanová, Marounek et al. 2005). Lauric acid is the most active MCFA inhibiting the majority of microbes at lower concentrations than capric or caprylic acids. Lauric acid is also more active against enveloped viruses, including HIV (Hornung, Amtmann et al. 1994; Enig 1998; Bartolotta, Garci et al. 2001). An exception to lauric acid’s bactericidal superiority is when tested against Staphylococcus aureus, where capric acid is more inhibitory than lauric acid (Bergsson, Arnfinnsson et al. 2001).

Many studies have been performed on viruses, yeasts and bacteria displaying the antimicrobial effect of VCO or its medium chain fatty acids, but their effect on C. difficile have not been determined. The objective of this research was to evaluate the
possible antimicrobial effects of VCO and/or its MCFA in vitro on toxin A and B positive *C. difficile* ATCC 9689. Effects on *C. difficile* cell viability in culture, as well as evaluation of bacterial cell membranes and cytoplasm using Live/Dead fluorescence assay and electron microscopy following exposure were explored.

**Methods**

**Culture Media.**

Culture media was prepared using Becton Dickenson (BD) Brain Heart Infusion broth (BHIB) (REF# 211059, Becton Dickenson and Company, Sparks, MD) following the manufacturer’s directions. Ethyl alcohol (ETOH, Quantum Chemical Co., Newark, NJ) was added to the BHIB so that the final volume was equal to 1% (v/v) to increase the miscibility of fatty acids. Brain Heart Infusion agar plates were prepared following the manufacturer’s directions. The pH of the broth cultures was determined using an Accumet pH meter (Fisher Scientific Co., Pittsburgh, PA).

**Virgin Coconut Oil (VCO).**

Pure 100% organic virgin coconut oil (Garden of Life, West Palm Beach, FL USA) was digested with porcine lipase (Cat# 096K0747 Sigma-Aldrich, Co. St. Louis, MO) following the method of Dumitriu *et al.* (Dumitriu, Chornet et al. 1995). A reaction mixture was prepared containing by adding 1.25 mL of virgin coconut oil (VCO), 1.25 mL of sterile distilled water, and 2 mL of phosphate buffered saline (PBS) pH 7.4 to 200
mg porcine lipase. The mixture was continuously stirred for 30 minutes at 37°C. After incubation, the mixture was centrifuged (IEC Centra CL5R, rotor model IEC 6555C, Thermo Electron Corporation, Needham Heights, MA) for 10 minutes at 4,000 x g to form three layers. Following lipase hydrolysis, titration of the top clear layer with 0.1 M KOH resulted in the release of 10.2 mmol/g of free fatty acids indicating an efficiency of 23%. Two hundred milliliters of the top clear layer (lipid) were solubilized in 400 mL of dimethyl sulfoxide (DMSO), (Sigma Aldrich, St. Louis, MO) for a concentration of 33.3% (v/v) digested coconut oil. The digested VCO was then added to brain-heart infusion BHIB to provide final concentrations of digested coconut oil between 0.15% (v/v) and 1.2%(v/v) in BHIB.

**Fatty acids.**

Lauric acid (Sigma Aldrich, St. Louis, MO) was solubilized by diluting 100 mg solid lauric acid in 10 mL Dimethyl sulfoxide (DMSO) to make a stock solution of 50 mM lauric acid in DMSO. The stock solution was further diluted into BHIB to make final concentrations of 1,000 µM, 500 µM, and 250µM lauric acid resulting in 2% DMSO in BHIB.

Capric acid (Sigma Aldrich, St. Louis, MO) was treated similarly to lauric acid where 860 mg was placed in 5 mL of DMSO to make a stock solution of 500 mM capric acid in DMSO. The stock was then diluted into the BHIB for final concentrations of 2,000 µM, 1,000 µM, 500 µM, and 200 µM capric acid resulting in 2% DMSO in BHIB.
A stock solution of caprylic acid (Sigma Aldrich, St. Louis, MO) was prepared by adding 720 mg to 5 mL of DMSO for a concentration of 500 mM caprylic acid in DMSO. The stock solution was further diluted in DMSO to obtain final concentrations of caprylic acid 10,000 µM, 5,000 µM, 2,000 µM, and 1,000 µM caprylic acid, resulting in 2% DMSO in BHIB.

LIVE/DEAD® Bacterial Viability Kit (BacLight™).

Overnight cultures of *C. difficile* were diluted 1:50 into BHIB and grown in the presence or absence of solubilized lipolyzed VCO or fatty acids as described above. Cell viability was assessed using SYTO 9™ (Ex/Em 480/500nm) and propidium iodide (PI; Ex/Em 490/635nm), per the manufacturer’s directions (LIVE/DEAD® Bacterial Viability Kit, BacLight™, Molecular Probes, Inc. Eugene, OR). Briefly, 100 µL aliquots of well-mixed BHIB/1% ethanol inoculated with *C. difficile*, containing either 2% DMSO only or 2% DMSO plus solubilized fatty acids, were mixed with 30 µL of Live/Dead stain diluted to working concentration per manufacturer instructions. Cell suspensions were vortexed for 5 seconds, mounted on a slide, and immediately observed using an Olympus BX-61 microscope (Olympus America, Lake Success, NY) adapted for fluorescence microscopy, using Olympus U-MNB2 and U-MNG2 filter sets for SYTO-9™ and PI, respectively. Cells with intact membranes appear green using the U-MNB2 filter set, while those cells with compromised membranes appear red using the U-MNG2 filter set.
**Fatty Acids and *Clostridium difficile* Susceptibility Assay.**

An overnight culture of *C. difficile* ATCC 9689 (American Type Culture Collection, Manassas, Virginia) was grown in BHIB. The BHIB containing *C. difficile* bacteria, was pipetted (100 microliters) into inoculate 9.9 mL of BHIB (containing 1% ETOH) containing varying amounts of lauric, capric or caprylic fatty acids (dissolved in DMSO). The cultures in 15 mL disposable sterile centrifuge tubes (VWR International, Radnor, Pennsylvania) were incubated at 36°C in an anaerobic chamber (Bactron, Sheldon Manufacturing, Cornelius, Oregon), and mixed by inversion every two minutes, for a total of 30 minutes. Following incubation, 100 µl samples were removed from the respective subcultures and serial dilutions were prepared in sterile water blanks and plated on BHI agar (BHIA). The culture plates were incubated overnight in the anaerobic chamber at 36°C and colony counts performed the next day. Controls for lauric, capric and caprylic acid solutions included: BHIB alone, BHIB 1% EtOH, and BHIB 1% EtOH + 2% DMSO. *C. difficile* was added to the controls from an overnight culture to determine whether inhibition occurred due to the addition of ethyl alcohol or DMSO.
Lipolyzed VCO and Clostridium difficile susceptibility assay.

The DMSO solubilized, lipase digested coconut oil was added to the overnight cultures of C. difficile. The mixture was incubated for 30 minutes at 36°C and mixed by inversion every two minutes before diluting in sterile distilled water and plating onto BHIA. The newly inoculated BHIA was incubated overnight in an anaerobic chamber. Colony counts were performed on plates demonstrating bacterial growth. Controls for the digested coconut oil were BHIB, and BHIB with DMSO/lipase/PBS mixture added, resulting in a 4% (v/v) final concentration of DMSO and a final lipase concentration of 67 µg/mL (6.7% w/v). Controls testing for the undigested coconut oil included BHIB, and BHIB containing 4% DMSO.

Transmission Electron Microscopy.

Lauric acid’s effect on C. difficile was observed using transmission electron microscopy (TEM) following the methods of Skrivanova et al. (Skrivanová, Marounek et al. 2005). Briefly, overnight cultures of C. difficile ATCC 9689 were inoculated with either 2 mg/mL of lauric acid in DMSO or a control of DMSO only. The suspensions were continuously stirred for 30 minutes at 37°C. After incubation, 1 mL aliquots of the suspension were centrifuged at 2,000 x g for 2 minutes (IEC Centra CL5R, rotor model IEC 6555C, Thermo Electron Corporation, Needham Heights, MA). The pellets were fixed with 500 µl of 5% glutaraldehyde (Fisher Scientific Co., Pittsburgh, PA) in 0.2 M Na-Cacodylate buffer (pH 7.4,Sigma Aldrich, St. Louis, MO) and 500 µl of a solution of
ruthenium red (0.15% in 0.2 M Na-Cacodylate buffer, MP Biomedicals, LLC, Solon, OH) for one hour at room temperature.

The samples were again centrifuged at 2,000 x g for 2 minutes, and the pellets washed three times with 0.1 M Na-Cacodylate buffer. The samples were fixed with 400 µl of 4% OsO4 (Stevens Metallurgical Company, New York, NY) in 0.2 M Na-Cacodylate buffer with 400 µl of ruthenium red (0.15% in 0.2 M Na-Cacodylate buffer) for 4 hours at 4°C. They were subsequently centrifuged (2500 x g) and washed three times with 0.1 M Na-Cacodylate buffer. The fixed cells were suspended in molten 1% agar (Becton Dickenson and Company, Sparks, MD), allowed to solidify at 37°C, and cut into 1 mm cubes. The cubes were then dehydrated by through a graded series of ethanol in water. Once dehydrated with 100% ethanol (Quantum Chemical Co., Newark, NJ), the samples were treated with propylene oxide (Sigma Aldrich, St. Louis, MO) first with a 1:1 solution in EtOH, followed by 100% propylene oxide. The cubes were treated with a series of dilutions of Embed 812 (Electron Microscopy Sciences, Hatfield, PA) with propylene oxide until 100% of Embed 812 solution was achieved. The treated samples were placed in resin mold trays in 100% Embed 812, and hardened overnight in a 70°C oven (Fisher Isotemp Oven, Fisher Scientific Co., Pittsburgh, PA).

Sections of samples embedded in Embed 812 were cut at 100 µm thickness (Leica EMUC6 ultramicrotome, Leica, Vienna, Austria), placed on copper grids (Electron Microscope Sciences, Hatfield, PA), and stained with filtered (#1 Whatman filter paper, Fisher Scientific Co., Pittsburgh, PA) 2% aqueous uranyl acetate (Fisher Scientific Co.,
Pittsburgh, PA) for 15 minutes, followed by Reynold's lead citrate (Fisher Scientific Co., Pittsburgh, PA) for 2 minutes. The grids were rinsed in distilled water between each step and allowed to air dry. Sections were examined using a JEOL JEM 100-S transmission electron microscope (JEOL, Peabody, MA) and imaged with Kodak SO-163 electron image film (Electron Microscope Sciences, Hatfield, PA).

**Statistical Analysis**

Statistical significance of the effects of VCO and select fatty acids on viable colony counts of *C. difficile* was determined using one-way analysis of variance (ANOVA) or linear regression, as appropriate, using the statistical software package R version 2.14.1 (2011, [http://statpages.org/](http://statpages.org/)). Replicate data were used to generate arithmetic means and standard deviations for each treatment within an experiment. These group means were first compared using one-way ANOVA. Post-hoc tests for significant differences between group means were then calculated using Tukey’s Honestly Significant Differences (HSD) method. If the ANOVA or graphs indicated a possible linear relationship, then linear regression, using the ordinary least-squares estimate, was used to model the relationship of the effects of VCO and select fatty acids on viable *C. difficile* colony counts. Significance was set a priori at $p < 0.05$. 
Results

*Clostridium difficile* Susceptibility to Lipolyzed VCO

Virgin coconut oil that was not pre-digested with lipase had no bactericidal effect on *C. difficile* when added to overnight cultures (data not shown). VCO was therefore digested with lipase prior to its addition to *C. difficile* cultures. Exposure of *C. difficile* cells to lipolyzed VCO (LVCO) or its constituent fatty acids for 30 minutes at 36°C was used to assess the cidal effect of these lipids on *C. difficile*. LVCO at a concentration of 1.2% killed 99.9% of *C. difficile*, while 0.15% LVCO killed nearly 50% of the bacteria (Figure 19 a). BHIB containing 1.3% LVCO had a pH of 6.9. Controls containing DMSO, lipase and PBS were not significantly different from the BHIB alone in inhibiting the growth of *C. difficile*. ANOVA results indicated significant differences in group means. Post-hoc comparisons of group means demonstrated that all doses of LVCO displayed bactericidal activity. The log growth of *C. difficile* plotted as the independent variable with respect to the concentration of LVCO indicates a log linear relationship and is amenable to simple linear regression. Using a least-squares estimate, it was determined that for every 0.1% increase in the concentration of LVCO there will be a reduction of $\ln(7.61)$ colony counts of *C. difficile* ($p < 0.0001$). The log-linear nature of this relationship means that coconut oil has a diminishing effect on colony counts of *C. difficile*. For example, the reduction in colony counts between 0.1% to 0.2% concentrations of digested coconut oil is 163,031 CFU/mL, while the reduction in colony...
counts between 0.3% to 0.4% concentrations is 35,553 CFU/mL. This log-linear relationship is remarkably strong with a multiple $R^2$ of 0.97.

**Clostridium difficile Susceptibility to Fatty Acids**

**Lauric acid.**

Lauric acid (1,000 µM) inhibited nearly 100% of bacterial growth (Figure 19 b). Lauric acid produced strong inhibition even at concentrations as low as 250 µM, reducing growth by 90% as compared to controls. There was no inhibition to the growth of *C. difficile* in any of the controls. Therefore, the control results are reported as an average in the figures. The pH of the broth at 1,000 µM was 7.0. Data evaluating the effect of lauric acid also demonstrated a strong log-linear relationship to *C. difficile*, similar to that of digested VCO. The results of simple linear regression show that for every 100 µM increase in the concentration of lauric acid there will be a reduction of ln(0.62) colony counts of *C. difficile*, which is significant ($p < 0.0001$). The log-linear relationship is also remarkably strong, with a multiple $R^2$ of 0.94.

**Capric acid.**

Capric acid (2,000 µM) displayed bactericidal activity, while 1,000 µM displayed less bactericidal activity on *C. difficile* (Figure 19 c). The pH of broth cultures containing 2,000 µM concentration was 6.8. Capric acid at concentrations from 2,000 to 200 µM/mL demonstrated a nearly linear relationship with colony counts of *C. difficile*. Simple linear regression showed that for every 1 mM increase in the concentration of
capric acid, there was a statically significant decrease in 2,928 CFU/mL of *C. difficile* 
(*p* <0.001).

**Caprylic acid.**

Caprylic acid was also bactericidal on *C. difficile*, but its cidal effect was blunted when diluted 2-fold (5,000 µM). Further dilution of caprylic acid also failed to display bactericidal activity (**Figure 19 d**). Caprylic acid at concentrations from 10,000 to 1,000 
µM/mL, demonstrated neither a linear or log-linear relationship on the colony counts of 
*C. difficile*. The concentrations of caprylic acid were treated as categorical and a one-way ANOVA with multiple comparisons of the concentrations using Tukey’s HSD showed 
that only when the concentration of caprylic acid reached 10 mM was there a significant 
decrease in colony counts of *C. difficile* (*p* <0.001).
Figure 19. Bactericidal activity of lipolyzed VCO and MCFA on *C. difficile*. Colony counts of an overnight broth culture of *C. difficile* ATCC 9689 exposed to VCO or MCFA for 30 minutes and plated to Brain Heart Infusion agar. 

**A. Lipolyzed VCO** (n=3) Control with DMSO, Lipase and PBS buffer with no VCO. (*p < 0.0001).**

**B. Lauric acid.** (n=3). The control contained only BHIB with 2% DMSO and no lauric acid. (*p < 0.0001).**

**C. Capric acid.** (n=3). The control contained only BHIB with 2% DMSO and no capric acid. (**p < 0.001).**

**D. Caprylic acid.** (n=3). The control contained only BHIB with 2% DMSO and no caprylic acid. (**p < 0.001).**
**Transmission Electron Microscopy.**

Transmission electron micrographs (TEM) of lauric acid-treated *C. difficile* demonstrated substantial alteration of cell ultrastructure compared to untreated control TEMs (Figure 20). The cell membrane of the lauric acid-treated bacteria appeared to be eliminated; the lipid layers were not discernible, while the cytoplasm appeared to be disorganized with possible vacuoles, as compared to the untreated control.

**BacLight viability testing (Live/Dead Staining).**

Live/Dead™ staining performed on bacterial aliquots from controls with DMSO only, and treatments with solubilized fatty acids or lipolyzed coconut oil solubilized in DMSO, demonstrated a change in membrane permeability as measured by uptake of PI by bacteria in samples exposed to fatty acids. Bacteria treated with concentrations of lipids that reduced the colony forming units of *C. difficile* by greater than 2 logs (lipolyzed VCO 1.2%, lauric acid 1mM, capric acid 2 mM, and caprylic acid 10 mM), converted bacteria immediately from green (live) to red (dead). Controls containing bacteria with DMSO displayed only a few bacteria converting from green to red even after 30 minutes at room temperature (Figure 21).
Figure 20. Transmission Electron Microscopy image shows a sample of *C. difficile* treated for 30 minutes with 10mM lauric acid solubilized in DMSO. Top row whole cells, bottom row magnified images of whole cells. (Left a,b) Control treated with only DMSO, displaying normal membrane and cytoplasm arrangement. (Right c,d) Lauric acid treated sample. Of note are intracellular cytoplasmic vacuoles (c), and the disruption of cell membrane, vacuoles (black arrow) and disorganization of cytoplasm (d).
Figure 21. Live/Dead staining of *C. difficile* overnight culture following exposure of VCO and MCFA. **A.** 2% DMSO Control 30 minutes. **B.** 4% DMSO Control 30 minutes. **C.** 1mM lauric acid at 1 minute. **D.** 2mM capric acid at 1 minute. **E.** 10mM caprylic acid at 1 minute. **F.** 1.2% digest of virgin coconut oil at 1 minute.
Discussion

Virgin coconut oil and several of its constituent fatty acids: lauric, capric, and caprylic acid, inhibit replication of enveloped viruses as well as having a toxic effect on microbes including *Candida albicans* and *Clostridium perfringens* (Hornung, Amtmann et al. 1994; Enig 1998; Dayrit 2000; Bartolotta, Garci et al. 2001; Bergsson, Arnfinnsson et al. 2001; Bergsson, Arnfinnsson et al. 2001; Enig 2002; Skrivanová, Marounek et al. 2005; Ogbolu, Oni et al. 2007). VCO, when consumed, is digested by lipases of the mammalian digestive tract, releasing the individual fatty acids potentially killing select microorganisms in *vivo* (Jensen, Clark et al. 1982). Because *C. difficile* lacks lipases to digest coconut oil (Wilson, Kennedy et al. 1982), to mimic the *in vivo* environment, in vitro studies require that the oil be lipolyzed with porcine lipase prior to evaluating its effect. Results from this study demonstrate the ability of both the constituent fatty acids and lipolyzed virgin coconut oil to display antimicrobial toxicity towards *C. difficile in vitro*.

Examination of fatty acid and lipolyzed coconut oil treated *C. difficile* using transmission electron microscopy, and Live/Dead™ staining indicate that the cell membrane is disrupted (*Figures 20,21*). Free fatty acids are amphipathic with characteristic hydrophilic head groups that are soluble in water and a nonpolar hydrophobic tail that is water insoluble. The hydrophobic tails interact with bacterial cell membranes forming pores disrupting the bacterial cell membrane, leading to cell death by changing the cell’s permeability to water and other ions. At high concentrations at or above the critical micelle concentration, fatty acids form micelles that act as detergents
solubilizing the membrane removing parts of the lipid bilayer and its membrane associated proteins. The concentrations of fatty acids used displaying 99% bactericidal activity (caprylic 10mM, capric 2mM, and lauric 1mM) were less than the critical micelle concentration previously reported (caprylic CMC 360mM, capric CMC 98mM, lauric CMC 25mM at 35°C) needed for them to act as detergents (Campbell and Lakshminarayanan 1965).

Virgin coconut oil, that was not lipolyzed with porcine lipase, was not toxic to and had no effect on *C. difficile in vitro* (data not shown). Bacterial growth was not inhibited by porcine lipase, PBS or DMSO. Lipolyzed VCO significantly inhibited the number of colony forming units of *C. difficile in vitro* at levels as low as 0.15% v/v as compared to bacteria that had not been exposed to VCO (Figure 19a). These results suggest that humans harboring *C. difficile* might benefit from consuming VCO, thus inhibiting *C. difficile* from growing in the digestive tract.

Lauric acid is the most abundant component of virgin coconut oil (Ghosh and Bhattacharyya 1997) and, in this study was demonstrated to be the most effective inhibitor of *C. difficile*. The strong similarities in the log-linear relationships of the effects of both lipolyzed coconut oil and lauric acid, together with the fact that lauric acid makes up approximately 50% of VCO, suggest that lauric acid is the primary antimicrobial ingredient in VCO (Figure 19b). Capric acid and caprylic acid, which comprise between 5 to 10% of VCO (Intahphuak, Khonsung et al. 2010), were also found to display bactericidal activity on *C. difficile in vitro* but at concentrations greater than those of
lauric acid (Figure 19c-d). Twice as much capric acid (as compared to lauric acid) was needed to achieve a 99% cidal effect on *C. difficile*; similarly, caprylic acid was much less effective than lauric acid, requiring 5 times as much to achieve the same level of bactericidal activity as lauric acid. These results suggest that both capric acid and caprylic acid also play a role in VCO’s bactericidal effect on *C. difficile*, but to a much lesser extent than that of lauric acid.

Based on the amount of lauric acid found in breast milk (6.2% of total fat) (Beare-Rogers, Dieffenbacher et al. 2001) and the nutritional requirements of infants, an adult would need to consume approximately 24 grams of fat containing lauric acid, to achieve the same systemic antiviral and bactericidal protection afforded to nursing infants (Enig 1998; Dayrit 2000). Lauric acid constitutes approximately 48-52% of the total fatty acid content of coconut oil, suggesting that approximately 52 grams of coconut oil per day would be necessary to have systemic antimicrobial activity, similar to that observed in infants consuming breast milk (Enig 1998; Dayrit 2000). However, the amount of coconut oil required to prevent intestinal colonization of pathogenic microbes may differ *in vivo* due to absorption of fatty acids before reaching the colon. Further research, using an animal model to determine fatty acids levels in the colon following consumption of VCO is warranted.

Coconut oil is a natural substance and is used in many tropical countries as the preferred oil for eating and cooking. A study of patients with acute diarrhea in New Caledonia, where coconut oil is the preferred oil, found that *C. difficile* was isolated in
only 2 of the 2,088 patients (Germani, Morillon et al. 1994), far less than the 2 to 3% current carriage rate in the United States (Barbut and Petit 2001). The results from this study may indicate a protective effect, where consuming coconut oil may inhibit gut colonization by *C. difficile*. Coconut oil is considered safe and not regulated by the FDA. There are however, concerns that monoglycerides and MCFA might have detrimental effects on eukaryotic cells. Research demonstrating the safety of these products tested mouse vaginal mucosal cells and determined they were not adversely affected by hydrogels containing 10 mM or 20 mM of monocaprin, glycerol with a single capric fatty acid tail (Thormar, Bergsson et al. 1999). In another study, human cell cultures were also unaffected by levels less than 2.5 mM of either capric or lauric acids (Bergsson, Arnfinnsson et al. 2001). These levels are less than the 2mM and 1mM concentrations demonstrated by this research to reduce growth by 2 logs. Generally speaking, the safety of consuming of saturated MCFAs (such as those found in coconut oil) is viewed with skepticism in light of the connection between fat consumption, increased cholesterol levels and heart disease. (Prior, Davidson et al. 1981; Van Heek and Zilversmit 1991; Kaunitz and Dayrit 1992; Kumar 1997; Nevin and Rajamohan 2006). Other research found no connection between consumption of VCO and increased levels of cholesterol in rats as compared to other oils (Nevin and Rajamohan 2004).

Efforts are ongoing to identify more effective alternative treatments for CDI. The VCO and lauric acid results outlined in this study provide support for VCO as a potential effective and economical therapy against CDI, especially by financially-challenged patients. Since the proposed pathophysiology for the development of *C. difficile* diarrhea
is bacterial overgrowth and alteration of the normal gut microbiota, future studies should include the study of the effect of VCO against cultures of members of the normal microbiota of the gastrointestinal tract, and other intestinal pathogens to ensure that ingesting coconut oil will not have harmful effects on the normal GI tract microbiota, and will have selective toxicity toward pathogens.

The threat of CDI is a major health concern when caring for patients in health care facilities. Alternate methods to control *C. difficile*, such as hand-washing and contact isolation should continue to be used to prevent its spread. The use of alternative therapeutic agents may reduce health care providers’ dependence on metronidazole and other antibiotics as well to reduce costs associated with CDI. This in *vitro* study presents a limited analysis. Thus a more complete evaluation of VCO and its constituent components should be undertaken to study their impact on *C. difficile* in *vivo*.

With further study, there may also be potential for coconut oil to be consumed as a prophylactic measure against CDI for those patients requiring long-term hospitalization, extended stays in rehabilitation, or skilled nursing facilities. Such a measure may serve to decrease colonization in patients, and subsequently lead to fewer cases of hospital-acquired CDI.
References


CHAPTER IV

Visualization and Chemical Treatments of *C. difficile* 630 Strain Spore Surface

Abstract

*Clostridium difficile* disease is spread by infectious spores present in the feces of infected patients. Proteins found on the outer spore surface have been shown to promote adherence to many surfaces including mammalian epithelial cells. The protein distribution on spore surface is not uniform and varies between the spore midsection and the poles. Freeze-fracture transmission electron microscope images of *C. difficile* spores grown in liquid medium reveal mother cell cytoplasmic residues covering the spore poles. Fluorescent *C. difficile* spore coat antibodies bound to the central region of untreated spores forming a fluorescent band but failed to adhere at the poles where mother cell residues were present. Chemical treatments which alter or remove the spore coat surface proteins and mother cell surface residues increased or decreased antibody binding to the spore coat. Effects of treatments were visualized using indirect fluorescent microscopy, and quantitated using an indirect enzyme immunoassay. Dithiothreitol, proteinase K and extended treatment with 3% hydrogen peroxide removes mother cell residues as determined by increased antibody binding to the spore coat. Treatments using trypsin as well as 10% EDTA were found to decrease antibody binding to the spore coat. Understanding how chemical treatments alter these spore coat surface proteins may prove
valuable in interrupting adherence of *C. difficile* to surfaces and decrease disease transmission.

**Introduction**

*Clostridium difficile* infection (CDI) is the leading cause of antibiotic associated diarrhea and pseudomembranous colitis in hospitalized patients (Winston, Ho et al. 1991; McFarland 1999; Levy, Stergachis et al. 2000). The cost of treating patients with *C. difficile* disease has risen to over three billion dollars a year in the United States alone (O'Brien, Lahue et al. 2007). Patients left untreated may develop pseudomembranous colitis, toxic megacolon and possibly death. It is estimated that there are 500,000 cases of CDI per year, with 20,000 mortalities in the United States (Rupnik, Wilcox et al. 2009). This increase is primarily due to a endemic hypervirulent strain (NAP1/BI/027) that produces excessive toxins and has increased production of spores (Akerlund, Persson et al. 2008). The large clostridial toxins A and B, act as glucosyltransferases modifying small GTPases of the Rho family of proteins within the intestinal mucosal epithelial cells effecting actin polymerization, leading to loss of mucosal integrity and cell death (Voth and Ballard 2005). NAP1 strains additionally produce the binary ADP-ribosyltransferase toxin CDT (*Clostridium difficile* transferase), which ADP-ribosylates actin inhibiting actin polymerization (Stubbs, Rupnik et al. 2006; Schwan, Stecher et al. 2009). Additionally, CDT toxin generates the formation of microtubule projections at the epithelial cell surface allowing for increased bacterial adherence and promotes the
colonization of the *C. difficile* vegetative cells (Schwan, Stecher et al. 2009; Aktories, Schwan et al. 2012).

The transmission of *C. difficile* disease is proposed to be from the resistant spores contaminating surfaces of health care equipment and the skin of health care professionals themselves (Vonberg, Kuijper et al. 2008). The spores are resistant to disinfectants commonly used in healthcare settings (Dubberke and Wertheimer 2009) and are found to persist in these environments for many months (Jabbar, Leischner et al. 2010).

Adherence to skin and other surfaces has been attributed to the *C. difficile* exosporium or spore coat proteins on the spore surface (Paredes-Sabja and Sarker 2012). Hand washing studies have documented the failure of even rigorous hand washing to remove 100% of the spores regardless of the type of cleanser being used in volunteer trials (Oughton, Loo et al. 2009; Edmonds, Zapka et al. 2013). This adherence to epithelial cells is attributed to unique surface protein receptors found in the spore’s exosporium and spore coat, allowing the spore to firmly attach to human keratinocytes covering hands (Edmonds, Zapka et al. 2013) and enterocytes lining the gut mucosa (Paredes-Sabja and Sarker 2012). Understanding the interaction between the spore surface of *C. difficile* and proteins found in epithelial cells may prove valuable in determining treatments to block this attachment. If these spore ligands that bind to epithelial cells could be made nonfunctional by chemical treatment, the spores may be easily removed.

Other gram positive spore forming pathogenic bacteria including *Bacillus anthracis* (Escobar-Cortés, Barra-Carrasco et al. 2013) and *Bacillus cereus* (Henriques
and Moran 2007) contain an exosporium as the outer most layer. However, the structure of their exosporia differs significantly from the exosporium described for C. difficile. C. difficile’s exosporium lacks the paracrystalline basal layer and hair-like surface projections found in both Bacillus sp. spores (Henriques and Moran 2007; Lawley, Croucher et al. 2009). The exosporium of all these organisms is thought to contribute to their pathogenicity.

The exosporium of C. difficile is the least understood of the spore’s components (Henriques and Moran 2007). Conflicting reports describe the exosporium. Permpoonpattana et al., state that the exosporium is unstable and present only on spores harvested immediately, and will disappear overnight following refrigeration (Permpoonpattana, Tolls et al. 2011). Other groups state that the exosporium is stable and can be removed with trypsin or sonication (Paredes-Sabja and Sarker 2012; Escobar-Cortés, Barra-Carrasco et al. 2013). The methods used to produce (agar plate versus broth culture), process, and store spores may also have an effect on the presence and properties of the exosporium of C. difficile (Permpoonpattana, Tolls et al. 2011).

Methods

_Clostridium difficile spore preparation_

_C. difficile_ 630 strain (ATCC, Anapolis, MD) spores were inoculated to pre-reduced Columbia Sheep Blood Agar plates (BBL, Becton Dickinson Microbiology
Systems, Cockeysville, Md.), in the Bactron 2 anaerobic chamber (Bactron, Sheldon Manufacturing, Cornelius, Oregon) and allowed to grow for 3 days at 36°C. Clospore broth was prepared following the method of Perez et al. (Perez, Springthorpe et al. 2011). Clospore broth was prepared by adding 10 grams of special peptone mix (SPM, Oxoid, Ottawa, ON, Canada), 10 grams of yeast extract (Ameresco, Solon, OH), 2.6 g of KH$_2$PO$_4$, 3.48 g of K$_2$CO$_3$, 0.12 g of MgSO$_4$, 0.08 g of CaCl$_2$.2H$_2$O, and 0.6 g of (NH$_4$)$_2$SO$_4$ into 1 liter of distilled water. The pH was adjusted to 7.9 using concentrated hydrochloric acid, and the broth was sterilized by autoclaving. The medium was allowed to acclimate in the Bactron 2 anaerobic chamber (Bactron, Sheldon Manufacturing, Cornelius, Oregon) overnight before being inoculated with 2-3 colonies from the Columbia Sheep Blood plate. The broths were incubated at 36°C for 7 days. Aliquots from the broth were evaluated by gram stain for spore presence. The broths containing spores were concentrated following the method used by Helfinstine et al., with adjustment for broth cultures (Helfinstine, Vargas-Aburto et al. 2005). Clospore broth containing spores was aliquoted to 50 mL pre-sterilized centrifuge tubes (VWR International LLC, Radnor, PA). The spore suspensions were then centrifuged at 2500 x g for 10 minutes at 25°C (IEC Centra MP4R, Needham Heights, MA); the pellet was resuspended in 50 mL phosphate buffered saline (PBS, pH 7.4) supplemented with 0.05% Tween 20 (PBST, BP2938-10 Fisher Scientific, Pittsburgh, PA) to aid in resuspending the spores, and mixed for 5 minutes, and centrifuged as before. The spore pellet was then washed four times with 50 mL sterile distilled water to remove the detergent. Finally, the spores were suspended in 50 mL sterile distilled water and stored at 4°C until used for
testing. Following spore washing and resuspension in sterile distilled water, aliquots were removed and serially diluted to quantify the spores. Two microliters of diluted spore suspensions were fixed onto glass slides and counted in triplicate, manually, using an Olympus BX61 microscope (Olympus America, Lake Success, NY). Final spore concentrations were adjusted with sterile distilled water.

**Transmission Electron Microscopy**

Spores grown in CLOspore broth for 7 days were aliquoted (50 mL) centrifuged at 2500 x g for 10 minutes (IEC Centra CL5R, rotor model IEC 6555C, Thermo Electron Corporation, Needham Heights, MA). The pellet was resuspended in 50 mL of sterile distilled water and centrifuged as above to form a pellet. The pellets were fixed using 500 µl of 5% glutaraldehyde (Fisher Scientific Co., Pittsburgh, PA) in 0.2 M Na-Cacodylate buffer (pH 7.4, Sigma Aldrich, St. Louis, MO) for two hours at room temperature. The samples were again centrifuged at 2000 x g for 2 minutes, and the pellets suspended in sterile distilled water and refrigerated at 2-8°C until processed for imaging. A heavy spore suspension (3 x 10^7 spores/mL) was used to completely fill gold holders used for freeze fracturing (Leica Microsystems Inc. Buffalo Grove, IL). The samples were quickly frozen by submersion into liquid nitrogen. Fracture and replication were performed in a freeze-fracture instrument (BAF 60, BAL-TEC, Leica Microsystems Inc. Buffalo Grove, IL) at temperature of -150 °C. The sample was processed by coating with metal platinum/carbon at 45° and carbon at 90°. Replicas were thawed in water and applied to
copper 400 mesh grids (Leica Microsystems Inc. Buffalo Grove, IL) and examined using a 200 kV electron microscope (Tecnai G2 F20 FEI Company, Hillsboro, OR).

**Indirect Fluorescent Microscopy.**

The method of Duc *et al.* (2003), was used with modification. Freshly prepared *C. difficile* 630 spores were suspended in 0.01M phosphate buffered saline (pH 7.4) containing 0.05% Tween 20 (PBST, Fisher Scientific, Fair Lawn, NJ). The spore suspension (5 µL) were applied to wells of 12 well 5mm microscope slides (Thermo Scientific, Waltham, MA), air dried at room temperature, and heat-fixed for 10 minutes at 70°C. Anti-*C. difficile* antibodies were diluted in PBST at the following dilutions: CD1067, 1:50; CD3620, 1:20; and CD1581, 1:20; final concentrations of 58 µg/mL, 90 µg/mL, and 65 µg/mL, respectively. Diluted antibody (10 µL) was added to individual slide wells, in duplicate, and incubated at 37°C for 30 minutes. All incubations were performed in a slide moisture chamber. Following incubation, slides were rinsed with PBST, and washed in a Coplin jar with PBST for 10 min. Following the wash step, slides were blotted and held in the slide moisture chamber until the secondary antibody was added. Dylight 488 goat anti-rabbit secondary antibody (Thermo scientific, Rockford, IL) was diluted 1:200 in PBST for a final concentration of 50 ng/mL. Diluted secondary (10 µL) was added to each well, and slides were incubated for another 30 minutes at 37°C. Following incubation, slides were again rinsed and washed, as previously, using PBST. Wells were covered with Type B immersion oil (Cargille Labs, Cedar Grove, NJ) and viewed at 1000 magnification using an Olympus BX61 microscope (Olympus...
America, Lake Success, NY) adapted for fluorescence microscopy, using an Olympus U-MNB2 filter set.

Indirect fluorescent antibody (IFA) assay was also used on completed ELISA well bottoms to detect presence of spores. Following completion of the indirect ELISA assay, wells were rinsed with PBST, air dried and heat fixed. The IFA assay was completed the same as the slide assay, with the cut out well bottoms mounted to glass microscope slides before staining. Additionally, nigrosin (negative staining) was performed on completed ELISA well bottoms where detection levels were lowered (due to failure of antibodies binding to spores) to ensure spores remained bound to the wells.

**Preparation of Indirect ELISA Plates**

Indirect ELISA plates were prepared and processed following the methods of Duc *et al.*, 2003, with a few adjustments. Spore suspensions stored at 4°C in distilled water were vortexed until completely resuspended; one mL aliquots were then centrifuged (ISC, Bioexpress, Kaysville, UT) to pellet the spores. The supernatant was discarded and the pellet resuspended in coating buffer (1.5 g Na₂CO₃ and 2.93 g NaHCO₃ in 1L distilled water, with the final pH adjusted to 9.6). The spore stock in coating buffer was diluted to 3 x 10⁶ spores/mL. To each test well one hundred microliters of spore suspension was added. Wells were covered with adhesive polyethylene film (Titer-Tops, Diversified Biotech, Boston, MA) to prevent desiccation during incubation. The sealed microtiter wells were refrigerated overnight at 4°C.
Following the microplate well-coating step, wells were decanted into a flask to dispose of non-attached spores. Wells were washed using phosphate buffered saline pH 7.4 containing 0.05% Tween (PBST) by filling the wells and dumping them into a disposal flask. Washing was repeated thrice. Wells were never allowed to remain empty or to dry out.

Wells were then coated with 1% albumin (Blocker BSA 10% in PBS, Thermo Scientific, Rockford, Il) to block any remaining binding sites. Concentrated Blocker was diluted in PBST for a final concentration of 1% before completely filling the microtiter wells (400µL/well). Microtiter wells were covered with a plastic cover to prevent desiccation and incubated at room temperature for 60 minutes. Following incubation, wells were decanted into a disposal flask, and washed with PBST by completely filling and decanting the wells into a disposal flask. The washing step was repeated thrice.

Polyclonal antibodies were created by injecting either whole formalin treated C. difficile 630 strain spores or individual recombinant surface proteins into a rabbit. The resulting antibodies were purified using protein A separation, and preserved by freezing (Strategic Diagnostics Inc., Newark, DE). Primary anti-spore antibodies were generously provided by GOJO industries (Akron, Ohio). Antibodies were diluted with PBST to achieve optimal antibody to spore binding and placed in their respective wells. Antibody CD 1067 (2.9 mg/mL) was diluted for a final concentration of 58 µg/mL in PBST, and 100 µL of antibody added to each well, including the blank wells, to a final concentration of 5.8 µg/mL in each well. Antibody CD 3620 (1.8 mg/ml) was diluted in PBTS, with
100 µL pipetted into each well for a final concentration of 9.0 µg/mL in each well. Antibody CD 1581 (1.3 mg/mL) was diluted in PBST and 100 µL pipetted into each well for a final concentration of 6.5 µg/mL. Wells were covered with adhesive polyethylene film to prevent desiccation, and incubated for 60 minutes at 37°C. Following incubation, microtiter well contents were decanted into the disposal flask, and the microtiter wells were washed using PBST thrice.

Following the primary antibody step, HRP conjugated goat anti-rabbit IgG antibody (10µg/ml, Thermo Scientific, Rockford, IL) was diluted in PBST and added as 100 µl per well for a final concentration of 2.0 ng/mL in each well. Wells were covered with adhesive polyethylene film and incubated for 60 minutes at 37°C. Following incubation, microtiter well contents were decanted into the disposal flask, blotted using paper towels, and washed using PBST for a total of five washes; blotting with a new paper towel occurred between every wash. Blotting is very important in this step as any trace of unbound antibody must be removed to prevent false positive reactions.

Room temperature tetramethylbenzididine (1-Step Turbo TMB-ELISA, Thermo Scientific, Waltham, MA) substrate (100 µL), was added to each well of the microtiter plate followed by gentle tapping to mix the contents in the wells. Wells were incubated uncovered at room temperature for 30 minutes. Wells containing spore-antibody-HRP antibody complexes appeared blue upon the addition of substrate. Following the 30 minute incubation, the enzymatic reaction was stopped with the addition of 100 µL of 2M sulfuric acid into every well. Reactants in the wells changed from blue to a visible yellow
color. Microtiter plates were read using a spectrophotometer (BIO-TEK Instruments, Inc., Winooski, VT) at 450 nm.

**Spore Treatments**

Freshly prepared spores, less than 30 days old and stored at 4°C in distilled water, were pelleted by centrifugation in 2 mL centrifuge tubes at 2000 x g for two minutes and the supernatant decanted. Spores were then resuspended in various chemical reactants to determine their effects (**Table 7**). Exposure to treatment was for five minute at room temperature unless otherwise noted. Once the exposure to the treatment was stopped, the spore suspensions were centrifuged at 2,000 x g for two minutes, with the pelleted spores resuspended in coating buffer (pH 9.6), and 100 µL of the spore suspensions added to microtiter wells allowing the spores to bind to the wells. The inoculated microtiter plate was sealed with adhesive polyethylene film and incubated at 4°C overnight. The microtiter plate with treated spores was then tested by the indirect ELISA assay (as above) to determine the effect of the treatment on antibody attachment. Untreated spore controls diluted in PBST were included on every microtiter plate, in addition to evaluating blank wells containing no spores.

Trypsin 0.1% (w/v) was prepared by diluting 100 mg of powdered trypsin (Worthington, Lakewood, NJ) in 100 mL of sodium bicarbonate buffer (pH 8.3). Spores were incubated for 30 or 60 minutes at 37°C in 0.1% trypsin, followed by pelleting by centrifugation 2000 x g, and washing using sterile distilled water twice, before resuspending them in coating buffer as above. Additionally, treated spores were applied
to microscope slides, air dried, heat fixed at 70°C for 10 minutes, and stained by the IFA method to detect any change in the spore antibody binding.

The effect of proteinase K on spore binding was also evaluated using the IFA method. Spores were pelleted by centrifugation, resuspended in 2 mL of PBS containing 200 mM EDTA, 300 ng/mL proteinase K, and 1% Sarkosyl NL30. Spores were incubated for 1 to 2 hours with constant shaking at 37°C until the samples cleared (Lawley, Croucher et al. 2009). Spores were washed twice in distilled water and held at 4°C until tested by the IFA method.
Table 7. Chemical treatments with specific target and action

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Target</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Alcohol</td>
<td>Surface proteins</td>
<td>Denatures proteins by dehydration</td>
</tr>
<tr>
<td>70% Alcohol</td>
<td>Surface and cytoplasmic proteins</td>
<td>Denatures proteins by dehydration</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Peptidoglycan</td>
<td>Hydrolysis of 1,4-beta-linkages between N-acetylMuramic acid and N-acetyl-D-glucosamine</td>
</tr>
<tr>
<td>10% ethylene diamine tetraacetic acid (EDTA)</td>
<td>Surface proteins</td>
<td>Chelating agent removes calcium and magnesium altering protein shape and weakens phospholipid interactions</td>
</tr>
<tr>
<td>Dithiotreitol 0.55M (DTT)</td>
<td>Surface and spore coat proteins</td>
<td>Breaks disulfide bonds denaturing protein</td>
</tr>
<tr>
<td>3% Hydrogen peroxide</td>
<td>Oxidizes surface proteins</td>
<td>Converts carboxylic acids (RCOOH) into peroxo acids (RCOOOH)</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Hydrolysis of peptide bonds</td>
<td>Breaks peptide bonds destroying antigenic epitopes and proteins</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>Surface and spore coat proteins</td>
<td>Breaks peptide bonds removing surface and some coat proteins</td>
</tr>
</tbody>
</table>
Statistical Analysis

Spore treatments were evaluated using GraphPad Instat v.4.0 (Graphpad Software, Inc., San Diego, CA). Student’s paired \( t \) tests or analyses of variance (ANOVA) were used to compare treatment effects on antibody binding by comparing the treatment’s absorbance to the PBS control’s absorbance. All \( p \) value differences were considered statistically significant at \( p \leq 0.05 \).

Results

Microscopic Imaging of Untreated *C. difficile* 630 Spores

Visualization of the outer surface and exosporium of spores of *C. difficile* 630 was done using freeze-fracture transmission electron microscopy (TEM) imaging (Figure 22a). These images do not show a structured exosporium as seen in *Bacillus* spores; instead, the remains of the mother cell appears to be either attached to the poles of the spore or surrounding the spore like a sleeve. In order to determine the nature of this surface residue, these same spores were imaged by immunofluorescence microscopy using primary antibody CD1581 which is specific to a novel *C. difficile* spore surface protein (GOJO Industries, unpublished data) and counterstained with propidium iodide. IFM-imaged control spores (Figures 22b and 23a) showed two types of staining patterns. In one, antibody binding around the spore’s midsection (green fluorescence) while binding at the polar regions appeared to be blocked. The red fluorescence of the
propidium iodide counterstain at the poles (diagrammed in Figure 22c) in the other, antibody binding was not observed.
Figure 22. TEM, IFA, and Illustrations of *C. difficile* spores.  A. TEM of freshly grown spore displaying cytoplasmic residue from the mother cell.. B. IFM of freshly grown spore stained with CD1581 (scale bar 500 nm). C. Diagram showing multiple spore layers and central fluorescent band. D. Illustration of *C. difficile* depicting subterminal spore showing lack of mother cell cytoplasm near central spore region (white arrow).
Figure 23. IFA staining of control and treated spores. A. No treatment B. 10% EDTA. C. 0.1% Trypsin. D. DTT 0.55 mol/L. E. 3% H$_2$O$_2$ after 150 minutes. F. Proteinase K treated spores.
Effect of Chemical Treatment on IFA-imaged Spores

IFA images of spores treated with 0.1% trypsin and 10% EDTA displayed decreased fluorescence. Some of the spores maintained fluorescence in the central region (Figure 23). Dithiothreitol, 3% H₂O₂ and Proteinase K treatments increased antibody binding as displayed by the increased fluorescence demonstrated across the entire spore surface (Figure 23). This increase in antibody binding was directly related to the removal of mother cell residue from the spore surface giving the antibodies more access to the spore coat.

ELISA Optimization

The ELISA format was used to quantitate the effect of chemical treatment to the spore surface allowing for changes in antibody binding. Spores were grown in liquid cultures, pelleted and washed to remove culture medium, and bound to microtiter wells using coating buffer. To determine the quantity of spores and antibodies to be used in the assay, serial dilutions of spores versus serial dilutions of the three individual spore-specific antibodies were tested. The assay was optimized for a spectrophotometric absorbance of at least 1.50 at 450 nm for all antibodies. This was done to maximize detection of a reduction in antibody binding within the linear detection range of the plate reader used. A spore concentration of 3.5 x 10⁶ spores/mL met these requirements (Figure 24a) and was used in all subsequent experiments. Antibody titrations determined that the optimal concentrations of antibodies CD1067, CD3620, and CD1581 to be used
in the test wells were 5.8 µg/mL, 9.0 µg/mL, and 6.5 µg/mL, respectively (Figure 24b-d).

After each assay, wells were tested for bound spores by either fluorescence microscopy with a secondary goat anti-rabbit antibody conjugated with DyLight 488 or conventional light microscopy using a nigrosin negative stain. These images (Figure 25) show that spores remain bound to the wells through the end of the ELISA protocol.
Figure 24. Optimization of Spore Binding Assay. X axis values are a percentage of the original concentration. A. *C. difficile* 630 spore serial dilutions versus antibodies to determine the optimal spore concentration. Antibody concentrations were as follows: CD1581 6.5µg/mL, CD3620 9.0µg/mL, and CD1067 5.8µg/mL. (n = 3) B, C, and D. Serial dilutions of CD1067, CD3620 and CD1581 antibodies versus *C. difficile* 630 spores at 3.5 x10^{6}/mL (n = 4) (Statistical differences between group means P value *p < 0.01).
Figure 25. IFA and Nigrosin Stained ELISA wells. A. ELISA well bottom IFA image from completed ELISA with fluorescent spores. B. Nigrosin negatively stained spores on well bottom following ELISA.
Spore Treatment Effect on Antibody Binding

Ethyl alcohol (EtOH), isopropyl alcohol (IPA), lysozyme, 0.55 mol/L dithiothreitol (DTT), 10% ethylenediaminetetraacetic acid (EDTA), and 3% hydrogen peroxide (H$_2$O$_2$) were applied to *C. difficile* 630 spores to determine if binding to antibodies could be inhibited (Table 8). Spore treatments with EtOH had different effects on the binding of spores to antibodies depending on the concentration of EtOH and the antibody used. Pure EtOH significantly inhibited the spores binding to antibody CD1067 and antibody CD 1581, while not having a significant effect on spores binding to CD3620 antibody (Column 2). EtOH diluted to seventy percent, had a lesser effect on spores binding to CD1067, but had a significant effect on their binding to CD1581 and 3620 (Column 3). Spores treated with isopropyl alcohol had significant effect on the binding of spores to antibody CD3620 both at 100% and 70% concentrations, but had lesser effects on spore binding to the other two antibodies (Columns 4-5). Spore treatments using lysozyme at 40 mg/mL, and 4 mg/mL, had little statistically significant effect on blocking any of the antibodies tested to binding to the spores (Columns 6-7). DTT spore treatment surprisingly increased all antibody binding to spores (Column 8), while treating spores with 10% EDTA (Column 9) decreased the binding of all antibodies to the spores. Spore treatments using 3% H$_2$O$_2$ (Column 10) decreased the binding of CD3620 and CD1581 to spores when incubated for 5 minutes.

Treatment with 3% H$_2$O$_2$ for extended periods resulted in statistically significant increases (p <0.0001) in antibody binding to spores, but only at incubation times greater
than 90 minutes (Figure 26), an unexpected result given the slight inhibition of antibody binding seen in short (5 minute) exposures.

Treatment of spores with 0.1% trypsin (w/v) for 30 or 60 minutes significantly decreased binding of all three antibodies to spores (Figure 26). The inhibitory effect was also statistically significant upon comparison of the 30 minute treatment to the 60 minute treatments. Statistical significance with one-way ANOVA was CD1067 (p <0.0001) $R^2$ 0.99, CD3620 (p <0.0001) $R^2$ 0.97, CD1581 (p <0.0001) $R^2$ 0.998. IFA images demonstrated reduced binding of antibodies to the spores where greater than two thirds of the spores failed to fluoresce as compared to untreated controls (Figure 27).
**Table 8.** Effect of Spore treatments on antibody binding by ELISA

<table>
<thead>
<tr>
<th>Antibody</th>
<th>PBS Control</th>
<th>100% EtOH</th>
<th>70% EtOH</th>
<th>100% IPA</th>
<th>70% IPA</th>
<th>Lysozyme 40mg/mL</th>
<th>Lysozyme 4mg/mL</th>
<th>DTT 0.55M</th>
<th>10% EDTA</th>
<th>3% H₂O₂</th>
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<tr>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>CD1067</td>
<td>1.052</td>
<td>0.648</td>
<td>1.233</td>
<td>1.402</td>
<td>1.138</td>
<td>1.222</td>
<td>1.054</td>
<td>1.700</td>
<td>(3.400*)</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>P value</td>
<td>n 5</td>
<td>&lt;0.0001</td>
<td>0.006</td>
<td>0.0024</td>
<td>0.2254</td>
<td>0.0316</td>
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<td>&lt;0.0001</td>
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<td>CD3620</td>
<td>0.953</td>
<td>1.049</td>
<td>0.569</td>
<td>1.324</td>
<td>0.524</td>
<td>0.961</td>
<td>0.871</td>
<td>1.896</td>
<td>(3.791*)</td>
<td>0.269</td>
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<tr>
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<td>P value</td>
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<td>0.0002</td>
<td>0.0003</td>
<td>0.8794</td>
<td>0.1100</td>
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<tr>
<td>CD1581</td>
<td>1.153</td>
<td>0.713</td>
<td>0.664</td>
<td>1.379</td>
<td>0.896</td>
<td>0.974</td>
<td>0.990</td>
<td>2.905</td>
<td>(5.809*)</td>
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<tr>
<td>Absorbance</td>
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</tr>
<tr>
<td></td>
<td>P value</td>
<td>n 7</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
<td>0.0085</td>
<td>0.0011</td>
<td>0.0044</td>
<td>0.0336</td>
<td>&lt;0.0001</td>
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*Samples diluted 1:1 in 2M H₂SO₄ and calculated absorbance determined.
Figure 26. Effect of extended treatment with 3% hydrogen peroxide on antibody binding to spores by ELISA. Treatment in 3% H₂O₂ increased antibodies binding to spores when exposure to hydrogen peroxide was prolonged past 90 minutes at room temperature * (n=3, p <0.0001).
Figure 27. Indirect ELISA of spores treated with 0.1% trypsin (w/v). Treatments for 30 or 60 minutes resulted in a significant decrease in spore binding to antibodies. * (n=3, p value <0.0001)
Figure 28. IFA of spores treated with 0.1 % trypsin. **A.** Control spores lacking trypsin. **B.** Spores incubated with CD1067 following 60 minute treatment with 0.1% trypsin. Image stained with secondary DyLight 488-conjugated antibodies (green) and counterstained with propidium iodide (red). Blue circles indicate non-fluorescent spores.
Discussion

Freeze fracture TEMs of freshly prepared, untreated *C. difficile* 630 spores showed spores surrounded by a significant amount of material that appears to be the remains of the mother cell. The arrangement of this material (as seen in Figure 22a) conforms to the subterminal placement of *C. difficile* spores within the mother cell (Figure 22d). If the degradation of the mother cell is uniform following spore formation, one would expect a large amount of residual material from the mother cell at the poles with only a thin layer (or none at all) in the central spore region. This would cause the spore to have the appearance of a bowling pin, on one pole the residue forms a knob and on the other a bowl-like base (Figure 22c). Only this degraded mother cell material, and no defined exosporium, was observed in either these freeze-fracture TEMs or in conventional negative-stain TEMs (GOJO Industries, unpublished data). This conflicts with other reports of exosporium being present in *C. difficile* 630 spores (Lawley, Croucher et al. 2009; Permpoonpattana, Tolls et al. 2011; Escobar-Cortés, Barra-Carrasco et al. 2013). In TEM cross-sections in the above-cited works the putative exosporium appears as fuzzy amorphous debris surrounding the spore and may have been mistaken for exosporium. This is what we have determined to be the remnants of the dead mother cell. In addition, the spores are commonly shown in transverse section (down the long axis of the cell) where the debris layer would be the thinnest, suggesting that this amorphous “exosporium” may actually be a thin layer of mother cell residue rather than a true exosporium such as that found in spores of *B. anthracis* or *B. cereus*. Differences in growth conditions between studies is unlikely but cannot be completely ruled out as a
factor. Additional analyses replicating this work using other growth conditions are necessary to remove this possible confounding factor.

The indirect fluorescent antibody staining also showed material present on the spores that interfered with antibody binding to spore coat proteins. In the majority of control spores, the central spore region clearly bound the green-fluorescent antibodies but at the poles, where the majority of the remains of the mother cell was located, binding was blocked (Figures 22, 23). In some untreated control spores binding was blocked entirely displaying no fluorescence (Figure 23a) suggesting that some spores remain completely surrounded by mother cell remnants while in others degradation of mother cell material is sufficient to permit antibody binding to the spore coat.

Additionally, the layer of mother cell material was quite easy to remove. A variety of treatments (DTT, hydrogen peroxide, and proteinase K) removed the mother cell remnants and made the entire spore available for antibody binding (Figure 23d-f) and is especially evident in the IFA images of spores treated with proteinase K (Figure 23f). Proteinase K treatment has been previously shown to completely remove the proteins attached to the spore coat surface, leaving the spore coat stripped of spore surface residues (Escobar-Cortés, Barra-Carrasco et al. 2013). Removal of mother cell remnants with proteinase K from the spore surface allows for increased access to the spore surface and thus an increase in total antibody binding.

The ease with which these treatments removed mother cell residue from the spore surface is supported by prior reports that the purported “exosporium” would be lost by
refrigeration of the spores overnight (Permpoonpattana, Tolls et al. 2011) or by mechanical manipulation which does not occur in spores with a structured exosporium such as those found in Bacillus species (Escobar-Cortés, Barra-Carrasco et al. 2013). Given these findings, C. difficile 630 likely does not have a true exosporium and the adherence of spores is due primarily to the exposed proteins of the spore coat and not the mother cell residues. Any contribution by exosporium proteins can be ignored as they don’t exist.

In order to quantify changes in antibody binding due to chemical treatment, as well as to create a potential high-throughput assay for adherence-blocking compounds, an indirect ELISA scheme was designed and optimized. A statistically significant increase antibody binding occurred for spores treated with DTT (Table 8) confirming results from IFA images (Figure 23d). This likely occurs for the same reason as mother cell remnants are stripped providing greater surface area to be available for the spore to bind both to the plastic surface of the ELISA plate and to the primary antibodies. DTT treatment reduces proteins by breaking disulfide bonds, which are used extensively in the spore coat. The spore coat is arranged in concentric layers and these layers are held together by disulfide bonds formed between the proteins of each layer (Gould and Hitchins 1963). Reduction of the disulfide bonds allows for both removal of the outer spore coat layers and the mother cell cytoplasmic residues. This increases the surface area available for antibody binding to the spore coat. Interestingly, antibody binding decreased slightly with 5 minute exposure to 3% hydrogen peroxide (Table 8) but at prolonged treatment time, antibody binding increased as the mother cell remnants were removed increasing the
spore surface area for antibody binding (Figures 23e and 26). The mother cell remnants may also continue to degrade naturally, even after the hydrogen peroxide breaks down, exposing the spore coat surface over time.

Treatment with 0.1% trypsin resulted in the greatest inhibition of antibody binding (Figures 27), a 3-6 fold reduction. This was also reflected by the dramatic change in staining of spores in IFA images where the vast majority of spores were unable to bind antibody, indicated by the lack of green fluorescence (Figure 28). This change is likely due to the digestion of spore surface proteins by trypsin, destroying the epitopes to which the antibodies bind. Trypsin did not remove mother cell material as the other treatments did; however, the concentration of trypsin used and duration of treatment may have been insufficient.

Treatment with 10% EDTA also inhibited antibody binding to the spore surface, but to a lesser extent than 0.1% trypsin (Table 8, Figure 27). The IFA images of spores treated with 10% EDTA also displayed less fluorescence, with the localization of the bound antibodies remaining in the central spore region (Figure 23b-c). EDTA acts as a chelating agent removing calcium and magnesium which may have altered the protein’s conformational shape inhibiting antibody binding, while not removing mother cell cytoplasmic residues from the spore surface.

Caution should be used in developing treatments that alter the spore coat surface. Chemical treatments to the spore surface can increase the spore surface area for binding, which may make them harder to remove from surfaces such as mammalian epithelium.
Care must be taken to ensure new treatments result in spores with decreased binding to surfaces. Blocking the specific interaction between the spore coat and other surfaces, including human epithelium, may lead to decreased transmission in healthcare environments, or in the homes of patients suffering from CDI. Knowledge gained by researching treatments that can alter the spore surface will assist in developing usable products which can block the adhesion of spores to human epithelial cells.
References


CHAPTER V

General Conclusions

Detection of *C. difficile* in fecal samples is difficult. The significance of negative test results can be unclear due to degradation of *C. difficile* antigens in unpreserved (fecal) specimens. Anaerobic culture of patient feces, followed by proof of toxin production, is the new gold standard for all *C. difficile* testing (Knetsch, Bakker et al. 2011; Doing and Hintz 2012). PCR methods detecting the toxin B gene (*tcdB*) have the best specificity and sensitivity when compared to the toxigenic anaerobic culture method; however, when compared to the cell cytotoxin neutralization assays, many more “false” positives are being reported (Eastwood, Else et al. 2009; Stamper, Alcabasa et al. 2009). PCR methods are considered by many to be “too sensitive,” and may detect *C. difficile* carried asymptomatically (Riggs, Sethi et al. 2007). Acting on these results may increase the cost to healthcare facilities, as these asymptomatic carriers are placed in isolation rooms, treated unnecessarily with expensive antibiotics, and are put at risk for other infections caused by rotavirus (affecting infants) or Noravirus (affecting adults) (Barbut and Petit 2001; Bélanger, Boissinot et al. 2003; Wilkins and Lyerly 2003; Cohen, Gerding et al. 2010). PCR detection of the *tcdB* gene currently allows for quick results (2-3 hours), and can be performed by low-skilled technologists. Although the instrumentation and cost per test using PCR methods are relatively high ($25-50/test), the
timeliness and reliability of the results are welcome in many clinical microbiology laboratories (Humphries 2012).

The cell cytotoxin neutralization assay (CCNA) was the old gold standard by which all assays were once compared (Eastwood, Else et al. 2009). Clinical microbiology laboratories relied on the CCNA to identify *C. difficile* toxin in feces from patients suffering from CDI. The test cost ($30/test); high technologist skill level, slowest turn-around time (2-3 days), and additional or repeat assays (to rule out false test results), added to reporting delays and increased costs, now making the CCNA an undesirable test method (Humphries 2012). Additional costs are incurred due to maintainence of tissue culture cell lines for toxin assays.

The glutamate dehydrogenase (GDH) assay appears to be a sound screening method to rule out, but not detect toxin producing *C. difficile* infection. There are two different test formats, one flow-through, a low skill level test that typically provides results in less than one hour (Chin, Linder et al. 2012), or an enzyme immuno-assay (EIA) that can run high volumes of samples in less than two hours (Novak-Weekley, Marlowe et al. 2010). Testing for GDH alone is very sensitive for the presence of *C. difficile* bacteria in feces compared to bacterial culture; however, toxin testing still needs to be performed to confirm CDI on GDH positive samples (Novak-Weekley, Marlowe et al. 2010). Newer, flow-through GDH kits (C. Diff Quik Chek Complete, Techlab, Blacksburg, VA) have a toxin indicator, although the sensitivity of toxin detection is unacceptably low as compared to PCR or CCNA (Sharp, Ruden et al. 2010).
The *C. difficile* toxin EIA tests were once the rage of clinical laboratory testing, originally with sensitivity and specificity levels in the high nineties. However, they are now viewed as a poor choice for clinical lab use (Novak-Weekley, Marlowe et al. 2010). The sensitivities of these EIA toxin tests are now being reported as low as 38% (as compared to the cytotoxic culture method) indicating that nearly 2/3 of the positive patient samples are being reported as negative when tested by the EIA methods (Ticehurst, Aird et al. 2006). In response, physicians have adapted their ordering practices to require three fecal samples to be evaluated for EIA toxin studies, hoping to find at least one accurate reading for the presence of *C. difficile* toxin (Luo and Banaei 2010). This practice increases not only the laboratory workload and patient cost but also increases the false-positive rate with every additional sample tested. Restricted ordering often gets ignored as physicians become irate over test results that don’t match their empirical diagnosis. This is a concern since CDI is currently the leading cause of antibiotic-associated diarrhea (AAD) even though only 20 to 30% of AAD can be attributed to CDI (Cohen, Gerding et al. 2010).

Enteric Transport Medium (ETM) was designed to transport and maintain a variety of gastrointestinal pathogens and/or their antigens. ETM can be used to support culturing of *Salmonella, Shigella, E. coli O157:H7, Campylobacter, Yersinia*, and many other bacterial pathogens transmitted in patient feces. Clinical microbiologists appreciate the ability of using one transport medium to recover a variety of pathogens and their products, including *C. difficile*’s toxin and GDH. ETM is essentially a modified Cary-Blair medium (phosphate buffered solution) with a pH indicator added. The phosphate
buffering maintains a neutral pH, neutralizing acids present in the feces (Cary and Blair 1964). Strongly acidic samples (pH 4-5) can overpower the buffering capacity of ETM, causing the medium to change from pink to yellow. Yellow samples are considered invalid for transport and testing. Manufacturers of *C. difficile* tests acknowledge the acceptability of samples preserved and transported in Cary-Blair (ETM).

**Hypothesis 1:** Testing fecal samples by multiple methods will show that Enteric Transport Medium can maintain *C. difficile* antigens and toxins while being maintained at room temperature.

While *C. difficile* test manufacturers have allowed for Cary-Blair preserved samples like ETM to be used, the efficacy by which ETM preserves *C. difficile* toxins and GDH antigens at room temperature has not been documented. Transport of fecal samples for detection of *C. difficile* at room temperature has not been investigated. Investigators agree that the *C. difficile* toxins rapidly degrade at room temperature (Bowman and Riley 1986; Brazier 1993). Investigations into the cause of sample degradation have indicated that acidic pH, or the presence of fecal proteases are responsible (Tenover, Baron et al. 2011). The data of this dissertation disputes the manufacturer’s claim that ETM medium can maintain all *C. difficile* toxins for five days at room temperature without loss of test sensitivity. Interestingly, only one of 14 toxin positive samples, from the original 100 patient samples studied, lost its ability to preserve toxin in ETM, at room temperature, after 4 hours (Chapter 2, Tables 2, 3). In addition, this fecal sample was also negative for GDH, at its initial testing. The *C. difficile*
vegetative cells cultured from this sample tested strongly positive for GDH and Toxin A/B. It is likely that fecal proteases overwhelmed ETM’s ability to prevent toxin and GDH deterioration, in this lone sample. Of note, 8 of the 10 additional *C. difficile* GDH-positive samples collected, displayed a high level of fecal proteases, but toxin integrity and GDH was maintained in 6 of the samples tested while being preserved in ETM at room temperature (*Chapter 2, Table 5*).

The sub-hypothesis that fecal samples testing positive for *C. difficile* would have increased fecal trypsin levels, was confirmed in eight of the additional ten samples tested. Feces from these GDH positive samples displayed very high levels of total fecal proteases including trypsin. These fecal proteases and likely other bacterial proteases are suspected to degrade *C. difficile* toxins and its GDH antigen. Feces with high fecal trypsin concentrations had better toxin stability and thus detection when samples diluted in PBS were treated with antitrypsin (*Chapter 2, Figure 18*). Toxin detection from room temperature ETM-preserved stool samples remained stable regardless of fecal trypsin concentration. Furthermore, the addition of antitrypsin had no impact on toxin detection (*Chapter 2, Table 6*).

The sub-hypothesis that low fecal pH will affect the detection of toxin, proved not to be supported by the data, as acidic fecal samples were rarely submitted. Only one sample out of the initial 100 tested was found to be at a pH that would alter toxin detection. The effect of acidic pH has been shown previously to interfere with toxin testing (Taylor, Thorne et al. 1981). EIA testing for toxin, using a toxin A and B positive
control strain (ATCC 9689) displayed a dramatic decrease in toxin detection when the sample pH was decreased to pH 4 (Chapter 2, figure 17). When these samples were returned to neutral pH, the toxin detection remained low. However, in testing 100 fecal samples only one sample was below pH 5 (4.7), and another less than pH 6 (5.7). There may need to be a larger number of patient samples surveyed to better test this hypothesis. However, stool testing did indicate that *C. difficile* will not grow *in vitro* at pH levels less than 5. This knowledge may have a practical use where diets of suspected CDI patients could be supplemented with beneficial bacteria (i.e. *Lactobacillus acidophilus* or *Bidobacterium spp.*) with the ability to ferment carbohydrate food stuffs and create an acidic fecal environments to inhibit *C. difficile* growth (Tuohy, Kolida et al. 2001; Gibson, Probert et al. 2004). From these data we conclude that acidic stool samples are probably not responsible for the decreased rate of toxin detection by EIA as they are rarely submitted for testing (Chapter 2, Figures 14-17).

The data herein suggest that a two-step algorithm for testing stool samples be used, unlike the majority of laboratories that currently use only one Toxin EIA test. The first step should evaluate samples for GDH. The second step should evaluate GDH positive samples using a toxin-specific assay. This would reduce cost and decrease turnaround time for CDI diagnosis. This would also allow GDH-negative patients to be removed from expensive contact precautions mandated by a CDI diagnosis. Doctors could receive a preliminary GDH-positive report and be prepared to take action, even before the results of the toxin assay are known. The confirmatory result of toxin presence in stool samples may take several hours or several days depending on the test method
used. Historically, only 20 to 30% of the fecal samples will test positive for toxin, depending on the patient population. Screening for GDH will allow for laboratory savings by reducing the number of samples (70 to 80%) that would be required to undergo expensive testing for *C. difficile* toxin (e.g., toxigenic cultures, cell cytotoxin neutralization, or PCR). Additionally, fecal samples testing positive by EIA methods, as compared to PCR for GDH, indicate active growth of *C. difficile*, which may help reduce the hospital precautions for, and treatment of, asymptomatic carriers.

Today’s healthcare environment is designed to keep as many patients out of the hospital as possible (Bokhari, Caulkins et al. 1985). The risk of inpatients acquiring CDI increases with every day they are exposed to the hospital environment. CDI acquisition remains the responsibility of the hospital for up to 8 weeks following patient discharge. Beyond 8 weeks of discharge, CDI is considered community acquired (Cohen, Gerding et al. 2010). The number of outpatient fecal samples submitted for *C. difficile* testing from long-term healthcare facilities, patient homes, or doctor’s offices will continue to grow, and the use of sample transport systems such as ETM must be encouraged to maintain the stability and validity of the sample.

**Hypothesis 2**: As an alternative treatment, lipolyzed virgin coconut oil and its medium chain fatty acids will inhibit the growth of *C. difficile in vitro*.

The hypothesis that lipolyzed virgin coconut oil (VCO) would inhibit the growth of *C. difficile* was supported, as VCO demonstrated significant growth inhibition when tested *in vitro* against *C. difficile*. This research suggested that the *in vitro* inhibitory
effect of lipolyzed virgin coconut oil is primarily caused by lauric acid, which displayed a statistically significant log linear inhibition. Capric acid and caprylic acid also VCO components, were inhibitory to C. difficile, but to a much lesser extent than lauric acid.

The ideal antimicrobial agent would have a cidal effect on the targeted pathogenic microbes, while having no or a lesser effect on commensal organisms. Also the agent should have a neutral effect or beneficial effect on the host. Natural foods like virgin coconut oil match these goals. The fear associated with the continued dependency on vancomycin to treat CDI, is the generation of vancomycin-resistant organisms, currently occurring within other bacteria (i.e., vancomycin-resistant enterococci). Fortunately, the enterococci are not very pathogenic, but have been implicated in urinary tract infections and some septicemias (Low, Keller et al. 2001). Transference of the Van-A gene from vancomycin-resistant enterococci to S. aureus, enables vancomycin resistance in S. aureus; although this has rarely been documented before 2000, but recently has been found to be increasing. (Weigel, Clewell et al. 2003; Appelbaum 2006).

Alternative therapies utilizing natural products like virgin coconut oil to prevent colonization of pathogenic bacteria have been shown to be valid. Use of coconut oil has been documented to decrease the isolation of Escherichia coli from intestinal tracts of pigs being feed coconut oil (Dierick, Decuypere et al. 2002). C. difficile is constantly changing and adapting to efforts to thwart its spread, and the use of a natural product like VCO may help control further spread. Newer ideas for the prevention and treatment of
CDI are being pursued that don’t rely on antimicrobials, which ultimately inhibit normal fecal microbiota and their restoration after infection clearance.

**Hypothesis 3:** *C. difficile* spores are partially coated with mother cell residues on the spore surface.

The last hypothesis was data supported. Visualizing freshly grown spores using freeze-fracture transmission microscopy displayed material covering the entire spore surface with excess being present at the spore poles (Chapter 4 Figure 22a). This was determined to be remnants of the mother cell and consistent with what would be expected for bacteria that produce subterminal spores. Excess deposits from the dead mother cell would be present at the spore poles, while little or an absence of these deposits at the spore midsection. These deposits were also evident when spores were observed using fluorescent microscopy staining using antibodies that specifically bound to the spore coat proteins (Chapter 4 Figure 23). Observing spores treated with chemicals which removed the remnants from the spore surface, displayed increased antibody binding across the entire spore surface.

Using an indirect enzyme immunoassay, chemical treatments were quantified for their ability to alter or remove the spore surface proteins including mother cell remnants as indicated by increased or decreases in absorbances paralleling the changes in antibody binding (Chapter 4 Table 8). The changes in antibody binding to the spore surface proteins may be likened to the interaction between human epithelium and the spore surface proteins; where treatments determined to increase antibody binding might also
increase the spore binding to skin. Conversely, treatments resulting in decreased antibody binding may inhibit the binding of spores to skin. Understanding how chemical treatments affect the unique interaction between the spore surface proteins and skin may lead to usable treatments that block this specific interaction. Great care in developing treatments must be taken to ensure that this interaction is blocked and not encouraged.

**Future Studies**

Further studies using animal models are necessary to evaluate *C. difficile* transmission, toxin survival, and treatment options. To test the feasibility of using virgin coconut oil as a protective measure against colonization of *C. difficile*, guinea pigs, rabbits, or mice could be fed a diet containing virgin coconut products, (for example, lauric acid or monolaurin) for several days before the *C. difficile* challenge. Next they could be exposed to antibiotic agents (i.e., clindamycin, cephalosporins, or quinolones) to alter their protective normal gut flora. Toxigenic *C. difficile* spores could be mixed into their food and the animals could be evaluated for several days to check for any protective effects. Similarly, fatty acids could be added to the animals’ diet following the exposure to toxigenic *C. difficile*, or possibly as an addition to an antibiotic treatment, such as metronidazole or vancomycin. A 2002 study supplementing piglet diet with medium chain fatty acids resulted in increased health and weight gain, without the traditional use of antibacterial agents to inhibit gastrointestinal pathogens (Dierick, Decuypere et al. 2002). Studies by Preus et al reported the protective effect of feeding lauric acid to mice in which *S. aureus* septicemia was induced. They showed that lauric acid had a beneficial
effect preventing death, as compared to control groups (Preuss, Echard et al. 2005). Once animal studies have shown the benefit of supplementing diets with virgin coconut oil, human studies could follow by testing small groups of patients such as those found in a nursing home. Residents’ diet could be switched to contain more coconut oil products, with the possible reduction in CDI studied. Lauricidin is an adult nutritional supplement currently available on the commercial market. It contains monolaurate, which has been shown to exhibit antimicrobial properties and may enhance immune function (Haese and Minnerath 2009).

There is also an additional incentive to use natural products like virgin coconut oil or lauric acid to prevent or reduce the presence of detrimental microbes. Products would be able to be labeled organic, when antibiotics are replaced with coconut products. These organic products could be used on organic farms as a means of decreasing animal disease, in the same way antibiotics are used now. Also, medium chain fatty acids can be used as food preservatives or bactericides, and used to grow or maintain organic products (Wang and Johnson 1992; Ouattara, Simard et al. 1997).

Larger studies, using ETM for *C. difficile* toxin and GDH preservation, need to be conducted to determine the ability of ETM to preserve these antigens and improve test results. The use of ETM by healthcare facilities may improve the sensitivity and specificity of results when using EIA methods to detect *C. difficile* toxin in stool, thus reducing the number of asymptomatic carriers being treated because of misleading PCR - positive results.
Studying chemically-treated *C. difficile* spores using the indirect spore ELISA test may result in discovery of novel treatments that interrupt transmission of *C. difficile*. Effective spore treatments as determined by the ELISA could further be evaluated by using treated spores in cell cultures containing keratinocytes to test the treatment’s efficacy. Ideally, the treatment contact time needed to interrupt attachment would occur in a matter of seconds, rather than minutes-to-hour, as is seen in treatment based on 0.1% trypsin. Development of novel treatments into products, either through licensing agreements or direct public-private partnership, would help integrate these innovations into standard operating procedures within healthcare facilities for the control *C. difficile* transmission.
References


