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Detection of Antibiotic Resistance Clostridium difficile in Lettuce

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DETECTION OF ANTIBIOTIC RESISTANCE *CLOSTRIDIUM DIFFICILE* IN LETTUCE

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by
Yi Han
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ABSTRACT

Clostridium difficile (*C. difficile*) is regarded as the major cause of infectious diarrhea in humans after antimicrobial treatment. *C. difficile* has been reported to be widely isolated from food animals and meat. The main purpose of this study was to characterize *C. difficile* isolates from retail fresh vegetable (lettuce), test the antibiotic-resistance property using five common clinical-selected antibiotics (metronidazole, vancomycin, clindamycin, erythromycin, and cefotaxime). Lettuces (grown in California, Arkansas, and Louisiana) were purchased from retail stores. Toxigenic *C. difficile* was isolated from 13.8% (41/297) of the lettuce samples. Among the toxigenic isolates, 82.9% (34/41) only produce toxin B, and 17.1% (7/41) produced both toxin A and toxin B. Under the treatment of the five antibiotics, the virulence *C. difficile* isolates were identified as having antibiotic resistance to metronidazole, vancomycin, and erythromycin. The present study reports the highest toxigenic *C. difficile* yield rate from varieties of retail vegetables (lettuce) in the USA. The antibiotic resistance to metronidazole, vancomycin, and erythromycin of the isolated *C. difficle* from varieties of retail lettuces could lead to public health concerns.

CHAPTER I. Introduction to Clostridium difficile

1.1 *Clostridium difficile*

Clostridium difficile (*C. difficile),* a species of Gram-positive, spore-forming,

and anaerobic bacteria, causes diarrhea and more serious intestinal conditions such as

pseudomembranous colitis in humans (Monaghan et al. 2013). The name given to this

organism was because it was difficult to isolate and grew slowly when being cultured.

According to the Center for Disease Control and Prevention (CDC), *C. difficile* has

been listed as an immediate health threat, and *C. difficile* infection (CDI) induces

250,000 hospitalizations and 14,000 deaths per year. There are typically three

manifestations of CDI development: asymptomatic carrier state, colitis with or

without pseudomembranes, and fulminant colitis.

1.2 History of *C. difficile*

C. difficile was first identified in 1935 in the common intestinal colonic flora

of newborn infants(Hall and O'Toole, 390-402). At first, it was known as *Bacillus*

difficile, because it was difficult to grow outside of anaerobic broth culture. It was so

frequently isolated from the feces of newborn infants that it was regarded as part of

the infants' gut microbiota at that time. However, subsequent studies indicated its pure

broth cultures could lead to experimental animal death if being injected(Snyder 1937).

Thus, *C. difficile* was believed as non-pathogenic to humans. Not until the late 1970s

were the biological activities of its toxins defined, and *C. difficile* toxins were linked

to pseudomembranous colitis (PMC), proving it to be a human pathogen(Bartlett et al. 1978a).

In 1893, the definition of pseudomembranous colitis (PMC) was first raised by Finney (Bartlett et al. 1978b) as a post-operative complication. PMC (Figure 1) is described as multiple yellow discrete patches (Figure 1) growing on the surface of colon mucosal (Nelson et al. 1994) ,which microscopically reveals partially destroyed
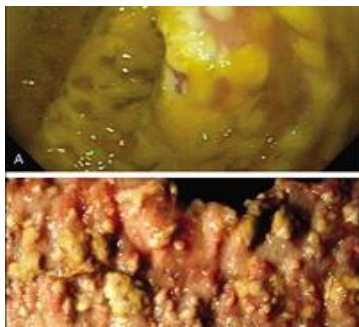
Figure 1. Pseudomembranous colitis.

mucosal layer glands covered with fibrin and inflammatory polymorph nuclear leukocytes (Nelson et al. 1994). The residues of colonic glands are able to become necrotic and inflamed. Besides, the submucosa may also become contaminated with the increasing amount of vasculature and inflammatory cells, which are responsible for water loss and subsequent diarrhea (Nelson et al. 1994). The pseudomembrane is considered as having the ability of growing over and replacing the normal colonic mucosa concerning bacterial toxins (Bartlett et al. 1978b).

PMC was not commonly diagnosed until the increasing usage of antimicrobial agents in the 1950s, when PMC was known as a common complication with high mortality rates (Nelson et al. 1994). At first, *Staphylococcus aureus* was regarded as

the reason to cause PMC by the Gram staining of plaques. Later, the stains revealed

many Gram positive cocci in clusters (Bartlett et al. 1978b).   This theory was also

supported by the knowledge of staphylococcal enterotoxins produced by *S. aureus*

causing enteric disease, and the clinical improvement when treating those patients

suffering from PMC with vancomycin instead (Bartlett et al. 1978b). Because of the

failure of imitating the effects that were occurring in humans using animal models

infected with *S. aureus*, other potential causes of the origin of PMC were pursued

continuously (Bartlett et al. 1978b). The true explanation of these infections stayed

unknown, and was not being associated with *C. difficile* until two decades later when

the connection between the toxins A and B of *C. difficile* and PMC was revealed

(Bartlett et al. 1978b).

In the 1960s, anaerobic bacteria were related to the formation of aposteme,

and several new antibiotics were developed to target them. Clindamycin was

especially effective for intestinal infections caused by anaerobic bacteria (Bignardi

1998). However, there was a common side effect of clindamycin treatment---diarrhea,

which some patients severely suffered from and progressed to PMC in a lot of cases

(Bignardi 1998). This antibiotic-associated diarrhea was not only seen with

clindamycin, but also with other broad spectrum antimicrobials, so it was named as

antibiotic associated diarrhea (AAD) (Bignardi 1998). After *C. difficile* toxins were

identified in patients suffering from colitis, the linkage among *C. difficile*, antibiotic

usage and PMC was established (Bartlett et al. 1978b).

Based on the association of antimicrobial use, PMC and *C. difficile*, researchers began to exploring a toxin responsible for the diarrhea associated with *C. difficile* infection (CDI). First, toxin B was confirmed by neutralizing the toxin's effect by using the antitoxin of *Clostridium sordellii*. Toxin B was lethal in animal testing, the ability of causing severe hemorrhage, and mucosal edema in the cecum of laboratory animals. During the process of purifying the toxin, a second toxin showed up, toxin A. However, injecting an equal amount of toxin A followed by the same route was not lethal in animal models, and only brought about focal hemorrhage (Taylor, Thorne, and Bartlett 1981). Purified toxin A was able to result in a disease pathology similar to CDI, while testing animals in the same condition, toxin B had no effect at that time. Thus, Toxin B was thought to have an effect on test animals only with the presence of toxin A (Mitchell et al. 1986b, Taylor, Thorne, and Bartlett 1981). However, a toxin A negative, toxin B positive strain was isolated in a CDI outbreak in Canada in 1998, and later these strains have been discovered frequently (Drudy, Fanning, and Kyne 2007). So it has been regarded that both toxins are now considered responsible for the pathology, although toxin A positive, toxin B negative strains have not been found to be naturally present. In 2009, laboratory created toxin-A positive, toxin B-negative mutants showing reduced pathology in a hamster model, where pathology in hamsters caused by toxin A-negative, toxin B positive mutants remained unaffected in severity (Lyras, O'Connor, et al. 2009). This result indicates that toxin B

is essential for *C. difficile* disease, and synergy effect along with toxin A may not be necessary (Lyras, O'Connor, et al. 2009).

1.3 Toxins of *C. difficile*

Those strains lack of the ability of producing toxins are not considered pathogenic. When nutrients are abundant, toxin production will be inhibited, while during the essential nutrients shortage, particularly biotin, toxin production will cause intestinal epithelial cells to rupture, releasing nutrients (Yamakawa et al. 1996). Pathogenic *C. difficile* produces two protein exotoxins, Toxin A, comprised of 2710 residues (308.0 kDa), and toxin B, comprised of 2366 residues (269.6 kDa) (Kelly and LaMont 1998). Toxin A and toxin B (also called TcdA and TcdB), belonging to the large clostridial cytotoxins (LCT), can cause animal death, while toxin A, called enterotoxin, has been proven to cause fluid accumulation, and severe epithelial damage with hemorrhage and diarrhea in rabbit ileal loops (Mitchell et al. 1986a, Torres and Lonnroth 1989). After toxin A and toxin B gain access into cells, they exert an alteration of Rho proteins (a family of GTP-binding protein associated with actin polymerization, cytoskeletal architecture, and cell movement) and finally result in the depolymerization of actin filaments, disruption of the cytoskeleton, cell rounding, and cell death (Kelly and LaMont 1998). Both A and B toxins contain four domains (Figure 2): a receptor binding domain; an enzymatic domain; an autoproteolytic cleavage during toxin-processing domain; and a hydrophobic translocation domain responsible for transferring the enzymatic domain into the cytosol (Belyi and Aktories

2010). The C terminal receptor binding domains of the two toxins are different,

allowing the toxins to attach to the corresponding intestinal epithelial cell receptors

toxin A to the apical surface receptors, and toxin B to the basolateral surface receptors

(Jank, Giesemann, and Aktories 2007).
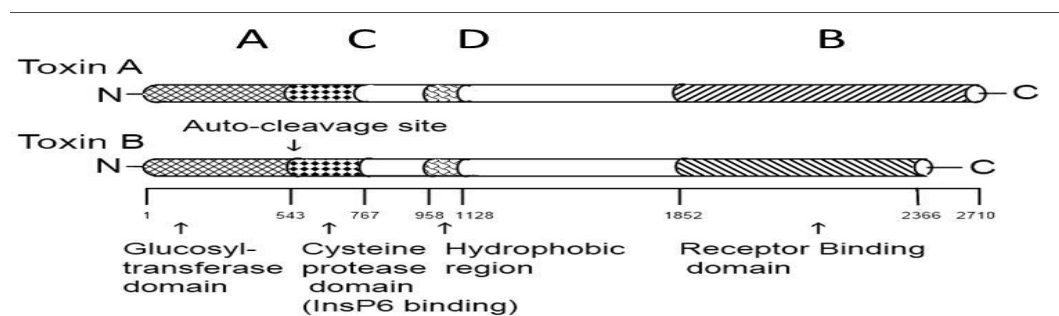
Figure 2. The model of toxin A and toxin B.

The genes encoding toxin A and toxin B are part of the pathogenicity locus

(PaLoc), a 19.6kb chromosomal segment consists of TcdA-E genes (Figure 3) which

provides a base for C. difficile PCR genotyping (Warny et al. 2005). These genes

encode toxin A (TcdA), toxin B (TcdB), a negative regulator (TcdC), a positive

regulator (TcdD), and a holing-like protein (TcdE). Transcription analysis studies

indicate that TcdA, B, D, E are transcribed both monocistronically and

polycistronically (Hundsberger et al. 1997). This mode of transcription is clearly the

prior condition of a high production of toxin A and toxin B (Hundsberger et al. 1997).

While, during the logarithmic phase, the TcdC gene has strong expression, and the

other four genes are in weak transcription. The inverse is observed during the

stationary phase, implicating that TcdC negatively regulates toxin expression

(Hundsberger et al. 1997). Yet, the presence of TcdC genes, including TcdA and TcdB,

has also been found outside the PaLoc in several C. difficile strains. As TcdD

increases with TcdA, B, E, TcdD is recognized as a positive regulation promoting

toxin expression. Thus, TcdC and TcdD might be key factors regulating the production

of the two toxins.
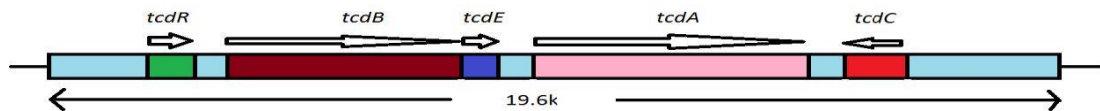
Figure 3. The pathogenicity locus.

Both toxins stay stable from -20 to 37^0C, but will lose activity at 56^0C

(Sullivan, Pellett, and Wilkins 1982). Yet, neither toxin has effect on intracellular

levels of cyclic AMP or GMP (Kelly and LaMont 1998). In addition, neither toxin is

found to have association with spore production (Arnon et al. 1984). One of the

physicochemical properties of toxin A, after purification, is its large size (Lyerly,

Krivan, and Wilkins 1988). It can cause huge damage to gut mucosa (Mitchell et al.

1986a).

Toxin B, as a cytotoxin, is believed to be as lethal as toxin A, but less stable

than toxin A (Arnon et al. 1984, Banno et al. 1984). It brings generous nonspecific

responses in mammalian cells, such as the loss of intracellular potassium, decrease in

protein synthesis, and decrease in synthesis of ribonucleic and deoxyribonucleic acids

(Pothoulakis et al. 1986, Rihn et al. 1985). It induces rounding cells 100-10000 times

more severe than toxin A in many cell types (Chaves-Olarte et al. 1997).

C. difficile transferase (CDT), a third toxin (aka, binary toxin), is an actin-specific ADP-ribosyltransferase; it was first proposed by Popoff et al. together with a few *C. difficile* strains in 1988 (Perelle et al. 1997, Popoff et al. 1988, Stubbs et al. 2000). Other similar toxins are produced by other species such as: iota toxin from *Clostridium perfringens* (Stiles and Wilkins 1986),*Clostridium botulinum* ADPribosyltransferase C3 (Aktories, Weller, and Chhatwal 1987),and *Clostridium spiroforme* toxin (CST) (Simpson 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. Cells contaminated by CDT toxin present depolymerization of F actin, leading to cell rounding. Additionally, the formation of surface microtubules displays in these cells that have been shown to raise the possibility of *C. difficile* adherence. This increased adherence to the cell surfaces may promote colonization, adding to the virulence of these CDT producing strains (Schwan et al. 2009). It was shown that *C. difficile* strains which produce CDT toxin in addition to toxins A and B tend to be more virulent, especially the hypervirulent PCR ribotype 027 and 078 strains. Nonetheless, some *C. difficile* strains only produce CDT toxin without toxins A and B; there is no strong evidence indicating a hypervirulent strain (Stubbs et al. 2000).

1.4 Sporulation of *C. difficile*

C. difficile's ability to produce infectious endospores assists both its survivability outside and inside of its host. Sporulation forms when reproduction of

vegetative cells suffers from nutrient deprivation or undesirable conditions. The

metabolically inactive property of the spores gives them the ability of being resistant

to antimicrobial treatments, heat, radiation, desiccation and chemical treatments. Thus,

spores are resistant to the majority of cleaning products used in healthcare facilities

(Gerding, Muto, and Owens 2008). The spore formation of *C. difficile* grants the

bacteria fecal oral transmission in healthcare facilities, either directly from patient to

patient, or by transmission from the hands of health care personnel (Lawley et al.

2009, Underwood et al. 2009). Researchers found spores are frequently spread by

flushing toilets or changing the bed sheets contaminated with feces from *C. difficile*

patients (Best et al. 2010, Donskey 2010, Roberts et al. 2008).

Prokaryote sporulation is an evolutionary performance to protect species

survival. Spore formation allows cells to wait until environmental conditions become

suitable for them to switch back to their vegetative form and continue growing (De

Hoon, Eichenberger, and Vitkup 2010). The process of sporulation has been

thoroughly studied in *Bacillus subtilis*; its process may also be applied to *C. difficile*.

Sporulation can be divided into seven separate stages (Figure 4). The process starts

with stage I, where the vegetative cell grows in size and replicates its DNA followed

normally by cell division resulting in stage 0. Different from a normal binary cell

division, the cell is divided into two unequal parts called stage II during the

sporulation process. The larger portion is considered as the mother cell, and the

smaller portion is the fore spore. In stage III, the fore spore then becomes immersed in

the mother cell, continuing the process of growing to a mature spore at the same time. In stage IV, peptidoglycan forms the cortex layer of the fore pore, separating the two cells. Calcium dipicolinate synthesized by the mother cell is concentrated in the spore core, stabilizing the DNA. Diplocolinic acid and calcium enable the spore to become resistance to heat and oxidizing agents (Setlow 2007). During stage V, an outer protein layer is formed to coat the outer membrane. The spore turns to be mature in stage VI, along with finishing coat synthesis, dehydration, and lysis of the mother cell wall by the action of lytic proteins. Accordingly, the dormant spore is released (De Hoon, Eichenberger, and Vitkup 2010, Paredes, Alsaker, and Papoutsakis 2005).
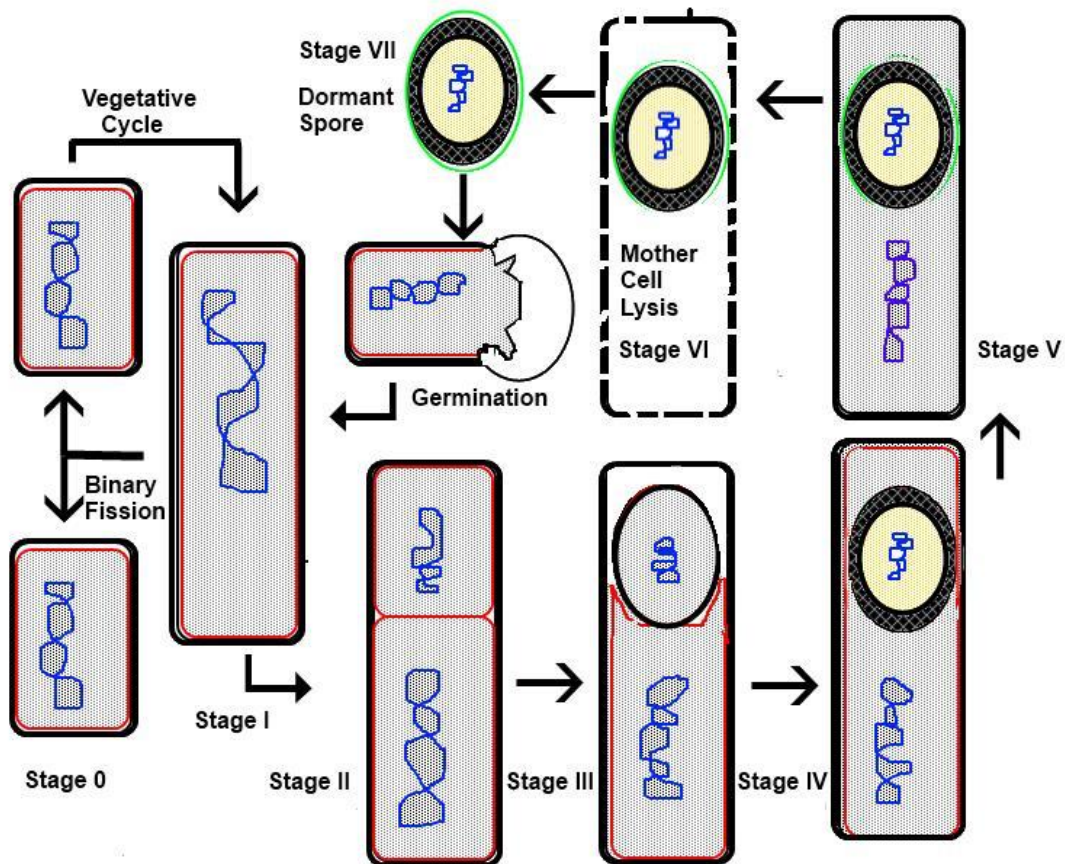
Figure 4. Stages of Sporulation.

1.5 Epidemiology

C. difficile infection stands in the first place in the cause of antibiotic associated diarrhea, having a percentage of 10-25% of all cases (Bartlett 2002). Carriage and colonization rates vary widely between different patient groups. Among the normal adult population, *C. difficile* asymptomatic carriage is estimated at 2-3%, but much higher in those frequently exposed to healthcare environments (Barbut and Petit 2001). Concerning the newborns, they are highly susceptible to acquiring *C. difficile,* given the fact that they have no protective normal gut flora to inhibit colonization. Normally, the acquisition of *C. difficile* in newborns is considered to be gained from the child's mother flora or the newborn nursery environment (TABAQCHALI et al. 1984). Carriage rates in newborns born in hospitals have been appeared to be as high as 70% (Kato et al. 1994, TABAQCHALI et al. 1984). Carriage rates are also showed to be higher in healthcare providers and high risk patients that have been hospitalized or received antibiotics (Barbut and Petit 2001, Giannasca and Warny 2004). It is reported that approximately one-third of the long-term healthcare facility residents asymptomatically carry *C. difficile* (Simor et al. 2002).

1.6 Risk factors associated with *C. difficile* infection (CDI)

The key risk factor associated with development of CDI is the treatment with antibiotics. More than 90% of all CDI cases happen during or following treatment with antibiotics (Barbut and Petit 2001). Apart from aminoglycosides, almost every

other antibiotic would lead to some risk of progression from asymptomatic colonization to CDI (Sunenshine and McDonald 2006). Exposure to antibiotics may develop to CDI, but the infection could be delayed for up to 8 weeks after antibiotics are consumed (Johnson and Gerding 1998b, Kelly, Pothoulakis, and LaMont 1994). A majority of CDI cases involved the use of clindamycin, fluoroquinolones, or third generation cephalosporins (Gerding 2004). It is supported that limiting the use of these antibiotics could significantly reduce the healthcare acquired infections (HAI) concerning *C. difficile* (Carling et al. 2003). It is vital for individual healthcare institutions to be aware of the sensitivity and resistance patterns of *C. difficile* isolates that rely on their environment and patient population. If an isolate in an institution shows its resistant to clindamycin, then the use of clindamycin should be highly restricted (Owens et al. 2008, Pear et al. 1994). On the other side, if a patient's *C. difficile* organisms are routinely sensitive to a specific antimicrobial, then the use of that antibiotic should be encouraged (Donskey 2010).

There are several high risk populations, whose who are the elderly, immune dysfunctional, pregnant, and those frequently exposed to healthcare environments. Patients older than 65 years of age or with severe underlying illness are vulnerable to CDI (Barbut and Petit 2001, Bignardi 1998). The reason of this may also be associated with more frequency of hospitalization, decreasing immune function, and an increased likelihood to be treated with antibiotics. Accordingly, patients living in

long-term healthcare facilities are generally older, and having higher chance of being exposed to increased antibiotics (Sunenshine and McDonald 2006).

The disease progression can vary from patient to patient, depending on their immune response to CDI. Those patients that produce high antibody titers to toxin A usually only develop diarrhea, and have a higher possibility of resolving without reoccurrence. On the contrary, failure to produce adequate antibodies to toxins results in increased risk for complications and recurrent infections (Kelly 1996, Kyne et al. 2001). Besides, asymptomatic carriers normally have high antibody titers to toxin A, and are unlikely to develop severe symptoms when exposed to antibiotics (Salcedo et al. 1997).

Prior to the emergence of hypervirulent strains of *C. difficile*, pregnant women share an unusual high occurrence rate of CDI, with some minor symptoms not involving hospitalization. Now there is an increased frequency and severity of disease is associated with CDI in peripartum women, leading to increased colectomies, stillbirths and maternal deaths (Rouphael et al. 2008). Those patients admitted to hospitals have increased rates of asymptomatic colonization despite of progress to infection. Cultures on a variety of surfaces in healthcare facilities have been tested and demonstrated high levels of *C. difficile* contamination (McFarland et al. 1989). In screenings of patients upon admission, it has been found that carriage rates have a range between 5.9 to 11%, which is higher than the normal distribution in adults of 2 to 3%. Infection rates differ among patient groups depending on other risks factors,

but overall the rate is between 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 increased to 50% based on the hospitalized patients for more than a

month (Calabi et al. 2002). Approximately 63% of these patients would become

asymptomatic carriers. What's more, studies of outbreaks have found acquisition rates

can rise to as high as 32%, especially when highly susceptible patients are involved

(Barbut and Petit 2001). It is found that the time needed to treat patients, those who

developed CDI while being treated for another issue at the same time, could be

increased by 3.6 days in the hospital, at an estimated cost of over 1 billion dollars in

the U.S. per year (Kyne et al. 2002).

1.7 Treatment for CDI

The first step in treatment of CDI is to stop the current offending antibiotic if

possible, or switch to another antibiotic with a narrower spectrum. Using this method,

nearly 25% of diarrhea from mild CDI would be resolved without further

complication (Barbut et al. 2000). Subsequently, supportive therapy should be

followed to rehydrate and replace electrolytes, especially in patients with severe

diarrhea. Antiperistaltic drug therapy should be avoided to limit patients' exposure to

C. difficile toxins, which may result in the development of toxic megacolon

(McFarland 2005). Because of the rise of hypervirulent strains, some physicians may

treat with antibiotics until CDI has been ruled out by laboratory testing (Bartlett and

Gerding 2008).

Clindamycin is an effective treatment for serious anaerobic infection, but was used so widely that it has been gradually losing its efficiency (Kabins and Spira 1975). To date, when diarrhea and colitis caused by *C. difficile* are severe, there are some other common effective treatments which are oral metronidazole and oral vancomycin (Kelly and LaMont 1998). Besides, ampicillin, amoxicillin, and cephalosporins are also some common alternatives concerned with *C. difficile* (Kelly and LaMont 1998).

C. difficile strains produce a binary toxin (CDT), apart from toxin A and toxin B, and they exhibit a resistance to fluroquinolones and erythromycin (Cartman et al. 2010). Moreover, treating asymptomatic carriers with metronidazole or vancomycin is not ideal, for the treatment may extend the carrier state (Johnson et al. 1992). Antibiotics are regarded as the most important risk factor for *C. difficile*-associated diarrhea by reducing 'colonization resistance' of the bowel, allowing subsequent colonization, and infection with C. difficile (Johnson and Gerding 1998a). Thus, using proper antibiotic is of crucial importance, and is also the most effective treatment of *C. difficile* infection.

Besides, probiotics containing one or several living beneficial microbes have also been a popular treatment, either serving as addition to antibiotic therapy or as a replacement for antibiotic treatment. The advantages of probiotics are: multiple mechanisms of acting on pathogens, benefit to host immune system, survival to host colon, no drug interaction, and low risk to the patient, while the drawbacks of probiotics are: poor quality control, poor standardization, few clinical trials, possible

infections with immunocompromised patients, and sometimes adverse reactions in

patients (McFarland 2009).

1.8 *C. difficile* in food

   C. difficile infection (CDI) essentially occurs in clinic environments, while the

majority of patients infected by *C. difficile* are asymptomatic carriers, and one of the

known risk of infection is sharing a hospital room with an infected patient (Johnson et

al. 1990). However, in the community, *C. difficile* is increasingly found among young

and relatively healthy individuals (Hensgens et al. 2012). In the environment, such as

soil and water, *C. difficile* commonly exists. Nevertheless, its presence in numerous

animals is also ubiquitous, and similar PCR ribotypes are found, particularly ribotype

027 and ribotype 078 (Rodriguez et al. 2013, Hensgens et al. 2012). Accordingly,

there may be a potential for transmission from food to humans.

   Community-associated *C. difficile* infection is increasingly regarded as a

potential foodborne disease, especially in food animal. A study from USA, examining

samples from stores in the Tuscon, Arizona area, found isolation of *C. difficile* from

37 of 88 samples, including ground beef (13/26), summer sausage (1/7), ground pork

(3/7), braunschweiger (10/16), chorizo (3/10), pork sausage (3/13), and ground turkey

(4/9) (Songer et al. 2009). Ribotype 078 was the majority strain, and the rest belonged

to ribotype 027 (Songer et al. 2009).

   Though infection with retail meat is most compelling, infection with other

food products may be equally fatal, particularly for those that are not cooked before

eating. A research from South Wales described 71% of *C. difficile* isolates from

vegetables were toxigenic (al Saif and Brazier 1996b). Reported CDIs associated with

salad-used-vegetables have also been found in France and Scotland (Bakri et al. 2009,

Eckert, Burghoffer, and Barbut 2013).

CHAPTER II. Prevalence and antibiotic resistance of *C. difficile* in lettuce

2.1 Introduction

Clostridium difficile (*C. difficile*)*,* a species of Gram-positive, spore-forming,

and anaerobic bacteria, is the causative reason of *Clostridium difficile*-associated

diarrhea (CDAD) and can lead to more serious disease such as pseudomembranous

colitis, toxic megacolon, and even death in humans (Monaghan et al. 2013).

Pathogenic *C. difficile* produces two protein exotoxins, toxin A, comprised of 2710

residues (308.0 kDa), and toxin B, comprised of 2366 residues (269.6 kDa) (Kelly and

LaMont 1998). Toxin A and B (also called TcdA and TcdB), the primary makers of

Clostridium difficile infection (CDI), belong to the large clostridial cytotoxins (LCT).

In addition to toxin A and B, *C. difficile* strains produce a binary toxin, called *C.*

difficile toxin (CDT). However, only about 6% of *C. difficile* isolates produce the binary toxin, and these are toxinotype variants (Geric et al. 2004).

In earlier studies, toxin A was regarded as the predominant virulence factor, and toxin B alone, without the present of toxin A did not cause disease (1994, Pothoulakis et al. 1986) (Voth and Ballard 2005).   As a result, the mechanism of toxin B in disease is not well explored as the role of toxin A. With the discoveries of some toxinA⁻toxinB⁺ strains (King, Mackin, and Lyras 2015), toxin B was reported to contribute to the *C. difficile*–associated diseases, and it was regarded as the essential virulence contributor (Lyras, O'Connor, et al. 2009).

C. difficile infection (CDI) essentially has occured in a clinic environment, however, community-associated *C. difficile* infection is increasingly regarded as a potential foodborne disease, especially in food animals (Rodriguez et al. 2014). Though infection with retail meat is most compelling but has not been proven (Rodriguez et al. 2013), infection with other food products may be equally fatal, particularly for those that are not cooked before eating (Weese 2010). Pathogenic *C. difficile* isolated from vegetables has been reported in Europe (al Saif and Brazier 1996b), while in United States, there is limited research focusing on ready-to-eat vegetable such as lettuce. Modified atmphosere packing and storage condition of lettuce could promote the growth of anaerobic bacteria such as *C. difficile* (Doulgeraki, Paramithiotis, and Nychas 2011). Furthermore, there are various possible sources of lettuce contamination with *C. difficile*, all of which are likely to be ultimately human

or animal, such as soil, fertilizer (manure), water, processing environments, and human hands (Simango 2006, Rodriguez et al. 2013, Weese 2010).

People are more likely to become infected with *C. difficile* with the use of antibiotics, not only because antibiotics disrupt the normal intestinal flora, resulting in *C. difficile* colonization (Kyne et al. 2002), but also *C. difficile* has been found to be resistance to several antibiotics (Owens et al. 2008, Gerding 2004). Antibiotics are used to treat bacterial infections, but some antibiotics are found to be ineffective in treating the infection of anaerobic bacteria including *C. difficile* (Lyerly, Krivan, and Wilkins 1988). Clindamycin is an effective treatment for serious anaerobic bacterial infections, but has been used so widely that it is now gradually losing its efficiency (Kabins and Spira 1975). To date, when diarrhea and colitis caused by *C. difficile* are severe, the common effective treatments are oral metronidazole and vancomycin (Kelly and LaMont 1998).

In this study we determined the prevalence and antibiotic resistance of *C. difficle* in lettuce.

2.2 Materials and Methods

2.2.1 Sample preparation

Lettuces, harvested in California, Arkansas, and Louisiana, were continuously purchased from retail stores and tested from September 2014 to March 2015. The lettuce samples were grown in Salinas, California; Bentonville, Arkansas and Baton Rouge, Louisiana. The types of lettuce sample purchased from California, Arkansas,

and Louisiana were iceberg lettuce, butter lettuce, and romaine lettuce, respectifully. In all, 297 lettuce samples were tested, and for each state, 99 samples were tested (8 lettuce samples for per month in September, Ocotober, November 2014; 10 lettuce samples in December 2014; 15 lettuce samples in January 2015; 20 lettuce samples in Feberary 2015; 30 lettuce samples in March 2015)

Brain Heart Infusion broth (BHI) (BD) supplemented with 0.1% sodium taurocolic acid and *C. difficile* selective supplement (Sigma-Aldrich), containing cefoxitin (8 µg/ ml) and D-cycloserine (250 µg/ ml), was used to enrich *C. difficile* isolates, for isolation from the lettuce samples. For each lettuce sample, 60 ml sterile BHI supplemented broth and 40 g lettuce were blended together in a filter bag; the collection from each sample was done in duplicate. Every filter bag was incubated anaerobically by a GasPak™ EZ Anaerobe Pouch System at 37 ^{0}C for 10 days.

2.2.2 Isolation *C. difficile*

After the samples were incubated for 10 days, the sample broth in the filter bag was transferred into sterile test tubes. To detect the presence of *C. difficle*, BBL™ *Clostridium difficile* Selective Agar (BD) plates were used, 2 plates per filter bag. The plates were reduced anaerobically under room temperature for 24 hours before use. Then 0.1 ml of the collected supernatant was streaked onto the selective plates under a certified bacteria safety hood and the inverted plates were incubated anaerobically, with the anaerobe pouch system mentioned above, at 37 ^{0}C for 48 hours. *C. difficile* colonies were identified by their morphological and fluorescence

properties under long wavelength UV (380 nm) within one hour in the presence of oxygen. The positive *C. difficile* colonies emitted a yellow fluorescence. For further research, the *C. difficile* colonies of each lettuce sample isolated from the *C. difficile* selective plates were collected and stored in –80ºC freezer.

2.2.3 DNA extraction

Right after the colonies of *C. difficile* were observed on the plates, DNA extraction was conducted according to the instructions of a commercial DNA extraction kit (MO-BIO UltraClean® Microbial DNA Isolation Kit). Three colonies from each plate were collected into a sterile 2 ml centrifuge tube. Then the microbial cells were resuspended in provided bead solution, they were added to a bead beating tube containing beads, followed by lysis solution. With a combination of heat, detergent, and mechanical force against specialized beads, the cellular components were lysed. *C. fifficile* DNA was release from the lysed cells, and bound to a silica Spin Filter. After washing the filter several times, the DNA was recovered in the provided DNA-free Tris buffer. Extracted DNA was stored at $-20\ ^0$C until real-time PCR was performed.

2.2.4 Real-time PCR assays for toxin A and B

Non-repeat regions on toxin A and toxin B genes are commonly chosen as amplifying-segments in real-time PCR assays. For toxin A detection assay, the primers and the probe described by Luna et al. were utilized (Luna et al. 2011); for toxin B detection, the real-time PCR method was performed with the primers and the probe

specific to determine the virulence of *C. difficile* isolates in lettuce, which is described by van den Berg et al (van den Berg et al. 2005)(Table 1). The total volume of each reaction mixture for the Real-time PCR was 25 µl. For the toxin A assay, each amplification mixture consisted 12.5 µl Bio-Rad iQ^{TM} Supermix (2x), 0.6 µM forward primer (tcdAF), 0.6 µM reverse primer (tedAR), 0.1 µM hydrolysis probe (tcdATM), PCR grade water, and a 6.25 µl DNA sample. For the toxin B assay, each final reaction mixture included 12.5 µl Bio-Rad iQ^{TM} Supermix (2x), 0.25 µl of 10 µM forward primer (398CLDs), 0.5 µl of 10 µM reverse primer (399CLDs), 0.5 µl of 10 µM 551CLD-tq-FAM probe, 0.25 µl of 0.1M MgCl$_2$, 8.5 µl PCR water, and 2.5 µl DNA template. Amplification was performed using a Cepheid SmartCycler II system (Sunnyvale). The cycling program of the toxin A assay was as following: 1 cycle of 95°C for 10 min; 45 cycles of 95°C for 10 min, 57°C for 20 s, and 72°C for 10 s. For toxin B assay, after the reaction mixtures were initially heated for 3 minutes at 95 ^{0}C, they went through 45 cycles. Each cycle possessed a 30 s denaturation step at 94 ^{0}C, a 30 s annealing step at 57 ^{0}C, and a 30 s extension step at 72 ^{0}C. Positive and negative controls were run in each trial. The extracted DNA (2.5 µl) from a toxin A positive toxin B positive *C. difficile* strain (ATCC 43255) was employed as the positive control, and the PCR grade water (2.5 µl) was served as the negative control.

Table 1. Primers and probes for real-time PCR detection of *C. difficile* toxin A and B

Primers and probe	Nucleotide sequence (5'- 3')

Toxin A	tcdAF	GGTAATAATTCAAAAGCGGCT
	tcdAR	AGCATCCGTATTAGCAGGTG
	tcdATM	FAM-AGCCTAATACAGCTATGGGTGCGAA-AMRA
Toxin B	398CLDs	GAAAGTCCAAGTTTACGCTCAAT
	399CLDas	GCTGCACCTAAACTTACACCA
	551CLD-tq-FAM	FAM-ACAGATGCAGCCAAAGTTGTTGAATT-TAMRA

2.2.5 Antibiotic resistance detection

The standard NCCLS (National Committee for Clinical Laboratory Standards) broth microdilution MIC (minimal inhibitory concentration) test was performed for the toxigenic isolates to determine the effect of following antibiotics, clindamycin, vancomycin, metronidazole, erythromycin, cefotaxime. For each toxigenic isolates, the broth microdilution MIC test was conducted in duplicate. The Mueller-Hinton broth (BD) was used, and the pH was adjusted between 7.2 and 7.4. Within 15 min of adjusting the inoculum broth to the turbidity of a 0.5 McFarland standard, the inoculum suspension was diluted to a final concentration of 5×10^4 CFU/ 0.1 ml well. Results were recorded after 20-24 h incubation, and NCCLS interpretive criteria was

used to interpret the results: clindamycin, susceptible, ≤2 μg/ml, resistant, ≥ 8 μg/ml;

vancomycin, susceptible, ≤2 μg/ml, resistant, >2 μg/m; metronidazole, susceptible, ≤8

μg/ml, resistant, ≥ 32 μg/ml; erythromycin, susceptible, ≤0.5 μg/ml, resistant, ≥ 8

μg/ml; cefotaxime, susceptible, ≤8 μg/ml, resistant, ≥ 64 μg/ml.

2.3 Results and Discussions

2.3.1 Isolation *C. difficile*

From *Clostridium difficile* Selective Agar (CDSA) test, 52 (52.5%) *C. difficile*

isolates were detected in 99 California lettuce samples; 44 (44.4%) *C. difficile* isolates

were detected 99 Arkansas lettuce samples; 61 (61.6%) *C. difficile* isolates in 99

Louisiana lettuce samples (Table 2). Since the selective plates do not have the ability

to differ the toxigenic isolates from the non-toxigenic ones. The detection of toxin A

and B was followed to identify the toxigenic strains.

Table 2. *Clostridium difficile* isolated from California, Arkansas, and Louisiana
lettuce samples between September 2014 and March 2015

State tested		Sep	Oct	Nov	Dec	Jan	Feb	Mar	Total (%)
California	POS	2	5	7	3	7	11	17	52 (52.5%)
	N	8	8	8	10	15	20	30	99
Arkansas	PO	0	5	7	2	8	6	16	44 (44.4%)

	S								
	N	8	8	8	10	15	20	30	99
Louisiana	PO	0	3	6	3	8	16	25	61 (61.6%)
	S								
	N	8	8	8	10	15	20	30	99

POS, the number of the sample having positive result from CDSA test. N, the total number of the sample tested.

2.3.2 Real-time PCR assays for toxin A and B

Since naturally occurring toxin A positive B negative isolates have not been reported so far (Lyras, O'Connor, et al. 2009), to determine the prevalence of the *C. difficile* isolates found in lettuce samples, toxin B real-time PCR assay was conducted. According to the results from toxin B detection (Table 3), 15 *Clostridium difficile* toxigenic isolates were detected in 99 samples from California; 10 *Clostridium difficile* toxigenic isolates were detected in 99 samples from Arkansas; 16 *Clostridium difficile* toxigenic isolates were detected in 99 samples from Louisiana.

The samples were continuously purchased and tested from September 2014 to March 2015. To determine whether there is a close relationship between the temperatures of the months (Figure 5) and the frequency of the toxigenic isolates presence from California, Arkansas, and Louisiana, a linear regression model was tested. The p values of the results from California, Arkansas, and Louisiana were

0.645, 0.0561, and 0.6659 larger than 0.05. The hypnosis was denied, meaning there

was no direct relationship between these two.

Table 3. The positive result from real-time PCR for toxin B detection of *C. difficile* isolates in California, Arkansas, and Louisiana between September 2014 and March 2015

State tested		Sep	Oct	Nov	Dec	Jan	Feb	Mar	Total (%)
California	POS	2	1	3	1	0	5	3	15 (15.2%)
	N	8	8	8	10	15	20	30	99
Arkansas	POS	0	2	1	1	2	4	0	10 (10.1%)
	N	8	8	8	10	15	20	30	99
Louisiana	POS	0	2	0	1	0	11	2	16 (16.2%)
	N	8	8	8	10	15	20	30	99

POS, the number of the sample having positive result from toxin B real-time PCRassay. N, the total number of the sample tested.
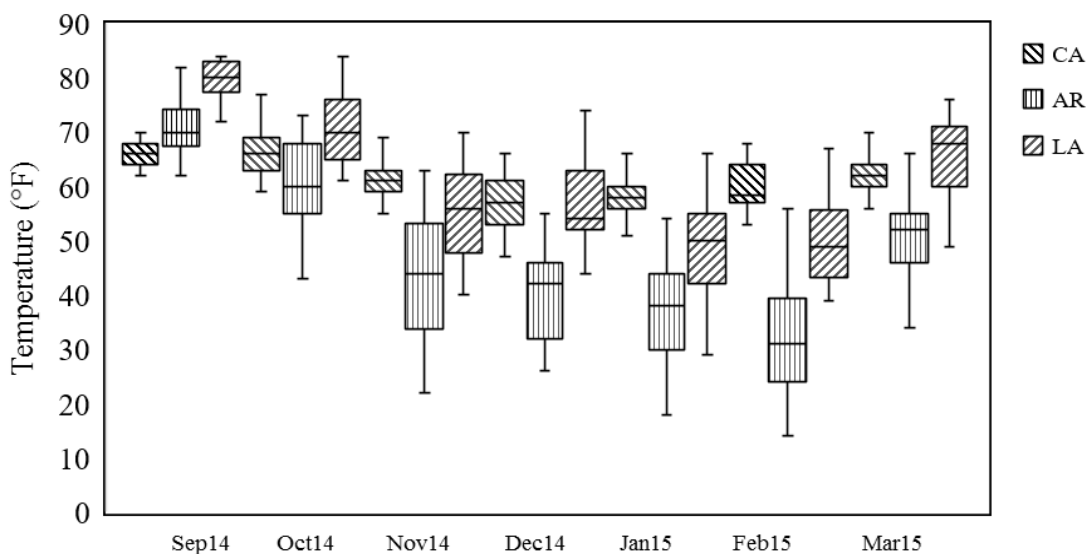
Figure 5. The growth temperature of the sample lettuces harvested from California, Arkansas, and Louisiana between September 2014 and March 2015.

The toxin A real-time PCR results were recorded, combined with the results from toxin B detection assay (Table 4). Overall, 41 toxigenic isolates were detected from 297 samples; and among the 41 toxigenic isolates, there were 7 toxin A negative toxin B positive isolates. The totally percentage of the toxigenic *C. difficle* isolates found in lettuce samples was 13.8%, this was higher than the other reported results (Al Saif and Brazier 1996a, Bakri et al. 2009, Metcalf et al. 2010, Rodriguez-Palacios, Ilic, and LeJeune 2014). Previous scientific studies have concentrated on testing several types of vegetables including lettuce for toxigenic *C. difficle*. A study conduction in 2014, tested 125 different vegetables that included 41 lettuce samples for toxigenic *C. difficle*. The vegetable samples were from several retail stores located in Ohio and had originated from several states in the USA and Mexico. The results of their study found 1 positive toxigenic *C. difficle* isolate in the 41 lettuce samples

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tested. (Rodriguez-Palacios, Ilic, and LeJeune 2014). Another study, reported 7.5% *C. difficile* prevalence in ready-to-eat salad in Scotland, they collected 40 packaged lettuce samples over one month from 7 different supermarkets (Bakri et al. 2009). These previous studies detected lower *C. difficile* prevalence in lettuce than this study possibly due to a small sample size and purchase location. (Bakri et al. 2009, Al Saif and Brazier 1996a, Metcalf et al. 2010, Rodriguez-Palacios, Ilic, and LeJeune 2014).

In addition, contamination of lettuce with *C. difficile* spores would not only be due to attachment on the leaves from contaminated water or soil (Simango 2006), but also would widely exist in the downstream production chain including storage, transportation, and handling environments (Rodriguez et al. 2013). Since the same varieties and brand of lettuces tested for toxigenic *C. difficile* came from the same processor located in California, Arkansas, and Louisiana, if the processing environment was exposed to toxigenic *C. difficile* spores, it would largely increase the presence of the *C. difficile* spores on the lettuce samples and thus increase the prevalence.

Table 4. Real-time PCR profile of *C. difficile* isolates in lettuce samples among the states tested

State tested	Toxin A - Toxin B +	Toxin A + Toxin B+	Total positive samples (%)
Californa, n=99	3	12	15 (15.1%)

Arkansas, n=99	2	8	10 (10.1%)
Louisiana, n=99	2	14	16 (16.2%)
Total	7/297 (2.4%)	34/297 (11.4%)	41/297 (13.8%)

2.3.3 Antibiotic resistance detection

Five antibiotics, metronidazole, vancomycin, clindamycin, erythromycin, and cefotaxime (Table 5), were tested for the toxigenic *C. difficile* isolates. Among the antibiotics we tested, all the 41 isolates were resistant or intermediately resistant to all the 5 antibiotics, while 37 isolates and 26 isolates showed intermediately resistance to clindamycin and cefotaxime. The resistance and intermediately resistance properties of the isolates for clindamycin and cefotaxime were in accord with the findings from other *C. difficile* vegetable isolates (Bakri et al. 2009). However, metronidazole, vancomycin, and erythromycin did not have the susceptive effect to the isolates as being reported (Bakri et al. 2009, Metcalf et al. 2010). Since the antibiotic resistance property of *C. difficile* would vary depending on the location (Metcalf et al. 2010), the different antibiotics effect would be understandable. Since no antibiotic resistance pattern remain consistent enough to be used as a *C. difficile* strain marker (Tenover, Tickler, and Persing 2012), the different antibiotics effect in this study would be

understandable. Besides, two same toxigenic *C. difficile* isolates from the same kind

of vegetable were reported to have different antibiotic susceptibility to a certain

antibiotic (Metcalf et al. 2010).

Table 5. Susceptibility of the *C. difficile isolates* to 5 antibiotics

Agent	MIC (µg/ml)			No. (%) of isolates		
	Range	50%	90%	Susceptible	Intermediate	Resistant
Metronidazole	0.125-80	>80	>80	0 (0)	0 (0)	41 (100)
Vancomycin	0.25–4	4	>4	0 (0)	0 (0)	41 (100)
Clindamycin	1-16	4	8	0 (0)	37 (90.2)	4 (9.8)
Erythromycin	1-16	16	>16	0 (0)	0 (0)	41 (100)
Cefotaxime	6-64	12	24	0 (0)	26 (63.4)	15 (36.6)

50%, the antibiotic concentration when 50% growth of the tested *C. difficle* isolate
inhibited. 90%, the antibiotic concentration when 90% growth of the tested *C. difficle*
isolate inhibited.

2.4 Conclusions

The *C. difficile* isolated from the retail lettuce has a high possibility to be

toxigenic. Although the public health relevance is still unclear, consumption retail

vegetables as salad vegetable or without high-temperature processed might be a

source of *C. difficile* infection. Treated with five different antibiotics, the *C. difficile*

sample isolates expressed strong resistance to metronidazole, vancomycin, and

erythromycin. This present research contributes in revealing a possible source of

community-associated *C. difficile* infection.

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