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Detection of *Clostridium difficile* in Louisiana oysters, harvesting water and sewage

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DETECTION OF CLOSTRIDIUM DIFFICILE IN LOUISIANA OYSTERS,
HARVESTING WATER AND SEWAGE

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

School of Nutrition and Food Sciences

by

Da Liu

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ABSTRACT

Clostridium difficile infection (CDI) was generally considered as a hospital-associated disease; however, recent studies indicated that foods might also play a role in its epidemical chain. From August-2013 to February-2014, oyster samples and harvesting water samples were collected monthly from 6 commercial oyster harvesting areas along Louisiana Gulf Coast and analyzed for total aerobic bacteria, fecal coliforms, coliphage and pathogenic *C. difficile*. In this study, *C. difficile* were isolated from 9 (47.37%) of 19 oyster samples and 3 (37.5%) of 8 harvesting water samples and all isolations were carrying the toxin B-encoding gene (tcdB). Toxin B positive *C. difficile* was detected in all influent and effluent samples collected from a waste water treatment plant (WWTP) located in New Orleans, LA. However, the PCR-ribotyping showed that different strains of *C. difficile* were found in sewage effluent and oysters/harvesting water, suggesting the discharge of treated sewage might not contribute to the transmission of *C. difficile* into the harvesting areas. No statistical significance was found between the density of fecal coliform/*E. coli* in oysters and the occurrence of *C. difficile*, according to a binary logistic regression model (*odds ratio* = 1.025 and 0.997). The concentration of Male-specific (F+) Coliphage and Somatic Coliphage in oysters was also found not to be directly related to the occurrence of *C. difficile* in oysters due to the low detection rate.

CHAPTER 1 INTRODUCTION

Clostridium difficile was originally regarded as part of the natural micro-flora of the intestinal tract until research on germ free rats showed decisive evidence that the microorganism was pathogenic to mammals in the absence of competing micro biota(Lessa and others 2012). *C. difficile* was later demonstrated to cause diarrhea, colitis, and occasionally could cause deaths, among human (Barbut and Petit 2001). The demonstration that *C. difficile* is pathogenic catalyzed a series of studies in the 1970s and several significant characteristics of *C. difficile* were revealed. First Green (1974) found a *C. difficile*-associated cytotoxin in the stools of guinea pigs that developed gastroenteritis after receiving ampicillin (Green 1974). In the same year, Tedesco and others. found that the use of clindamycin was also associated with the development of pseudomembranous colitis (PMC) caused by *C. difficile* (Tedesco and others 1982). Later Barlett and others (1977) isolated *C. difficile* species from hamsters with clindamycin-induced colitis and finally confirmed the role of *C. difficile* in antibiotic associated gut diseases.

Epidemiology studies soon followed and showed astounding facts on the prevalence of this pathogen. Based on hospital patients, 10~25% of all cases of antibiotic-associated diarrhea in industrialized countries are caused by *C. difficile*, a microorganism once regarded as a common resident in the human intestinal tract (Burkhardt and Calci 2000). In healthy infants, the carriage rate of *C. difficile* ranged from 2~60% according to several reports and up to 17.5% of healthy adults were reported as asymptomatic carriers of *C. difficile*. Additionally, *C. difficile* was proved to be highly contagious between carrier and non-carrier patients in hospitals and health-care facilities. According to Riggs and others., the transmission rate

could reach up to 21.4% (Riggs and others 2007). The data from European studies reported a mean incidence of healthcare-associated *C. difficile* infection (CDI) of 4.1 per 10,000 hospital patients. However, the numbers vary greatly in response to the hygiene and instruments in surveyed hospitals and those located in less-developed areas may have an infection rate as high as 36.8 per 100,00 patients. CDI used to be a healthcare-acquired infection while in advanced areas, such as in Europe and North America, CDI associated with hospitals and nursing facilities has dropped to 25% of the total CDI cases due to proper surveillance.

Community-acquired *C. difficile* infection (CA-CDI) has been becoming a more common CDI entity than previously perceived. In the USA, incidence rates of CA-CDI ranged from 6.9 to 46 cases per 100,000 person per year. Worldwide, approximately 11–28% of patients with CDI acquire the infection in the community, which does not vary much between countries and areas (Barbut and Petit 2001). Besides a wider spread region, *C. difficile* also gained higher virulence and has been causing more severe diseases. The increasing severity in the disease was partly related to the emergence of hypervirulent strains, for instance, NAP1/BI/027, which stands for North American pulse-typed type 1 by PFGE, restriction endonuclease analysis group BI and PCR-ribotype 027. Research in recent decades has proven a significant correlation between NAP1 and increased morbidity and mortality of CDI, especially in the case of neopathies, such as toxic megacolon, septic shock, perforation, a higher fatality rate, and a poor clinical response to antibiotics such as metronidazole. The infection of NAP1 was also demonstrated to cause a much higher relapse rate (69%) than other *C. difficile* strains (15%~35%) (Marsh and others 2012). The increased virulence of this strain results from different mechanisms that may include the increased sporulation rate, the

production of a new binary toxins and the increased production of toxins resulting from impaired toxin-producing regulator gene clusters. NAP1 has different distributions among countries and was especially active in North America. In the most recent outbreak in May, 2011 in Ontario, Canada, 26 health care facilities were involved and the report afterwards pointed out that it was closely related to NAP1 *C. difficile*.

Other emerging hypervirulent genotypes, such as ribotype 078, were also isolated in several *C. difficile* outbreaks. Now *C. difficile* has overcome norovirus to become the most significant cause of deaths associating with gastroenteritis. In 2011, there were 17,414 reported *C. difficile* related deaths in the UK, accounting for 1.1% of all deaths in England and Wales between 2006 and 2010. In the United States, *C. difficile* causes 14,000 deaths each year and have caused heavy financial burdens to the government. Generally, each case of *C. difficile* infection will result in a health-care cost of \$3,600 and these costs have been exceeding more than 3.2 billion annually from the year of 2002 through 2010 (Warny, Pepin and others 2005; Dubberke, Haslam and others 2011). The occurrence of new hypervirulent strains, such as ribotype 027 and 078, not only has brought about more CDI cases each year, but added extra debt to the government due to the high relapse rate.

Many diagnosis methods had been developed since *C. difficile* became a growing threat for public health. Generally these methods can be divided into two types in terms of detection target: toxin or gene. The most representative toxin-detecting method is the cytotoxin assay (CYT), which was developed by Chang and others (2000). This method specifically detects *C. difficile* toxins with antitoxin-protected and nonprotected cell monolayers. CYT has been regarded as the golden standard method for comparison in toxin kit evaluations and giving

highly sensitive and specific detection of *C. difficile*. Other toxin-targeting methods may include cytotoxigenic culture (CYTGC), which is a similar method to CYT with a modified sample preparation procedure. Enzyme immunoassays (EIAs) and Lateral-flow assays use different enzymes to rapidly detect *C. difficile*-specific glutamate dehydrogenase (GDH) antigen and toxin A/B. The DNA-targeting methods are mainly based on the polymerase chain reaction (PCR). Using specific primers, the gene segments in pathogenicity locus, a 19.6kb chromosomal segment containing *tcdA-E* gene, can be amplified and further specified by size through electrophoresis. The most widely used target gene is *tcdB* gene that encodes *C. difficile* toxin B, a highly prevalent toxin produced by virulent *C. difficile* strains. In comparison to traditional toxin typing methods, gene-targeting based methods showed higher specificity and sensitivity. An evaluation of the PCR assay detecting *tcdC* gene of *C. difficile* reported a sensitivity and specificity values of 86% and 97%, respectively (Eastwood and others 2009).

The isolation of high virulence, epidemic ribotype 078 *C. difficile* from calves and pigs indicated the possibility of interspecies transmission of *C. difficile* from animals to humans (Jhung and others 2008). In 2007, a study conducted in the USA found forty-two percent of retail meat products had toxigenic *C. difficile* strains that were ribotype 078/toxinotype V (73%) and 027 (27%). There were also surveys of *C. difficile* in waters and shellfish products from Europe claiming a higher prevalence of virulent *C. difficile* than formerly perceived (Pasquale and others 2012; Yam and others 2000). Therefore, foods could be a source of *C. difficile* and may have been involved in the increased relapse rate of CDI. N.AL Saif and J.S. Brazier (1996) reported the prevalence of *C. difficile* in water in South Wales and their

observation was confirmed by other researchers in different locations (Zidaric, Beigot and others 2010; Pasquale, Romano and others 2012). *C. difficile* spores were also isolated from soil, unwashed raw vegetables, shellfish, animals and livestock (Metcalf, Avery and others 2011; Pasquale, Romano and others 2012).

The occurrence of *C. difficile* in foods and water might have contributed to the increased numbers of carriers who possess a higher chance of getting CDAD when treated with antibiotics. On the other hand, although *C. difficile* is not likely to induce infections among healthy people, elder people and those who newly received antimicrobial treatment are still at high risk of receiving CDAD or relapse when exposed to contaminated foods and water.

Oysters, belonging to the bivalve molluscs family, are one of the most important seafood products in the United States. Louisiana produces around 520 million oysters each year, which accounts for around 60% of oysters in the Gulf Coast region and about one-quarter of the nation's supply. Therefore, the safety of these oysters is crucial to both public health and the Louisiana oyster harvesting industry. As filter feeders, oysters are able to concentrate micro particles to folds higher than their exposure in the surrounding waters thus are ideal reservoir of many bacterial and viral pathogens.

C. difficile can spread through the fecal-oral route and its spores are highly resistant to normal chlorination-based or UV treatment. In Louisiana, most sewage will be treated first in the wastewater treatment plant (WWTP) and then discharged to wetlands for further purification. The Thibodaux site, one of several wetlands located in the Mississippi river delta, has been receiving secondarily treated municipal wastewater at the average rate of 15,140 m³ per day since 1992. Therefore, with such a large amount of sewage eluted each day, the

spread of *C. difficile* may become a health threaten since the spores can survive and accumulate in environment for a long time period (Barbut and Petit 2001). Romano et.al has isolated 13 profiled and 8 unknown strains of *C. difficile* from both influent and effluent of nine waste water treatment plants (WTTP) in Switzerland, indicating that treated human sewage as another source of environmental *C. difficile* contamination (Vincenza Romano 2012). In addition, there are studies showing that the efficacy of removing microbes in WTTP drops drastically during storm and rainy weather, as the water drainage becomes too much to be treated properly.

For the entire United States, excluding Hawaii and Alaska, the average amount of moisture falling as rain and snow is 30.21 inches (80cm). In terms of Louisiana, the annual rainfall can reach 64.2 inches (163 cm) and stormy weather can be seen occasionally in the summer. Therefore, there can be a higher chance for *C. difficile* to overpass WTTP and enter the outside water body. On the other hand, Louisiana annually sells more than 330,000 hunting licenses and 900,000 fishing licenses that allows people to hunt, fish, boat, swim, camp, hike and paint in wetlands area. Considering the high carriage rate of *C. difficile* among human, the disposal of waste and sewage by the tourists may contribute to the spread of *C. difficile* in the water system. In order to properly control the hazard, monitoring of the *C. difficile* level in the environment, particularly in food-producing areas, is needed. This study will focus on detecting *C. difficile* in the oysters, oyster-bed water and sewage water in and near the Louisiana oyster harvest areas.

The oysters and harvesting water were periodically collected and processed according to the standard method described by APHA's *Recommended Procedures for the Examination of*

Sea Water and Shellfish, 4th ed., 1970. A two-step bacteria culture method was used to propagate and preliminarily detect *C. difficile* in collected samples. Further confirmation and virulence determination was realized by a Real-time PCR method described by van den Berg and coworkers, which aims to amplify *C. difficile* toxin B encoding gene, tcdB (van den Berg and others 2005). Total aerobic bacteria was enumerated as a background and used to determine the abundance of microorganisms in samples upon each sampling activity. Traditional fecal indicators: coliforms and *E. coli*, which are standard markers of fecal contamination, was partly evaluated to investigate their potential as an indicator of *C. difficile* contamination. The count of indicator bacteria will be performed with a five-tube serial dilution most probable number (MPN) method, according to the *Bacteriological Analytical Manual*, FDA.

Two novel fecal indicators, Male-specific (F+) RNA coliphage and somatic coliphage, which have been suggested as an advanced alternative of fecal coliform for the evaluation of fecal contamination, was evaluated. These two types of coliphage have been successfully used in the indication of viral contamination since they are able to better simulate reaction of viruses to waste water treatment and harsh environmental conditions. *C. difficile* will be largely eliminated in WWTP as other bacterial pathogens, but the spores may persist as viruses do: although chlorination and UV could eliminate most vegetative cells, they are less effective against spores and viruses. Therefore, the coliphages, which are highly resistant to waste water treatment and can live longer in environment conditions, might be a more suitable indicators of *C. difficile*, in comparison to fecal coliforms. The count of fecal indicators was performed with a double-layer plating method. To determine if the discharge

of treated sewage is associated with the presence of *C. difficile* in the harvesting area, the harvested *C. difficile* both from sewage treatment plant and oysters and harvesting water was tested with a modified PCR-ribotyping method. The aim of this PCR-ribotyping is to discriminate if the same strains of *C. difficile* appear in the sewage and harvesting area and give a preliminary conclusion about the effect of sewage discharge on *C. difficile* spread in oyster harvesting areas. Also by comparing the *C. difficile* type between oysters and seawater, a clue might be obtained if the oyster bio-accumulate *C. difficile* from water body.

The sampling sites of oyster and water were six neighboring areas selected from 28 commercial harvesting areas defined by Department of Health and Hospitals, Louisiana. These areas are located near the exit of Mississippi River to the sea thus are good references for examining possible sewage contamination from upstream discharges. A representative municipal WWTP in New Orleans was selected for this study. This facility is permitted to treat 122 million gallons per day (MGD) as an Annual Average Daily Flow and saw an average flow of 98 MGD for 2013. Both influent and effluent sewage was sampled and tested for *C. difficile* to determine the efficacy of *C. difficile* removal from waste water treatment.

Briefly, the objective of this study was to determine the prevalence of *C. difficile* in Louisiana oysters, harvesting water and sewage. To the best of our knowledge, this is the first survey about *C. difficile* in Louisiana oysters and waters and it may give usable data for further work to control this pathogen.

CHAPTER 2 LITERATURE REVIEW

2.1 *Clostridium difficile*

Clostridium difficile is a gram-positive, strictly anaerobic and spore-forming bacillus. It was first discovered in the intestinal flora of a fetus in the 1930s and was designated as a major cause of antibiotic-related diarrhea in the 1970s (Dubberke and others 2010). Toxins are the main virulence factors of *C. difficile*: mainly toxin A and B from the large clostridial toxin family and sometimes an additional binary toxin. In the form of spores, *C. difficile* manages to survive high dose of antimicrobial drugs (which are usually employed to cure other infections), and out compete regular bowel flora and cause *C. difficile* infections (CDI). There are typically three manifestations of CDI development: asymptomatic carrier state, colitis with or without pseudomembranes and fulminant colitis. Asymptomatic state is quite prevalent among the population while no clinical treatment by far has been proved to be effective in elimination of *C. difficile* spores carried by healthy people; pseudomembranes colitis occurs in more than 50% of *C. difficile* infection cases with symptoms of fever, chills, and tenesmus in addition to common CDI symptoms like pain (usually crampy), malaise, nausea and vomiting; Although only representing less than 10% of symptomatically infections, fulminant colitis is the most dangerous state of CDI that may require the infected patients to take colectomy (Dubberke, Haslam and others 2011).

Vegetative cells of *C. difficile* are extremely sensitive to oxygen thus the generally accepted pathogenic form of *C. difficile* are spores. Upon releasing, the spores remains dormant and have a strong resistance to undesirable environment conditions with a prolonged

survival period. Once an ideal growth environment is available, for instance, when they are ingested by humans or animals, the spores germinate, become vegetative cells and start producing toxins. *C. difficile* spores can survive routine environmental cleaning with detergents (Gerding, Muto and others 2008) and are capable of persisting on hard surfaces for as long as 5 months. Since *C. difficile* spores are shed from feces, any device that becomes contaminated with feces can be a reservoir.

The invasion of *C. difficile* can be briefly described by an established model. When a healthy adult intakes *C. difficile* spores, the spores will survive its path through stomach into the duodenum and jejunum. The high concentration of bile salts and nutrients in the jejunum will aid in the germination of spores. The germinated spores will further travel to cecum, which is a strict anaerobic environment, through the ileum. Here most normal gut flora metabolize cholate derivatives, which is a promoter of *C. difficile* growth, and produce deoxycholate. Deoxycholate inhibits the growth of vegetative *C. difficile* cells thus no colonies of *C. difficile* can be formed. The spores may still germinate but without colony forming no toxin can be produced and released.

However, once the normal flora are killed with antibiotics, the reduction of species that are able to dehydroxylate cholate will lead to the overgrowth of *C. difficile*. Deoxycholation of primary bile salts is the most important and effective defensive mechanism against *C. difficile* and even after standard therapy, such as vancomycin or metronidazole, the patients and practitioners still need to be very careful about relapse before normal gut flora is fully recovered (Sorg and Sonenshein 2008).

The strains of concern recently are ribotype 027/NAP1/toxinotype III and ribotype 078/toxinotype V due to their prevalence in multiple outbreaks. Ribotype 027 is characterized with increased virulence and relapse rate, which is thought to be associated with a one base pair deletion at position 117 of the *tcdC* gene, which impaired the negative regulation mechanism of toxin production. The malfunction of negative regulator further leads to an increased or prolonged production of toxins A and B. Production of an extra binary toxin was also determined in this ribotype. Ribotype 078, on the other hand, present a closer relation to our daily foods and multiple studies have pointed out the prevalence of *C. difficile* ribotype 078 in livestock and retail meat products (Jhung and others 2008; Lessa and others 2012).

C. difficile nowadays is still a big threat to public health and has imposed financial pressure on government. The presence of new, hypervirulent epidemic strains has been making prevention, diagnosis and therapy more difficult and more costly. The evidence that *C. difficile* can transmit through foods and water are warning us of its potential hazard if no action is taken to monitor and control its spread.

2.2 Toxins

There are two major toxins produced by *C. difficile*, enterotoxin A consists of 2710 residues (308.0kDa) and cytotoxin B consists of 2366 residues (269.6kDa) (Sullivan, Pellett and others 1982; Reinert, Jank and others 2005; Jank, Gieseemann and others 2007). The structures of the two toxins are showed in Figure 2. Both toxins are glycosyltransferases that could modify small GTPase in host cells (Geric, Carman and others 2006). Toxin A had been proven to cause severe epithelial damage with hemorrhage and diarrhea in rabbit ileal loops

tests (Lyerly, Lockwood and others 1982; Mitchell, Ketley and others 1986). Hecht and coworkers further concluded that toxin A can directly diminish the barrier effect of human intestinal epithelial cells by exerting an alteration to the permeability of tight junction and affecting the function of F actin (Hecht, Pothoulakis and others 1988). Their conclusions were confirmed later in investigations concerning the interferences toxin A and B exert on Rho proteins, a family of proteins closely associated with actin cytoskeleton regulation, epithelial barrier function and immune cell migration of human intestinal epithelial cells (Just, Fritz and others 1994; Jank, Gieseemann and others 2007).

Toxin B, on the other hand, does not cause mucosal damage in in-vivo animal models though it does elicit alterations in the cytoskeleton of cultured mammalian cells and cause inflammation (Riegler, Sedivy and others 1995). Lacking of direct enterotoxic effects has led to an underestimation of toxin B until investigators recently found its significant role in the virulence of *C. difficile* (Riegler, Sedivy and others 1995; Lyras, O'Connor and others 2009). The discovery of several strains of toxin A negative but toxin B positive strains of *C. difficile* also supports the theory that toxin B, instead of toxin A, is the key virulence factor (Depitre, Delmee and others 1993; Alfa, Kabani and others 2000).

In addition to large clostridial toxin A and B, a novel binary toxin consists of two unlinked molecules: CDTa (enzymatic component) and CTDb (receptor-binding component) were isolated from new strains of *C. difficile* (Warny, Pepin and others 2005; Geric, Carman and others 2006). Binary toxin is a type of ribosyltransferase that disrupts actin cytoskeleton of host cells and the presence of which is highly suspected relating to the increasing severity of CDI symptoms (Barbut, Decré and others 2005; Geric, Carman and others 2006). Binary

toxin CDT genes are found in less than 10% of clinical *C. difficile* isolates and its role in the development of disease was hard to determine since most CDT gene carrying *C. difficile* strains also carry the clostridial toxin encoding genes (i.e. tcdA and tcdB). In a recent study, Geric and coworkers reported that the CDT gene plays an important role in the accelerated development of CDI and can cause gastroenteritis independently (Geric and others 2006). Therefore, the emergence of CDT gene carrying *C. difficile* strains has raised a new task for the practitioners.

All three toxins invade cells through endocytosis and the procedure can be described by a model. First the toxins bind to the receptors on the surface of a target cell and get endocytosed; then the low pH of endosome acidifies the toxins and induces a conformational change – the hydrophobic region of the toxins is exposed and the whole toxin-containing endosome is inserted through the membrane; Finally the toxins form pores on the endosome and are released to cytosol (Thelestam, Florin and others 1997; Jank, Gieseemann and others 2007). The structure of toxin A and B are showed in Figure 2.1.

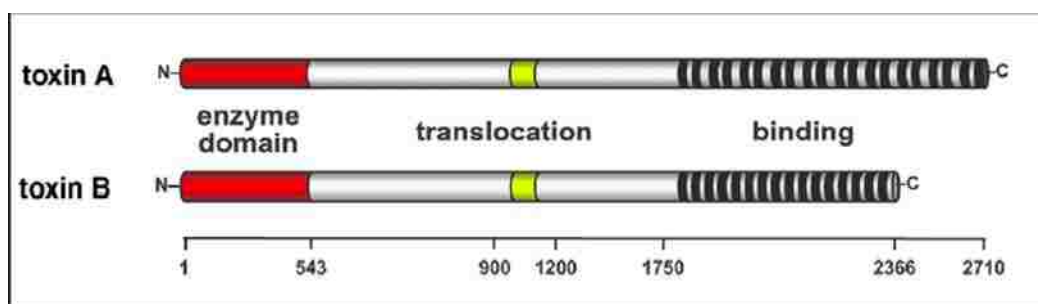


Figure 2.1. Structure of toxin A and toxin B (Jank, Gieseemann and others 2007)

The relapse of *C. difficile* partly attributes to the promoting effect of the toxins in *C. difficile* colonization and neutrophil chemotaxis activation. The use of wide spectrum

antibiotics as therapy such as vancomycin and metronidazole will also allow the invasion of other intestinal pathogens, for instance, *Staphylococcus aureus* and *Enterococcus spp.* to colonization in digestive tracts. Therefore, antitoxin might be a good aid through the therapy of *C. difficile*. Kink et al. have reported their success in the development of avian antibodies that neutralize both toxin A and B. They found that antibodies held the same ability in inhibiting *C. difficile* and, by neutralization of these toxins with antibodies, the pathogenic mechanism of the organism is blocked, its ability to thrive in the gut can be diminished. However, the cost of antibodies is still a block of wide application of antibiotics worldwide to deal with CDI.

The production of multiple toxins by *C. difficile* can happen rapidly in 24 hours following infection. The speed of toxin accumulation varies from different *C. difficile* strains, with a generally higher toxin-producing activity observed in NAP1/BI/027 than other strains. According to Warny et al., under ideal conditions, the maximum toxin production speed was reached at 24h following incubation, when the cell density achieved maximum and the growth entered stationary phase. With little difference in the maximum cell concentration (17%, showed in Figure 2.2), the peak median concentration of toxin A was 16 times higher in hypervirulent strain NAP1/BI/027 (toxintype III) than in toxintype 0; toxin B concentrations were 23 times higher in the toxintype 0, as is showed in Figure 2.3 (Warny and others 2005).

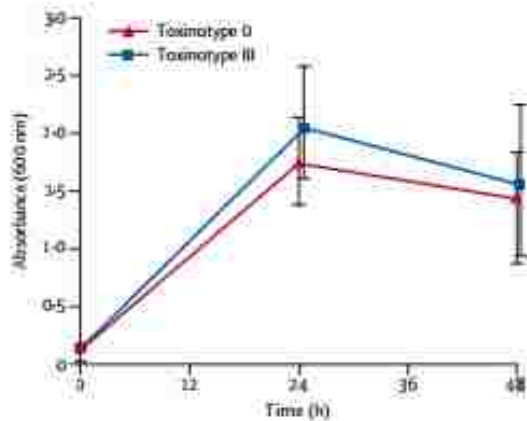


Figure 2.2 Growth curves of toxinotype 0 and toxinotype III (Warny and others 2005).

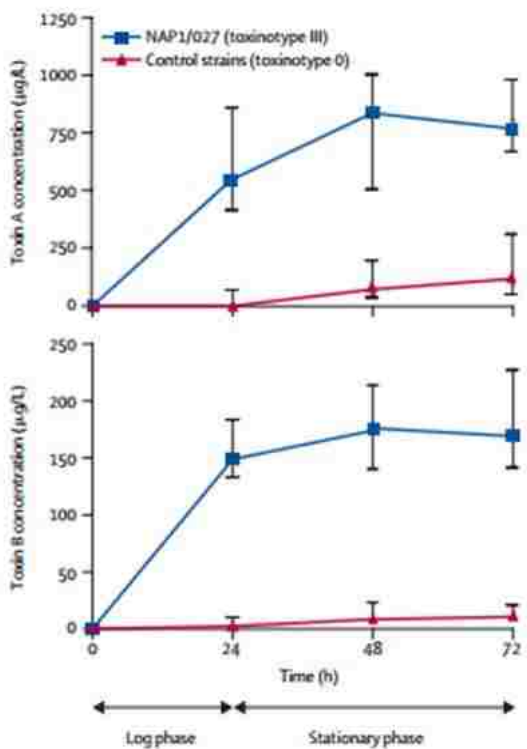


Figure 2.3: In-vitro production of toxins A and B by *C. difficile* isolates (Warny and others 2005).

2.3 Epidemiology

C. difficile is currently the most impending nosocomial diarrhea causer that has led to many large outbreaks in health-care facilities. In more than 95% PMC cases and 15-25% antibiotic-associated diarrhea (AAD) cases, *C. difficile* toxin B was isolated from the stool of

patients (Barbut and Petit 2001). More than 90% of *C. difficile* infections happen during or after antibiotic treatment when the colonic flora of patients is disrupted, allowing *C. difficile* to colonize and release toxins. In a 10-year study performed by Olson et al., 93% of a total of 908 cases were associated with health-care facilities, where antibiotic treatments are implicated (Olson, Shanholtzer and others 1994). Epidemiology studies also suggested a high carriage rate (5.9%-11%) of people admitted to hospital and a high acquisition rate (4%-32%, basically through the oral-fecal pathway) in hospitals, indicating hospitals as an important source of *C. difficile* contagion (Barbut and Petit 2001). Besides hospital settings, *C. difficile* also causes diarrhea in the community and an increase in the community-associated CDI was seen. According to a recent study conducted by Beaujerie et al., *C. difficile* infections are responsible for approximately 1.5% of post- antibiotic treatment diarrhea in the community (Beaujerie, Flahaut and others 2001). Almost all antibiotics could contribute to the development of *C. difficile*-associated diseases and orally-taken broad spectrum drugs (such as penicillin and clindamycin) are usually the direct causes of infections. The overall acquisition rate of CDAD by people older than age 65 was several fold higher in people age 45-65 thus CDAD is particularly dangerous to people older than 65 years or with other potential illness due to the enfeebled immune system brought by diarrhea and tissue inflammation (McDonald and others 2006; Barbut and Petit 2001)

The attributable mortality rate of CDI varies from the infected population and epidemic *C. difficile* strains. (Warny, Pepin and others 2005). A 6-month study among 1430 Canadian patients in the year of 2009 gave a high death rate of 5.7%, which was almost 4-fold higher than the death rate given by a similar surveillance performed in 1997 (Gravel, Miller and

others 2009). Specifically, severely infected people acquiring colectomy will face a much higher mortality of 35-50% (Morris, Zollinger and others 1990). The epidemiological studies of *C. difficile* over the past decade were not showing promising results as was previously expected. The worldwide spread of novel, hypervirulent *C. difficile* strains like ribotype 027 and 078 were largely responsible for the unsatisfying results. These new strains have been proven to induce more massive, hospital-associated, severer and poorer treatment-response infections. Community-associated incidences also increased with more young individuals and other formerly-considered low CDI-risk population involved (Weese 2010). The detection of *C. difficile*, especially ribotype 078, in foods, the environment and recreational waters, also suggested that *C. difficile* might have found a new way, without our perception, to invade and cause diseases.

Another challenge to clinicians and epidemiologists is the high reoccurrence rate of CDI. Approximately 15 to 35% of patients with a first episode of CDAD relapse within 2 months. The mechanism of the re-infection remains unclear but some prospective studies pointed out that almost half of the reoccurrences are re-infections of a different strain of *C. difficile* (Johnson, Adelman and others 1989; Barbut and Petit 2001). Therefore, proper prevention could be the most effective way of reducing *C. difficile* infections and re-infections (Barbut and Petit 2001).

The main risk factors of *C. difficile* include increasing age (excluding infancy, when *C. difficile* presents a high carriage rate but does not cause disease), severity of underlying diseases, non-surgical gastrointestinal procedures, anti-ulcer medications, duration of hospital stay, duration of antibiotic course and administration of multiple antibiotics (Bignardi 1998).

In a survey between the years of 2000 to 2009, the CDI case per 1000 discharges from the hospital of patients older than 65 years old is significantly higher than that of patients less than 44 years old (Lessa and others 2012). Figure 2.4 shows their investigation results among patients differing by age, ranging from 18 to 85+ years old.

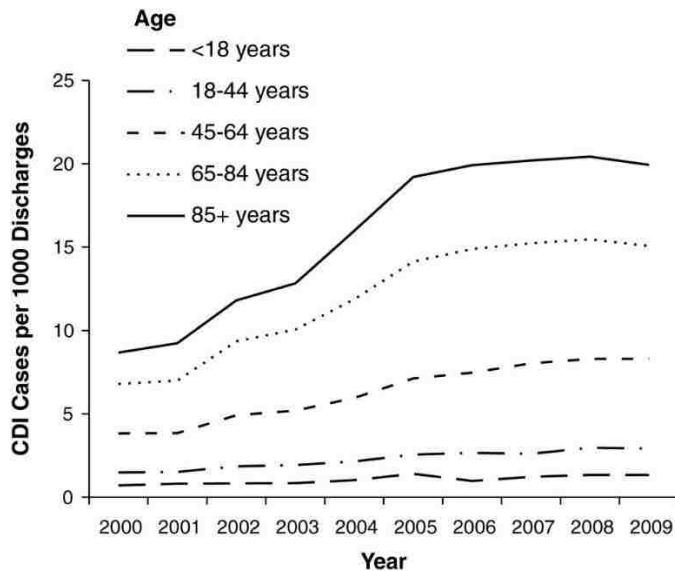


Figure 2.4 Discharge rate for *Clostridium difficile* infection from US short-stay hospitals by age (Lessa and others 2012)

Other information received from the survey is that there was a marked increase in CDI incidence and mortality across the United States in the last decade. In comparison to the year of 2000, there was nearly a 20% increase in CDI discharge for people older than 85 and *C. difficile* has become the 18th leading cause of death among people older than 65 years old (Lessa and others 2012). The emergence of a new strain of *C. difficile*, classified as restriction endonuclease analysis type BI, North American pulsed-field gel electrophoresis type 1 (NAP1), PCR ribotype 027 (BI/NAP1/07) contributed largely to the current epidemiological status of *C. difficile*. Ribotyp027 possesses polymorphisms in an important toxin production down regulatory gene, *tcdC* that increases toxin production. Polymorphisms in *tcdB* that could result in improved toxin binding were also reported (Lessa and others 2012). A new

toxin, binary toxin was detected with Ribotype 027 and *cdtA/cdtB* gene located outside the traditional pathogenicity locus was determined as the encoding gene. Moreover, recent whole-genome comparative analyses have demonstrated that a number of genetic rearrangements have happened to epidemic ribotype027. Stabler and coworkers reported that the genome of the epidemic ribotype 027 added 5 more genetic regions compared with its historic counterpart (Stabler, He and others 2009). These additional genes may have contributed to the phenotypic differences between these strains relating to motility, survival, antimicrobial resistance, and toxicity.

2.4 Treatment

Successful treatment of *C. difficile* infections requires a series of optimal management methods towards the case: prompt recognition, timely therapeutic actions, keeping up with observations and implementation of infection control measures (Pépin, Valiquette and others 2005; Gerding, Muto and others 2008). Occurrence of more virulent strains of *C. difficile* has shortened the ideal time interval for most effective treatments to be applied thus the significance of rapid recognition is greatly emphasized. All clinicians, particularly nursing staff who can usually realize CDI signs 1-2 days ahead of physicians, should be fully alerted to any possible development of CDIs. Timely recognition could be as crucial as to prevent the development of deadly fulminant colitis and fast clinical deterioration of patients (Gerding, Muto and others 2008).

Once CDI is diagnosed, the choice of initial therapy will depend on the severity of the infection: metronidazole (Mtz) is optimal for most cases while vancomycin is recommended

for severely ill patients and those with complicated or fulminant infections or multiple recurrences of CDI (Olson, Shanholtzer and others 1994). Monitoring of therapeutical responses (WBC count, temperature, abdominal examination, number of bowel movements, etc.) will be done during the treatment and usually patients will show some improvements within 1-2 days after the initiation of therapy. If the patient does not improve or even gets worse, the therapy should be immediately shifted to orally giving vancomycin with further examination performed. Any signs of ileus, obstruction, perforation, toxic mega-colon, colonic-wall thickening, or ascites should be seriously evaluated. If the situation is impending, a consultation of colectomy might be made as the only life-saving option at this phase of infection (Pasic, Jost and others 1993).

Re-infection or relapse of *C. difficile* may happen 7-14 days after therapy and in some patients they may occur continuously year by year. An explanation of the frequently reoccurrence of CDI in the same patients is the weakened immune response to toxin A and the disruption of colonization protection of the normal colonic flora (Aas, Gessert and othres 2003). Besides vancomycin and metronidazole, there are several other options for CDI treatment: probiotics and anion-exchange resins, intravenous immunoglobulin, nitazoxanide and rifximin.

However, the application of robotic, anion-exchange resins and intravenous immunoglobulin are trapped by their high costs and limited efficacy; Nitazoxanide and rifximin, which have been successfully used in CDI treatment, lack the approval of FDA (Gerding, Muto and others 2008). Although many guidelines of CDI treatment are available currently, the prevention of multiple recurrences of CDI is still challenging. More tests and

data are needed to verify these novel therapies and the costs of the drugs need to be further lowered for a wider application against the rising complexity of the disease.

2.5 Detection of *C. difficile*

The diagnosis of *C. difficile* generally relies on detecting the organism, its toxins, other cellular antigens, and toxin-specific genes. The cytotoxicity assay (tissue culture assay) for toxin B has been considered the "gold standard," for its high specificity and sensitivity. Generally it gives a 100~1000 fold higher sensitivity than the rapid enzyme linked immunosorbent assay (ELISA) methods and a 99%~100% specificity. This method is based on a characteristic cytopathic effect that can be observed and could be neutralized with anti-*Clostridium sordellii* antiserum. However, in exchange to the high performance, the tissue culture takes at least 48 h to produce the result and requires laborious work.

The stool culture is considered to be the most sensitive detection method for *C. difficile* while it requires longer incubation time (72~96h) to yield reliable results and the lab work is more intense. An advantage of this method is that it provides a chance for further strain typing but non-virulent *C. difficile* may in turn interfere with the results. The most widely used method, both in laboratory and healthcare facilities, for diagnosis of *C. difficile* is ELISA. This group of methods are based on the immunoassays for detecting toxins and antigens of *C. difficile*. ELISA has proven its usefulness in discriminating toxin A-negative, toxin B-positive (A-/B+) *C. difficile* variants and the ability to yield results in a few hours. This is very meaningful in the practical use in clinical diagnosis and many commercial available ELISA-based *C. difficile* detection kits were developed. However, the sensitivity

and specificity of these kits was not satisfying in comparison to traditional laboratory methods. According to several studies, the sensitivity of the rapid immunoassays to detect *C. difficile* fall below 60% in comparison to PCR-based detection of toxins A and B genes (van den Berg and others 2005).

PCR-based detection of *C. difficile*, on the other hand, gives better performance than ELISA and requires a reduced amount of time and work when compared to traditional culture assays. Moreover, PCR methods can be applied to the typing of different *C. difficile* strains and thus are able to satisfy multiple experimental intends simultaneously. There are by far more than 150 identified *C. difficile* PCR ribotypes that are marked with 3-digital numbers (003, 005, 027, 078 etc.) and the establishment of online *C. difficile* ribotype library has provided great convenience for the researchers to study the epidemiological properties of the microorganism.

The toxicity of a *C. difficile* is basically decided by the structure of its pathogenicity locus, a 19.6kb chromosomal segment consists of tcdA-E genes. These genes encode toxin A (tcdA), toxin B (tcdB), a negative regulator (tcdC), a positive regulator (tcdD) and a pore-forming protein (tcdE). During the logarithmic phase, strong expression of tcdC gene but restrained transcription of others could be observed; inversed trend is seen in the stationary phase which confirms the tcdC as the negative regulator in production of *C. difficile* toxins. The 18-bp deletion in tcdC gene characterizes the new deadly strain of *C. difficile* NAP1/027, which has been causing severe outbreaks across Europe and North America.

Besides the malfunction of tcdC gene, a ctd gene encoding binary toxin is also designated on the chromosome of NAP1/027. Cdt genes, including cdtA and cdtB, are located outside

the pathogenicity locus and have been found in several *C. difficile* strains. The presence of *cdt* gene and binary toxin is also speculated to relate severe CDIs. The variances in the structure of pathogenicity locus provide a base for *C. difficile* genotyping. Two most variable regions, A3 region in *tcdA* and B1 region in *tcdB*, have been used to detect variants or/and discriminate different *C. difficile* strains. Sole amplification of the A3 region possesses the ability to recognize 13 of 15 known toxin types and a full screen of both regions will further allow an allocation of tested strains into specific toxin types. However, although it is rarely seen for strains that do not generate toxin A and B but binary toxin (like *C. difficile* toxintype X), amplification of *ctdB* gene is recommended (Stubbs, Rupnik and others 2000). The detection of *ctdB* gene could also be a less time-consuming alternative of A3 segment screen in any binary toxin-producing strains, allowing the recognition 10 of 15 toxintypes (Stubbs, Rupnik and others 2000).

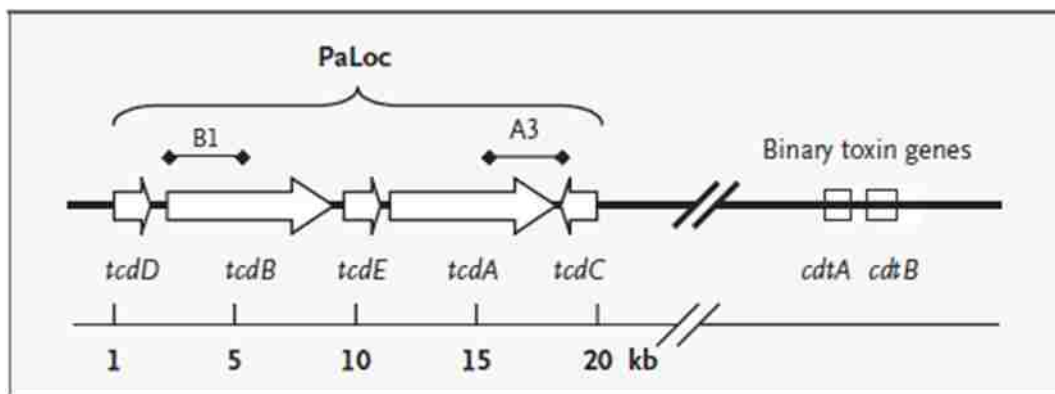


Figure 2.5 Major gene in PaLoc of *C. difficile* and CDT genes (Warny, Pepin and others 2005)

2.6 PCR-based typing

The most traditional typing method used was the slide agglutination with rabbit antisera, together with the protein profiles obtained by polyacrylamide gel electrophoresis (PAGE). By

far there were 14 serogroups determined by this method and are characterized by capitalized letters and numbers (A, B, C, D, F, G, I, K, S1, S2, S3, S4, and X) (Delmee and others 1986).

Toxinotyping is a PCR-based assay used to type *C. difficile* isolates. It is a PCR-RFLP (i.e. Restriction fragment length polymorphism) analysis of a 19 kb region encompassing the *C. difficile* pathogenicity locus. PCR products are digested by restriction enzymes and the resulting restriction fragments are separated according to their lengths by gel electrophoresis. There are 16 *C. difficile* toxintypes (0-XV) reported and more toxintypes might be found. This method could be easily performed in routine laboratory practice and further applied to detect and characterize variant strains.

Genotyping is based on the isolation and sequencing of specific gene segments in different *C. difficile* strains. One genotyping method is to target the *tcdC* gene, which functions in toxin regulation and secretion. Genotype *tcdC*-A is characterized by a nonsense mutation (C184T) and a 39-bp deletion from nucleotides 341 to 379. Genotype *tcdC*-A was identified in *C. difficile* isolates of toxintypes V and VI. *TcdC*-B and *tcdC*-C were marked by 18-bp deletions at nucleotides 330 to 347. This deletion was found in *C. difficile* ribotype 027 and was proved to be responsible for the increased pathogenicity and infectivity of this strain.

Besides toxinotyping and genotyping, a PCR-ribotyping method developed by Kostman and coworkers (1992) was also shown to be reproducible, easy to perform and cost-effective. This typing method is based on the amplification of several alleles of the rRNA operon differing by the length of the intragenic spacer region located between the 16S and the 23S rRNA genes (Bidet, Barbut and others 1999). Thus, a PCR using one primer in the 16S rRNA gene and the other in the 23S rRNA gene will yield a series of amplified DNA

fragments with different length and molecular weight. The difference in sizes of DNA fragments from one sample to the other will make the banding pattern specific for certain strains of *C. difficile*.

The establishment of *C. difficile* ribotyping library has been done by many laboratories using standard *C. difficile* strains isolated from feces of patients. For *C. difficile*, the bands usually located from 225 base pairs (bp) to 700 bp and nearly all the serogroups show different fingerprint-like patterns in banding. The novel PCR-ribotyping showed an extremely high reproducibility thus reliable results can be generated at any lab through a comparison between sample gel and established standards. Another advantage of this method lies in the situation that exact ribotype cannot be identified effectively (e.g. environmental sample with low concentration/less robust *C. difficile*). Observing the differences/similarities between banding patterns may also, at least, allow the researchers to determine if they discovered same/different strains of *C. difficile* under various sampling conditions.

Figure 2.6 shows a gel image obtained from the PCR products of 10 different *C. difficile* strains by a modified PCR-ribotyping method developed by O' Neil et al (1996). The banding patterns were well distinguishable due to the modification to the primer pairs, which generate smaller DNA fragments in response to a narrower interspace region between the binding sites of upstream and downstream primers. Generally, the smaller the DNA fragments generated by PCR-ribotyping, the more easily they are separated on an agarose gel matrix, which actually lacks resolution to differentiate between two high molecular mass DNAs with similar size.

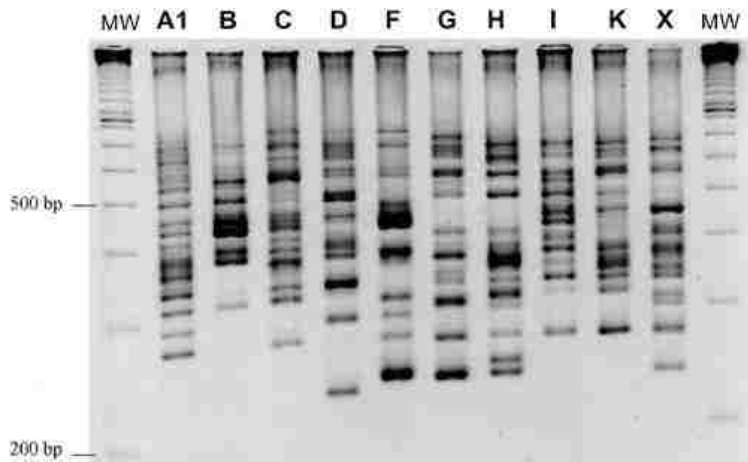


Figure 2.6 PCR-ribotyping results for 10 *C. difficile* strains (Bidet, Barbut and others 1999)

2.7 Fecal indicators

Fecal pollution has been a serious environmental problem that affects many coastal regions in the United States (Sinton and others 2002). Pathogens associated with feces will cause diseases if contaminated food products, such as shellfish and vegetables, are consumed. The sources of contamination may include inefficient sewage treatment plants, leaking septic systems, agricultural runoff, human activity or wildlife (Bernhard and Field 2000). Epidemiological study has indicated its presence as a fecal-associated pathogen from various sources, and most importantly, from human and animal feces (Barbut and Petit 2001). Although few comprehensive surveys were performed about the prevalence of epidemic *C. difficile* in human populations, the common asymptomatic carriage of *C. difficile* has been proved by a series of epidemiological research within healthcare facilities. Of the patients examined 5.9%~13% were carrying epidemic *C. difficile* strains upon admission to the hospital (Barbut and Petit 2001; Riggs, Sethi and others 2007) and a 4%~21% acquisition rate of *C. difficile* by non-carriers were also determined. Therefore, it is reasonable to presume that *C. difficile* has a higher presence rate among human now than it used to be (3%

in adults) and the daily discharge of human feces would contribute to the presence of *C. difficile* in sewage.

The presence of *C. difficile* in surface water was also detected and this might lead to the transmission of *C. difficile* to farm produce since 74,900 million gallons of surface water is drawn per day in U.S for irrigation purpose. According to a research performed in South Wales, England, epidemic *C. difficile* strains were isolated from 14 (87.5%) of 16 samples from four rivers and 7 (44%) of 15 from seawater samples. In addition, 7 (46.7%) of 15 samples of lake water were also positive (Al Saif and Brazier 1996). The most widely used detection method for fecal pathogens in water body is to quantify indicator bacteria by culturing techniques.

Coliforms and fecal streptococci are two main groups of bacteria that are fecal indicators in water samples. They indicate the possible presence of pathogenic (disease-causing) bacteria, viruses, and protozoans that also live in human and animal digestive systems. Coliforms comprise a group of microorganisms that utilize lactose to produce acid and gas, or contain β -D-galactosidase, which is capable of using a chromogenic galactopyranoside substrate (Edberg and others 2000). *Klebsiella*, *Serratia*, *Citrobacter*, *Enterobacter* and *Escherichia* are the main members of this group while except for *Escherichia spp.*, all other genera are widely found in the environment and are not necessarily related to fecal contamination. Many studies have reported *E. coli* as the only coliform that inhabits the gastrointestinal tract of human and animals. However, the routine test of *E. coli* used to be costly and laborious thus fecal coliforms, a group of facultative anaerobic, rod-shaped, gram-negative, non-sporulating bacterium, have long been used as the surrogate of *E. coli* in monitoring fecal contamination.

Although the majority of fecal coliforms were proven to be members of the genus *Klebsiella* (Edberg and others 2000), instead of *E. coli*, enumeration of fecal coliforms are still standard methods documented by the FDA (*Chapter4, Bacteriological Analytical Manual, FDA*).

The emergence of a new technology, The Defined Substrate Technology (DST) in the 1980s, permitted for the first time, the direct detection and identification of *E. coli* in environmental waters. The method is mainly based on detecting the metabolism of 4-methy-umbelliferyl-8-D-glucuronide (MUG), a compound that can only be utilized by a specific enzyme system carried by *E. coli*. This enzyme system, which was later proved to be, β -glucuronidase, is present in more than 95% of all isolates of profiled *E. coli*. The DST method allowed direct detection and enumeration of *E. coli* and coliforms with as low as only 1CFU/100ml water sample. Traditional fecal indicators (e.g. fecal coliforms and *E. coli*) have been proven reliable in many occasions but they are non-spore formers thus may not be able to provide the most accurate information about *C. difficile*, especially through waste water treatment plants, where most vegetative cells are eliminated. In this case, two novel fecal indicators, F+-specific RNA coliphage and Somatic coliphage have been suggested as alternative indicators for enteric viruses and bacteria spores.

Coliphages are a group of nonpathogenic viruses that infect coliform bacteria, and act more similarly to bacterial spores with respect to environmental persistence, and resistance to treatment processes than are indicator bacteria (Sinton and others 2002). Varying from the level of sewage treatment, the consistent presence of coliphages in raw and treated sewage and have been reported in concentrations ranging from 10^3 to 10^7 PFU/liter. In addition, a variety of domestic and feral animals, especially seasonal birds, also shed coliphages in their

feces. The male-specific (F+) coliphage received its name from their morphological characteristics that they infect only F+ male hosts through the F sex pilus. Besides F+ male specific coliphage, there are five more major groups of coliphage differing from morphological characters. Two F+ coliphage families are the Leviviridae (small, icosahedral, single-stranded RNA phages) and the Inoviridae (filamentous, single-stranded DNA phages) and both of which have been proven to be associated with viral contamination in raw and treated sewage, drinking water, and recreational waters. On the other hand, serotyping or DNA oligoprobing of the two F+ coliphage types have been used in identification and to distinguish between human and nonhuman fecal contamination sources. This is of great significance in the trace of source of fecal contamination and control the spread of enteric pathogens. In consideration to the fact that *C. difficile* spores are similar to enteric virus in environmental water and waste water treatment, coliphages might be a better alternative to traditional fecal coliforms as an indicator of *C. difficile* spread. To the best of my knowledge, this was the first study to look into the relationship between fecal indicators and *C. difficile* and the use of coliphages as indicators of pathogenic *C. difficile* groups.

CHAPTER 3 METHODOLOGY

3.1 Sampling

Five commercial harvesting areas (Area 9-13) defined by the Louisiana Department of Health and Hospitals (DHH) were selected for this study. Oyster and seawater samples were randomly collected twice a month by DHH from these six areas. Sewage sample were collected on a monthly basis from a municipal waste water treatment plant (WWTP) in New Orleans. This facility is permitted to treat 122 million gallons per day (MGD) as an Annual Average Daily Flow and had an average flow of 98 MGD for 2013. The exact sampling sites are shown in Figure 3.1.

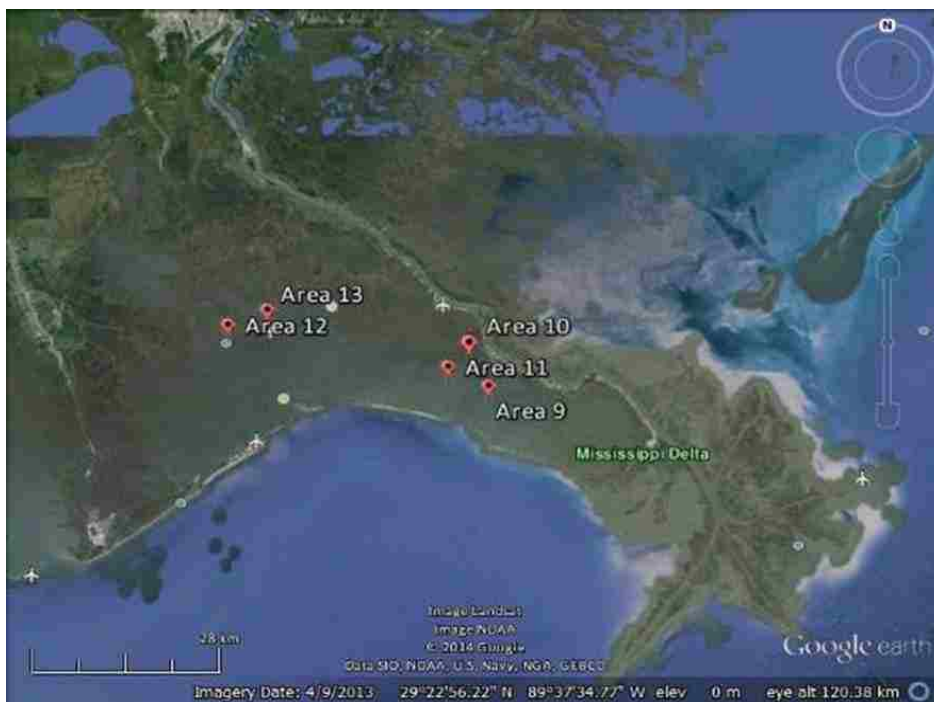


Figure 3.1 Five commercial oyster harvesting sites along Louisiana Gulf Coast region (Google Earth v. 7.1.2.2041)

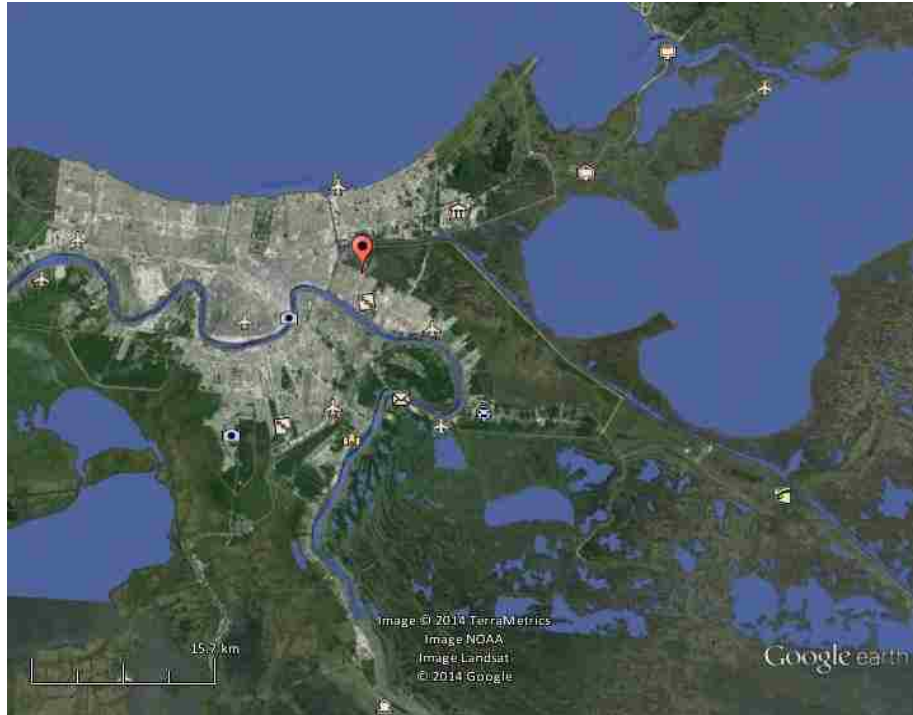


Figure 3.2 Municipal Waste Water treatment plant in New Orleans, LA (Google Earth v. 7.1.2.2041).

Both influent and effluent (i.e. untreated and treated waste water) were collected with sterile capped polypropylene bottles. Oysters and water samples were stored on ice under 4°C in a walk-in cooler for no longer than 24 hours upon sample preparation.

3.2 Sample preparation

3.2.1 Oysters

Prior to shucking, oysters were cleaned with a clean brush and flowing water to purge mud and parasites. Oysters were taken in pairs from sampling bags with approximately even sizes for uniformity. Oysters were shucked with sterile shucking knives and both meat and juice were gathered in a sterile filter bag. Homogenate was prepared in a sterile blender by blending the muscle and juice at full speed for 1 minute. The blender was cleaned with 10% bleach and sterilized under UV for 5 min between each sample. Prepared homogenate was placed on ice until used.

3.2.2 Harvesting water and sewage

One liter of harvesting water sample was filtered through a Millipore filtration system (Millipore, Billerica, MA) with a vacuum pump. Bacteria was blocked and concentrated on a 47mm (diameter), 0.45um-pore size cellulose filtration membrane (Millipore, Billerica, MA) and the filtrate was discarded. For sewage samples, 15ml of each influent and effluent water were filtered and concentrated with same instrument. The membranes were used immediately for further test.

3.3 Detection of *C. difficile* in oyster samples

Non-repeat regions on *tcdA*, *tcdB* and CDT genes (encoding binary toxin) are usually the choices of amplification targets in strain-specific PCR assays. Considering the emergence of toxin A-negative and toxin B-positive epidemic *C. difficile* strains, sole amplification of *tcdB* gene would be a feasible and economic choice. Therefore, a *tcdB*-targeting real-time PCR method described by Van den Berg and coworkers (van den Berg, Bruijnesteijn van Coppenraet and others 2005) was applied to determine the virulence of *C. difficile* in oyster and water samples.

The copy number of *C. difficile* spores in environmental samples could be really low (Pasquale and others 2012) and might be hard to detect. Therefore, preliminary incubation was performed with BHI broth supplemented with 0.1% sodium taurocolic acid and *C. difficile* selective supplement (Sigma-Aldrich). Following processing, 15 g of oyster homogenate was added to 40 ml of Brain Heart Infusion broth (BHI) supplemented with cefoxitin (8 µg/ml) and D-cycloserine (250 µg/ml). Cefoxitin and D-cycloserine selectivity supported growth of *C. difficile*, while inhibiting growth of the majority of

Enterobacteriaceae, *Streptococcus faecalis*, *Staphylococci spp.*, Gram negative, non-sporing anaerobic bacilli and *Clostridia spp.* Taurocolic acid sodium salt hydrate (1mg/ml) was added together with cefoxitin and D-cycloserine under sterile conditions into the autoclave BHI broth. The supplemented broth was stored in 50 ml polypropylene tubes under 4°C.

Upon adding oyster samples, the BHI tubes were incubated anaerobically by a GasPak™ EZ Anaerobe Pouch System under 37°C for 10 days. A loose cap was applied to the tubes to ensure an anaerobic environment in the space between the liquid surface and tube cap. Ethanol shock was performed to eliminate all vegetative cells except *C. difficile* spores for DNA extraction. Specifically, 2 ml of 96% ethanol was combined with 2 ml incubated broth and the mixture stayed at room temperature for 50 min. After ethanol shock, the sample was centrifuged at $6000 \times g$ for 10 min and the supernatant was collected in sterile 2 ml centrifuge tubes.

For the confirmation of *C. difficile*, a commercial *Clostridium difficile* selective agar (CDSA) was used (BBL™ *Clostridium difficile* Selective Agar, BD) following the instructions of manufacturer. Briefly, the plates were first reduced anaerobically under room temperature for 24 hours before use. Then the supernatant collected from supplemented BHI broth was streaked on the plates under a certified bacteria hood and the inverted plates were incubated anaerobically (with the Gaspak system) at 37°C for 24-48 hours. The formation of yellowish, round and elevated colonies indicated *C. difficile* and the colonies were further observed under long wavelength UV (380nm) to positively confirm. Specifically, *C. difficile* colonies on CDSA emit a glowing yellow fluorescence under long wavelength UV within approximately one hour in the presence of oxygen. DNA extraction was conducted right after

colonies of *C. difficile* were confirmed on CDSA plates and three colonies from each sample with approximately identical sizes were collected in a sterile 2 ml centrifuge tube.

The DNA extraction was performed with a commercial DNA extraction kit according to the instructions of the manufacturer. Extracted DNA was stored under -80°C if not used for PCR immediately. The primers used in the real-time PCR were specifically designed to amplify a 177 bp non-repeat region of *tcdB* gene (van den Berg and others 2005). The amplification reactions were performed in a 25 µl final volume, containing 12.5 µl IQ supermix (Bio-Rad, Veenendaal, The Netherlands), 2.5 pmol of the forward primer (398CLDs), 5 pmol of the reverse primer (399CLDas), 4 mM MgCl₂, 0.2 µM probe, and 2.5 µl of sample DNA. After an enzyme activation step of 3 min at 95 °C, the protocol consisted of 50 cycles of 30 s at 94 °C for denaturation, 30 s at 57 °C for annealing, and 30 s at 72 °C for elongation. The PCR was performed using a Cepheid Smart Cycler PCR instrument (Cepheid, Sunnyvale, CA). The sensitivity of the method was 1 × 10³CFU/ml (van den Berg and others 2007). Table 3.1 shows the primers and probe used in this method.

Table 3.1. Primers and probes used for real-time PCR detection of *C. difficile*

Primers and probe	Sequence (5'- 3')	Reference
398CLDs	GAAAGTCCAAGTTTACGCTCAAT	Van den Berg and others 2005
399CLDas	GCTGCACCTAAACTTACACCA	Van den Berg and others 2005
551CLD-tq-F AM	FAM-ACAGATGCAGCCAAAGTTGTTGA ATT-TAMRA	Van den Berg and others 2005

3.4 Detection of *C. difficile* in sea water and sewage samples

Similar PCR procedure as was described above was used with some modifications in the enrichment step. Following filtration, the membrane filter was immersed in 40 ml BHI broth

supplemented with sodium taurocolic acid (0.04g/ml), cefoxitin (8µg/ml) and D-cycloserine (250 µg/ml). Incubation and DNA extraction was performed as was described above in section 3.3. For sewage samples, 15 ml of effluent and 15 ml of pre-filtered (Whatman filter 40) influent water was filtered through 0.45-µm pore size filter nitrocellulose membrane filter (Millipore, Billerica, MA).

3.5 Quantification of Fecal indicators in oyster

Microbial indicators were analyzed in collected oyster meat samples according to *APHA's Recommended Procedures for the Examination of Sea Water and Shellfish, 4th ed, 1970*. Total aerobic bacteria, Male-specific (F+) and Somatic coliphages, fecal coliforms, and *E. coli* were determined. A standard method agar (Acumedia, U.S.) was used for the aerobic plate count. A 10-fold dilution of oyster homogenate was prepared with phosphate-buffered saline buffer and the highest dilution was 1 to 1000. Briefly, 1ml of each diluted oyster homogenate was poured with 15ml standard method agar in duplicate. Two blank standard agar plates were incubated along each test as Then the plates were incubated inverted at 35° C for 48 hours. All colonies were enumerated, regardless of size, color, or morphology. Fecal coliforms and *E. coli* were enumerated using a 5-tube, 3-dilution most probable number (MPN) method with 1.5× Lauryl tryptose broth, *Escherichia coli* (EC) broth with or without 4-methylumbelliferyl-β-D-glucuronide (MUG).

Following processing, a 10-fold dilution (highest dilution 10⁻³) was prepared and 2ml of each dilution was added to each tube and incubated for 24 hours for gas formation. Gas formation in Durham tubes were regarded as positive for fecal coliform. Gas-negative LTB

tubes were incubated for another 24-hour period to confirm negatively. The 48-hour positive tubes were also recorded as positive and used for *E. coli* test. The calculation of CFU/g was determined by MPN calculator software. Positive LTB tubes (gas formation in Durham tubes) were then tested for *E. coli* and were transferred to EC broth tubes and incubated under 45°C for 24 hours. Gas formation in EC broth within 24 hours indicated a high possibility of *E. coli* presence and samples were transferred to EC broth with MUG for final confirmation. EC MUG tubes were incubated for 24 hours under 45°C for gas formation and were observed under 366nm UV light.

Male specific (F+) and Somatic coliphage are viruses that infect *E. coli* thus the enumeration of the coliphage needed prepared *E. coli* cultures. *E. coli* F-amp, carrying a specific resistance to streptomycin and ampicillin, and *E. coli* CN13, which is engineered to be resistant to nalidixic acid, were used for the culture of Male specific and Somatic coliphage, respectively. Either streptomycin/ampicillin or nalidixic acid provided a selective pressure against microorganisms other than *E. coli* to ensure an ideal condition for the growth of coliphage. To achieve maximum number of *E. coli* cells, a log-phase incubation was applied. Overnight culture of each *E. coli* strain in antibiotics-supplemented (50µg/ml streptomycin/ampicillin and 334µg/ml nalidixic acid) Tryptic Soy broth (TSB) was further incubate for 4-6 hours in the same culture under 37°C before plating. A double-layer method was used to quantify the coliphage in oyster samples. Approximately 15.0 g of oyster homogenate aliquots from each sample were placed into a sterile centrifuge tubes and in 1:3 dilution with 30ml growth broth. Then the tube was centrifuged at 10,000 × g for 15 min at 4 °C. The supernatant was collected and weighed in a new, sterile 50ml centrifuge tube then

allowed to warm to room temperature for around 20 min. Then 2.5 ml of the supernatant will be combined with 2.5 ml of pre-autoclaved double-strength soft agar (tempered to 52°C) and 200 µl of log-phase *E. coli* F-amp or *E. coli* CN-13.

The mixture was mixed well by rolling the tube generally for around 10 times and overlaid onto a tryptone agar plate containing streptomycin/ampicillin (50µg/ml) for the Famp mixture or a tryptone agar plate containing nalidixic acid (334µg/ml) for the CN-13 mixture. After the upper layer is hardened, the plates were inverted and incubated for 24 hours at 37°C. A 10⁻⁹ and 10⁻⁸ dilutions of positive Somatic and F-amp coliphage sample collected from previous tests (sewage sample) were plated along with each sample as positive controls. Negative controls of 2.5ml of growth broth were used with the tested of oysters. The average PFU per 100 g of oyster meat and juice was calculated using the average PFU/plate, quantity of supernatant/plate, and the total weight of oyster meat and the formula:

$$PFU/100g \text{ oyster} = \frac{\text{Average PFU/plate}}{\text{supernatant/plate (ml)}} \times \frac{\text{Total supernatant recovered (g)}}{\text{Total homogenate (g)}} \times 100$$

The relationship between the indicator and the occurrence of *C. difficile* in samples was determined by SPSS Statistics software, using a binary logistic regression process. The model was validated by a Chi-Square test hypnotizing significant deviances between the predicted and observed values. The insignificance of the test indicates the statistical model is effective.

3.6 PCR ribotyping

The ribotyping of *C. difficile* depends on PCR amplification of several rRNA operon differing by length. Through an agarose gel electrophoresis, the PCR products formed unique banding patterns varying from one ribotype to the other, thus provide a typing ability between different species of *C. difficile*. Similar processing and DNA extraction was used as is described in the Real-time PCR detection of *C. difficile*. Differently, the instrument used in this part was a traditional PCR machine (Bio-Rad) thus no probe was included. Table 3.3 shows the primers used in this method.

Table 3.3 Primers for PCR-ribotyping of *C. difficile*

Primer	Sequence	Reference
16S primer	5'-GTG CGG CTG GAT CAC CTC CT-3'	Bidet and others 1999
23S primer	5'-CCC TGC ACC CTT AAT AAC TTG ACC-3'	Bidet and others 1999

The 1.0% agarose gel was prepared with 1.0× Tris-acetate-EDTA (TAE) buffer and molecular agarose gel powder (Seakem). Briefly, 25 ml of 1.0× TAE buffer and 0.25 g of agarose gel powder will be mixed and heated to a slight boil in a 150 ml capped flask. Then the flask was incubated in a 50°C water bath for 10 min to cool down to approximately 60°C. Ethidium Bromide was added to the gel before pouring with a final concentration of 50ug/L and mixed well. Pre-mixed 3 µl of loading dye and 15 µl of PCR amplifications was added to each sample loading slots after the gel was hardened. Then the DNA was fractioned by electrophoresis for 2 h at 35V in 1.0× TAE buffer for 2 hours with a distance of 10 cm between the two electrodes.

3.7 Data processing

All analyses were performed in duplicate and reported with as mean per 100g or +/- (C. difficile). A binary logistic regression model was used to measure the contribution of each variables (TPC, fecal coliform, E. coli and coliphage) to the occurrence of *C. difficile*. Significant differences among odds ratios were determined using Kruskal-Wallis test ($\alpha = 0.05$). Analysis of Variances (ANOVA) was performed on a 0.05 confidential level to determine significance among data points. Software IBM SPSS Statistics (v. 19.0.0) was used for statistical analyses.

CHAPTER 4

RESULTS

4.1 Aerobic plate count

The results for aerobic plate count are presented by area in Fig. 4.1. Between January, 2013 and February, 2014, 19 oyster samples were obtained from area 9, 10, 11, 12 and 13. Highest count in area 9 was 6.46CFU/100g and the lowest was 4.70 CFU/100g. From area 10, two samples were obtained, the aerobic counts were 5.01 and 6.46logCFU/100g. Maximum counts for samples from area 11, 12 and 13 were 5.80, 6.03 and 6.65logCFU/100g and minimum were 4.50, 4.78 and 4.55logCFU/100g, respectfully. A relation between Aerobic plate counts and the occurrence of *C. difficile* in oysters was observed according to the statistical analysis (*odds ratio* = 3.211).

4.2 Fecal coliform and *Escherichia coli*

The fecal coliform and *E. coli* counts are showed in Table 4.1. Nineteen samples were collected and enumerated for fecal coliform and *E. coli* during January, 2013 – February, 2014. Eleven of 19 (57.9 %) samples were tested positive for fecal coliform and the obtained MPN ranged from 20 to 104 per 100 gram of oyster sample. Six of 11 (54.5%) fecal coliform-positive samples were confirmed with *E. coli* and the most probable numbers (MPN) were ranged from 10 to 35 per 100 gram of oyster sample. Fecal coliforms were found at least once in each area while *E. coli* was not confirmed in oyster sample from area 10. Despite the sampling area, there were more fecal coliforms observed in colder months. Seven of 10 positive samples were obtained in January-April and November and the average fecal coliform MPN/100g was higher in comparison to that obtained from June to October (52 vs.

6.2). Generally, fecal coliform and *E. coli* showed a different behavior than did aerobic plate count. A binary logistic regression model was used to evaluate the influence of Fecal coliform in the occurrence of *C. difficile* while no statistical significance was found (Table 4.3). The model was proved to be effective by a Chi-square test ($p > 0.05$).

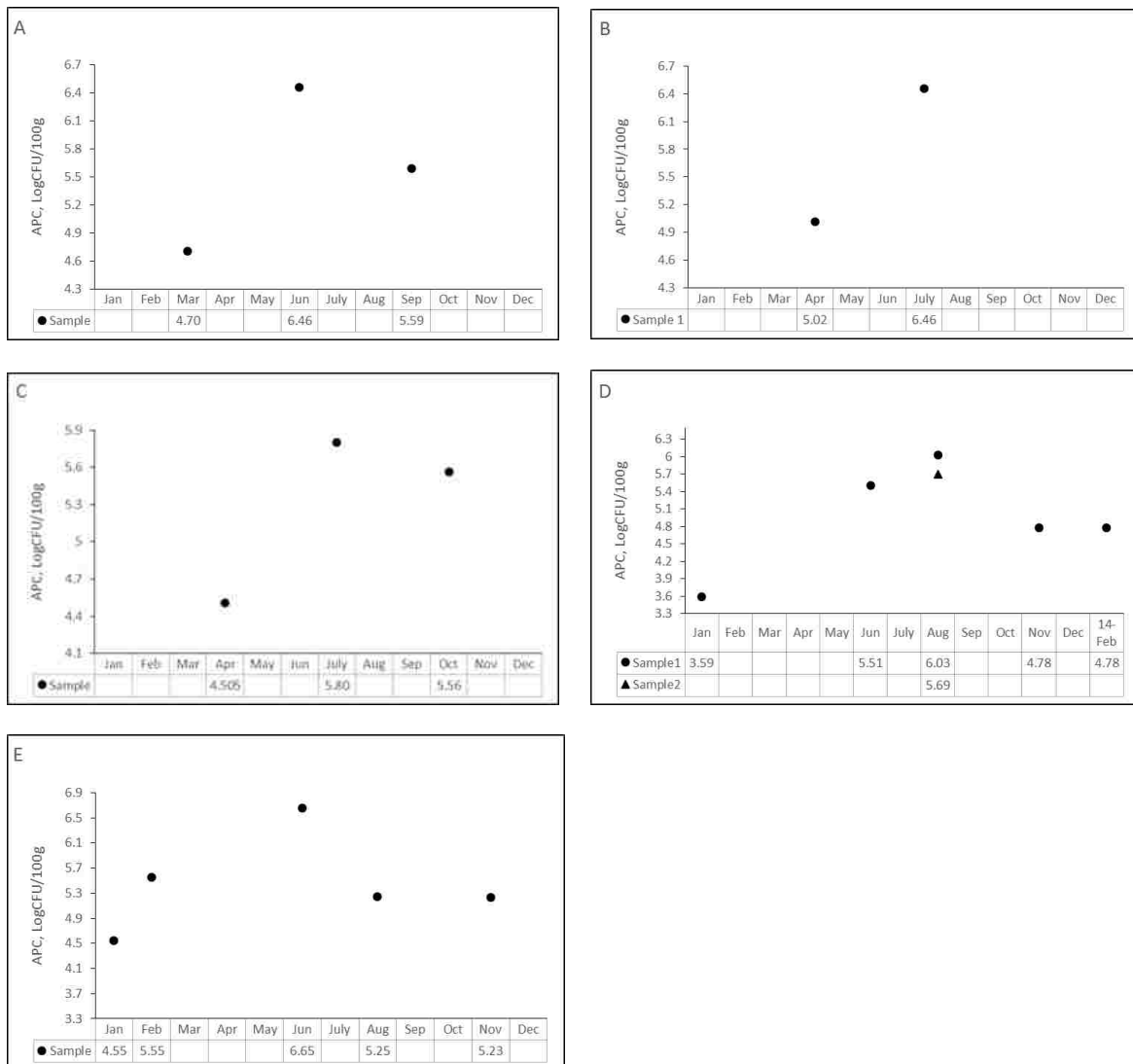


Figure 4.1 Average aerobic plate counts for oyster samples collected from harvesting areas along the Louisiana Gulf Coast. (A) Area 9; (B) Area 10; (C) Area 11; (D) Area 12; (E): Area 13.

Table 4.1 *C. difficile*, fecal coliform, *E. coli* and coliphage in oysters and harvesting water ^a

Date	<i>C. difficile</i>		Fecal coliform	<i>E. coli</i>	Male-specific(F+) Coliphage	Somatic Coliphage
	Oysters	Harvesting water				
13-Jan	+	NAb	94	35	< 6.0	< 6.0
13-Jan	-	NA	95	20	7.7	32
13-Feb	-	NA	35	< 2	< 6.0	< 6.0
13-Mar	-	NA	60	< 2	< 6.0	< 6.0
13-Apr	+	NA	104	< 2	< 6.0	< 6.0
13-Apr	-	NA	50	35	< 6.0	< 6.0
13-Jun	-	NA	20	20	20.8	< 6.0
13-Jun	-	NA	10	10	10.8	< 6.0
13-Jul	+	NA	< 2	< 2	< 6.0	< 6.0
13-Jul	+	NA	< 2	< 2	< 6.0	< 6.0
13-Jul	+	NA	< 2	< 2	< 6.0	< 6.0
13-Aug	-	-	< 2	< 2	< 6.0	< 6.0
13-Aug	+	-	< 2	< 2	< 6.0	< 6.0
13-Sep	+	+	32	10	< 6.0	< 6.0
13-Sep	-	-	< 2	< 2	< 6.0	< 6.0
13-Oct	-	-	< 2	< 2	< 6.0	< 6.0
13-Nov	+	+	30	20	< 6.0	11.4
13-Nov	+	+	< 2	10	< 6.0	< 6.0
14-Feb	-	-	< 2	< 2	< 6.0	< 6.0

^a the results are expressed in the following manners: *C. difficile* (+/-); Fecal coliform and *E. coli* (CFU/100g); Male-specific (F+) and Somatic Coliphage (PFU/100g)

^b Date: year-month

^c NA: not available

Table 4.2. Presence of *C. difficile* in sewage

Date ^a	Influent Sewage	Effluent Sewage
13-Sep	+	+
13-Oct	+	+
13-Nov	+	+
14-Jan	+	+
14-Feb	+	+

^a Date: year-month

Table 4.3. Exp (B) (odds ratio)of microbial indicators

Microbial indicator	Exp (B)
Fecal coliform	1.025 ^a
<i>E. coli</i>	0.997 ^b
APC	3.211 ^c

^a per unit increase in fecal coliform count will result in a 2.5% higher possibility of *C. difficile* occurrence;

^b per unit increase in *E. coli* count will result in a 0.3% lower possibility of positive *C. difficile*

^c per unit increase in Aerobic plate count will result in a 221.1% higher possibility of positive *C. difficile*

4.3 Coliphage

The Male-specific (F+) Coliphage and Somatic Coliphage test results are showed in table 4.1. For Male-specific (F+) Coliphage, 3 positive results were seen in 19 oysters; for Somatic Coliphage, only 2 positive responses were seen in 19 oysters. The Male-specific (F+) Coliphage counts were 7.7, 20.8 and 10.8 PFU per 100g oysters. The Somatic Coliphage counts were 32.0 and 11.4 PFU per 100g oysters.

4.4 Presence of *C. difficile* in oysters and waters

Figure 4.3-4.6 and Table 4.1, 4.2 show the real-time PCR results obtained from oysters, harvesting water and sewage. From January, 2013 to February, 2014, 19 oysters, 8 harvesting waters and 5 sewage samples were collected and tested for pathogenic *Clostridium difficile* strains by CDSA and real-time PCR. For oysters collected prior to August-2013, frozen homogenates being kept at -20°C were used. The frozen temperature (-20°C) was proved to have little impact on the survival of *C. difficile* spores (Freeman and Wilcox 2003).

Nine out of 19 oyster samples (47.3%) were tested positive for *C. difficile* by CDSA and 100% (9/9) of the *C. difficile* collected from CDSA were positive for tcdB gene by real-time

PCR. Three of 8 (37.5%) collected harvesting water samples were tested positive by CDSA and all were confirmed of virulence by real-time PCR. All five sewage samples, influent or effluent, tested positive in both tests. Pathogenic *C. difficile* were found in harvesting waters (except one sample from area 12) when the oyster samples from same harvesting area were tested positive.

Oyster samples collected between warm months (June - September) showed more positive (5 vs. 4) in than those collected in other months, but the difference was not significant. In real-time PCR, oyster and harvesting water samples mostly crossed the threshold at 38~40 cycles while the sewage samples tended to cross the threshold at 24~26 cycles. This might indicate that the *C. difficile* strains found in sewage were more robust than those in environmental samples and presented higher copy numbers in each colony on CDSA plates. The similar behavior of influent and effluent sewage sample in real-time PCR also showed that waste water treatment might have limited impact on the robustness of *C. difficile* spores.

Our observation was partly in accordance to the report of Romano et.al in the year of 2012, in which 85% *C. difficile* isolates from nine waste water treatment plants were found to be toxigenic (Romano and others 2012). To validate the method, an internal positive control was used. *C. difficile* ATCC 43255, a toxin B-positive *C. difficile* strain was used and gave a stable threshold cycle of around 25 cycles in each test.

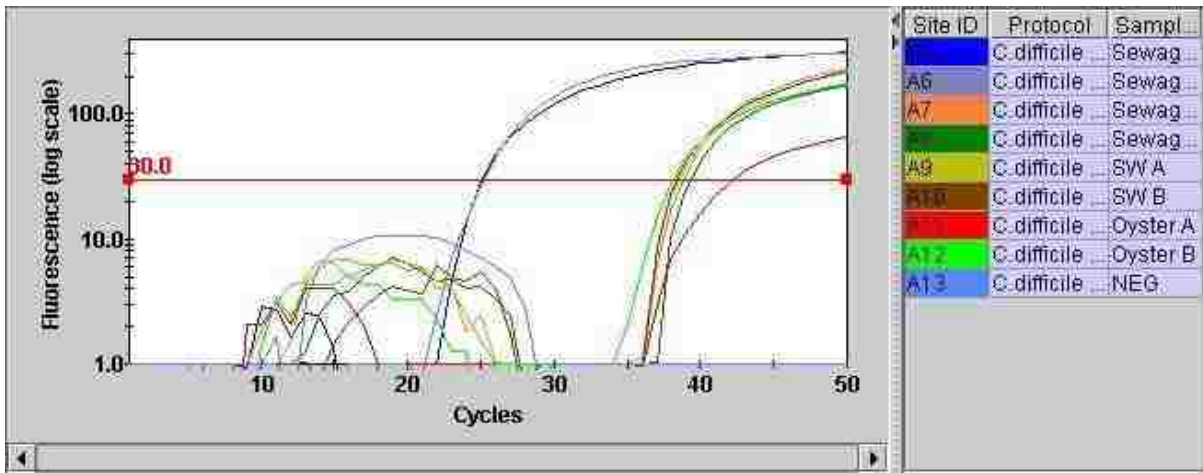


Figure 4.3. Real-time PCR results for sewage sample collected in September, 2013 (A5, A6), October, 2013 (A7, A8), November, 2013 (A9, A10) and Oyster samples collected in January, 2013 (A11, A12).

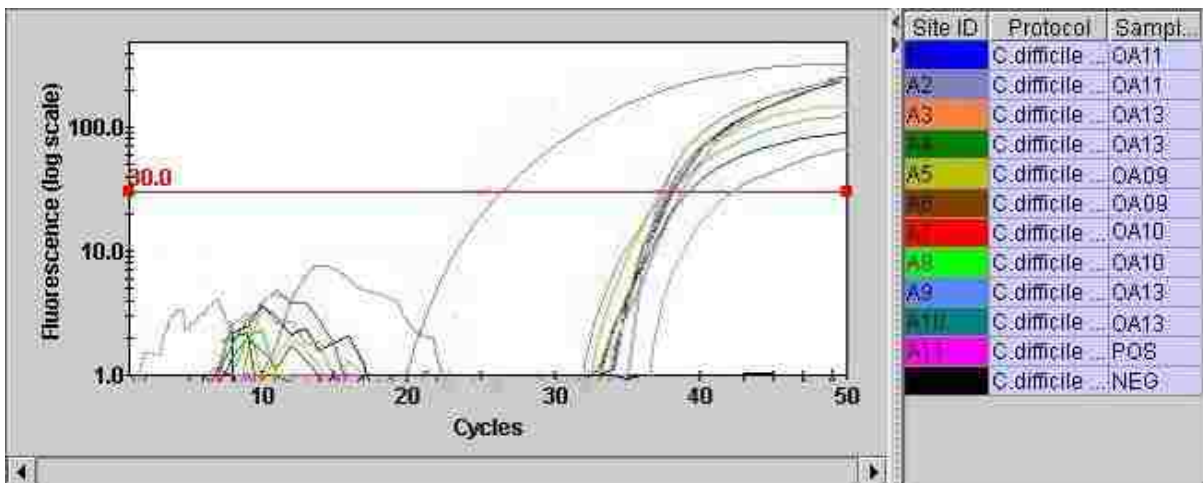


Figure 4.4 Real-time PCR results for Oyster samples from area 9 (A5, A6), 10 (A7, A8), 11 (A1, A2), 13 (A3, A4: August, 2013; A9, A10: November, 2013).

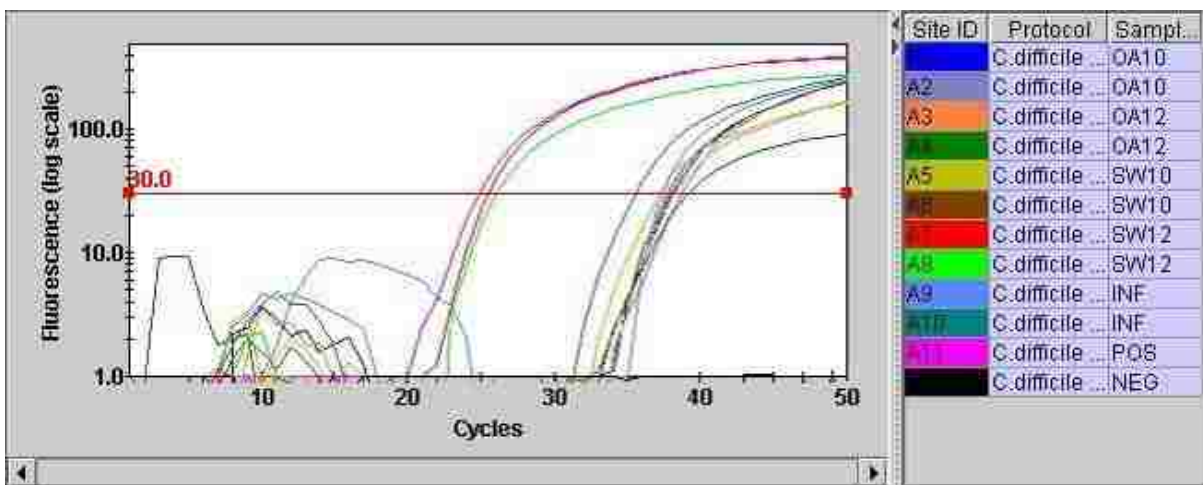


Figure 4.5 Real-time PCR results for oysters from area 10 (A1, A2) and 12 (A3, A4), harvesting water from area 10 (A5, A6) and 12 (A7, A8), influent sewage (A9, A10).

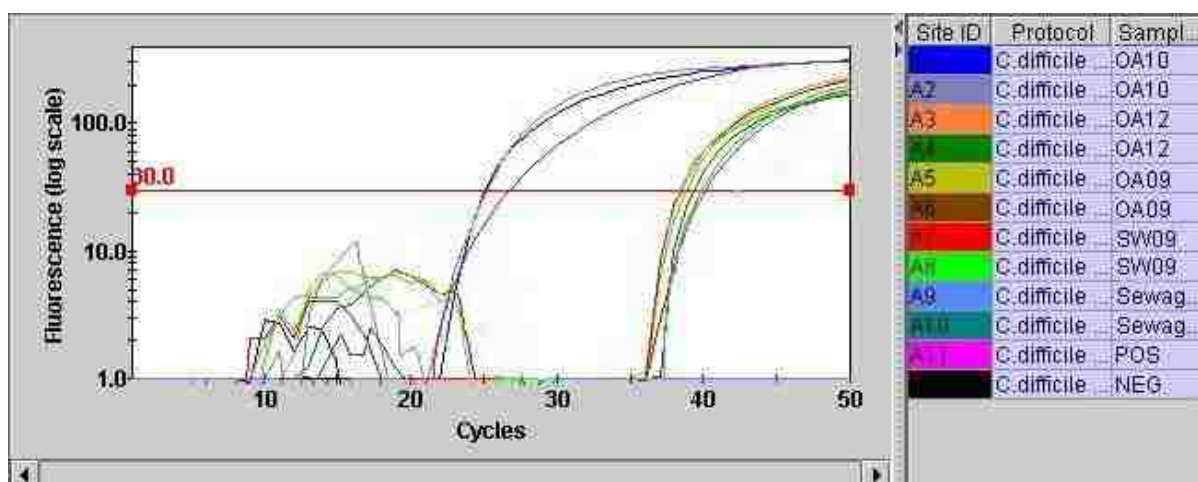


Figure 4.6 Real-time PCR results for oysters from area 9 (A5, A6), 10 (A1, A2), 12 (A3, A4) and harvesting water from area 9 (A7, A8). And sewage water sample received in February (A10, A11).

4.5 PCR-ribotyping of *C. difficile*

The PCR ribotyping was performed on extracted DNAs that gave positive results in CDSA and real-time PCR tests. Nine oyster samples, 3 harvesting samples and all sewage samples were involved in the test. The gel images are shown in Figure 4.3-4.6. All sewage samples (both influent and effluent) were characterized with two bright bands between 200-500bp, which was obviously different from the banding pattern obtained from oysters and harvesting water. Multiple pale bands were observed between 200bp-400bp for most oyster and water samples but with one exception: harvesting water sample from area 12, collected in November, 2012 showed a similar banding pattern to sewage samples.

Oyster and water samples collected from same areas tended to present same banding pattern (Figure 4. 7, C, D vs. E, F; Figure 4.8 E, F vs. G, H) but different banding patterns were also found in harvesting waters and oyster in samples from area 12, November, 2012. Oyster samples coming from same harvesting area but different sampling time (e.g. Area 9,

July vs. September, in Figure 4.7, lane C and D vs. Figure 4.9, lane D and E) showed different banding patterns, indicating the existence of various *C. difficile* strains in one sampling location.

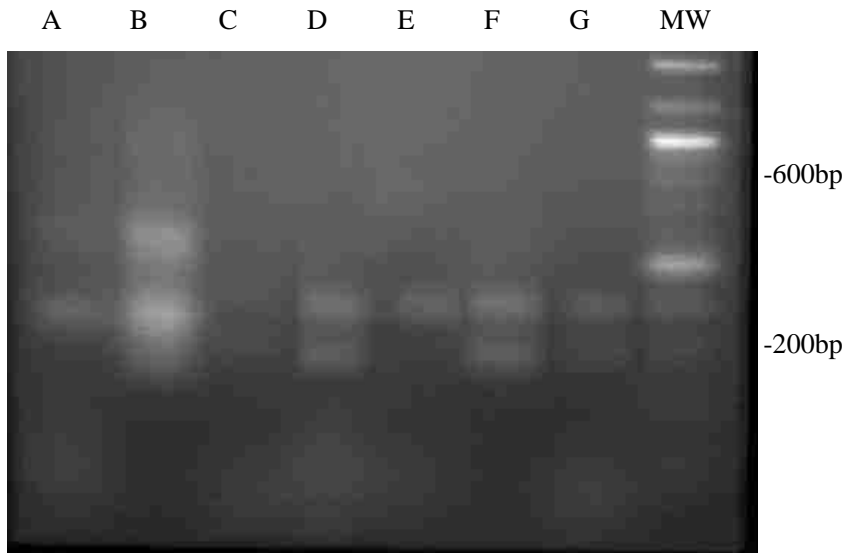


Figure 4.3 PCR ribotyping results for oysters and waters. Lanes: A and B: sewage influent\effluent, area 9, 13-Sep; C and D, oysters, area 9, 13-Sep; E and F, harvesting water, area 9, 13-Sep; G: oysters, area 12, 13-Nov; MW: 1000bp molecular marker.

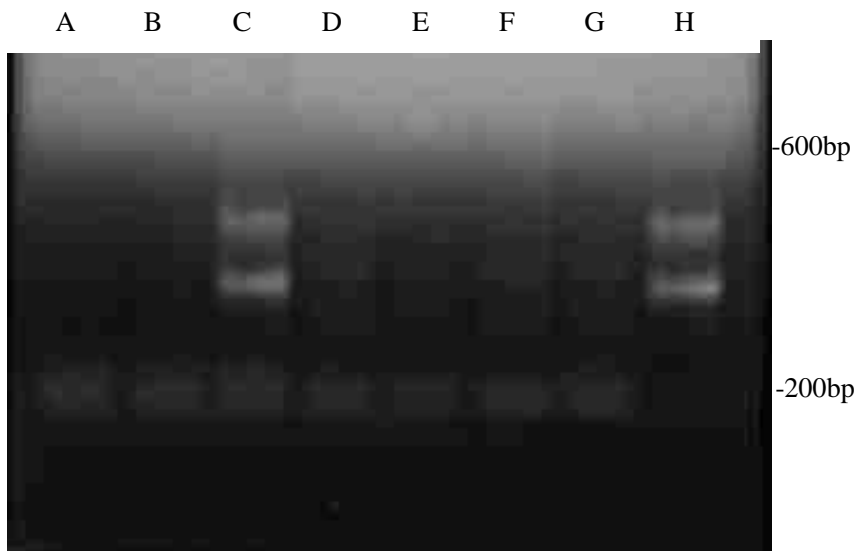


Figure 4.4 PCR ribotyping results for oysters and waters. Lanes: A, oysters; area 12 13-Nov; B and C, harvesting water, area 12, 13-Nov; D and E, oysters, area 13, 13-Nov; F and G, harvesting water, area 13, 13-Nov; H: sewage influent, 13-Nov;

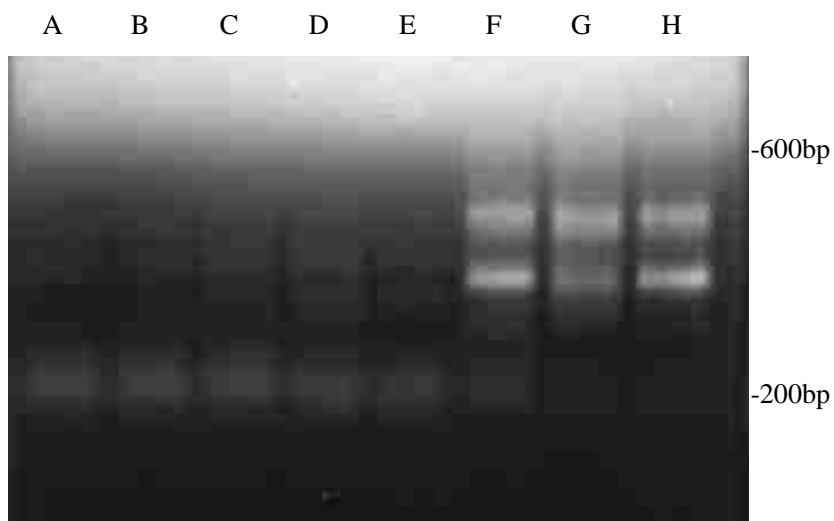


Figure 4.5 PCR ribotyping results for oysters and waters. Lanes: A, oysters; area 12 13-Jan; B and C, oysters, area 10, 13-Apr; D and E, oysters, area 9, Jul/2013; F and G, sewage influent/effluent, 13-Oct; H: sewage influent, 13-Nov.

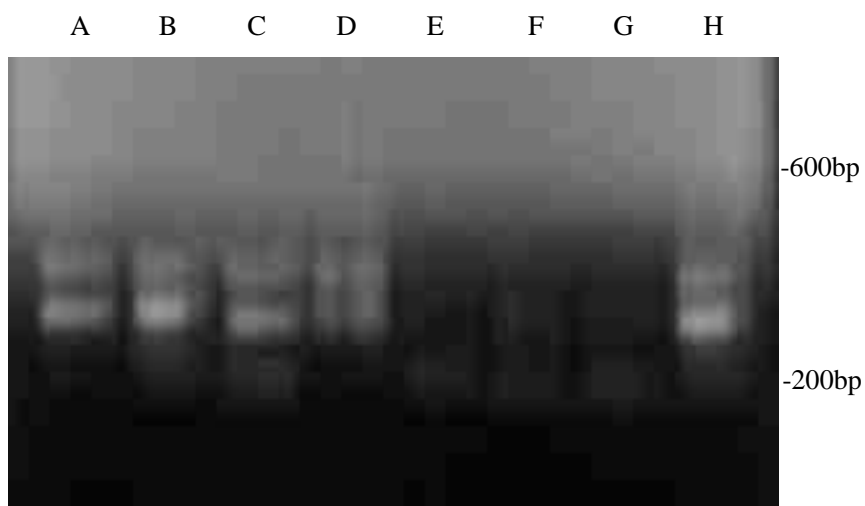


Figure 4.6 PCR ribotyping results for oysters and waters. Lanes: A and B, sewage influent/effluent, 14-Jan; C and D, sewage influent/effluent, 14-Feb; E and F, oysters, area 10, 13-Jul; G and H, oysters, area 11, 13-Jul; H: sewage effluent, 13-Nov

CHAPTER 5 DISCUSSION

The purpose of this study was to provide a general idea about the prevalence of epidemic *C. difficile* in Louisiana oysters, harvesting water and sewage, and investigate the potential of fecal coliforms and coliphage as indicators for *C. difficile*.

As a common resident of intestinal tracts and a spore former, *C. difficile* can enter the environment through various routes and survive theoretically a longer lifespan than common fecal pathogens (Freeman and Wilcox 2003). In a survey performed in the waste water treatment plants in Southern Switzerland, pathogenic *C. difficile* was found in both raw sewage influents and treated effluents, which revealed the possible contamination of water bodies that receive waste water treatment plant effluents (Romano and others 2012). As the distribution of *Clostridium spp.* by WWTPs in water ecosystems has been determined, the possible spreading of *C. difficile* in water bodies through treated sewage discharge should be of particular concern.

Besides river water, which is usually first to receive sewage discharge, the presence of *C. difficile* in seawater was also reported by AI Saif and coworkers (1996), who found *C. difficile* in 7 (44%) of 15 sea water samples collected from Europe coastal areas, in the year of 1996. Oysters, on the other hand, are brackish filter-feeders and have a strong ability to concentrate micro particles, including pathogens and spores, within their bodies. V. Pasquale and coworkers reported that 12 known different PCR ribotypes of *C. difficile* were found in 49% of the 53 marine bivalve molluscs in Europe.

Although many studies have proven the effective defense of *C. difficile* by healthy intestinal flora, the consumption of contaminated oysters still impose great health risk to

vulnerable population (e.g. elder people who receive antibiotic treatment frequently), as *C. difficile* become highly contagious within health care facilities.

In this study, 9 (47.4%) of 19 oyster samples collected from five commercial harvesting areas were positive for toxin-B positive *C. difficile* by a two-step culturing-PCR method. Our results are comparable to those (49% and 42.8%) obtained by two independent studies with shellfish (Pasquale and others 2012) in Europe. The high detection rate indicates the accumulation of pathogenic *C. difficile* in oysters and the potential of oysters as a transmission source of *C. difficile* among people who consume oysters raw.

A slightly lower proportion of positive results were obtained from harvesting waters, in 3 (37.5%) of 8 samples, in comparison to the detection rate of *C. difficile* in oysters (47.4%). The difference might partly resulted from the oysters' ability to largely concentrate spore particles, which are originally distributed sparsely in water bodies. In addition, the presence of *C. difficile* in oysters and harvesting water was almost identical, with 7(87.5%) of 8 harvesting water samples showed same real-time PCR results as oysters collected from same area. Moreover, Willis and coworkers (1957), has reported a seasonal fluctuation of *Clostridium Perfringens*: generally higher counts were obtained in the colder months than in the summer in treated water (Willis 1957). However, no such trend was observed in this study since the number of positive *C. difficile* detected in cold months (5) was very close to that in warm months (4), indicating temperature might not be a decisive factor of the presence of *C. difficile* in oysters.

Aerobic plate count was performed with each test and was used to evaluate microbial abundance, which can be largely affected by temperature, weather and human/animal activities. According to our results, all sampling areas were affected similarly throughout the study since no significant fluctuation in the aerobic counts were observed. The high aerobic plate counts were obtained mostly in warm months (From June to October), during which 5 (50%) of 10 collected oyster samples were tested positive for *C. difficile*. Moreover, pathogenic *C. difficile* was always found in samples with high aerobic plate count (e.g. oysters from area 9, 10, 11 in July/2013) from each sampling area. The statistical analysis revealed a relation between the aerobic plate count and the occurrence of *C. difficile* in oysters (*odds ratio* = 3.221), while still APC can hardly be regarded as a bio-marker of *C. difficile* due to the lack of specificity.

The open or closure of shellfish harvest areas is decided by the counts of fecal coliforms present in their surface waters. According to FDA's *National shellfish sanitation program manual of operations, 1997 revision*, unrestricted shellfish harvesting areas should have a mean fecal coliform MPN of less than 14/100 ml, with 10% of the samples not to exceed MPN of 43/100 ml in a five-tube MPN test. It was also reported that the accumulation of fecal coliforms by oysters in the Gulf Coast averaged 4.4 (SD = 4.0) times greater than their exposure in the waters. The maximum acceptable fecal coliform count in oysters could be calculated by multiplying the maximum acceptable fecal coliform count in water (14 MPN/100ml) by 8.4 (mean accumulation factor + SD). Therefore, fecal contamination might have happened if the tested fecal coliform count rose over 118 MPN/100g oysters. The highest MPN of fecal coliform was observed in oysters collected in April, 2013, that had

108MPN/100g. Therefore, no fecal contamination might have happened in the harvesting areas throughout the study. In addition, according to past data obtained in coastal region of Louisiana, the mean counts of fecal coliforms in water peaked in late winter (January/February) reaching 120 MPN (February 1990), 165 MPN (January 1992), and 86 MPN (January 1997), and then decreased considerably during spring and summer (1.2 - 19 MPN). Our results agreed with previous data had an average fecal coliform count of 28 MPN/100g, with peak values recorded as 104MPN/100g in April and 95MPN/100g in January. In comparison to the counts in colder months, the fecal coliforms counts in summer (From June to October) were significantly lower and gave an average counts of 6.9 MPN/100g, with peak values recorded as 32MPN/100g. Eight of 10 samples collected between June and October were giving less than 2MPN/100g fecal coliform. Most (7/10, 70.0%) positive fecal coliform in oyster samples were further confirmed as *E. coli*. Two = higher fecal coliform counts found in January and April were found to yield positive *C. difficile* as well. This showed that the increased environmental fecal coliforms and *E. coli* concentration might relate to the presence of *C. difficile*. However, in summer, the fecal coliform counts naturally became pretty low and failed to give ideal overlap (1 of 5, 20.0%) for *C. difficile*. This may partly attributed to the ability of *C. difficile* to form resistant spores that are better adaptive to changing environmental conditions, and may thus live a longer lifespan in summer in comparison to fecal indicators. Overall, the overlap of positive fecal coliform/*E. coli* and *C. difficile* were 4 (44.4%) of 9 samples. No significant relation was observed between the fecal coliform count and the occurrence of *C. difficile*. For fecal coliforms, increase in per unit of count only increase 2.5% of the possibility to observe *C.*

difficile; For *E. coli*, per unit increase in count will decrease 0.3% of the possibility of positive *C. difficile*. Therefore, the fecal coliform actually affect very little on the occurrence of *C. difficile*.

The aim of PCR-ribotyping in this study was to determine whether the presence of *C. difficile* in oysters harvesting areas is related to the discharge of treated sewage from upstream WWTP. According to our results, similar *C. difficile* ribotype were found in all five sewage samples collected from the studied WWTP. Therefore, if the outflow of treated sewage does contribute to the accumulation of *C. difficile* spores in harvesting areas, similar *C. difficile* ribotype should be found in oysters and harvesting waters from these areas. However, except for one harvesting water sample from area 12, all other oysters and water samples formed very obvious different banding patterns to those obtained from sewage samples.

The results suggested that the presence of *C. difficile* in oyster harvesting areas does not necessarily related to the sewage effluent discharged by upstream WWTP. Similar conclusion were also given by Willis, who found two samples of river water taken upstream from the studied WWTP were also positive for *C. difficile* (Willis 1957). Theoretically, the source of *C. difficile* found in WWTP was limited to human sewage, while the *C. difficile* found in oysters and harvesting water might derive from multiple sources: soil runoff, storm, sewage, feces of human, birds or other animals. Therefore, it is not so surprising to observe that *C. difficile* found in WWTP were similar while those found in environmental samples vary. This explanation was partly supported by our results: an inconsistency of *C. difficile* ribotype was found in same sampling area at different times (Figure 4.9 Lane A vs. Figure 4.8 Lane A),

which suggests that multiple sources might be responsible for the spread of *C. difficile* in harvesting waters and oysters.

We found a randomized pattern of Male-specific (F+) Coliphage and Somatic Coliphage distributions in oyster samples collected. The overall PFU too low to fit in statistical models. The overlap of positive Male-specific (F+) Coliphage and positive *C. difficile* were low, with only 1 in 9 samples. As for Somatic Coliphage, the overlap was only 2 of 9. The lack of PFU higher than 6/100g samples has prevented us in further investigating possible relations between the emergence of *C. difficile* and the abundance of coliphage in oysters.

Our observation were partly in accordance to a previous study reported that no Male-specific (F+) Coliphage was found in oyster samples they collected from Gulf Coast areas(Burkhardt and Calci 2000). The lack of coliphage in shellfish was also reported in a study about the microbiological quality of shellfish-growing waters(Chai and others 1994). Hereby we regarded coliphage as an inappropriate indicator of *C. difficile* in oysters, on one hand due to its low detection rate in oysters (only in 3 samples had higher PFU than the detection limit of this method, 6 PFU/100g, in total 19 oyster samples) and on the other hand its low overlap rate to *C. difficile*. Actually, the lack of correlation between coliphage and *Clostridium Perfringens*, a close relative to *C. difficile*, has been reported in a previous study(Burkhardt and Calci 2000).

CHAPTER 6 CONCLUSION

The epidemiology of *C. difficile* has been changing, due in part to the emergence of more virulent *C. difficile* strains (NAP1/BI/027) in North America and Europe and the increasing proportion of *C. difficile*-associated diarrhea in the community. Moreover, *C. difficile* appears to be a growing concern in food producing animals and has been isolated from food products. Therefore, CDAD is no longer simply a health care-associated phenomenon, but a much more complicated epidemic disease that causes public health problems and financial loss. Foods, recreational waters, risk factors other than antimicrobial exposure should draw more attention as we begin to understand the broader scope of this microorganism. In this study, a high percentage of occurrence of pathogenic *C. difficile* strains in oysters, harvesting water and sewage flows was observed. The potential of fecal bacteria and coliphage as bio-indicators of *C. difficile* was evaluated, while neither of them showed an ability to mark the presence of *C. difficile* in changing weather conditions. The lack of reliable indicator thus raises a task on the development of more sensitive and rapid quantification method of *C. difficile* in complicated weather and geological environments. Although epidemic *C. difficile* strains were detected frequently in both raw and treated sewage, no significant linkage was found in this study, between the occurrence of *C. difficile* in oysters, harvesting waters and discharge of treated sewage from upstream. This suggested the existence of possible alternative sources of *C. difficile*, besides sewage outflows, attributing to the transmission of *C. difficile* into harvesting areas.

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VITA

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