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The presence of Clostridium difficile on environmental surfaces in healthcare facilities pre- and post-decontamination of patient rooms

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THE PRESENCE OF *CLOSTRIDIUM DIFFICILE* ON ENVIRONMENTAL SURFACES IN HEALTHCARE FACILITIES PRE- AND POST-DECONTAMINATION OF PATIENT ROOMS

By

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ABSTRACT

The presence of *Clostridium difficile* on environmental surfaces in healthcare facilities pre- and post-decontamination of patient rooms

By

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Healthcare-associated infections (HAIs) are infections related to receiving medical care. HAIs are responsible for an excess of morbidity and mortality among hospitalized patients. Though most HAIs rates are on the decline, *Clostridium difficile* infection rates are at an all-time high, primarily due to the persistence of *C. difficile* spores in the environment. In the United States, *Clostridium difficile*-related mortality rates per million have increased from 5.7 in 1999 to 23.7 in 2004, with an estimated 26,642 deaths due to *Clostridium difficile* infections (CDIs). *Clostridium difficile* is transmitted via the fecal-oral route or aerosolized endospores, but it can also be transmitted from high touch surfaces in healthcare facilities, such as door handles, bed rails, and bed pans contaminated with *C. difficile* spores. Various methods of detection have been established since the 1970s, but they have limitations, such as cost, time, and availability. The use of a molecular method of detection, such as polymerase chain reaction (PCR), could provide more rapid and sensitive results for the detection of

Clostridium difficile. The objective of this study was to determine the presence of *Clostridium difficile* pre- and post-decontamination of patients' rooms in a healthcare facility environment using culture and PCR analysis of surface samples. No culturable *C. difficile* were detected; however, the culture analysis results showed a significant difference between the number of facultative and anaerobic bacteria in pre-decontamination samples and post-decontamination samples ($Z = -5.852$, $p = 0.000$). Of the 128 samples tested using PCR analysis, five samples were positive for *Clostridium difficile* DNA (3.9%); three were from pre-decontamination samples and two were from post-decontamination samples. Reducing the rate of transmission of *Clostridium difficile* infections in hospitals is dependent on a number of factors (e.g., proper use of antibiotics, environmental decontamination, and proper hand-hygiene). The results of this study indicate decontamination methods used at these facilities were effective in preventing environmental contamination of hospital rooms with facultative and anaerobic bacteria such as, *C. difficile*.

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for making me laugh through the most stressful situations. Thank you for distracting me from my studies…even though I still got A's.

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CHAPTER 1

BACKGROUND

From birth, individuals are continuously colonized with microorganisms throughout the gastrointestinal tract. The normal human flora is complex in nature, yet crucial to an individual's health. Approximately 1,000 species of bacteria can be found in the gastrointestinal tracts of humans (Jernberg, Lofmark, Edlund, & Jansson, 2010). These bacteria interact and contribute to physiological processes of the body, such as the immune system (Jernberg et al., 2010). The normal human flora is influenced by age, health status, and diet. Despite the importance of the gut microbiota, some of these bacteria are potentially pathogenic. The normal flora of an individual is believed to be a protective barrier against unwanted microorganisms; however, antibiotic use can lead to a disruption in the protective barrier of the gut microbiota. This allows potentially pathogenic bacteria to proliferate in the gut and can result in an increase in toxicogenic bacteria and antibiotic resistant bacteria (Guarner & Malagelada, 2003; Jernberg et al., 2010).

Since the late 1940s, gastrointestinal complications have been associated with the use of antibiotics. In the first thirty years of antibiotic use, most gastrointestinal complications were believed to be caused by the microorganism, *Staphylococcus aureus* (Bartlett, 2010). During the 1970s, after the introduction of the antibiotic, clindamycin, there was a noticeable increase in antibiotic-associated colitis, also referred to as clindamycin-associated colitis. Physicians reported a 20% incidence of diarrhea in patients who received clindamycin and a 10% incidence of pseudomembranous colitis

(Luciano & Zuckerbraun, 2014). It was not until 1978 when Bartlett and his colleagues determined *Clostridium difficile* to be the causative agent of clindamycin-associated colitis, which was later called antibiotic-associated pseudomembranous colitis (Luciano & Zuckerbraun, 2014). The colonization of *C. difficile* was initially seen in patients using clindamycin; however, *Clostridium difficile* infections (CDIs) are associated with the use of antibiotics in general, but especially after the administration of clindamycin, cephalosporins, fluoroquinolones, and β-lactams (Luciano & Zuckerbraun, 2014; Surawicz, 2015; Walters & Zuckerbraun, 2014).

Clostridium difficile is the most common cause of infectious hospital acquired diarrhea and is known to cause 10% to 35% of all cases of antibiotic-associated diarrhea (Khan & Elzouki, 2014). Within the last two decades, the incidence of CDIs has increased significantly (To & Napolitano, 2014). The incidence of CDIs has more than tripled from 1996 to 2005, and continued to increase through 2008 (Luciano & Zuckerbraun, 2014). Recent data from 2008, documented 350,000 CDI cases at the time of discharge from acute care hospitals (Walters & Zuckerbraun, 2014). Another concern with *Clostridium difficile* is the emergence of community-acquired CDIs. These infections are becoming more common among younger individuals who are at low risk and lack the traditional risk factors associated with CDIs (Khanna, Pardi, Aronson, Kammer, & Baddour, 2012). Most CDI cases occur in the United States, Canada, and Europe. These outbreaks are attributed to the increased use of broad-spectrum antibiotics, the evolving demographics in hospitalized patients (elderly vs. young), contaminated hospital surfaces, community-acquired CDIs, and the emergence of the hypervirulent *C. difficile* strain, BI/NAP1/027 (Khan & Elzouki, 2014).

CHAPTER 2

INTRODUCTION

Healthcare Associated Infections

Healthcare-associated infections (HAIs), or hospital/nosocomial infections, occur in hospitals and other healthcare settings, and are considered infections that are not present or incubating at the time of patient admission (World Health Organization; WHO, n.d.). Healthcare-associated infections are considered the most frequent adverse health event in healthcare settings (WHO, n.d.). HAIs are accountable for an excess of morbidity and mortality among hospitalized patients annually (Office of Disease Prevention and Health Promotion, 2014). In 2011, roughly 75,000 hospitalized patients with an HAI died during their hospitalization (Centers for Disease Control and Prevention; CDC, 2014). Each year, these infections cost the U.S. health care system billions of dollars in excess healthcare costs, in addition to the thousands of lives lost. Hospital-onset *Clostridium difficile* infections (CDIs) are estimated to cost anywhere between $5000 to $7200 per case in annual excess healthcare costs (Walters & Zuckerbraun, 2014). The national estimate in excess healthcare costs due to CDIs range from $897 million to $1.3 billion (Walters & Zuckerbraun, 2014). A survey on acute care hospitals in the United States determined that 1 in 25 hospitalized patients have at least one HAI (CDC, 2014). At any given time, 7 out of 100 hospitalized patients in developed countries will acquire at least one healthcare-associated infection during their hospitalization (WHO, n.d.). In 2011, there were approximately 722,000 HAIs in acute care hospitals in the United States. Of those HAI cases, about 123,000 were due to

gastrointestinal illness, which is the second most common HAI, after pneumonia (CDC, 2014). According to the Centers for Disease Control and Prevention (2013), while most HAIs are declining, one HAI in particular, *Clostridium difficile* infection, is on the rise. *Clostridium difficile* infections remains at an all-time high and has currently replaced methicillin-resistant *Staphylococcus aureus* (MRSA) as the most common HAI (Lessa, Gould, & McDonald, 2012). Approximately 80% of all *Clostridium difficile* infections are HAIs, but community-acquired *Clostridium difficile* infections are becoming more common (Khan & Elzouki, 2014). Millions of patients around the world are affected by HAIs each year (WHO, n.d.). Healthcare-associated infections are not only emotionally devastating, but can also result in catastrophic medical and financial consequences.

Epidemiology of *Clostridium difficile* Infections

During the 1990s, the incidence of *Clostridium difficile* infections in acute care hospitals in the United States was relatively low, with a rate of 30 to 40 cases per 100,000 (Kelly & LaMont, 2008). According to the Agency of Healthcare Research and Quality, the amount of CDI cases at the time of patient discharge doubled in the United States during the early 2000's from 139,000 to 301,200 cases (Walters & Zuckerbraun, 2014). Due to the increase in CDI incidence, the mortality rate increased from 5.7 to 23.7 deaths per million persons (Drekonja, 2014). Since the 1990s, the rate of hospital discharges that list CDI as the main diagnosis increased from 3.82 per 1,000 discharges in 2000 to 8.75 per 1,000 discharges in 2008. This increase was most prevalent among patients 65 years and older. In 2009, the rate of CDI hospital discharges began stabilizing with a 2.5% decrease, and the hospital discharge estimate decreased to 8.53 per 1,000 discharges. However, these hospital rates do not include CDI cases that are managed in

an outpatient setting without the need of hospitalization. In 2006, Ohio made all inpatient and outpatient CDI reporting mandatory. From this information the CDI burden in Ohio was generalized to the entire U.S. population, which suggested that 333,000 initial and 145,000 recurrent healthcare facility-onset CDI cases occur annually within the United States (Lessa et al., 2012).

Data collected from 28 community hospitals in the southern United States have suggested that *Clostridium difficile* is 21% more common than methicillin-resistant *Staphylococcus aureus* (Weber, Anderson, Sexton, & Rutala, 2013). Vital records within the United States specify that the number of deaths with *C. difficile* enterocolitis listed as the primary cause of death increased from 793 in 1999 to 7,483 in 2008. The age-adjusted death rate for *C. difficile* showed a 15% increase from 2007 to 2008 (Lessa et al., 2012). As of 2008, a total of 93% of deaths from *C. difficile* occurred in those 65 years and older, and CDI was reported as the 18th leading cause of death in this age group. In 2014, *Clostridium difficile* was estimated to cause more than 500,000 infections per year (Walters & Zuckerbraun, 2014). As the morbidity and mortality rates increase for CDIs it is more important than ever to alleviate the burden of this infection.

Despite the high rates of hospital-acquired CDIs, community-acquired CDIs are becoming more common, accounting for more than one-third of all CDIs (Leffler & Lamont, 2011). In 1994, the incidence of community-acquired *C. difficile* infections was estimated at less than 7 per 100,000 patients per year. More recent data from 2005 suggest that community-acquired CDIs have increased to roughly 40 cases per 100,000 persons (Leffler & Lamont, 2011).

Clostridium difficile

Clostridium difficile is an anaerobic, gram-positive, spore forming rod (van den Berg, Vaessen, Endt, Schülin, van der Vorm, & Kuijper, 2007). Hall and O'Toole first isolated *Clostridium difficile* in 1935 from meconium and feces of newborn infants (Khan & Elzouki, 2014). While most vegetative cells are susceptible to environmental stressors, bacterial endospores can survive in harsh environments. One of the most important events for *C. difficile* infection and disease transmission is sporulation. The production of endospores allows *Clostridium difficile* to remain dormant in the environment for a prolonged period of time without the necessary nutrients for survival. Once the environment becomes more favorable, *C. difficile* becomes vegetative and capable of causing infection. *Clostridium difficile* endospores are resistant to the gastric acid within the stomach and will germinate once they reach the bowel (To & Napolitano, 2014). Certain characteristics of *C. difficile* promote environmental survival and transmission, which include sporulation, a low infectivity dose, and resistance to disinfectants (Weber et al., 2013). Although vegetative *C. difficile* cells can only survive for 15 minutes in dry environments, endospores are persistent for five months in the environment (Weber et al., 2013).

Since the 1990's, the severity of CDIs has increased due to the increase in virulence. The frequency and severity of CDIs is the result of the hypervirulent *Clostridium difficile* strain, BI/NAP1/027 or NAP-1/027. This particular strain has been linked to changes in CDI disease pathogenesis in the United States, Canada, and Europe (Walters & Zuckerbraun, 2014). NAP-1/027 has been known to cause increased

mortality in those experiencing fulminant colitis and has been recovered from more than 35% of all CDI cases (Walters & Zuckerbraun, 2014). *Clostridium difficile* strain NAP-1/027 has mutations in the *tcdC* gene that inhibit toxin transcription. A mutation in this regulatory gene leads to toxin production 10 times greater than less virulent strains (Walters & Zuckerbraun, 2014). This particular strain also has higher rates of germination and sporulation, which is known to contribute to the virulence factor.

Pathogenesis of *Clostridium difficile*

The normal gut flora of an individual is the first line of defense against infection. However, after antibiotic treatment, the normal gut flora is depleted, allowing *C. difficile* to proliferate and adhere to host tissue. Adhesion is an important mechanism for the full expression of virulence in a microorganism (Borriello, 1998). The pathogenicity of *Clostridium difficile* is dependent on the production of two toxins, enterotoxin A (*tcdA*) and cytotoxin B (*tcdB*) (van den Berg, Juijper, Bruijnesteijn van Coppenraet, & Claas, 2006). Approximately, 50% to 60% of *C. difficile* strains are considered toxicogenic. The majority of *C. difficile* strains produce both toxin A and B, but 1% to 2% only produce toxin B (Bartlett, 2010).

Toxins A and B are transcribed from a 19.6 kb pathogenicity locus that consists of five genes: Toxin A and B, and three regulatory genes. Of the three regulatory genes, *tcdR* is a positive regulator of transcription; *tcdC* inhibits toxin transcription, while the role of *tcdE* is uncertain. It is believed that *tcdE* facilitates the release of toxins by lysing the cytoplasmic membrane (Kelly & LaMont, 2008). Toxin A was generally regarded as the most important factor causing enteropathogenic disease; however, recently there has

been an increase in toxin A negative, toxin B positive disease-causing *C. difficile* strains (van den Berg, et al., 2006). It is more evident that toxin B is an essential element for the onset of CDI (Bartlett, 2010). In non-toxicogenic strains of *C. difficile*, the pathogenicity locus is replaced with 127 bases of non-coding DNA (Borriello, 1998).

Toxin A acts primarily on the intestinal epithelium, which triggers the immune response causing local tissue damage or necrosis, fluid secretion, and inflammation (Khan & Elzouki, 2014). Toxin B signals the release of cytotoxins causing the destruction of the cytoskeleton, which supports cell function. Toxin B is considered 1,000 times more potent than toxin A (To & Napolitano, 2014). Toxins A and B are internalized after binding to receptors of the large bowel. The toxins then disrupt intracellular signaling pathways regulated by Rho GTPases, a family of small signaling G proteins. Toxins A and B disrupt the integrity of the colon by activating epithelial cell apoptosis, which recruits polymorhponuclear neutrophils to the site of toxin action. In the presence of polymorhponuclear neutrophils, CDIs are then considered pseudomembranous colitis (Khan & Elzouki, 2014).

Clinical Manifestations

Clostridium difficile infections occur almost exclusively in the large bowel (Voth & Ballard, 2005). *Clostridium difficile* is the causative agent of antibiotic-associated diarrhea (CDAD), pseudomembranous colitis (PMC), and toxic megacolon (O'Neill, Ogunsola, Brazier, & Duerden, 1996). However, CDIs can also progress to fulminant colitis, which is often due to poor medical management (Walters & Zuckerbraun, 2014). Fulminant colitis refers to the sudden and quick onset of colitis. It is considered so

intense and severe that it can lead to death. Fulminant colitis only occurs in about 5% of CDI patients, but is associated with a mortality rate of 35% to 80% (Walters & Zuckerbraun, 2014). Patients who develop diarrhea or colitis during or after treatment with an antibiotic are believed to have *C. difficile*-induced diarrhea or colitis (Kato, Ou, Kato, Bartley, Brown, Dowell, & Ueno, 1990).

The disease associated with *Clostridium difficile* can range from mild self-limiting diarrhea to severe diarrhea, PMC, and fulminant colitis which can be fatal (Khan & Elzouki, 2014; Noren, 2010). The onset of CDI symptoms is often sudden and an individual will experience watery, foul-smelling diarrhea, as well as abdominal pain, elevated white blood cell counts, and possible fever (Borriello, 1998). Mild cases of CDIs are defined by three or more loose stools in a 24 hour period and are generally self-limiting, but for cases that persist symptoms will worsen and can result in fulminant colitis (Noren, 2010; To & Napolitano, 2014). Fulminant colitis is characterized by PMC with white fibrin-covered protrusions throughout the colonic wall (Noren, 2010). Fulminant colitis and PMC can lead to multiple organ dysfunction syndrome and ultimately death (To & Napolitano, 2014). Severe cases of CDI may show improvement after diarrhea has resolved, but this is often a sign of paralytic ileus or toxic megacolon (Khan & Elzouki, 2014; Noren, 2010).

Diagnosing *Clostridium difficile* Infections

Since the 1970s, scientists have been discovering ways to identify and diagnose CDIs. The cytotoxin assay was the initial test used to diagnose CDIs in 1978. The cytotoxin assay is about 95% sensitive and 98% specific for detecting CDI in patients

with pseudomembranous colitis, making it the gold standard for *C. difficile* detection. Although the cytotoxin test is considered the standard, there are some disadvantages associated with this test. This test is considered a demanding and expensive test, which generally takes about 24 to 72 hours to obtain results, and the reagents are not readily available. Despite the fact that there are faster, easier, and less expensive ways to diagnose CDIs, the cytotoxin assay is still the gold standard (Bartlett, 2010).

The latex agglutination assay (LAT) was introduced in the early 1980s to detect the *Clostridium difficile* toxin A. However, it did not actually detect the toxin it was designed to detect; instead, it detected a protein of *C. difficile*. The LAT is now commercially available to detect *C. difficile* itself and not toxin A. The LAT is typically used in combination with the enzyme immunoassay (EIA) to detect CDIs. The EIA test for the diagnosis of *Clostridium difficile* was also introduced in the 1980s. The EIA test is used to detect *C. difficile* toxins, as well as *C. difficile* itself. It is used to detect either toxin A or the combination of toxin A and B. Presently, testing for both toxins A and B is preferred over toxin A alone. The EIA test has become the most frequently used CDI detection method in the U.S. and the world due to its rapid results, and relatively low cost. Some of the disadvantages surrounding this test include decreased sensitivity resulting in possible false-negatives (Bartlett, 2010).

The culture-toxin test is rarely used in U.S. laboratories. This test requires alcohol-shocked or heat-shocked stool to select for Clostridia. The cultures are incubated, and then tested for *C. difficile* toxins. This test is highly sensitive and is also considered a gold standard for CDI diagnosis. As with any detection method, the culture-

toxin test can also result in false-positives (Bartlett, 2010). The most recent test introduced for the detection of *C. difficile* toxins is polymerase chain reaction (PCR). PCR detects the genes responsible for the production of *C. difficile* toxin A and B. It is advantageous because it provides quick results, and PCR reagents from commercial sources are readily available. The greatest advantage of PCR testing is the high sensitivity of PCR technology (Bartlett, 2010). However, PCR cannot distinguish between viable and nonviable cells, and inhibition can limit the detection of positive samples.

The United States currently uses EIAs for toxin A and B, but it will eventually be replaced by Glutamate Dehydrogenase (GDH)-based combination tests or PCR for the detection of toxicogenic *Clostridium difficile*. GDH-based combination tests detect the GDH enzyme, which is produced in significantly higher quantities than the *C. difficile* toxin itself and yields a more sensitive assay than toxin EIAs. However, GDH detection does not distinguish between toxicogenic and non-toxicogenic *C. difficile* strains (Bassetti, Villa, Pecori, Arzese, & Wilcox, 2012). GDH-based combination tests are a highly specific assay that can rule out the presence of *C. difficile* in stool samples (Goldenberg, Cliff, & French, 2010). GDH-based combination tests and PCR are rapid, sensitive, and specific within noted limitations (Bartlett, 2010).

Treating *Clostridium difficile* Infections

The first step in treating a CDI is to stop all current antibiotic treatments and provide supportive care, such as electrolyte replacement. A *Clostridium difficile* infection can be treated with the use of CDI specific antibiotics. Oral vancomycin and

metronidazole are the most commonly used antibiotics to treat CDIs (Bartlett, 2010). These antibiotics tend to be equally effective; however, patients with a more serious case of CDI respond better to oral vancomycin because oral vancomycin goes directly to the site of infection (Bartlett, 2010). In recent years, CDIs were showing an increased resistance to fluoroquinolones. Any previous use of fluoroquinolones or clindamycin to treat a CDI is now correlated with the development of high-level *C. difficile* resistance to those antibiotics (Keller, 2010).

Treatment for CDIs is based on the severity of infection. Mild to moderate CDIs are treated with 500 mg oral metronidazole, taken three times a day for 10 days. Severe CDIs require 125 mg oral vancomycin, taken four times a day for 10 days. Severe-complicated CDIs are treated with 500 mg intravenous metronidazole every 8 hours and 125 to 500 mg of oral vancomycin four times a day. In the presence of ileus or abdominal distention, the administration of 500 mg vancomycin in 500 ml of saline per rectum four times a day is required (Luciano & Zuckerbraun, 2014).

In 2011, the U.S. Food and Drug Administration approved fidaxomicin for use in the treatment of CDIs, and studies have shown a decrease in the recurrence of CDIs compared with vancomycin use (Ritter & Petri, 2013). Fidaxomicin is one of the first RNA polymerase inhibiting antibiotics with a very narrow spectrum of antibiotic activity (Hostler & Chen, 2013). Fidaxomicin is effective against *Clostridium difficile* and *C. perfringens*. This particular antibiotic has little to no activity on the normal enteric flora (Hostler & Chen, 2013). Fidaxomicin is administered in doses ranging between 100 to 400 mg/day for 10 to 48 days, depending on CDI severity. *Clostridium difficile*

infections were cured 100% of the time in individuals receiving 400 mg of fidaxomicin per day (Hostler & Chen, 2013).

Recurrent *Clostridium difficile* Infections

Recurrent CDIs are infections that occur within 8 to 12 weeks of a previous infection due to the altered microbial flora within the gastrointestinal tract (Luciano & Zuckerbraun, 2014; Surawicz, 2015). An estimated 10% to 25% of patients develop recurrent CDIs within 12 weeks of the initial CDI (Luciano & Zuckerbraun, 2014). Treatment for first recurrent CDI is the same as an initial CDI. A second recurrent CDI requires a pulsed and tapered vancomycin treatment consisting of 125 mg of oral vancomycin for seven weeks. A third episode of CDI can be treated with fecal microbiota transplantation or fecal microbiota therapy (FMT) in an attempt to recolonize the colonic flora (Drekonja, 2014; Luciano & Zuckerbraun, 2014).

FMT consists of the infusion of healthy donor stool into the gastrointestinal tract of another individual to cure a specific illness (Aroniadis & Brandt, 2013). The effectiveness of FMT is dependent on the relationship to the stool donor, the route of administration, the volume administered, and previous treatment. FMT is effective in 92% of patients with no adverse reactions (Walters & Zuckerbraun, 2014). Several studies have shown that FMT has great therapeutic potential for CDIs (Aroniadis & Brandt, 2013); however, FMT has not been approved by the Food and Drug Administration (Luciano & Zuckerbraun, 2014). The pharmaceutical company, Sanofi Pasteur, is currently developing an oral vaccine using nonpathogenic endospores to

stimulate *C. difficile* antigen immunity. Clinical trials are expected to begin in 2015 (Luciano & Zuckerbraun, 2014).

CDI Risk Factors

The vast majority of CDI cases are seen in individuals on antibiotic treatments, those undergoing medical procedures, hospitalized patients, elderly patients, and individuals with compromised immune systems. However, the risk for CDIs in patient populations that were previously unrecognized has increased. More recent cases of CDIs have shown an increased morbidity in patients not receiving antibiotics, pediatric patients, pregnant women, and community-acquired CDIs (Bartlett, 2010; Khan & Elzouki, 2014).

The most important risk factor for CDIs is the recent administration of antibiotics, within the last three months. All antibiotics have been linked to CDIs; however, clindamycin, fluoroquinolones, cephalosporins, and β-lactams are most often implicated in CDIs (Luciano & Zuckerbraun, 2014; Surawicz, 2015; Walters & Zuckerbraun, 2014). Frequent use of broad-spectrum antibiotics is the most widely recognized modifiable risk factor (Walters & Zuckerbraun, 2014). Antimicrobials that are active against anaerobic bacteria, such as *Clostridium difficile*, are considered the greatest risk for the development of a CDI because of their ability to alter gastrointestinal flora (Khan & Elzouki, 2014). Other risk factors include age, the use of proton pump inhibitors, immunosuppressants, inflammatory bowel disease, recent surgery, nasogastric tube feeding, and prolonged hospitalization (Khan & Elzouki, 2014).

Community-acquired CDI cases are seen in individuals that did not receive antibiotic treatment prior to the development of symptoms (Khan & Elzouki, 2014). The transmission of community-acquired CDIs is not widely studied, but it is believed to be transmitted through contaminated food and water. Another theory suggests there is an increase in asymptomatic *Clostridium difficile* carriers, which leads to an increase in person-to-person transmission (Khanna et al., 2012)

CDI Control and Prevention

Preventing CDIs is an ongoing challenge for healthcare facilities. Infection control measures such as good hand-hygiene, early contact isolation, and barrier precautions are practiced to help alleviate the risk of CDI transmission (Surawicz, 2015). Environmental cleaning programs require the use of bleach-based disinfectants or EPA approved sporicidal disinfectants, which contain at least 5000 ppm chlorine (Luciano & Zuckerbraun, 2014; Surawicz, 2015). Antibiotic stewardship programs can decrease the incidence of CDIs in hospitals by promoting the appropriate use of antimicrobials. Prompt CDI patient identification is a key factor in infection control because the majority of CDI patients will respond to antimicrobial therapy if the antimicrobials are administered promptly (Khan & Elzouki, 2014). The United Kingdom's National Health Service was able to reduce the rate of CDIs by 60% by limiting the use of broad-spectrum cephalosporins and fluoroquinolones in conjunction with other control and prevention measures (Luciano & Zuckerbraun, 2014).

Clostridium difficile on Environmental Surfaces

Clostridium difficile is mainly transmitted via the fecal-oral route or aerosolized endospores, but it can be transmitted from patient-to-patient, healthcare worker-to-patient, or from contaminated surfaces in the hospital, such as door handles, bed rails, and bed pans (Mutters, Nonnenmacher, Susin, Albrecht, Kropatsch, & Schumacher, 2008). In a hospital setting, there are three main modes of transmission aside from fecal-oral transmission (Figure 1). The first mode of transmission is the direct transfer of *C. difficile* via hands to a non-infected patient. The second mode of transmission is the transfer of *C. difficile* through the contaminated environment with direct inoculation into the mouth or into the colon. The final mode of transmission is from the contaminated environment to a healthcare worker and the indirect transfer to a non-infected patient (Weber, Rutala, Miller, Huslage, & Sickbert-Bennett, 2010).

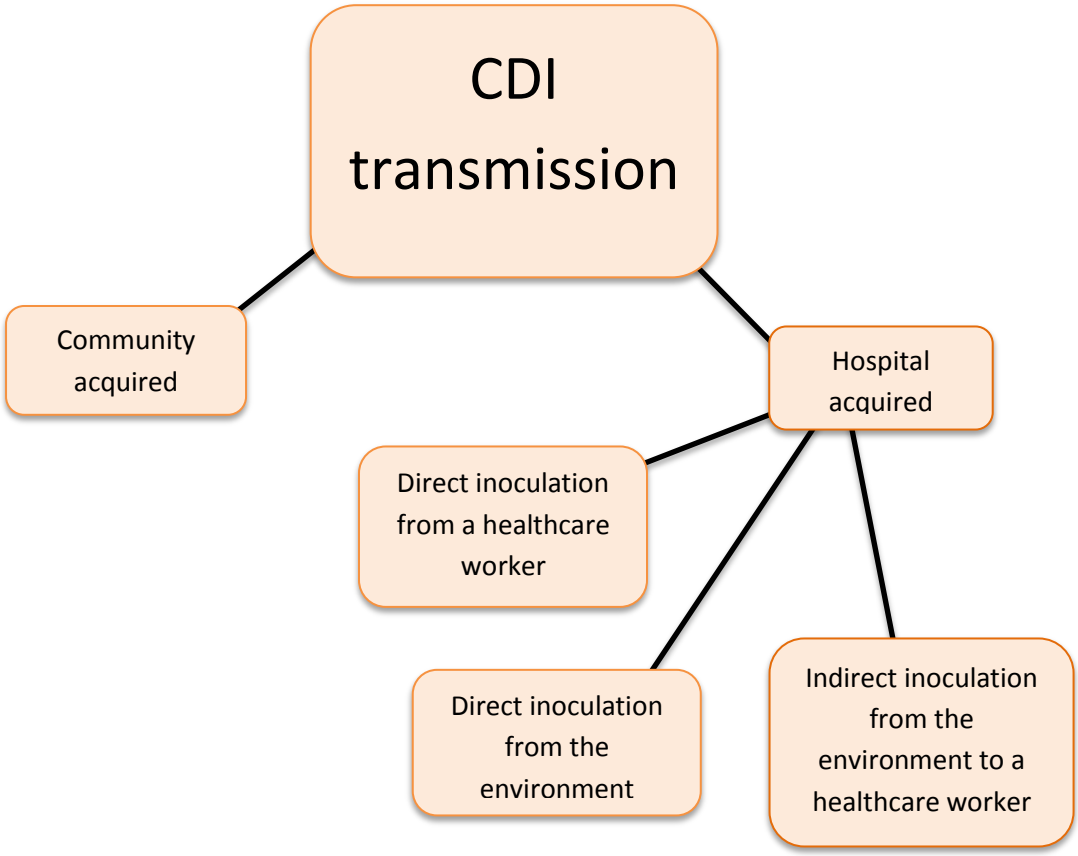


Figure 1. CDI modes of transmission (Weber et al., 2010)

An infected individual with acute diarrhea may defecate 10^7 or 10^9 microorganisms per gram of stool (Mutters et al., 2008). These microorganisms can contaminate the environment with vegetative cells and spores. Asymptomatic carriers can also contribute to environmental contamination and may be one of the causes of endemic diseases in hospitals (Mutters et al., 2008). There are between 1% and 2% of healthy adults who harbor *C. difficile* asymptomatically, and approximately 10% to 30% of hospitalized patients are colonized with this organism (Bartlett, 2010). The prevalence of *C. difficile* spores in the environment is relatively high in long-term healthcare facilities and hospitals (Bartlett & Gerding, 2008). The presence of *C. difficile* spores can

range between 10% and 15% in areas where infected patients are present (Mutters et al., 2008). *Clostridium difficile* spores are a major concern for HAIs because spores are not easily destroyed with regular decontamination procedures; therefore, they require special infection control strategies to avoid transmission throughout the hospital environment.

Contaminated environmental surfaces are considered a potential source of transmission for many healthcare associated infections (Donskey, 2013). Generally, surfaces in the surrounding area of an infected patient are more contaminated than more distant surfaces; however, patients with diarrhea can cause widespread contamination (Otter, Yezli, Salkeld, & French, 2013). Numerous studies have shown that environmental surface cleaning interventions can not only increase the effectiveness of cleaning, but also reduce the amount of contamination on environmental surfaces (Donskey, 2013). Ineffective decontamination after patient discharge can result in an increased risk of infection of the same pathogen in the next patient room occupant. This has been shown for many microorganisms including, *C. difficile*, methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, and other multidrug-resistant microorganisms (Otter et al., 2013). It is currently believed that environmental contamination contributes to the transmission of *Clostridium difficile* in a hospital setting (Weber et al., 2010).

In a study conducted by Mutters et al. (2008), a total of 531 environmental samples were collected. The environmental samples were collected and analyzed for the presence of *C. difficile* from the hands of patients and health care workers, toilets, beds, and other surfaces near the patients using a flocked swab. These samples were then

classified into three different groups based on patient and hospital ward status for *C. difficile*. The three groups were: 1) *C. difficile* positive, ward positive, 2) *C. difficile* negative, ward positive, and 3) *C. difficile* negative, ward negative. PCR analysis determined that the presence of *C. difficile* in the environment was greater near *C. difficile* patients. In addition, the environment of *C. difficile*-positive patients had significantly higher counts of bacteria on the floor and the near environment (<40 inches) (Mutters et al., 2008).

Recently, the interest in *Clostridium difficile* has grown due to the considerable increase of CDI cases and the increase in morbidity and mortality (Bartlett, 2010). Important factors in controlling the rate of *Clostridium difficile* infection are through infection control, antibiotic control, and hand hygiene (Bartlett, 2010). Determining the presence of *Clostridium difficile* spores in the hospital environment is important to evaluate decontamination protocols after a patient with a CDI is discharged. *Clostridium difficile* spores are difficult to eliminate from the environment with detergent based cleaners (Mutters et al., 2008). In addition, many healthcare facilities may not be cleaning rooms thoroughly. One study showed that only 47% of high touch surfaces in three hospitals were being cleaned (Carling, Briggs, Perkins, & Highlander, 2005). If healthcare facilities were able to eliminate *C. difficile* spores after a known case of CDI is discharged from the hospital, this would further reduce the risk of *Clostridium difficile* infections.

Objective

The objective of this study was to determine the presence of *Clostridium difficile* in healthcare facility environments pre- and post-patient room decontamination. Environmental samples were collected from seven high touch surfaces (i.e., floor, call bell/TV remote, telephone, bathroom doorknob, toilet flush lever, bed rail, and bedside table) and analyzed using culture and real-time PCR analysis with species-specific primers and probes. The results were used to assess the effectiveness of decontamination practices to reduce the prevalence of *C. difficile* in patient rooms.

Research Questions and Hypotheses

1) Will *Clostridium difficile* be detected on high touch environmental surfaces in the rooms of infected patients pre- and post-decontamination?

2) Are the decontamination procedures used in healthcare facilities effective in reducing the prevalence of *Clostridium difficile*?

Therefore, the proposed hypotheses were:

H^1_0: There is no difference in the prevalence of *Clostridium difficile* on environmental surfaces pre- and post-decontamination of patient rooms.

H^1_a: There is a difference in the prevalence of *Clostridium difficile* on environmental surfaces pre- and post-decontamination of patient rooms.

H^2_0: There is no difference between the effectiveness of decontamination procedures used in healthcare facilities to reduce the prevalence of *Clostridium difficile*.

H^2_a: There is a difference between the effectiveness of decontamination procedures used in health care facilities to reduce the prevalence of *Clostridium difficile*.

CHAPTER 3

MATERIALS AND METHODS

Study Design

This study was conducted at three healthcare facilities in Southern Nevada. Environmental swab surface samples were collected on six high touch surfaces from healthcare facilities in rooms of patients with diagnosed cases of *C. difficile*. No patient identifying information was provided to UNLV personnel. An Institutional Review Board (IRB) application was submitted and approved for exemption from IRB review (IRB Protocol # 1408-4890M; Appendix A). Prior to patient room decontamination, seven surface samples were collected from high touch surfaces. Following patient room decontamination, the seven corresponding post-decontamination samples were collected for a total of 14 samples per room (Figure 2). A total of 10 rooms were sampled for a total of 128 environmental samples collected. Following sample collection, the samples were processed and analyzed for the presence of *Clostridium difficile* using culture analysis and real-time PCR.

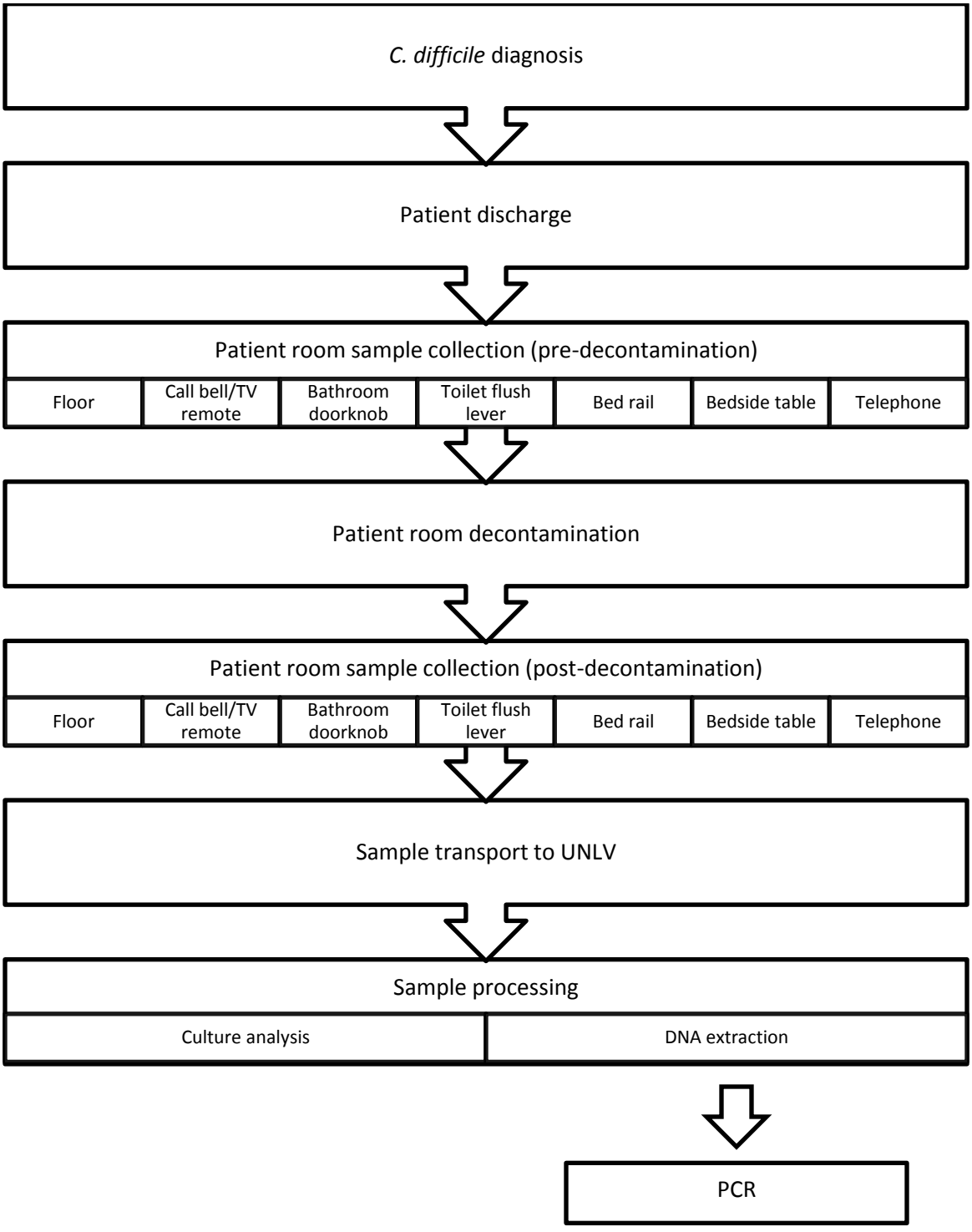


Figure 2. Study design flow chart

Sample Collection

Pre-labeled sampling kits were distributed to the participating healthcare facilities along with environmental investigation data sheets (Appendix B). Hospital personnel were trained in the procedures of the surface sampling protocol. The environmental swabs and transport containers were pre-labeled with a number and letter designation to match the sample location and whether the sample was pre- or post-decontamination. The first set of surface samples were taken after patient discharge, pre-patient room decontamination using the Centers for Disease Control and Prevention's (CDC) surface sampling procedures for *Bacillus anthracis* spores from smooth, non-porous surfaces (Appendix C). The second set of environmental surface samples were taken post-patient room decontamination using the same method. All environmental samples collected were stored at 4°C and transported within 24 hours to the Emerging Diseases Laboratory (EDL) at the University of Nevada, Las Vegas (UNLV) for analysis. The high touch surfaces tested were determined based on previous research, and consisted of: the floor, call bell/TV remote, telephone, bathroom doorknob (inside and outside), toilet flush lever, bed rail, and bedside table (Mutters et al., 2008).

Hospital Decontamination Methods

Post patient discharge, patient room decontamination procedures required a systematic cleaning of rooms from cleanest to dirtiest surfaces. Two facilities use the disinfectant, Dispatch®, which contains sodium hypochlorite (0.5%), to perform terminal cleaning. One facility utilizes timed terminal cleaning, meaning hospital personnel clean surfaces for 45 minutes followed by disinfection using Dispatch®. The other facility uses similar cleaning procedures; however, they do not utilize timed terminal cleaning

before disinfection with Dispatch®. The third facility uses a quaternary ammonium compound for terminal cleaning followed by the use of a UV (Tru-D SmartUVC, Memphis, TN) light for 35 minutes. Once terminal cleaning is completed, the UV light is then placed in the room to ensure the entire room and bathroom is exposed. If it is not possible to cover the room and bathroom simultaneously, the light is run in each area for 35 minutes separately.

Sampling Methods

The bed rail, call bell/TV remote, bathroom doorknobs, and toilet flush lever samples were collected from an area of 26 cm^2 by trained hospital personnel using sterile, foam tipped swabs (Puritan ™, Guilford, ME). All sterile swabs were transported in 10 ml of neutralizing buffer (Hardy Diagnostic, Santa Maria, CA). Wearing clean gloves, hospital personnel aseptically removed the sterile swab from the transport container and expressed any excess buffer using the side of the transport container. Applying light pressure, the surface was swabbed in an overlapping 'S' pattern to cover the entire surface with horizontal strokes. The swab was then rotated 180° and the same area was swabbed again using vertical 'S' strokes. The head of the exposed swab was broken off into the transport container. The floor and bedside table samples were collected from an area of 645 cm^2 using sterile, sponge-sticks (3M™, St. Paul, MN). Wearing clean gloves, hospital personnel aseptically removed the sterile sponge from the packaging and placed the sponge into a transport container where 10 ml of neutralizing buffer was added to moisten the sponge. Applying light pressure, the surface was sampled using a push-pull method in an overlapping 'S' pattern to cover the entire surface with horizontal strokes. The sponge was then rotated 180° and the same area was sampled again using

vertical ‘S’ strokes. Using the narrow side of the sponge, the area was sampled using diagonal ‘S’ strokes. The perimeter of the sampling area was wiped using the tip of the sponge. The head of the exposed sponge was broken off into the transport container. Upon completion of sample collection all samples were stored at 4°C until sample retrieval. Sample analysis procedures were adapted from the Laboratory Response Network (LRN), *Bacillus anthracis* Spore Environmental Swab and Wipe Processing Procedure (2014).

Sample Processing

Once the samples were transferred to the EDL at UNLV, all the samples were processed in the biological safety cabinet within 24 hours. The sponges were transferred from the original specimen container to a sterile, labeled Stomacher® 80 bag (Seward Laboratory Systems Inc., Davie, FL) using sterile forceps. Approximately 10 ml of neutralizing buffer was added to each stomacher bag (adapted from LRN, 2014). The sponges were then homogenized on the high setting of a Stomacher® 80 for one minute (Seward Laboratory Systems Inc.). From the outside of the bag, the sponges were moved to the top of the bag and the excess liquid was squeezed from the sponges. Using sterile forceps, the sponges were removed from the stomacher bags and discarded in a biohazard bag. The stomacher bags sat for 10 minutes to allow the elution suspension foam to settle. Approximately 7 to 10 ml of neutralizing buffer was recovered from each sponge sample. The sample was then divided for culturing, DNA extraction, and storing. The elution suspension was gently mixed in the stomacher bag and 3 ml of the elution suspension was pipetted into a sterile, labeled 4 ml cryovial to be transferred to a local commercial laboratory for culture analysis (Forensic Analytical, Las Vegas, NV). A 2 ml

aliquot of the elution suspension was pipetted into a sterile, labeled 2 ml microcentrifuge tube for concentration of the sample and DNA extraction. An additional remaining elution suspension was pipetted into a sterile, labeled cryovials and stored at -20°C.

Swab samples were processed in a biological safety cabinet. The handles of the swabs were cut off using sterile scissors for the swab to fit into a sterile, labeled 15 ml centrifuge tube. The swabs were then vortexed in 10-second intervals for two minutes to dislodge spores from the swabs. Excess fluid was expressed from the swabs and the swabs were then removed from the 15 ml centrifuge tubes using sterile forceps and discarded in a biohazard bag. Approximately 9 ml of sample was recovered from each swab. The elution suspension was vortexed and 3 ml of the elution suspension was pipetted into a sterile, labeled 4 ml cryovial for culturing. Two milliliters of elution suspension was pipetted into a sterile, labeled 2 ml microcentrifuge tube for concentration of the sample and DNA extraction. An additional 5 ml of the elution suspension was pipetted into a sterile, labeled 15 ml centrifuge tube and stored at -20°C.

Culture Analysis

Approximately 3 ml of the processed *C. difficile* sample was transferred to Forensic Analytical Laboratories, Inc. (Las Vegas, NV). For culture analysis, 100 µl was plated on tryptic soy agar (TSA; Hardy Diagnostic) and incubated at 37°C for 24 hours under anaerobic conditions. Indicator strips were used to ensure no oxygen was present. Visible colony forming units (CFU) were then transferred to BioLog Universal Anaerobe (BUA; Oxyrase, Inc., Mansfield, OH) agar and incubated for an additional 24 to 48 hours. Colonies were then placed into AN inoculating fluid (BioLog, Hayward, CA) and placed on an anaerobic AN MicroPlate (BioLog), which was incubated for 24 to 48 hours

in anaerobic conditions. The AN MicroPlate was then read by BioLog ELx808BLG software (BioTek Instruments, Inc., Winooski, Vermont) to identify the anaerobic colonies. If the sample was negative for *C. difficile*, the CFU were classified as facultative bacteria or unrelated anaerobic bacteria. Indicator strips were used to ensure no oxygen was present. Results were reported as CFU/ml and converted to CFU/sample.

DNA Extraction and Purification

For concentration of the sample, 2 ml was filtered through a 0.45 µm HAWG filter membrane (EMD Millipore, Billerica, MA) using a swinnex and a 10 ml sterile syringe. The filter was then rinsed with 8 ml of 0.01M phosphate buffer with tween (pH 7.0) and aseptically removed from the syringe and placed in a bead beater tube containing 50 mg of 425-600 µm and ≤ 106 µm diameter glass beads to begin the DNA extraction process. The DNA was extracted from the concentrated environmental samples using the Amicon DNA extraction and purification kit (EMD Millipore) following the manufacturer's protocol. Upon completion, this procedure provided approximately 100 µl of DNA, which was stored at -70ºC for future use.

Real-Time Polymerase Chain Reaction

PCR analysis of the DNA was conducted in duplicate using the 7900 HT Fast PCR System (Applied Biosystems, Foster City, CA). Positive and negative controls were included with each PCR. The primers and probe targeted a 157 base pair species-specific highly conserved region from the 16S rRNA gene sequence (Mutters et al., 2008). The sequence of the forward primer was 5'TTGAGCGATTTACTTCGGTAAAGA3'and the sequence of the reverse primer was 5'CCATCCTGTACTGGCTCACCT3'. A TaqMan® probe was used, and the sequence was as follows, 6-FAM-

5’CGGCGGACGGGTGAGTAACG3’-TAMRA. An internal positive control (IPC) was included in the reaction to test for inhibition in the samples (Applied Biosystems). Quantitative PCR (QPCR) was used to determine the concentration of the target gene in environmental samples. QPCR standards consisted of serial dilutions with known *C. difficile* DNA (ATCC BAA-1871-D-5, ATCC, Manassas, VA) concentrations amplified in duplicate with environmental samples. Each PCR reaction contained a total volume of 25 µl, that included nuclease free water (Promega, Madison, WI), 1X of TaqMan® Universal PCR Master Mix (Applied Biosystems), 0.5 µM of the *C. difficile* forward primer (Eurofins MWG Operon, Huntsville, AL), 0.5 µM of the *C. difficile* reverse primer (Eurofins MWG Operon), 0.15 µM of the *C. difficile* probe (Applied Biosystems) and 5µl of template DNA. The instrument was operated in standard mode with the following parameters: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C followed by 1 min at 60°C. After amplification, the DNA was analyzed using the 7900 HT Fast PCR system (Applied Biosystems). The software constructed a standard curve of cycle threshold (C_T) values versus DNA concentration. The C_T value is the number of PCR cycles required for detectable amplification and is inversely proportional to the concentration of DNA in the sample. The standard curve was then used to determine the number of cells in the reaction and the number of cells per sample was calculated.

Data Analysis

A total of 128 samples were collected from 10 rooms during this study. The mean CFU were calculated before and after decontamination. The percent reduction was also determined. The data were analyzed using the Shapiro Wilks W test to test for normality of the distribution. The data were then analyzed using a means comparison statistical

method dependent upon the distribution of the data. The Wilcoxon Rank Sum test was the non-parametric statistical method used. This statistical method is used to compare two matched samples, related samples, or repeated measurements on a sample that does not display a normal distribution. This statistical method will determine whether the populations' means differ pre- and post-decontamination of patient rooms. Descriptive statistics were calculated using IBM SPSS Statistics version 22.

CHAPTER 4

RESULTS

Culture Analysis

A total of 128 samples were taken from 10 patient rooms, 65 pre-decontamination and 63 post-decontamination (Table 1). The detection limit for a 10 ml sample was estimated to be 100 colony forming units (CFU) per sample. Pre-decontamination samples had an average of 6,532 facultative CFU/sample. Post-decontamination samples had an average of 400 facultative CFU/sample. No viable *Clostridium difficile* was detected on any of the surfaces tested. Two related species, *Ruminococcus torques* and *Fusobacterium nucleatum*, from the class Clostridia were cultured from two of the 65 pre-decontamination samples. There were no viable organisms in the class Costridia from the 63 post-decontamination samples.

Table 1. Surface sampling design for patient rooms		
Pre decontamination:		
Letter designation:	**Location:**	**Total samples:**
A	Bed rail	9
B	Call bell/TV remote	10
C	Telephone	9
D	Bathroom doorknob	9
E	Toilet flush lever	8
F	Floor	10
G	Bed side table	10
Total pre-decontamination samples:		65
Post decontamination:		
H	Bed rail	7
I	Call bell/TV remote	10
J	Telephone	9
K	Bathroom doorknob	9
L	Toilet flush lever	8
M	Floor	10
N	Bed side table	10
Total post-decontamination samples:		63
	Total samples:	**128**

The facultative bacteria CFU count were also analyzed to determine the effectiveness of patient room decontamination (Figure 3). The bedside table had the highest CFU count pre-decontamination with an average of 23,150 CFU/sample, followed by the call bell/TV remote, and the floor. The toilet flush lever had the lowest CFU count pre-decontamination with an average of 1,114 CFU/sample. The percent reduction of facultative bacteria at each location was calculated between the average CFU/sample pre- and post-decontamination (Table 2). The percent reduction of the bathroom doorknob and toilet flush handle pre- and post-decontamination was 100%. The percent reduction between the remaining samples ranged between 83% and 97%. Two samples (4F and 9E) had a 100% reduction rate of CFU in the class Clostridia post-decontamination.

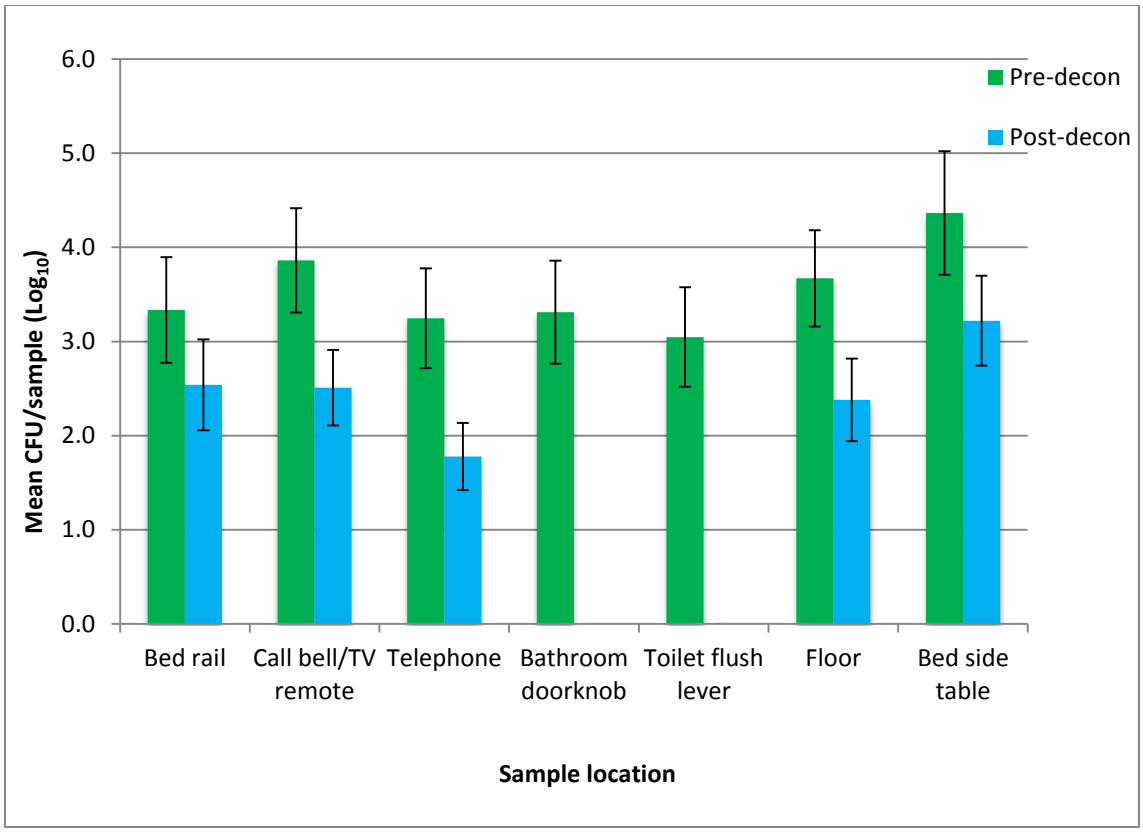


Figure 3. Mean colony forming units (CFU ± 1 standard error) from surface samples pre- and post-decontamination in patient rooms

Table 2. The percent reduction of facultative bacteria (average CFU/sample ± 1 S.E.) pre- and post-decontamination

Location:	Pre-decontamination:	Post-decontamination:	Percent reduction:
Bed rail	2160 (± 898)	347 (± 347)	83.9%
Call bell/TV remote	7254 (± 5727)	324 (± 295)	95.5%
Telephone	1770 (± 1033)	60 (± 40)	96.6%
Bathroom doorknob	2050 (± 1261)	< LDL	100.0%
Toilet flush lever	1114 (± 961)	< LDL	100.0%
Floor	4680 (± 1678)	240 (± 147)	94.9%
Bed side table	23150 (± 11333)	1660 (±1594)	92.8%

The three facilities utilized different decontamination methods post-patient discharge, and the percent reduction of facultative bacteria for each method was determined (Table 3). Decontamination by UV light had a 96.3% reduction in the concentration of viable bacteria per sample. The use of Dispatch® after terminal cleaning had a 95.8% reduction, while the use of Dispatch® following a timed terminal cleaning of 45 minutes had a 92.9% reduction in CFU/sample.

Table 3. Decontamination method and average CFU/sample (± 1 S.E.) pre- and post-decontamination

Decontamination Method:	**Pre-decontamination:**	**Post-decontamination:**	**Percent Reduction:**
UV Light (n=28)	2239 (± 1161)	84 (± 48)	96.3%
Dispatch & Time (n=66)	8289 (± 3760)	593 (± 487)	92.9%
Dispatch (n=34)	6650 (± 3201)	279 (± 202)	95.8%

No culturable *C. difficile* were detected. Therefore, culturable facultative bacterial concentrations were compared. The Shapiro Wilks W test was performed to test for normality of the distribution. The results showed a distribution that is not normal at the $\alpha = 0.05$ level ($p = 0.000$). Therefore, the non-parametric Wilcoxon Rank Sum test statistic was performed to compare the prevalence of CFU/sample in pre-decontamination samples and post-decontamination samples using SPSS version 22. The results showed a significant difference at the $\alpha = 0.05$ level between the number of CFU/sample in pre-decontamination samples and post-decontamination samples ($Z = -5.852$, $p = 0.000$). The Wilcoxon Rank Sum test was also performed for each decontamination method. Decontamination by UV light showed a significant difference between pre- and post-decontamination samples ($Z = -2.036$, $p = 0.042$). One facility required 45 minute timed

cleaning with Dispatch® (Z = -3.791, p = 0.000), while the other facility did not use timed cleaning (Z = -2.803, p = 0.005). Both facilities that utilized Dispatch® also had a significant difference between pre- and post-decontamination samples.

PCR Assay

Of the 128 samples tested using PCR analysis, five samples were positive for the *Clostridium difficile* 16S rRNA gene sequence (3.9%) (Table 4). The detection limit was estimated to be 90 DNA template copies per sample. Each sample was initially analyzed using an undiluted concentration with an internal positive control (IPC) to test for inhibition. Two undiluted samples of the 128 samples were positive. Eighty-three of the samples exhibited either partial or complete inhibition and necessitated a 10^{-1} dilution to resolve inhibition. Inhibition was determined by a three C_T increase in the IPC detector. Inhibition resolved at a 10^{-1} dilution for all samples. After the 10^{-1} dilutions were analyzed, three additional samples were positive. In total, five samples were PCR positive. From the five positive samples, three (4G, 11A, and 11F) were from pre-decontamination samples and two (6I and 6M) were from post-decontamination samples. Rooms with post-decontamination positive samples were negative pre-decontamination. Samples were considered positive if the C_T value was less than 40. The five PCR positive samples were not culture positive for *C. difficile*.

A standard curve was constructed from known *C. difficile* DNA concentrations versus C_T values. The equation of the line (y = -0.2656x + 9.9617) was then used to determine the number of cells in the reaction, and the estimated number of cells per sample was calculated.

Table 4. The average Ct values for the presence of the *Clostridium difficile* 16S rRNA gene sequence		
Sample	**Mean Ct Value (± 1 S.E.; n=4)**	**Estimated cells/sample**
4G	38.15 (± 0.56)	150
6I	37.31 (±0.32)	246
6M (1:10)	38.89 (± 0.32)*	970
11A (1:10)	37.23 (± 0.25)	2,580
11F (1:10)	35.33 (± 0.24)	7,940

*n=2

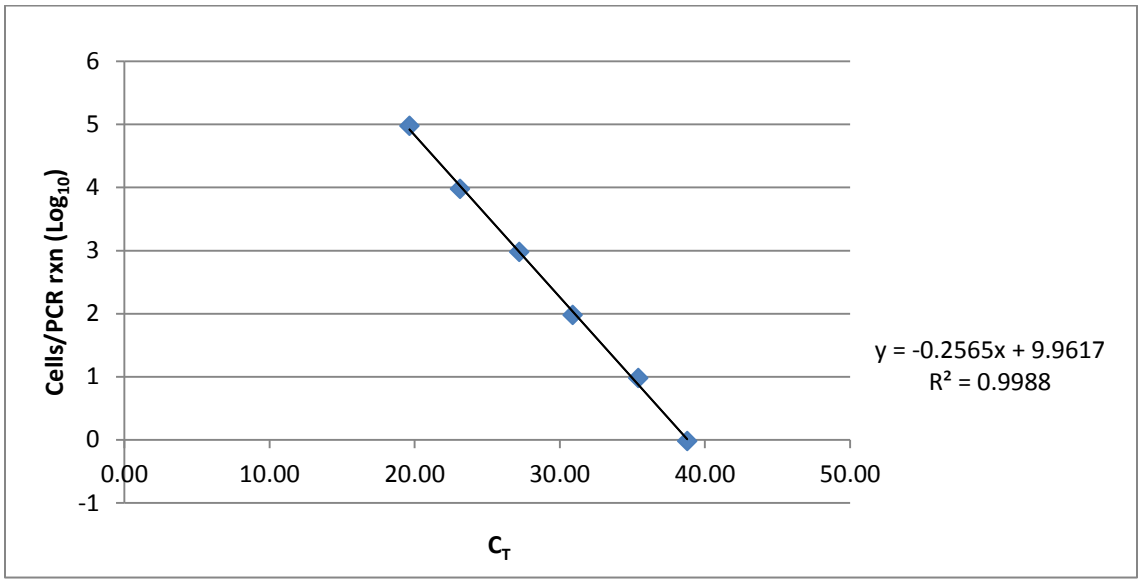


Figure 4. Standard curve of known *Clostridium difficile* DNA concentrations versus C_T values (C_T = Cycle threshold)

CHAPTER 5

DISCUSSION

Prevalence of *Clostridium difficile*

The main objective of this study was to determine the prevalence of *Clostiridum difficile* on environmental surfaces pre- and post-decontamination in patient rooms with a confirmed clinical case of CDI. Culture analysis results showed no culturable *C. difficile*. Therefore, the hypotheses could not be tested. Other studies have shown a high prevalence of *C. difficile* on the floor and in the near environment (Kaatz, Gitlin, & Schaberg, 1988; Mutters et al., 2008; Riggs, Sethi, Zabarsky, Eckstein, Jump, & Donskey, 2007). Widespread environmental contamination has been seen in up to 75% of rooms previously occupied by a CDI patient (McFarland, Mulligan, Kwok, & Stam, 1989; Weber et at., 2010). The five PCR positive samples obtained in this study did not have any culturable *C. difficile*. Research has shown that vegetative cells of *Clostridium difficile* are only viable on dry surfaces for 15 minutes (Weber et at., 2010). *Clostridium difficile* is also considered a fastidious organism in its vegetative state, meaning it will only grow under certain conditions, further limiting positive culture analysis results. The DNA detected in the PCR assay in this study could have been obtained from non-viable cells or spores after decontamination.

The two samples with viable counts from the Clostridia family were identified using the Biolog system, but were not PCR positive. This could be due to the fact that *Ruminococcus torques* and *Fusobacterium nucleatum* are not closely related to *Clostridium difficile* and do not share the same target DNA sequence. The primers and

probe set used for our PCR assay was a species specific targeting a highly conserved region of the 16S rRNA from *Clostridium difficile* (Mutters et al., 2008).

Prevalence of Facultative Bacteria

The BioLog system correctly identified the *Clostridium difficile* positive control strain. However, culture analysis of the samples was inconclusive. It is possible that the BioLog technology was unable to identify *Clostridium difficile* or it was present in low concentrations below the detection limit of the assay. In a study conducted by Simmon and colleagues, it was determined that anaerobic isolates were misidentified at the species level 24% of the time and were deemed inconclusive 10% of the time (Simmon, Mirrett, Reller, & Petti, 2007). In this study, the viable organisms present were either facultative anaerobes or strictly anaerobic bacteria not related to *Clostridium difficile*. Facultative bacteria are organisms that are able to grow in conditions with or without oxygen.

Given the symptoms of a CDI, it is possible that the bacteria cultured were coliform bacteria, which is a group of facultative bacteria most commonly found in the feces of warm-blooded animals. Common coliform bacteria include *Escherichia coli* and species from the genera, *Citrobacter*, *Enterobacter*, and *Klebsiella*. *Escherichia coli* is a well-known coliform bacterium that is part of the normal human flora; however, pathogenic strains are known to cause severe gastrointestinal illness. Another facultative bacterium of concern is *Staphylococcus aureus*, and the causative agent of the HAI methicillin-resistant *Staphylococcus aureus*. MRSA infections are generally seen on the skin and surgical sites, but it can also infect the lungs, urinary tract, and blood stream. Anaerobic bacteria of concern in hospital environments include members of the genera

Peptostreptococcus and *Bacteroides*, as well as *Clostridium perfringens*, which is known to cause gas gangrene.

Facility Differences

The three facilities tested used three different decontamination methods. From the ten rooms tested, two used a UV light post-decontamination; eight of the rooms used the EPA-registered disinfectant, Dispatch®, which is equivalent to a 1:10 bleach solution. From the eight sites utilizing Dispatch®, five of the sites required a 45 minute timed cleaning procedure prior to using Dispatch®, while the remaining two sites did not. According to the Clorox Company, Dispatch® requires a five minute contact time to kill *Clostridium difficile* spores. The three methods all proved to be effective in patient room decontamination. However, due to the low sample size for the decontamination methods, no conclusions can be made about which decontamination method is more effective.

Study Limitations

There are various limitations to this study. One limitation is the relatively small sample size. This was due to low CDI rates and long hospital admissions; this limits the strength of the conclusions obtained by comparing pre- and post-decontamination data, especially between decontamination methods. The second limitation is the potential for sampling bias. While hospital personnel were trained on sample collection, sampling bias could have occurred with different hospital personnel taking the samples. The third limitation to this study is that the PCR analysis does not determine if the DNA detected was from viable or non-viable *C. difficile* cells and endospores. Culture analysis was used to assess the presence of viable *C. difficile*, but no culturable *C. difficile* were

detected. It is possible that *Clostridium difficile* may have been present below the detection limit of the assays used.

Significance

This study showed a decontamination percent reduction of facultative bacteria of 83% to 100% in post-decontamination samples. The data suggest that each of the decontamination methods proved to be effective and patient rooms were not contaminated with *Clostridium difficile*. PCR analysis was able to detect *C. difficile* DNA on environmental surfaces. PCR provides fast results and can be used with culture analysis of environmental samples to assess the presence of target microorganisms.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

Due to the ubiquitous nature of bacteria, hospital acquired infections will continue to be a concern in hospital environments. There are an estimated 1.7 million HAIs each year and 99,000 deaths as a result (Weber et al., 2010). Although the prevalence of CDI is decreasing, it is still one of the main HAIs. The estimated annual direct cost in the USA is nearly $3.4 billion dollars for the treatment of CDIs. The Centers for Disease Control and Prevention has designated *C. difficile* as one of three microorganisms with the highest threat level (Drekonja, 2014). *Clostridium difficile* infections cause approximately 10% to 35% of all cases of antibiotic-associated diarrhea. *Clostridium difficile* associated diarrhea is the most common nosocomial diarrhea, which results in substantial morbidity and mortality, as well as increased healthcare costs (Khan & Elzouki, 2014).

The main objective of this study was to determine the prevalence of *Clostridium difficile* on environmental surfaces pre- and post-decontamination. One of the main modes of CDI transmission is through the contaminated environment; therefore, seven high touch surfaces were tested pre- and post-terminal cleaning. While no *C. difficile* was cultured, decontamination of patient rooms showed a significant difference in the amount of bacteria on surfaces before and after terminal cleaning. The three decontamination methods used proved to be comparable in effectiveness. PCR analysis was able to detect *C. difficile* DNA in five samples (3.9%), the bedside table, bed rail, and the floor pre-decontamination, as well as the call bell/TV remote and floor post-

decontamination. This study demonstrates the sensitivity of PCR for enhanced detection of microorganisms.

Reducing the rate of *Clostridium difficile* infections is dependent on a number of factors, including the proper use of antibiotics, environmental decontamination, early detection, and proper hand-hygiene. Four environmental decontamination methods that could potentially reduce the rate of CDIs are: 1) improved terminal cleaning, 2) daily decontamination of high touch surfaces in isolation rooms, 3) decontamination of portable equipment, and 4) improved decontamination of all rooms in cases of asymptomatic carriers (Donskey, 2013). The CDC recommends the use of a 1:10 dilution of sodium hypochlorite as a disinfectant due to its sporicidal effects. The decontamination methods used in this study conform to the CDC recommendations. The results of this study suggest that the three decontamination methods used are effective at reducing surface contamination.

Early detection is the first step in preventing CDI transmission in hospitals. The Hospital Infection Control Practice Advisory Panel of the Centers for Disease Control and Prevention recommends that suspected CDI cases be placed in contact precaution isolation until a patient is asymptomatic for 48 to 72 hours (Walters & Zuckerbraun, 2014; Weber et al., 2010). Environmental contamination plays an important role in the transmission of various HAIs. Therefore, improved compliance with infection control can insure the delivery of the safest health care possible.

Appendix A
IRB Exclusion Form

UNLV
UNIVERSITY OF NEVADA LAS VEGAS

Biomedical IRB
Notice of Excluded Activity

DATE: August 14, 2014

TO: **Dr. Mark Buttner,** Environmenal and Occupational Health

FROM: Office of Research Integrity – Human Subjects

RE: Notification of IRB Action
Protocol Title: **The Presence of Clostridium difficile on Environmental Services in Healthcare Facilities Pre- and Post-Decontamination of Patient Rooms**
Protocol# 1408-4890M

This memorandum is notification that the project referenced above has been reviewed as indicated in Federal regulatory statutes 45CFR46.

The protocol has been reviewed and deemed excluded from IRB review. It is not in need of further review or approval by the IRB.

Any changes to the excluded activity may cause this project to require a different level of IRB review. Should any changes need to be made, please submit a Modification Form.

If you have questions or require any assistance, please contact the Office of Research Integrity – Human Subjects at IRB@unlv.edu or call 895-2794.

Appendix B
Environmental Investigation Data Sheet

UNIVERSITY OF NEVADA, LAS VEGAS
EMERGING DISEASES LABORATORY, SCHOOL OF COMMUNITY HEALTH SCIENCES
ENVIRONMENTAL INVESTIGATION DATA SHEET

Facility Name: _____ Site Number: _____

Sample Collector: _____ Decontamination Date: _____

	Site number	**Sample ID**	**Sample Location/Description**
Pre-Decontamination		**A**	bed rail (swab)
		B	call bell/television remote control (swab)
		C	telephone (swab)
		D	bathroom doorknobs (inside and outside) (swab)
		E	toilet flush lever (swab)
		F	floor (sponge)
		G	bed side table (sponge)
Post-decontamination		**H**	bed rail (swab)
		I	call bell/television remote control (swab)
		J	telephone (swab)
		K	bathroom doorknobs (inside and outside) (swab)
		L	toilet flush lever (swab)
		M	floor (sponge)
		N	bed side table (sponge)

Instructions:

1. Enter information on data sheet
2. Wearing cleaning gloves, aseptically remove sterile swab, open transport tube, and inset swab in transport tube; squeeze tube to express transport buffer from the sponge and moisten the swab in the buffer
3. Remove moistened swab, close transport tube and perform surface sampling with swab. Applying light pressure, swab the surface in an overlapping side-to-side pattern over an area of approximately 2 in. by 2 in. Rotate the swab 180º and sample the same area from another (i.e., perpendicular) orientation
4. Open transport tube, insert exposed swab until it is in contact with the transport buffer, close tube
5. Enter sample information on the sample transport tube label (i.e., site number, sample ID, date)
6. Store transport tube containing swab at 4ºC and contact Theresa Trice (Email: theresa.trice@unlv.edu) to arrange sample pickup

Appendix C

Centers for Disease Control and Prevention's Surface Sampling Procedures for *Bacillus anthracis* spores from smooth, non-porous surfaces

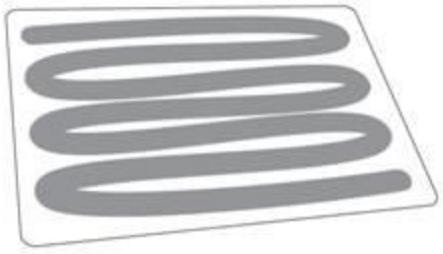
Macrofoam Swab Procedure

Swab Materials

1. Gloves, nitrile
2. Ruler, disposable, and masking tape
   or
   Sample template, disposable, sample area size 4 in^2 (26 cm^2)
3. Macrofoam swab, sterile, 3/16 inch thick medical-grade polyurethane foam head, 100 pores per inch, thermally bonded to a polypropylene stick (such as the Sterile Foam Tipped Applicators Scored with Thumb Stop [Puritan, Guilford, Maine; catalog number 25-1607 1PF SC] or equivalent)
4. General neutralizing buffer that will inactivate halogen disinfectants and quaternary ammonium compounds, 10 milliliter (ml), sterile (such as the Neutralizing Buffer [Hardy Diagnostics, Santa Maria, California; catalog number K105] or equivalent)
5. Screw-cap centrifuge tubes, sterile, 15 ml (such as 15 ml High-Clarity Polypropylene Conical Centrifuge Tube [Becton Dickinson, Franklin Lakes, New Jersey; catalog number 352097] or equivalent)
6. Sample labels
   or
   Permanent marker
7. Re-sealable plastic bag, 1-quart or smaller
8. Re-sealable plastic bag, 1-gallon or larger

Swab Sampling Procedure

1. Wearing a clean pair of gloves over existing gloves, place the disposable template over the area to be sampled and secure it. If the template cannot be used, measure the sampling area with a disposable ruler, and delineate the area to be sampled with masking tape.
2. Remove the sterile swab from its package. Grasp the swab near the top of the handle. Do not handle below the thumb stop.
3. If the sterile swab is not pre-moistened, moisten the sterile swab by dipping it in the 10 ml container of neutralizing buffer solution. Remove any excess liquid by pressing the swab head on the inside surface of the neutralizing buffer solution container.
   Note: Once a sterile swab has been moistened, the remaining neutralizing buffer solution and container must be discarded.
4. Swab the surface to be sampled using the moistened sterile swab. Use an overlapping 'S' pattern to cover the entire surface with horizontal strokes.



Note: Depending on the design of the swab, a rolling motion can be used when swabbing the surface to maximize swab contact with the surface.

5. Rotate the swab and swab the same area again using vertical ‘S’-strokes.

6. Rotate the swab once more and swab the same area using diagonal ‘S’-strokes.

7. Place the head of the swab directly into a sterile screw-capped centrifuge tube. Break off the head of the swab by bending the handle. The end of the swab handle, touched by the collector, should not touch the inside of the tube. Securely tighten the screw-cap and label the tube (e.g., unique sample identifier, sample location, initials of collectors and date and time sample was collected). Collection tubes and re-sealable bags may be pre-labeled to assist with sampling efficiency.
8. Place the sample container in a re-sealable 1-quart plastic bag. Securely seal and label the bag (e.g., sample location, date and time sample was collected, and name of individual collecting the sample).

   Note: Remove excessive air from the re-sealable plastic bags to increase the number of samples that can be shipped in one container.

9. Dispose of the template, if used.
10. Remove outer gloves and discard. Clean gloves must be worn for each new sample.

Cellulose Sponge Procedure

Cellulose Sponge Materials

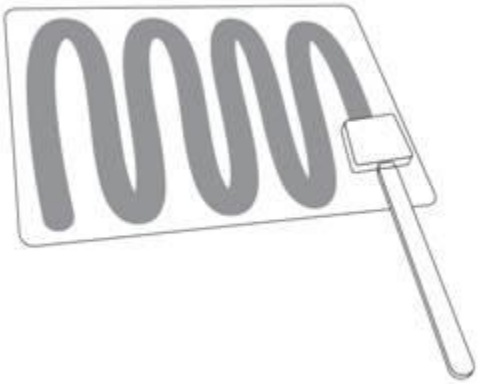
1. Gloves, nitrile
2. Ruler, disposable, and masking tape
   or
   Sample template, disposable, sample area size 100 in^2 (645 cm^2)
3. Sponge, sterile, pre-moistened with 10 ml neutralizing buffer solution, 1.5 by 3 inches cellulose sponge folded over a handle (such as the 3M™ Sponge-Stick [3M, St. Paul, Minnesota; catalog number SSL-10NB] or equivalent)[1]
   or
   Sponge, sterile, dry, 1.5 by 3 inches cellulose sponge folded over a handle (such as the 3M™ Sponge-Stick [3M, St. Paul, Minnesota; catalog number SSL-100] or equivalent)
   and
   General neutralizing buffer that will inactivate halogen disinfectants and quaternary ammonium compounds, sterile, 10 ml (such as the Neutralizing Buffer [Hardy Diagnostics, Santa Maria, California; catalog number K105] or equivalent)
4. Screw-cap specimen container, sterile, individually wrapped 4 ounce (such as General Purpose Specimen Container [Kendall Healthcare, Mansfield, Massachusetts; catalog number 8889-207026] or equivalent)
5. Sample labels
   or
   Permanent marker
6. Re-sealable plastic bag, 1-quart or smaller
7. Re-sealable plastic bag, 1-gallon or larger

Cellulose Sponge Sampling Procedure

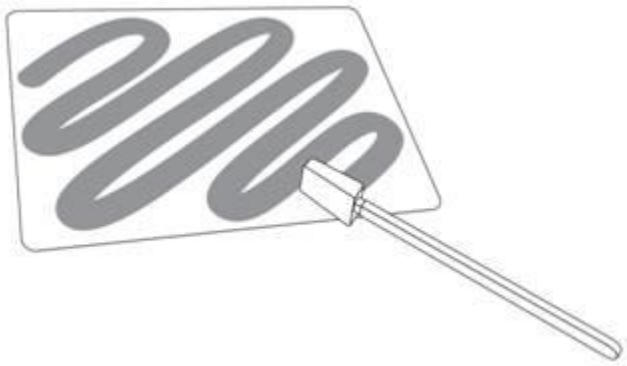
1. Wearing a clean pair of gloves over existing gloves, place the disposable template over the area to be sampled and secure it. If a template cannot be used, measure the sampling area with a disposable ruler, and delineate the area to be sampled with masking tape. The surface area sampled should be less than or equal to 100 in^2 (645 cm^2).
2. Remove the sterile sponge from its package. Grasp the sponge near the top of the handle. Do not handle below the thumb stop.
3. If the sterile sponge is not pre-moistened, moisten the sponge by pouring the 10 ml container of neutralizing buffer solution over the dry sponge.
   Note: The moistened sponge should not be dripping neutralizing buffer solution.

   Note: Any unused neutralizing buffer solution **must** be discarded.
4. Wipe the surface to be sampled using the moistened sterile sponge by laying the widest part of the sponge on the surface, leaving the leading edge slightly lifted. Apply gentle but firm pressure and use an overlapping ‘S’ pattern to cover the entire surface with horizontal strokes.

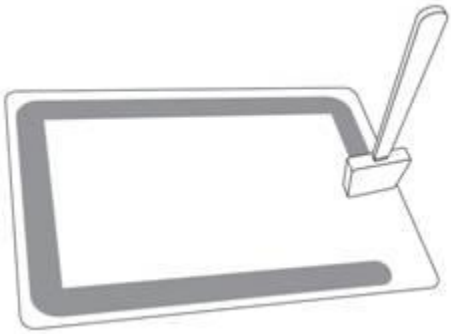
5. Turn the sponge over and wipe the same area again using vertical ‘S’-strokes.



6. Use the edges of the sponge (narrow sides) to wipe the same area using diagonal ‘S’-strokes.



7. Use the tip of the sponge to wipe the perimeter of the sampling area.



8. Place the head of the sponge directly into a sterile specimen container. Break off the head of the sponge by bending the handle. The end of the sponge handle, touched by the collector, should not touch the inside of the specimen container. Securely seal and label the container (e.g., unique sample identifier, sample location, initials of collector and date and time sample was collected).
9. Place the sample container in a re-sealable 1-quart plastic bag. Securely seal and label the bag (e.g., sample location, date and time sample was collected, and name of individual collecting the sample). Specimen containers and re-sealable bags may be pre-labeled to assist with sampling efficiency.

   Note: Remove excessive air from the re-sealable plastic bags to increase the number of samples that can be shipped in one container.

10. Dispose of the template, if used.

    Remove outer gloves and discard. Clean gloves should be worn for each new sample

Appendix D
Culture Analysis Data

PRE Site/ location:	Volume:	Dilution:	CFU:	CFU/ ml:	CFU/ sample:	Log CFU/ Sample:	Organism:
4A	**.1 ml**	**1:30**	**17**	**510**	**4,590**	**3.662**	**Facultative bacteria**
4B	**.1 ml**	**1:30**	**0**	**0**	**0**	**0.000**	
4C	**.1 ml**	**1:30**	**1**	**30**	**270**	**2.431**	**Facultative bacteria**
4D	**.1 ml**	**1:30**	**1**	**30**	**270**	**2.431**	**Facultative bacteria**
4E	**.1 ml**	**1:30**	**1**	**30**	**270**	**2.431**	**Facultative bacteria**
4F	**.1 ml**	**1:30**	**5**	**150**	**1,500**	**3.176**	**Facultative bacteria**
			35	**1,100**	**11,000**	**4.041**	***Ruminococcus torques***
4G	**.1 ml**	**1:30**	**3**	**90**	**900**	**2.954**	**Facultative bacteria**
5A	**.1 ml**	**1:30**	**1**	**30**	**270**	**2.431**	**Facultative bacteria**
5B	**.1 ml**	**1:30**	**0**	**0**	**0**	**0.000**	
5C	**.1 ml**	**1:30**	**0**	**0**	**0**	**0.000**	
5D	**.1 ml**	**1:30**	**43**	**1300**	**11700**	**4.068**	**Facultative bacteria**
5E	**.1 ml**	**1:30**	**1**	**30**	**270**	**2.431**	**Facultative bacteria**
5F	**.1 ml**	**1:30**	**1**	**30**	**300**	**2.477**	**Facultative bacteria**
5G	**.1 ml**	**1:30**	**0**	**0**	**0**	**0.000**	
6A	**.1 ml**	**1:30**	**3**	**90**	**810**	**2.908**	**Facultative bacteria**
6B	**.1 ml**	**1:30**	**215**	**6,500**	**58500**	**4.767**	**Facultative bacteria**
6C	**.1 ml**	**1:30**	**23**	**690**	**6210**	**3.793**	**Facultative bacteria**
6D	**.1 ml**	**1:30**	**10**	**300**	**2700**	**3.431**	**Facultative bacteria**
6E	**.1 ml**	**1:30**	**0**	**0**	**0**	**0.000**	
6F	**.1 ml**	**1:30**	**13**	**390**	**3900**	**3.591**	**Facultative bacteria**
6G	**.1 ml**	**1:30**	**30**	**900**	**9000**	**3.954**	**Facultative bacteria**

PRE Site/ location:	Volume:	Dilution:	CFU:	CFU/ ml:	CFU/ sample:	Log CFU/ Sample:	Organism:
7A	nd	x	x	x	x	x	
7B	.1 ml	1:30	3	90	810	2.908	Facultative bacteria
7C	.1 ml	1:30	3	90	810	2.908	Facultative bacteria
7D	.1 ml	1:30	3	90	810	2.908	Facultative bacteria
7E	.1 ml	1:30	1	30	270	2.431	Facultative bacteria
7F	.1 ml	1:30	19	570	5700	3.756	Facultative bacteria
7G	.1 ml	1:30	107	3,200	32000	4.505	Facultative bacteria
8A	.1 ml	1:30	2	60	540	2.732	Facultative bacteria
8B	.1 ml	1:30	2	60	540	2.732	Facultative bacteria
8C	.1 ml	1:30	1	30	270	2.431	Facultative bacteria
8D	.1 ml	1:30	0	0	0	0.000	
8E	nd	x	x	x	x	x	
8F	.1 ml	1:30	50	1,500	15,000	4.176	Facultative bacteria
8G	.1 ml	1:30	300	9,000	90,000	4.954	Facultative bacteria
9A	.1 ml	1:30	4	120	1080	3.033	Facultative bacteria
9B	.1 ml	1:30	0	0	0	0.000	
9C	.1 ml	1:30	1	30	270	2.431	Facultative bacteria
9D	.1 ml	1:30	0	0	0	0.000	
9E	.1 ml	1:30	29	870	7830	3.894	*Fusobacterium nucleatum*
9F	.1 ml	1:30	24	720	7200	3.857	Facultative bacteria
9G	.1 ml	1:30	291	8,700	87000	4.940	Facultative bacteria
10A	.1 ml	1:30	18	540	4860	3.687	Facultative bacteria
10B	.1 ml	1:30	1	30	270	2.431	Facultative bacteria

PRE Site/ location:	Volume:	Dilution:	CFU:	CFU/ ml:	CFU/ sample:	Log CFU/ Sample:	Organism:
10C	.1 ml	1:30	0	0	0	0.000	
10D	.1 ml	1:30	0	0	0	0.000	
10E	.1 ml	1:30	0	0	0	0.000	
10F	.1 ml	1:30	4	120	1200	3.079	Facultative bacteria
10G	.1 ml	1:30	2	60	600	2.778	Facultative bacteria
11A	.1 ml	1:30	0	0	0	0.000	
11B	.1 ml	1:30	20	600	5400	3.732	Facultative bacteria
11C	.1 ml	1:30	30	900	8100	3.908	Facultative bacteria
11D	.1 ml	1:30	10	300	2700	3.431	Facultative bacteria
11E	.1 ml	1:30	1	30	270	2.431	Facultative bacteria
11F	.1 ml	1:30	0	0	0	0.000	
11G	.1 ml	1:30	0	0	0	0.000	
13A	.1 ml	1:30	0	0	0	0	
13B	.1 ml	1:30	11	330	2970	3.473	Facultative bacteria
13C	nd	x	x	x	x	x	
13D	nd	x	x	x	x	x	
13E	nd	x	x	x	x	x	
13F	.1 ml	1:30	0	0	0	0	
13G	.1 ml	1:30	0	0	0	0	
14A	.1 ml	1:30	27	810	7290	3.863	Facultative bacteria
14B	.1 ml	1:30	15	450	4050	3.607	Facultative bacteria
14C	.1 ml	1:30	0	0	0	0.000	
14D	.1 ml	1:30	1	30	270	2.431	Facultative bacteria
14E	.1 ml	1:30	0	0	0	0.000	
14F	.1 ml	1:30	41	1200	12000	4.079	Facultative bacteria
14G	.1 ml	1:30	41	1200	12000	4.079	Facultative bacteria

POST Site/ location:	Volume:	Dilution:	CFU:	CFU/ ml:	CFU/ sample:	Log CFU/ Sample:	Organism:
4H	.1 ml	1:30	0	0	0	0.000	
4I	.1 ml	1:30	0	0	0	0.000	
4J	.1 ml	1:30	0	0	0	0.000	
4K	.1 ml	1:30	0	0	0	0.000	
4L	.1 ml	1:30	0	0	0	0.000	
4M	.1 ml	1:30	0	0	0	0.000	
				0	0	0.000	
4N	.1 mL	1:30	0	0	0	0.000	
5H	.1 ml	1:30	0	0	0	0.000	
5I	.1 ml	1:30	0	0	0	0.000	
5J	.1 ml	1:30	1	30	270	2.431	Facultative bacteria
5K	.1 ml	1:30	0	0	0	0.000	
5L	.1 ml	1:30	0	0	0	0.000	
5M	.1 ml	1:30	1	30	300	2.477	Facultative bacteria
5N	.1 ml	1:30	2	60	600	2.778	Facultative bacteria
6H	nd	x	x	x	x	x	
6I	.1 ml	1:30	0	0	0	0.000	
6J	.1 ml	1:30	0	0	0	0.000	
6K	.1 ml	1:30	0	0	0	0.000	
6L	.1 ml	1:30	0	0	0	0.000	
6M	.1 ml	1:30	0	0	0	0.000	
6N	.1 ml	1:30	0	0	0	0.000	
7H	nd	x	x	x	x	x	
7I	.1 ml	1:30	1	30	270	2.431	Facultative bacteria
7J	.1 ml	1:30	0	0	0	0.000	
7K	.1 ml	1:30	0	0	0	0.000	
7L	.1 ml	1:30	0	0	0	0.000	
7M	.1 ml	1:30	1	30	300	2.477	Facultative bacteria
7N	.1 ml	1:30	0	0	0	0.000	

POST Site/ location:	Volume:	Dilution:	CFU:	CFU/ ml:	CFU/ sample:	Log CFU/ Sample:	Organism:
8H	.1 ml	1:30	0	0	0	0.000	
8I	.1 ml	1:30	0	0	0	0.000	
8J	.1 ml	1:30	0	0	0	0.000	
8K	.1 ml	1:30	0	0	0	0.000	
8L	nd	x		x	x	x	
8M	.1 ml	1:30	0	0	0	0.000	
8N	.1 ml	1:30	0	0	0	0.000	
9H	.1 ml	1:30	0	0	0	0.000	
9I	.1 ml	1:30	0	0	0	0.000	
9J	.1 ml	1:30	0	0	0	0.000	
9K	.1 ml	1:30	0	0	0	0.000	
9L	.1 ml	1:30	0	0	0	0.000	
9M	.1 ml	1:30	1	30	300	2.477	Facultative bacteria
9N	.1 ml	1:30	0	0	0	0.000	
10H	.1 ml	1:30	9	270	2430	3.386	Facultative bacteria
10I	.1 ml	1:30	0	0	0	0.000	
10J	.1 ml	1:30	1	30	270	2.431	Facultative bacteria
10K	.1 ml	1:30	0	0	0	0.000	
10L	.1 ml	1:30	0	0	0	0.000	
10M	.1 ml	1:30	0	0	0	0.000	
10N	.1 ml	1:30	0	0	0	0.000	
11H	.1 ml	1:30	0	0	0	0.000	
11I	.1 ml	1:30	0	0	0	0.000	
11J	.1 ml	1:30	0	0	0	0.000	
11K	.1 ml	1:30	0	0	0	0.000	
11L	.1 ml	1:30	0	0	0	0.000	
11M	.1 ml	1:30	0	0	0	0.000	
11N	.1 ml	1:30	52	1,600	16000	4.204	Facultative bacteria
13H	.1 ml	1:30	0	0	0	0.000	
13I	.1 ml	1:30	0	0	0	0.000	

POST Site/ location:	Volume:	Dilution:	CFU:	CFU/ ml:	CFU/ sample:	Log CFU/ Sample:	Organism:
13J	nd	1:30	x	x	x	x	
13K	nd	1:30	x	x	x	x	
13L	nd	1:30	x	x	x	x	
13M	.1 ml	1:30	0	0	0	0.000	
13N	.1 ml	1:30	0	0	0	0.000	
14H	nd	x	x	x	x	x	
14I	.1 ml	1:30	11	330	2970	3.473	Facultative bacteria
14J	.1 ml	1:30	0	0	0	0.000	
14K	.1 ml	1:30	0	0	0	0.000	
14L	.1 ml	1:30	0	0	0	0.000	
14M	.1 ml	1:30	5	150	1500	3.176	Facultative bacteria
14N	.1 ml	1:30	0	0	0	0.000	

Appendix E

Internal positive control (IPC) PCR Data

(NAC = No amplification control, NTC = No template control, 10^{-5} = positive control)

Location	Site number	Detector name	Ct	Mean Ct
4A	**4**	**IPC**	**29.18**	**29.22**
4A	**4**	**IPC**	**29.25**	
5A	**5**	**IPC**	**28.76**	**29.76**
5A	**5**	**IPC**	**30.76**	
6A	**6**	**IPC**	**28.87**	**28.92**
6A	**6**	**IPC**	**28.97**	
8A	**8**	**IPC**	**Undetermined**	
8A	**8**	**IPC**	**Undetermined**	
8A (1:10)	**8**	**IPC**	**29.02**	**29.21**
8A (1:10)	**8**	**IPC**	**29.40**	
9A	**9**	**IPC**	**Undetermined**	
9A	**9**	**IPC**	**Undetermined**	
9A (1:10)	**9**	**IPC**	**29.34**	**29.41**
9A (1:10)	**9**	**IPC**	**29.47**	
10A	**10**	**IPC**	**Undetermined**	
10A	**10**	**IPC**	**Undetermined**	
10A (1:10)	**10**	**IPC**	**28.77**	**28.75**
10A (1:10)	**10**	**IPC**	**28.73**	
11A	**11**	**IPC**	**Undetermined**	
11A	**11**	**IPC**	**Undetermined**	
11A (1:10)	**11**	**IPC**	**29.09**	**29.02**
11A (1:10)	**11**	**IPC**	**28.94**	
11A (1:10)	**11**	**IPC**	**28.97**	**29.01**
11A (1:10)	**11**	**IPC**	**29.05**	
13A	**13**	**IPC**	**29.26**	**29.33**
13A	**13**	**IPC**	**29.41**	
14A	**14**	**IPC**	**Undetermined**	
14A	**14**	**IPC**	**Undetermined**	
14A (1:10)	**14**	**IPC**	**28.50**	**28.40**
14A (1:10)	**14**	**IPC**	**28.31**	
4B	**4**	**IPC**	**39.61**	**39.55**
4B	**4**	**IPC**	**39.49**	
4B (1:10)	**4**	**IPC**	**28.89**	**28.86**

Location	Site number	Detector name	Ct	Mean Ct
4B (1:10)	4	IPC	28.83	
5B	5	IPC	29.32	29.40
5B	5	IPC	29.47	
6B	6	IPC	28.91	28.90
6B	6	IPC	28.89	
7B	7	IPC	34.11	33.27
7B	7	IPC	32.42	
7B (1:10)	7	IPC	29.25	29.20
7B (1:10)	7	IPC	29.15	
8B	8	IPC	Undetermined	
8B	8	IPC	Undetermined	
8B (1:10)	8	IPC	29.11	29.24
8B (1:10)	8	IPC	29.37	
9B	9	IPC	Undetermined	
9B	9	IPC	Undetermined	
9B (1:10)	9	IPC	29.25	29.35
9B (1:10)	9	IPC	29.44	
10B	10	IPC	Undetermined	
10B	10	IPC	Undetermined	
10B (1:10)	10	IPC	29.32	29.08
10B (1:10)	10	IPC	28.83	
11B	11	IPC	Undetermined	
11B	11	IPC	Undetermined	
11B (1:10)	11	IPC	29.04	28.95
11B (1:10)	11	IPC	28.87	
13B	13	IPC	29.17	29.13
13B	13	IPC	29.08	
14B	14	IPC	Undetermined	
14B	14	IPC	Undetermined	
14B (1:10)	14	IPC	28.58	28.71
14B (1:10)	14	IPC	28.84	
4C	4	IPC	29.67	29.66
4C	4	IPC	29.65	
5C	5	IPC	30.28	30.17
5C	5	IPC	30.05	
6C	6	IPC	28.82	28.90
6C	6	IPC	28.98	

Location	Site number	Detector name	Ct	Mean Ct
7C	7	IPC	31.98	31.61
7C	7	IPC	31.24	
7C (1:10)	7	IPC	28.96	29.01
7C (1:10)	7	IPC	29.07	
8C	8	IPC	Undetermined	
8C	8	IPC	Undetermined	
8C (1:10)	8	IPC	29.25	29.09
8C (1:10)	8	IPC	28.92	
9C	9	IPC	Undetermined	
9C	9	IPC	Undetermined	
9C (1:10)	9	IPC	29.37	29.08
9C (1:10)	9	IPC	28.79	
10C	10	IPC	Undetermined	
10C	10	IPC	Undetermined	
10C (1:10)	10	IPC	29.03	29.14
10C (1:10)	10	IPC	29.25	
11C	11	IPC	Undetermined	
11C	11	IPC	Undetermined	
11C (1:10)	11	IPC	29.00	28.84
11C (1:10)	11	IPC	28.68	
14C	14	IPC	34.67	34.35
14C	14	IPC	34.03	
14C (1:10)	14	IPC	28.31	28.28
14C (1:10)	14	IPC	28.25	
4D	4	IPC	30.98	31.53
4D	4	IPC	32.08	
4D (1:10)	4	IPC	28.94	28.86
4D (1:10)	4	IPC	28.77	
5D	5	IPC	30.28	30.50
5D	5	IPC	30.73	
6D	6	IPC	28.68	28.67
6D	6	IPC	28.65	
7D	7	IPC	31.98	31.62
7D	7	IPC	31.27	
7D (1:10)	7	IPC	28.87	28.84
7D (1:10)	7	IPC	28.82	
8D	8	IPC	Undetermined	

Location	Site number	Detector name	Ct	Mean Ct
8D	8	IPC	Undetermined	
8D (1:10)	8	IPC	28.83	28.62
8D (1:10)	8	IPC	28.41	
9D	9	IPC	34.19	34.21
9D	9	IPC	34.23	
9D (1:10)	9	IPC	28.88	29.18
9D (1:10)	9	IPC	29.47	
10D	10	IPC	Undetermined	
10D	10	IPC	Undetermined	
10D (1:10)	10	IPC	28.96	28.85
10D (1:10)	10	IPC	28.74	
11D	11	IPC	30.85	30.69
11D	11	IPC	30.54	
14D	14	IPC	Undetermined	
14D	14	IPC	Undetermined	
14D (1:10)	14	IPC	28.53	28.43
14D (1:10)	14	IPC	28.33	
4E	4	IPC	30.81	30.56
4E	4	IPC	30.31	
5E	5	IPC	30.63	30.37
5E	5	IPC	30.10	
6E	6	IPC	28.90	29.11
6E	6	IPC	29.32	
7E	7	IPC	30.42	30.60
7E	7	IPC	30.78	
9E	9	IPC	30.58	30.30
9E	9	IPC	30.01	
10E	10	IPC	Undetermined	
10E	10	IPC	Undetermined	
10E (1:10)	10	IPC	28.90	28.87
10E (1:10)	10	IPC	28.84	
11E	11	IPC	32.62	32.51
11E	11	IPC	32.39	
11E (1:10)	11	IPC	28.68	28.70
11E (1:10)	11	IPC	28.72	
14E	14	IPC	Undetermined	
14E	14	IPC	Undetermined	

Location	Site number	Detector name	Ct	Mean Ct
14E (1:10)	14	IPC	28.51	28.30
14E (1:10)	14	IPC	28.09	
4F	4	IPC	30.05	30.04
4F	4	IPC	30.03	
4F	4	IPC	30.04	30.14
4F	4	IPC	30.23	
5F	5	IPC	36.52	36.78
5F	5	IPC	37.04	
5F (1:10)	5	IPC	29.12	28.87
5F (1:10)	5	IPC	28.62	
6F	6	IPC	Undetermined	
6F	6	IPC	Undetermined	
6F (1:10)	6	IPC	28.78	28.71
6F (1:10)	6	IPC	28.63	
7F	7	IPC	Undetermined	
7F	7	IPC	Undetermined	
7F (1:10)	7	IPC	29.30	29.27
7F (1:10)	7	IPC	29.25	
8F	8	IPC	32.88	32.84
8F	8	IPC	32.79	
8F (1:10)	8	IPC	28.27	28.39
8F (1:10)	8	IPC	28.51	
9F	9	IPC	29.60	29.58
9F	9	IPC	29.56	
10F	10	IPC	Undetermined	
10F	10	IPC	Undetermined	
10F (1:10)	10	IPC	28.66	28.45
10F (1:10)	10	IPC	28.25	
11F	11	IPC	Undetermined	
11F	11	IPC	Undetermined	
11F (1:10)	11	IPC	29.08	29.18
11F (1:10)	11	IPC	29.28	
11F (1:10)	11	IPC	29.46	29.28
11F (1:10)	11	IPC	29.09	
13F	13	IPC	30.43	30.27
13F	13	IPC	30.11	
14F	14	IPC	Undetermined	

Location	Site number	Detector name	Ct	Mean Ct
14F	14	IPC	Undetermined	
14F (1:10)	14	IPC	28.26	28.26
14F (1:10)	14	IPC	28.26	
4G	4	IPC	29.49	29.60
4G	4	IPC	29.72	
4G	4	IPC	29.09	28.92
4G	4	IPC	28.75	
4G	4	IPC	29.71	29.61
4G	4	IPC	29.52	
5G	5	IPC	30.46	30.60
5G	5	IPC	30.75	
6G	6	IPC	28.63	28.64
6G	6	IPC	28.65	
7G	7	IPC	Undetermined	
7G	7	IPC	Undetermined	
7G (1:10)	7	IPC	Undetermined	
7G (1:10)	7	IPC	30.34	
7G (1:10)	7	IPC	29.91	29.42
7G (1:10)	7	IPC	28.93	
8G	8	IPC	Undetermined	
8G	8	IPC	Undetermined	
8G (1:10)	8	IPC	28.72	28.74
8G (1:10)	8	IPC	28.77	
9G	9	IPC	Undetermined	
9G	9	IPC	Undetermined	
9G (1:10)	9	IPC	30.16	29.91
9G (1:10)	9	IPC	29.66	
10G	10	IPC	Undetermined	
10G	10	IPC	Undetermined	
10G (1:10)	10	IPC	28.43	28.48
10G (1:10)	10	IPC	28.52	
11G	11	IPC	35.69	35.19
11G	11	IPC	34.70	
11G (1:10)	11	IPC	28.67	28.62
11G (1:10)	11	IPC	28.56	
13G	13	IPC	29.40	29.26
13G	13	IPC	29.11	

Location	Site number	Detector name	Ct	Mean Ct
14G	14	IPC	35.64	35.31
14G	14	IPC	34.98	
14G (1:10)	14	IPC	26.81	
14G (1:10)	14	IPC	Undetermined	
14G (1:10)	14	IPC	28.69	28.49
14G (1:10)	14	IPC	28.28	
4H	4	IPC	31.15	31.15
4H	4	IPC	31.15	
4H (1:10)	4	IPC	29.09	28.92
4H (1:10)	4	IPC	28.75	
5H	5	IPC	32.56	32.27
5H	5	IPC	31.99	
5H (1:10)	5	IPC	28.61	28.52
5H (1:10)	5	IPC	28.42	
8H	8	IPC	31.24	31.02
8H	8	IPC	30.81	
8H (1:10)	8	IPC	28.36	28.35
8H (1:10)	8	IPC	28.35	
9H	9	IPC	36.46	36.24
9H	9	IPC	36.03	
9H (1:10)	9	IPC	29.34	29.26
9H (1:10)	9	IPC	29.19	
10H	10	IPC	34.22	33.68
10H	10	IPC	33.13	
10H (1:10)	10	IPC	28.33	28.36
10H (1:10)	10	IPC	28.39	
11H	11	IPC	30.78	31.24
11H	11	IPC	31.69	
11H (1:10)	11	IPC	28.71	28.56
11H (1:10)	11	IPC	28.41	
13H	13	IPC	29.94	29.80
13H	13	IPC	29.66	
4I	4	IPC	31.59	31.42
4I	4	IPC	31.26	
4I (1:10)	4	IPC	28.73	28.83
4I (1:10)	4	IPC	28.93	
5I	5	IPC	30.80	30.61

Location	Site number	Detector name	Ct	Mean Ct
5I	5	IPC	30.42	
6I	6	IPC	29.10	29.32
6I	6	IPC	29.53	
6I	6	IPC	29.66	29.40
6I	6	IPC	29.14	
6I	6	IPC	29.95	29.81
6I	6	IPC	29.68	
7I	7	IPC	Undetermined	
7I	7	IPC	39.71	
7I (1:10)	7	IPC	29.02	29.02
7I (1:10)	7	IPC	29.03	
8I	8	IPC	Undetermined	
8I	8	IPC	Undetermined	
8I (1:10)	8	IPC	29.13	29.35
8I (1:10)	8	IPC	29.56	
9I	9	IPC	30.42	30.32
9I	9	IPC	30.21	
10I	10	IPC	36.16	35.33
10I	10	IPC	34.50	
10I (1:10)	10	IPC	28.68	28.54
10I (1:10)	10	IPC	28.40	
11I	11	IPC	30.59	30.37
11I	11	IPC	30.15	
13I	13	IPC	30.97	30.27
13I	13	IPC	29.58	
14I	14	IPC	34.38	33.87
14I	14	IPC	33.37	
14I (1:10)	14	IPC	28.32	28.14
14I (1:10)	14	IPC	27.95	
4J	4	IPC	31.15	31.14
4J	4	IPC	31.14	
4J (1:10)	4	IPC	28.88	28.64
4J (1:10)	4	IPC	28.40	
5J	5	IPC	29.26	29.26
5J	5	IPC	29.27	
6J	6	IPC	28.98	28.82
6J	6	IPC	28.66	

Location	Site number	Detector name	Ct	Mean Ct
7J	7	IPC	32.59	32.29
7J	7	IPC	31.99	
7J (1:10)	7	IPC	28.95	28.97
7J (1:10)	7	IPC	29.00	
8J	8	IPC	Undetermined	
8J	8	IPC	Undetermined	
8J (1:10)	8	IPC	28.94	28.85
8J (1:10)	8	IPC	28.77	
9J	9	IPC	Undetermined	
9J	9	IPC	Undetermined	
9J (1:10)	9	IPC	29.97	29.87
9J (1:10)	9	IPC	29.77	
10J	10	IPC	31.30	31.44
10J	10	IPC	31.58	
10J (1:10)	10	IPC	28.43	28.43
10J (1:10)	10	IPC	28.43	
11J	11	IPC	30.50	30.48
11J	11	IPC	30.47	
14J	14	IPC	Undetermined	
14J	14	IPC	Undetermined	
14J (1:10)	14	IPC	27.69	27.25
14J (1:10)	14	IPC	26.81	
4K	4	IPC	31.05	30.90
4K	4	IPC	30.76	
4K (1:10)	4	IPC	28.92	28.85
4K (1:10)	4	IPC	28.78	
5K	5	IPC	32.55	32.07
5K	5	IPC	31.59	
5K (1:10)	5	IPC	28.54	28.55
5K (1:10)	5	IPC	28.56	
6K	6	IPC	28.63	28.52
6K	6	IPC	28.42	
7K	7	IPC	34.44	33.95
7K	7	IPC	33.46	
7K (1:10)	7	IPC	30.2	30.10
7K (1:10)	7	IPC	30.0	
8K	8	IPC	30.00	30.23

Location	Site number	Detector name	Ct	Mean Ct
8K	8	IPC	30.45	
9K	9	IPC	29.71	29.39
9K	9	IPC	29.08	
10K	10	IPC	Undetermined	
10K	10	IPC	Undetermined	
10K (1:10)	10	IPC	28.33	28.27
10K (1:10)	10	IPC	28.20	
11K	11	IPC	29.68	29.89
11K	11	IPC	30.11	
14K	14	IPC	36.35	36.36
14K	14	IPC	36.37	
14K (1:10)	14	IPC	28.33	28.30
14K (1:10)	14	IPC	28.26	
4L	4	IPC	30.65	30.64
4L	4	IPC	30.64	
5L	5	IPC	31.55	31.49
5L	5	IPC	31.42	
5L (1:10)	5	IPC	28.64	28.63
5L (1:10)	5	IPC	28.61	
6L	6	IPC	29.60	29.64
6L	6	IPC	29.67	
7L	7	IPC	37.68	37.74
7L	7	IPC	37.80	
7L (1:10)	7	IPC	28.48	28.50
7L (1:10)	7	IPC	28.51	
9L	9	IPC	30.43	30.21
9L	9	IPC	29.98	
10L	10	IPC	39.90	39.92
10L	10	IPC	39.94	
10L (1:10)	10	IPC	28.41	28.50
10L (1:10)	10	IPC	28.58	
11L	11	IPC	Undetermined	
11L	11	IPC	Undetermined	
11L (1:10)	11	IPC	28.86	28.79
11L (1:10)	11	IPC	28.72	
14L	14	IPC	39.71	39.20
14L	14	IPC	38.69	

Location	Site number	Detector name	Ct	Mean Ct
14L (1:10)	14	IPC	28.02	28.11
14L (1:10)	14	IPC	28.21	
4M	4	IPC	28.91	29.18
4M	4	IPC	29.45	
4M	4	IPC	29.45	29.39
4M	4	IPC	29.32	
5M	5	IPC	32.08	31.57
5M	5	IPC	31.05	
5M (1:10)	5	IPC	28.46	28.33
5M (1:10)	5	IPC	28.19	
6M	6	IPC	35.61	35.52
6M	6	IPC	35.44	
6M (1:10)	6	IPC	28.60	28.49
6M (1:10)	6	IPC	28.38	
6M (1:10)	6	IPC	28.54	28.45
6M (1:10)	6	IPC	28.37	
7M	7	IPC	31.26	30.85
7M	7	IPC	30.44	
7M (1:10)	7	IPC	28.37	28.38
7M (1:10)	7	IPC	28.39	
8M	8	IPC	33.63	33.51
8M	8	IPC	33.38	
8M (1:10)	8	IPC	28.57	28.38
8M (1:10)	8	IPC	28.19	
9M	9	IPC	Undetermined	
9M	9	IPC	Undetermined	
9M (1:10)	9	IPC	30.05	30.08
9M (1:10)	9	IPC	30.11	
10M	10	IPC	Undetermined	
10M	10	IPC	Undetermined	
10M (1:10)	10	IPC	28.51	28.78
10M (1:10)	10	IPC	29.04	
11M	11	IPC	30.43	30.20
11M	11	IPC	29.97	
13M	13	IPC	30.67	30.65
13M	13	IPC	30.64	

Location	Site number	Detector name	Ct	Mean Ct
14M	14	IPC	Undetermined	
14M	14	IPC	Undetermined	
14M (1:10)	14	IPC	28.25	28.15
14M (1:10)	14	IPC	28.05	
4N	4	IPC	31.51	31.37
4N	4	IPC	31.23	
4N (1:10)	4	IPC	28.44	28.41
4N (1:10)	4	IPC	28.38	
5N	5	IPC	33.20	32.91
5N	5	IPC	32.62	
5N (1:10)	5	IPC	28.54	28.55
5N (1:10)	5	IPC	28.56	
6N	6	IPC	28.38	28.29
6N	6	IPC	28.20	
7N	7	IPC	34.53	34.74
7N	7	IPC	34.95	
7N (1:10)	7	IPC	29.01	28.84
7N (1:10)	7	IPC	28.68	
8N	8	IPC	32.26	32.26
8N	8	IPC	32.25	
8N (1:10)	8	IPC	28.56	28.58
8N (1:10)	8	IPC	28.60	
9N	9	IPC	32.23	32.02
9N	9	IPC	31.82	
9N (1:10)	9	IPC	28.48	28.49
9N (1:10)	9	IPC	28.51	
10N	10	IPC	Undetermined	
10N	10	IPC	Undetermined	
10N (1:10)	10	IPC	30.36	29.43
10N (1:10)	10	IPC	28.51	
11N	11	IPC	24.04	27.70
11N	11	IPC	31.35	
11N	11	IPC	35.70	33.52
11N	11	IPC	31.34	
11N (1:10)	11	IPC	28.76	28.61
11N (1:10)	11	IPC	28.47	

Location	Site number	Detector name	Ct	Mean Ct
13N	13	IPC	30.15	30.08
13N	13	IPC	30.01	
14N	14	IPC	34.05	34.03
14N	14	IPC	34.01	
14N (1:10)	14	IPC	28.07	28.04
14N (1:10)	14	IPC	28.02	
10^{-5}		IPC	28.25	28.23
10^{-5}		IPC	28.21	
10^{-5}		IPC	27.96	28.02
10^{-5}		IPC	28.07	
10^{-5}		IPC	28.27	28.28
10^{-5}		IPC	28.28	
10^{-5}		IPC	27.91	27.86
10^{-5}		IPC	27.82	
10^{-5}		IPC	28.30	28.31
10^{-5}		IPC	28.32	
10^{-5}		IPC	28.06	28.19
10^{-5}		IPC	28.32	
10^{-5}		IPC	28.25	28.30
10^{-5}		IPC	28.34	
10^{-5}		IPC	28.19	28.08
10^{-5}		IPC	27.96	
10^{-5}		IPC	28.13	27.96
10^{-5}		IPC	27.80	
10^{-5}		IPC	28.27	28.21
10^{-5}		IPC	28.15	
10^{-5}		IPC	28.02	27.87
10^{-5}		IPC	27.71	
10^{-5}		IPC	28.26	28.25
10^{-5}		IPC	28.25	
10^{-3}		IPC	39.02	38.11
10^{-3}		IPC	37.20	

Location	Site number	Detector name	Ct	Mean Ct
NTC		IPC	28.19	28.15
NTC		IPC	28.11	
NAC		IPC	Undetermined	
NAC		IPC	Undetermined	
NTC		IPC	27.99	27.98
NTC		IPC	27.97	
NAC		IPC	Undetermined	
NAC		IPC	Undetermined	
NTC		IPC	28.55	28.50
NTC		IPC	28.46	
NAC		IPC	Undetermined	
NAC		IPC	Undetermined	
NTC		IPC	28.45	28.33
NTC		IPC	28.21	
NAC		IPC	Undetermined	
NAC		IPC	Undetermined	
NTC		IPC	28.49	28.50
NTC		IPC	28.52	
NAC		IPC	Undetermined	
NAC		IPC	Undetermined	
NTC		IPC	28.57	28.51
NTC		IPC	28.44	
NAC		IPC	Undetermined	
NAC		IPC	Undetermined	
NTC		IPC	30.33	30.01
NTC		IPC	29.69	
NAC		IPC	Undetermined	
NAC		IPC	Undetermined	
NTC		IPC	28.28	28.30
NTC		IPC	28.31	
NAC		IPC	Undetermined	
NAC		IPC	Undetermined	
NTC		IPC	28.28	28.33
NTC		IPC	28.38	
NAC		IPC	Undetermined	
NAC		IPC	Undetermined	
NTC		IPC	28.45	28.46

Location	Site number	Detector name	Ct	Mean Ct
NTC		**IPC**	**28.46**	
NAC		**IPC**	**Undetermined**	
NAC		**IPC**	**Undetermined**	
NTC		**IPC**	**28.27**	**28.37**
NTC		**IPC**	**28.47**	
NAC		**IPC**	**Undetermined**	
NAC		**IPC**	**Undetermined**	
NTC		**IPC**	**28.27**	**28.39**
NTC		**IPC**	**28.50**	
NAC		**IPC**	**Undetermined**	
NAC		**IPC**	**Undetermined**	
NTC		**IPC**	**28.28**	**28.26**
NTC		**IPC**	**28.24**	
NAC		**IPC**	**Undetermined**	
NAC		**IPC**	**Undetermined**	

Appendix F

Clostridium difficile (Cdif) PCR Data

(NAC = No amplification control, NTC = No template control, 10^{-5} = positive control)

Location	Site number	Detector name	Ct	Mean Ct
4A	**4**	**Cdif**	**Undetermined**	
4A	**4**	**Cdif**	**Undetermined**	
5A	**5**	**Cdif**	**Undetermined**	
5A	**5**	**Cdif**	**Undetermined**	
6A	**6**	**Cdif**	**Undetermined**	
6A	**6**	**Cdif**	**Undetermined**	
8A	**8**	**Cdif**	**Undetermined**	
8A	**8**	**Cdif**	**Undetermined**	
8A (1:10)	**8**	**Cdif**	**Undetermined**	
8A (1:10)	**8**	**Cdif**	**Undetermined**	
9A	**9**	**Cdif**	**Undetermined**	
9A	**9**	**Cdif**	**Undetermined**	
9A (1:10)	**9**	**Cdif**	**Undetermined**	
9A (1:10)	**9**	**Cdif**	**Undetermined**	
10A	**10**	**Cdif**	**Undetermined**	
10A	**10**	**Cdif**	**Undetermined**	
10A (1:10)	**10**	**Cdif**	**Undetermined**	
10A (1:10)	**10**	**Cdif**	**Undetermined**	
11A	**11**	**Cdif**	**Undetermined**	
11A	**11**	**Cdif**	**Undetermined**	
11A (1:10)	**11**	**Cdif**	**36.49**	**37.02**
11A (1:10)	**11**	**Cdif**	**37.55**	
11A (1:10)	**11**	**Cdif**	**37.51**	**37.44**
11A (1:10)	**11**	**Cdif**	**37.38**	
13A	**13**	**Cdif**	**Undetermined**	
13A	**13**	**Cdif**	**Undetermined**	
14A	**14**	**Cdif**	**Undetermined**	
14A	**14**	**Cdif**	**Undetermined**	
14A (1:10)	**14**	**Cdif**	**Undetermined**	
14A (1:10)	**14**	**Cdif**	**Undetermined**	
4B	**4**	**Cdif**	**Undetermined**	
4B	**4**	**Cdif**	**Undetermined**	
4B (1:10)	**4**	**Cdif**	**Undetermined**	

Location	Site number	Detector name	Ct	Mean Ct
4B (1:10)	4	Cdif	Undetermined	
5B	5	Cdif	Undetermined	
5B	5	Cdif	Undetermined	
6B	6	Cdif	Undetermined	
6B	6	Cdif	Undetermined	
7B	7	Cdif	Undetermined	
7B	7	Cdif	Undetermined	
7B (1:10)	7	Cdif	Undetermined	
7B (1:10)	7	Cdif	Undetermined	
8B	8	Cdif	Undetermined	
8B	8	Cdif	Undetermined	
8B (1:10)	8	Cdif	Undetermined	
8B (1:10)	8	Cdif	Undetermined	
9B	9	Cdif	Undetermined	
9B	9	Cdif	Undetermined	
9B (1:10)	9	Cdif	Undetermined	
9B (1:10)	9	Cdif	Undetermined	
10B	10	Cdif	Undetermined	
10B	10	Cdif	Undetermined	
10B (1:10)	10	Cdif	Undetermined	
10B (1:10)	10	Cdif	Undetermined	
11B	11	Cdif	Undetermined	
11B	11	Cdif	Undetermined	
11B (1:10)	11	Cdif	Undetermined	
11B (1:10)	11	Cdif	Undetermined	
13B	13	Cdif	Undetermined	
13B	13	Cdif	Undetermined	
14B	14	Cdif	Undetermined	
14B	14	Cdif	Undetermined	
14B (1:10)	14	Cdif	Undetermined	
14B (1:10)	14	Cdif	Undetermined	
4C	4	Cdif	Undetermined	
4C	4	Cdif	Undetermined	
5C	5	Cdif	Undetermined	
5C	5	Cdif	Undetermined	
6C	6	Cdif	Undetermined	
6C	6	Cdif	Undetermined	

Location	Site number	Detector name	Ct	Mean Ct
7C	7	Cdif	Undetermined	
7C	7	Cdif	Undetermined	
7C (1:10)	7	Cdif	Undetermined	
7C (1:10)	7	Cdif	Undetermined	
8C	8	Cdif	Undetermined	
8C	8	Cdif	Undetermined	
8C (1:10)	8	Cdif	Undetermined	
8C (1:10)	8	Cdif	Undetermined	
9C	9	Cdif	Undetermined	
9C	9	Cdif	Undetermined	
9C (1:10)	9	Cdif	Undetermined	
9C (1:10)	9	Cdif	Undetermined	
10C	10	Cdif	Undetermined	
10C	10	Cdif	Undetermined	
10C (1:10)	10	Cdif	Undetermined	
10C (1:10)	10	Cdif	Undetermined	
11C	11	Cdif	Undetermined	
11C	11	Cdif	Undetermined	
11C (1:10)	11	Cdif	Undetermined	
11C (1:10)	11	Cdif	Undetermined	
14C	14	Cdif	Undetermined	
14C	14	Cdif	Undetermined	
14C (1:10)	14	Cdif	Undetermined	
14C (1:10)	14	Cdif	Undetermined	
4D	4	Cdif	Undetermined	
4D	4	Cdif	Undetermined	
4D (1:10)	4	Cdif	Undetermined	
4D (1:10)	4	Cdif	Undetermined	
5D	5	Cdif	Undetermined	
5D	5	Cdif	Undetermined	
6D	6	Cdif	Undetermined	
6D	6	Cdif	Undetermined	
7D	7	Cdif	Undetermined	
7D	7	Cdif	Undetermined	
7D (1:10)	7	Cdif	Undetermined	
7D (1:10)	7	Cdif	Undetermined	
8D	8	Cdif	Undetermined	

Location	Site number	Detector name	Ct	Mean Ct
8D	8	Cdif	Undetermined	
8D (1:10)	8	Cdif	Undetermined	
8D (1:10)	8	Cdif	Undetermined	
9D	9	Cdif	Undetermined	
9D	9	Cdif	Undetermined	
9D (1:10)	9	Cdif	Undetermined	
9D (1:10)	9	Cdif	Undetermined	
10D	10	Cdif	Undetermined	
10D	10	Cdif	Undetermined	
10D (1:10)	10	Cdif	Undetermined	
10D (1:10)	10	Cdif	Undetermined	
11D	11	Cdif	Undetermined	
11D	11	Cdif	Undetermined	
14D	14	Cdif	Undetermined	
14D	14	Cdif	Undetermined	
14D (1:10)	14	Cdif	Undetermined	
14D (1:10)	14	Cdif	Undetermined	
4E	4	Cdif	Undetermined	
4E	4	Cdif	Undetermined	
5E	5	Cdif	Undetermined	
5E	5	Cdif	Undetermined	
6E	6	Cdif	Undetermined	
6E	6	Cdif	Undetermined	
7E	7	Cdif	Undetermined	
7E	7	Cdif	Undetermined	
9E	9	Cdif	Undetermined	
9E	9	Cdif	Undetermined	
10E	10	Cdif	Undetermined	
10E	10	Cdif	Undetermined	
10E (1:10)	10	Cdif	Undetermined	
10E (1:10)	10	Cdif	Undetermined	
11E	11	Cdif	Undetermined	
11E	11	Cdif	Undetermined	
11E (1:10)	11	Cdif	Undetermined	
11E (1:10)	11	Cdif	Undetermined	
14E	14	Cdif	Undetermined	
14E	14	Cdif	Undetermined	

Location	Site number	Detector name	Ct	Mean Ct
14E (1:10)	14	Cdif	Undetermined	
14E (1:10)	14	Cdif	Undetermined	
4F	4	Cdif	Undetermined	
4F	4	Cdif	Undetermined	
4F	4	Cdif	Undetermined	
4F	4	Cdif	Undetermined	
5F	5	Cdif	Undetermined	
5F	5	Cdif	Undetermined	
5F (1:10)	5	Cdif	Undetermined	
5F (1:10)	5	Cdif	Undetermined	
6F	6	Cdif	Undetermined	
6F	6	Cdif	Undetermined	
6F (1:10)	6	Cdif	Undetermined	
6F (1:10)	6	Cdif	Undetermined	
7F	7	Cdif	Undetermined	
7F	7	Cdif	Undetermined	
7F (1:10)	7	Cdif	Undetermined	
7F (1:10)	7	Cdif	Undetermined	
8F	8	Cdif	Undetermined	
8F	8	Cdif	Undetermined	
8F (1:10)	8	Cdif	Undetermined	
8F (1:10)	8	Cdif	Undetermined	
9F	9	Cdif	Undetermined	
9F	9	Cdif	Undetermined	
10F	10	Cdif	Undetermined	
10F	10	Cdif	Undetermined	
10F (1:10)	10	Cdif	Undetermined	
10F (1:10)	10	Cdif	Undetermined	
11F	11	Cdif	Undetermined	
11F	11	Cdif	Undetermined	
11F (1:10)	11	Cdif	35.24	35.13
11F (1:10)	11	Cdif	35.01	
11F (1:10)	11	Cdif	36.02	35.54
11F (1:10)	11	Cdif	35.06	
13F	13	Cdif	Undetermined	
13F	13	Cdif	Undetermined	
14F	14	Cdif	Undetermined	

Location	Site number	Detector name	Ct	Mean Ct
14F	14	Cdif	Undetermined	
14F (1:10)	14	Cdif	Undetermined	
14F (1:10)	14	Cdif	Undetermined	
4G	4	Cdif	36.90	37.22
4G	4	Cdif	37.54	
4G	4	Cdif	Undetermined	
4G	4	Cdif	Undetermined	
4G	4	Cdif	39.29	39.08
4G	4	Cdif	38.87	
5G	5	Cdif	Undetermined	
5G	5	Cdif	Undetermined	
6G	6	Cdif	Undetermined	
6G	6	Cdif	Undetermined	
7G	7	Cdif	Undetermined	
7G	7	Cdif	Undetermined	
7G (1:10)	7	Cdif	Undetermined	
7G (1:10)	7	Cdif	Undetermined	
7G (1:10)	7	Cdif	Undetermined	
7G (1:10)	7	Cdif	Undetermined	
8G	8	Cdif	Undetermined	
8G	8	Cdif	Undetermined	
8G (1:10)	8	Cdif	Undetermined	
8G (1:10)	8	Cdif	Undetermined	
9G	9	Cdif	Undetermined	
9G	9	Cdif	Undetermined	
9G (1:10)	9	Cdif	Undetermined	
9G (1:10)	9	Cdif	Undetermined	
10G	10	Cdif	Undetermined	
10G	10	Cdif	Undetermined	
10G (1:10)	10	Cdif	Undetermined	
10G (1:10)	10	Cdif	Undetermined	
11G	11	Cdif	Undetermined	
11G	11	Cdif	Undetermined	
11G (1:10)	11	Cdif	Undetermined	
11G (1:10)	11	Cdif	Undetermined	
13G	13	Cdif	Undetermined	
13G	13	Cdif	Undetermined	

Location	Site number	Detector name	Ct	Mean Ct
14G	14	Cdif	Undetermined	
14G	14	Cdif	Undetermined	
14G (1:10)	14	Cdif	Undetermined	
14G (1:10)	14	Cdif	Undetermined	
14G (1:10)	14	Cdif	Undetermined	
14G (1:10)	14	Cdif	Undetermined	
4H	4	Cdif	Undetermined	
4H	4	Cdif	Undetermined	
4H (1:10)	4	Cdif	Undetermined	
4H (1:10)	4	Cdif	Undetermined	
5H	5	Cdif	Undetermined	
5H	5	Cdif	Undetermined	
5H (1:10)	5	Cdif	Undetermined	
5H (1:10)	5	Cdif	Undetermined	
8H	8	Cdif	Undetermined	
8H	8	Cdif	Undetermined	
8H (1:10)	8	Cdif	Undetermined	
8H (1:10)	8	Cdif	Undetermined	
9H	9	Cdif	Undetermined	
9H	9	Cdif	Undetermined	
9H (1:10)	9	Cdif	Undetermined	
9H (1:10)	9	Cdif	Undetermined	
10H	10	Cdif	Undetermined	
10H	10	Cdif	Undetermined	
10H (1:10)	10	Cdif	Undetermined	
10H (1:10)	10	Cdif	Undetermined	
11H	11	Cdif	Undetermined	
11H	11	Cdif	Undetermined	
11H (1:10)	11	Cdif	Undetermined	
11H (1:10)	11	Cdif	Undetermined	
13H	13	Cdif	Undetermined	
13H	13	Cdif	Undetermined	
4I	4	Cdif	Undetermined	
4I	4	Cdif	Undetermined	
4I (1:10)	4	Cdif	Undetermined	
4I (1:10)	4	Cdif	Undetermined	
5I	5	Cdif	Undetermined	

Location	Site number	Detector name	Ct	Mean Ct
5I	5	Cdif	Undetermined	
6I	6	Cdif	39.13	
6I	6	Cdif	Undetermined	
6I	6	Cdif	36.66	36.98
6I	6	Cdif	37.29	
6I	6	Cdif	38.18	37.64
6I	6	Cdif	37.09	
7I	7	Cdif	Undetermined	
7I	7	Cdif	Undetermined	
7I (1:10)	7	Cdif	Undetermined	
7I (1:10)	7	Cdif	Undetermined	
8I	8	Cdif	Undetermined	
8I	8	Cdif	Undetermined	
8I (1:10)	8	Cdif	Undetermined	
8I (1:10)	8	Cdif	Undetermined	
9I	9	Cdif	Undetermined	
9I	9	Cdif	Undetermined	
10I	10	Cdif	Undetermined	
10I	10	Cdif	Undetermined	
10I (1:10)	10	Cdif	Undetermined	
10I (1:10)	10	Cdif	Undetermined	
11I	11	Cdif	Undetermined	
11I	11	Cdif	Undetermined	
13I	13	Cdif	Undetermined	
13I	13	Cdif	Undetermined	
14I	14	Cdif	Undetermined	
14I	14	Cdif	Undetermined	
14I (1:10)	14	Cdif	Undetermined	
14I (1:10)	14	Cdif	Undetermined	
4J	4	Cdif	Undetermined	
4J	4	Cdif	Undetermined	
4J (1:10)	4	Cdif	Undetermined	
4J (1:10)	4	Cdif	Undetermined	
5J	5	Cdif	Undetermined	
5J	5	Cdif	Undetermined	
6J	6	Cdif	Undetermined	
6J	6	Cdif	Undetermined	

Location	Site number	Detector name	Ct	Mean Ct
7J	7	Cdif	Undetermined	
7J	7	Cdif	Undetermined	
7J (1:10)	7	Cdif	Undetermined	
7J (1:10)	7	Cdif	Undetermined	
8J	8	Cdif	Undetermined	
8J	8	Cdif	Undetermined	
8J (1:10)	8	Cdif	Undetermined	
8J (1:10)	8	Cdif	Undetermined	
9J	9	Cdif	Undetermined	
9J	9	Cdif	Undetermined	
9J (1:10)	9	Cdif	Undetermined	
9J (1:10)	9	Cdif	Undetermined	
10J	10	Cdif	Undetermined	
10J	10	Cdif	Undetermined	
10J (1:10)	10	Cdif	Undetermined	
10J (1:10)	10	Cdif	Undetermined	
11J	11	Cdif	Undetermined	
11J	11	Cdif	Undetermined	
14J	14	Cdif	Undetermined	
14J	14	Cdif	Undetermined	
14J (1:10)	14	Cdif	Undetermined	
14J (1:10)	14	Cdif	Undetermined	
4K	4	Cdif	Undetermined	
4K	4	Cdif	Undetermined	
4K (1:10)	4	Cdif	Undetermined	
4K (1:10)	4	Cdif	Undetermined	
5K	5	Cdif	Undetermined	
5K	5	Cdif	Undetermined	
5K (1:10)	5	Cdif	Undetermined	
5K (1:10)	5	Cdif	Undetermined	
6K	6	Cdif	Undetermined	
6K	6	Cdif	Undetermined	
7K	7	Cdif	Undetermined	
7K	7	Cdif	Undetermined	
7K (1:10)	7	Cdif	Undetermined	
7K (1:10)	7	Cdif	Undetermined	
8K	8	Cdif	Undetermined	

Location	Site number	Detector name	Ct	Mean Ct
8K	8	Cdif	Undetermined	
9K	9	Cdif	Undetermined	
9K	9	Cdif	Undetermined	
10K	10	Cdif	Undetermined	
10K	10	Cdif	Undetermined	
10K (1:10)	10	Cdif	Undetermined	
10K (1:10)	10	Cdif	Undetermined	
11K	11	Cdif	Undetermined	
11K	11	Cdif	Undetermined	
14K	14	Cdif	Undetermined	
14K	14	Cdif	Undetermined	
14K (1:10)	14	Cdif	Undetermined	
14K (1:10)	14	Cdif	Undetermined	
4L	4	Cdif	Undetermined	
4L	4	Cdif	Undetermined	
5L	5	Cdif	Undetermined	
5L	5	Cdif	Undetermined	
5L (1:10)	5	Cdif	Undetermined	
5L (1:10)	5	Cdif	Undetermined	
6L	6	Cdif	Undetermined	
6L	6	Cdif	Undetermined	
7L	7	Cdif	Undetermined	
7L	7	Cdif	Undetermined	
7L (1:10)	7	Cdif	Undetermined	
7L (1:10)	7	Cdif	Undetermined	
9L	9	Cdif	Undetermined	
9L	9	Cdif	Undetermined	
10L	10	Cdif	Undetermined	
10L	10	Cdif	Undetermined	
10L (1:10)	10	Cdif	Undetermined	
10L (1:10)	10	Cdif	Undetermined	
11L	11	Cdif	Undetermined	
11L	11	Cdif	Undetermined	
11L (1:10)	11	Cdif	Undetermined	
11L (1:10)	11	Cdif	Undetermined	
14L	14	Cdif	Undetermined	
14L	14	Cdif	Undetermined	

Location	Site number	Detector name	Ct	Mean Ct
14L (1:10)	14	Cdif	Undetermined	
14L (1:10)	14	Cdif	Undetermined	
4M	4	Cdif	Undetermined	
4M	4	Cdif	Undetermined	
4M	4	Cdif	Undetermined	
4M	4	Cdif	Undetermined	
5M	5	Cdif	Undetermined	
5M	5	Cdif	Undetermined	
5M (1:10)	5	Cdif	Undetermined	
5M (1:10)	5	Cdif	Undetermined	
6M	6	Cdif	Undetermined	
6M	6	Cdif	Undetermined	
6M (1:10)	6	Cdif	39.30	
6M (1:10)	6	Cdif	Undetermined	
6M (1:10)	6	Cdif	38.65	
6M (1:10)	6	Cdif	Undetermined	
7M	7	Cdif	Undetermined	
7M	7	Cdif	Undetermined	
7M (1:10)	7	Cdif	Undetermined	
7M (1:10)	7	Cdif	Undetermined	
8M	8	Cdif	Undetermined	
8M	8	Cdif	Undetermined	
8M (1:10)	8	Cdif	Undetermined	
8M (1:10)	8	Cdif	Undetermined	
9M	9	Cdif	Undetermined	
9M	9	Cdif	Undetermined	
9M (1:10)	9	Cdif	Undetermined	
9M (1:10)	9	Cdif	Undetermined	
10M	10	Cdif	Undetermined	
10M	10	Cdif	Undetermined	
10M (1:10)	10	Cdif	Undetermined	
10M (1:10)	10	Cdif	Undetermined	
11M	11	Cdif	Undetermined	
11M	11	Cdif	Undetermined	
13M	13	Cdif	Undetermined	
13M	13	Cdif	Undetermined	
14M	14	Cdif	Undetermined	

Location	Site number	Detector name	Ct	Mean Ct
14M	14	Cdif	Undetermined	
14M (1:10)	14	Cdif	Undetermined	
14M (1:10)	14	Cdif	Undetermined	
4N	4	Cdif	Undetermined	
4N	4	Cdif	Undetermined	
4N (1:10)	4	Cdif	Undetermined	
4N (1:10)	4	Cdif	Undetermined	
5N	5	Cdif	Undetermined	
5N	5	Cdif	Undetermined	
5N (1:10)	5	Cdif	Undetermined	
5N (1:10)	5	Cdif	Undetermined	
6N	6	Cdif	Undetermined	
6N	6	Cdif	Undetermined	
7N	7	Cdif	Undetermined	
7N	7	Cdif	Undetermined	
7N (1:10)	7	Cdif	Undetermined	
7N (1:10)	7	Cdif	Undetermined	
8N	8	Cdif	Undetermined	
8N	8	Cdif	Undetermined	
8N (1:10)	8	Cdif	Undetermined	
8N (1:10)	8	Cdif	Undetermined	
9N	9	Cdif	Undetermined	
9N	9	Cdif	Undetermined	
9N (1:10)	9	Cdif	Undetermined	
9N (1:10)	9	Cdif	Undetermined	
10N	10	Cdif	Undetermined	
10N	10	Cdif	Undetermined	
10N (1:10)	10	Cdif	Undetermined	
10N (1:10)	10	Cdif	Undetermined	
11N	11	Cdif	22.76	26.32
11N	11	Cdif	29.87	
11N	11	Cdif	Undetermined	
11N	11	Cdif	Undetermined	
11N (1:10)	11	Cdif	Undetermined	
11N (1:10)	11	Cdif	Undetermined	
13N	13	Cdif	Undetermined	
13N	13	Cdif	Undetermined	

Location	Site number	Detector name	Ct	Mean Ct
14N	14	Cdif	Undetermined	
14N	14	Cdif	Undetermined	
14N (1:10)	14	Cdif	Undetermined	
14N (1:10)	14	Cdif	Undetermined	
10^{-5}		Cdif	29.35	29.53
10^{-5}		Cdif	29.71	
10^{-5}		Cdif	28.77	28.84
10^{-5}		Cdif	28.91	
10^{-5}		Cdif	29.14	29.15
10^{-5}		Cdif	29.16	
10^{-5}		Cdif	29.35	29.39
10^{-5}		Cdif	29.42	
10^{-5}		Cdif	29.41	29.43
10^{-5}		Cdif	29.45	
10^{-5}		Cdif	29.45	29.48
10^{-5}		Cdif	29.50	
10^{-5}		Cdif	29.12	29.24
10^{-5}		Cdif	29.36	
10^{-5}		Cdif	28.80	28.74
10^{-5}		Cdif	28.69	
10^{-5}		Cdif	29.61	29.34
10^{-5}		Cdif	29.07	
10^{-5}		Cdif	28.39	28.47
10^{-5}		Cdif	28.56	
10^{-5}		Cdif	28.57	28.53
10^{-5}		Cdif	28.49	
10^{-5}		Cdif	28.27	28.35
10^{-5}		Cdif	28.44	
10^{-3}		Cdif	20.57	20.56
10^{-3}		Cdif	20.54	
NTC		Cdif	Undetermined	
NTC		Cdif	Undetermined	

Location	Site number	Detector name	Ct	Mean Ct
NAC		Cdif	Undetermined	
NAC		Cdif	Undetermined	
NTC		Cdif	Undetermined	
NTC		Cdif	Undetermined	
NAC		Cdif	Undetermined	
NAC		Cdif	Undetermined	
NTC		Cdif	Undetermined	
NTC		Cdif	Undetermined	
NAC		Cdif	Undetermined	
NAC		Cdif	Undetermined	
NTC		Cdif	Undetermined	
NTC		Cdif	Undetermined	
NAC		Cdif	Undetermined	
NAC		Cdif	Undetermined	
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NTC		Cdif	Undetermined	
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NAC		Cdif	Undetermined	
NTC		Cdif	Undetermined	
NTC		Cdif	Undetermined	
NAC		Cdif	Undetermined	

Location	Site number	Detector name	Ct	Mean Ct
NAC		Cdif	Undetermined	
NTC		Cdif	Undetermined	
NTC		Cdif	Undetermined	
NAC		Cdif	Undetermined	
NAC		Cdif	Undetermined	
NTC		Cdif	Undetermined	
NTC		Cdif	Undetermined	
NAC		Cdif	Undetermined	
NAC		Cdif	Undetermined	
NTC		Cdif	Undetermined	
NTC		Cdif	Undetermined	
NAC		Cdif	Undetermined	
NAC		Cdif	Undetermined	

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