THE BUILT ENVIRONMENT IMPLICATIONS OF *C. DIFFICILE* SPORE DEPOSITION FROM PATIENTS WITH HOSPITAL-ASSOCIATED *C. DIFFICILE* INFECTIONS

by

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A THESIS

Presented to the Department of Architecture
and the Robert D. Clark Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science

May 2023

An Abstract of the Thesis of

Liliana Barnatan for the degree of Bachelor of Science
in the Department of Neuroscience to be taken June 2023

Title: The Built Environment Implications of *C. Difficile* Spore Deposition from Patients with Hospital-Associated *C. Difficile* Infections

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Clostridioides difficile (C. difficile, C. diff.) is a spore-forming, bacterium that infects the gastrointestinal tract of humans. The bacterium is transmitted via a fecal-oral route and germinates in the large intestine. It proliferates in a dysbiotic gut microbiome, commonly found in the following populations, including the elderly, cancer patients, people undergoing antibiotic treatment, surgical patients, and immunocompromised patients. The bacterium is the most common nosocomial cause of infective diarrhea and is the most common hospital-associated infection (HAI). Shedding of spores by symptomatic and asymptomatic patients contributes to the deposition of *C. difficile* in the hospital and can establish *C. difficile* reservoirs in the hospital environments they occupy. Our study sampled seven frequently touched locations within the room of 13 patients with a *C. difficile* infection at diagnosis, before room cleaning, and after room cleaning. A two-way ANOVA demonstrated that there were no significant differences between room locations and time points. However, we found that in several locations, including the staff keyboard, visitor chair, and exhaust grille there was prevalence of *C. difficile* found in the samples after decontamination protocols. This emphasizes the need to better understand mechanisms of transmission between the environment-to-person as *C. difficile* reservoirs can persist after cleaning, and potentially infect the next patient to occupy the room.

Our study sought to elucidate the dynamics of spore deposition in patient rooms that influence the likelihood of future patients getting infections.

Acknowledgements

I am extremely grateful to Dr. Robert Martindale (M.D, Ph.D.), and Dr. Kevin Van Den Wymelenberg (Ph.D.) for providing the tools and collaboration necessary to complete this project. This endeavor would not have been possible without the assistance and communication with the research associates in the Department of Surgery at the Oregon Health and Science University. I would like to especially acknowledge Dr. Kevin Van Den Wymelenberg and Dr. Mark Fretz (MArch, D.D.S.) for serving as excellent mentors and challenging me throughout this process. Dr. Kevin Van Den Wymelenberg recognized my ability to complete this project, while Dr. Mark Fretz held me accountable for my work and motivated me to pursue this project. I am also grateful for the contributions from Charlie Borzy for collecting samples, and I am especially thankful for the work Leslie Dietz (M.S.) has performed in sample collection, processing, DNA extractions, PCR, and culturing. I could not have undertaken this project without the numerous lessons Leslie has taught me, her depth of knowledge, and without her inspiring passion for science that fostered my drive for this project.

I'd also like to thank the OHSU healthcare personnel for facilitating sample collection and providing excellent patient care. Gratitude should also go to the staff in the Biology of the Built Environment Laboratory, as they contributed to this project and have provided me opportunities to work in the lab and have formed a welcoming, safe environment to work in. I'd additionally like to recognize Vincent Moore and Patrick Horve, for assisting with culturing and other laboratory work. Lastly, I'd like to acknowledge my family and friends for supporting me through this process.

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Introduction

Clostridiodes difficile (*C. difficile*, *C. diff.*) is a spore forming, anaerobic, Gram-positive bacterium that causes *C. difficile* infection (CDI) in the intestinal tract of humans and animals (Crobach et al. 2018; Czepiel et al. 2019; Kazanowski et al. 2014). *C. difficile* is formerly known as *Clostridium difficile*, wherein after molecular analysis they transferred the genus classification in 2016 (CDC, 2019). The bacterium can be found as spores on surfaces, soil, air, and water (*Clostridium Difficile* (*C. Diff*), 2020). The *C. difficile* transmits to humans through a fecal-oral route in which its spores are resistant to the acidic environment of the stomach (Crobach et al. 2018; Czepiel et al. 2019; Kazanowski et al. 2014)). Since *C. difficile* is shed through feces, if the feces have contact with any surface or material, the area can become a reservoir for *C. diff.* spores (CDC, 2019). For this reason, *C. difficile* is often referred to as a hospital-associated infection (HAI) as spores can be transferred to the hands of hospital personnel, or deposited through hospital traffic. The infection is also common in community-healthcare settings. It is estimated that approximately two-thirds of CDIs begin in hospitals, long-term care facilities, assisted living facilities, or other healthcare settings (*Clostridium Difficile* (*C. Diff*), 2020).

C. difficile Spore Resistance and Proliferation in a Dysbiotic Gut

C. difficile spores are metabolically dormant. These spores are composed of 7 layers, ordering from the center to the outer layer; they include the core, inner membrane, germ cell wall, cortex, outer membrane, the spore coat, and the exosporium (Lawler, Lambert, and Worthington 2020). The core consists of molecules essential for the spore to return to metabolic activity, such as DNA, tRNA, ribosomes, and enzymes. The core has very low water content, ranging from 25%-55% in wet weight, this in turn makes spores more resistant to temperature (Lawler, Lambert, and Worthington 2020). The spore DNA is bound to protective enzymes that

spare the genome from heat, chemical, and ultraviolet damage. The inner membrane has very low permeability, preventing the entrance of harmful substances into the core. The germ cell wall, cortex, and the outer membrane are important for cell germination. The spore coat and exosporium provide further protection and resistance to enzymes, chemicals, lysozymes, ethanol, and heat (Lawler, Lambert, and Worthington 2020). Therefore, the internal properties of the *C. difficile* spore allows for resistance in extreme conditions, and survival for up to 5 months. Asymptomatic and symptomatic patients with CDI both shed spores throughout their infection.

People who are susceptible to CDIs are elderly people, those undergoing antibiotic treatment, immunocompromised, hospitalized patients, specifically cancer patients and people housed in surgical units. These populations tend to be vulnerable to CDIs because they are more likely to have gut dysbiosis, an imbalance of the naturally occurring bacteria within the intestines. Typically, a healthy abundance of microbiota provides the best protection against *C. difficile* colonization in the digestive tract (Crobach et al. 2018; Czepiel et al. 2019; Kazanowski et al. 2014). In a healthy abundance of gut microbiota, *C. difficile* proliferation is severely hindered, as it faces competition to grow in the presence of other bacteria. However, susceptible populations tend to have a lower species richness and species diversity in the microflora of their gut. This gut imbalance results in easy establishment of new bacteria species that are introduced to the gut, thereby allowing *C. difficile* to thrive in this environment due to the lack of competition. Furthermore, patients with a compromised immune system are less likely to be able to effectively produce antimicrobial compounds such as lysozymes and cationic antimicrobial peptides, which is the body's first defense against CDI (Giesemann, Guttenberg, and Aktories 2008; Smits et al. 2016).

The dysbiosis within the gut of susceptible patients, which enables *C. difficile* growth, results from the inability to produce the chemical substances needed to digest fats. In order for proper fat digestion, the liver produces bile acids that are secreted into the digestive system to break down fats. These acids are considered primary bile acids, which later undergo a chemical reaction that converts them into secondary bile acids. When the gut microbiota is unbalanced or unhealthy, meaning changes in abundance and diversity of naturally occurring bacteria (present in the vulnerable populations), the primary bile acids cannot efficiently be converted to secondary bile acids. This is important because the presence of a high amount of primary bile acids induces *C. difficile* spore germination and colonization in the colon (Crobach et al. 2018; Czepiel et al. 2019; Kazanowski et al. 2014). Therefore, not only is there less competition but also the chemical environment of the disturbed gut allows for the growth and prosperity of *C. difficile.*

C. difficile Infection and Treatment

C. difficile colonizes within the epithelial lining of the mucosa in the intestines, where *C. difficile* dominates over other microorganisms. During *C. difficile* growth, it becomes infectious and begins to produce its toxins A and B, a known enterotoxin (toxic to the intestines) and cytotoxin (toxic to individual cells), respectively. These toxins can enter the cytoplasm of cells and lead to cessation of normal cycles and apoptosis (cell death) (Kazanowski et al. 2014). Studies show that toxin A leads to more fluid secretion in feces while toxin B is responsible for the virulence of the infection and inflammatory response. Patients with CDI often present with symptoms ranging from mild to life threatening including, diarrhea, abdominal pain, fevers, dehydration, ileus, and eventually toxic megacolon (Crobach et al. 2018; Czepiel et al. 2019; Kazanowski et al. 2014). A patient is considered colonized with *C. difficile* when there is a

detection of *C. diff.* with an absence of CDI symptoms and is considered infected when presents with CDI symptoms and also test positive for the presence of either or both *C. difficile* toxin(s) (Crobach et al. 2018; Czepiel et al. 2019; Kazanowski et al. 2014).

Broad spectrum antibiotics such as Clindamycin, Cephalosporins, Penicillins, Fluoroquinolones often lead to CDI as they target a wide variety of bacteria, including the healthy microbiota in the gut, ultimately creating an optimal environment for *C. difficile* proliferation (*C. Difficile* Infection - Symptoms and Causes, 2021; (Czepiel et al. 2019)). According to the CDC, people undergoing antibiotic treatment are seven to ten times more likely to acquire a CDI during their treatment or a month following compared to healthy individuals (CDC, 2018). Treatments for a CDI include antibiotics such as vancomycin or fidaxomicin, and in extremely resistant cases, a fecal microbiota transplant. However, one in 6 patients who suffer from a CDI could be reinfected in the following two to eight weeks, and 1 in 11 people over the age of 65 diagnosed with a HAI of *C. difficile* will perish within one month (CDC, 2018). This recurrence develops because antibiotics target metabolically active cells, and if *C. difficile* spores have yet to germinate, they will persist in the gut. As a result, after the antibiotic dosage is completed, the probability of re-infection persists as the durable spores still present may germinate and colonize the colon once more (Smits et al. 2016).

Built Environment Implications

C. difficile spores are quite resistant and can survive harsh environments and as a result, these spores can survive on surfaces for up to five months despite cleaning procedures (Gharaibeh, Smith, and Conway 2021). *C. difficile* spores are resistant to heat, oxygen, ethanol-based disinfectants, and elimination of environmental depositions requires a 1:10 dilution of sodium hypochlorite (Sandhu and McBride 2018). The bacterium *C. difficile* is an established

nosocomial infection, and a strong relationship exists between an increase in CDI mortality rates and an aging population (Abdullatif and Noymer 2016). Evidence suggests that the rise in CDI mortality is due to the increase in length of hospital stay (Abdullatif and Noymer 2016; Crobach et al. 2018; Czepiel et al. 2019). This demonstrates a need to better understand how aspects of the built environment may contribute to *C. difficile* spore deposition, prevalence and ultimately lead to more HAIs. Research shows that high human occupancy, human use and movement, building arrangement and ventilation sources have a strong impact on the distribution and diversity of the microbial communities found in our built environment (Kembel et al. 2014). Furthermore, materials used to construct buildings such as timber, concrete, or gypsum have different impacts on species abundance, diversity, and volatile organic compound emissions within the environment (Mhuireach et al. 2021). This illustrates that architectural design can influence the chemical and microbial environments within a building (Mhuireach et al. 2021). However, little is known about the relationship between healthcare built environments and *C. difficile* distribution.

The origin of a hospital-associated CDI is complex and can be difficult to identify, as differentiating community-associated versus hospital-associated infections is not a definitive process. International guidelines dictate a CDI to be a HAI when symptoms arise two or more days after admission (McLure et al. 2018). However, current mathematical models suggest that two days is overestimating HAIs and researchers recommend the standard to be an optimal five days post admission (McLure et al. 2018). In a longitudinal study performed in Australia, researchers analyzed the ribotypes (strain variation) in samples collected from symptomatic and asymptomatic CDI cases that were either HAI or community-acquired over a three-year period. They found that 79% of the isolates found in the HAI group were also present in the community-

acquired infection group (Furuya-Kanamori et al., n.d.). This result elucidates an unknown relationship between the infections prevalent in the community and those found in the hospital. Thus, the built environment likely plays a role in the mode of transmission of CDIs, as asymptomatic and colonized patients can shed spores, depositing them in the building throughout their infections. Additional studies show that the abundance of *C. difficile* in the population dramatically increases when comparing community cases to within the hospital, and increases more with hospital stay length (Abdullatif and Noymer 2016; Crobach et al. 2018; Czepiel et al. 2019). For these reasons, research scientists and hospital personnel support the idea that *C. difficile* is the main cause of nosocomial infective diarrhea and that it is one the most common HAIs (Abdullatif and Noymer 2016; Chamchod and Palittapongarnpim 2019). In 2015, the Centers for Disease Control (CDC) estimated a total of 500,000 *C. difficile* infections and 15,000-30,000 deaths due to *C. difficile* hospital-associated infections in the United States.This demonstrates the importance for understanding *C. difficile* transmission within the hospital, as longer hospital stays increase *C. difficile* prevalence.

Chamchod et al. used mathematical models to examine the prevalence and persistence of *C. difficile* in the hospital based on admission rates of colonized and symptomatic patients and the control efforts of reducing *C. difficile*, which included cleaning protocols and use of personal protective equipment (PPE) . This study showed that the number of patients admitted with CDI had a stronger effect on CDI abundance when compared to the relationship between the control efforts to reduce infections and CDI prevalence (Chamchod and Palittapongarnpim 2019). This suggests that the presence of colonized patients increases the risks of other patients acquiring CDI. Furthermore, because the presence of CDI patients has a greater impact on *C. difficile* persistence than control measures, it implies a lack of efficacy in decontamination protocols. A

similar study was performed as a retrospective analysis on the prevalence of *C. difficile* using space and time clusters in an Italian hospital (Kroker and Azadian, 2000). In this study they found significant clusters between space and time, where they observed between-ward cross infections, suggesting that hospital traffic is involved in *C. difficile* transmission. However, the researchers found no significance between space and time within hospital wards, concluding that in-ward protocols must be efficient in preventing spread of *C. difficile* in this hospital. This further reveals the need to investigate the abundance of *C. difficile* environmentally to be able to understand these mechanisms of its transmission.

Other studies have investigated *C. difficile* abundance in healthcare settings as well. One study analyzed the presence of MRSA and *C. difficile* on hospital windows and window shades. The researchers found windows had similar microbial composition to other rooms that tend to harbor reservoirs of microbes (Horve et al. 2020). Horve et al. also found that rooms with higher degrees of connectivity (more central in ward), contained more viable bacteria than rooms that were located on the periphery (Horve et al. 2020). The research team justified this finding to the proximity of the nurse's station and increased foot traffic. Furthermore, rooms with less exposure to sunlight had significantly more viable bacteria than other rooms (Horve et al. 2020). This finding illustrates the importance of understanding the relationship between architectural design and composition of microbe environments, as certain conditions may foster persistence of unwanted bacteria. Another study investigated the possibility of *C. difficile* aerosolization and deposition through toilet flushes (E. L. Best, Sandoe, and Wilcox 2012). As *C. difficile* is shed through feces, the toilet often harbors significant reservoirs. In this study, the researchers simulated toilet flushes containing *C. difficile* with toilets that are often used in hospitals (with and without lids). In the lidless toilet, they found that *C. difficile* can suspend in the air and

gradually deposit on surfaces in the room over the course of 90 minutes. *C. difficile* can be resuspended and deposited in rooms through brief aerosolization in toilets, and potentially air currents. Understanding these environmental conditions that may play a role in *C. difficile* deposition can help us better understand the dynamics of *C. difficile* transmission.

Moreover, in the Montoya et al. investigation, the researchers analyzed the samples of microorganisms found on the hand of hospital personnel (Montoya et al. 2019). The authors of the systematic review included only two articles that looked for *C. difficile*, in which one found the presence of *C. difficile* in the hand sample. While there is very little data on the presence of *C. difficile* on staff hands and the environment, this emphasizes the need to investigate more on the prevalence of *C. difficile* in the environment surrounding a patient. There is a lack of research on the spatial distributions of *C. difficile* in the patient environment, and how it relates with patient and hospital staff movements. Being able to identify the strain of *C. difficile* that infects a patient and strains present in the patient's room would provide further evidence to support mechanisms of transmission of hospital-associated CDI's. This is important because preliminary research suggests that bacterial deposits are found in frequently touched areas which may contribute to the transmission on the hands, clothes, or body of healthcare personnel. The aim of our study was to (1) identify the *C. diff.* strain that infects the patient and the strain of the environmental samples, (2) determine the environmental relative abundances of *C. difficile* at diagnosis, before, and after cleaning within the room, and (3) identify potential reservoirs of *C. difficile* in frequently touched and traveled areas. Our hypotheses were that relative abundance of *C. difficile* would persist post cleaning within frequently touched areas, and that the strain identified from the patient stool sample would match that of one found in the patient room.

Methods

Population

A total of 13 people that were diagnosed with a *C. difficile* infection at OHSU were enrolled in the study. To be included in the study, participants needed to: (i) be diagnosed at OHSU with a hospital-associated infection of *C. difficile*, (ii) have the ability to give informed consent, (iii) be over 18 years or older, and (iv) speak English or Spanish. Participants were excluded if they were unable to comply with study procedures or had any other condition that precluded participation in the study. No vulnerable populations were enrolled. Protocols were approved by the Oregon Health and Science University (OHSU) Institutional Review Board. Subjects were identified by a positive test result of CDI two or more days post admission at OHSU and screened for eligibility. Informed consent was obtained and no remuneration was provided to subjects.

Field Collection

A stool sample was collected from each study participant as close to diagnosis as possible. Environmental samples of the patient room that the CDI patients occupied were collected at three different time points: (1) at diagnosis, (2) immediately after a patient was discharged before the room was cleaned (pre cleaning), and (3) after the room was cleaned and prior to another patient occupying the room (post cleaning). Patient rooms were cleaned with 10% sodium hypochlorite (bleach) and a UV-c TURDY robot. Researchers and hospital personnel wore appropriate protective personal equipment (PPE), which included gloves and a gown, when entering the room. Environmental samples were collected with sterile swabs (Puritan #25-806 1PD BT) and placed in 2mL of sterile phosphate-buffered saline (1X PBS).

Environmental sample locations included the alarm buttons, bed rails, floors, exhaust ventilation sources, chairs, keyboard, and wash station as these were the most frequently touched areas (Figure 1). The swabs were pre-moistened with 1X PBS and environmental samples were collected with an "S" stroke movement vertically, horizontally, and then diagonally, across each sampling location. The bedrail was sampled on the outer-side of the railing (away-from-patient side), and the chair was swabbed on the arms, back cushion, and seat cushion. The samples from the wash station were collected from swabbing the faucet handles, and the floors were sampled using a template where the patient places their feet when exiting the bed. After placing the swabs in the 2mL aliquot of 1X PBS, all sample tubes were sealed in a primary and secondary containment and stored at 4ºC until processing. Hospital staff movements (nurse, medical doctor, medical student, and allied health) within the patient were observed and noted by research personnel.

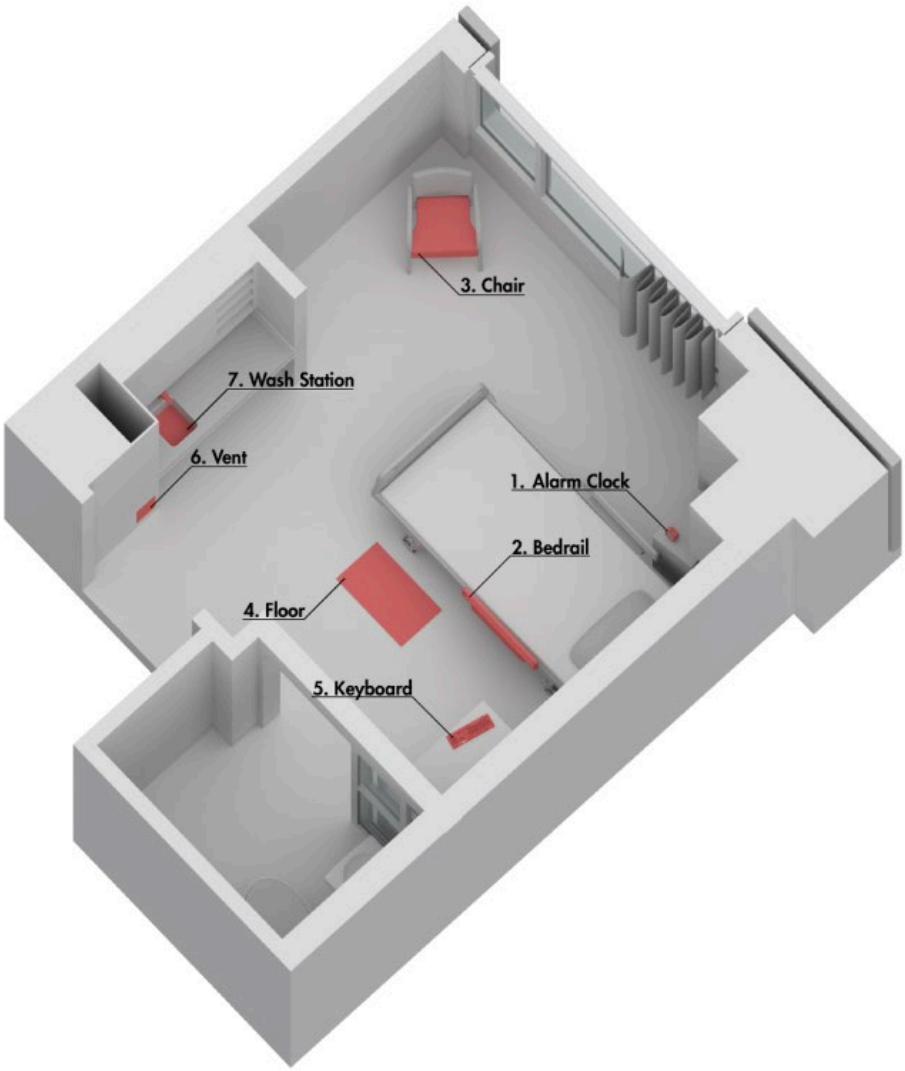

Figure 1. Sampling locations within the hospital room.

Sample Processing and Genomic Material Preparation

Samples were transferred to the Biology and the Built Environment (BioBE) BSL-2 laboratory located at the University of Oregon in Eugene, OR for processing. All samples were transported on ice and maintained at 4ºC until sample processing. The swabs tips and 1mL of the 1X PBS from the transport conical tubes were transferred into sterile 2mL Eppendorf tubes and vortexed to resuspend the sample collected in the swab tip. The swab tip was then removed from

the tube using sterile forceps, and the remaining 1mL of 1X PBS and suspended sample were used for DNA extraction using the DNeasy PowerLyzer PowerSoil Kit (Qiagen #12855-100), following the manufacturer's protocol.

Molecular Analysis and Culturing

To detect *C. difficile*, standards from Integrated DNA Technologies for qPCR were used. The primers used were for the *TcdA* gene and *TcdB* gene, including TcdA F (5′-CAGGACACACAGTGACTGGTAA-3′), TcdA R (5′- GAACTGCTCCAGTTTCCCAC-3′), TcdB F (5′-GTGTAGCAATGAAAGTCCAAGTTTACGC-3′) and TcdB R (5′-CACTTAGCTCTTTGATTGCTGCACCT-3′). All samples underwent thermocycling with the conditions of 95 °C for 5 min, 40 cycles of 95 °C for 15 s, 60 °C for 50 s, and 72 °C for 30 s. Culturing the *C. difficile* samples was attempted in order to identify the strain infecting the patient. The Cdifftox plate assay (CDPA) culturing protocol from (Darkoh, Dupont, and Kaplan 2011) was followed.

Data Analysis

The DADA2 version 1.12.1 in the R platform was used to filter, denoise, and trim the Raw Illumina Sequence Data and taxonomy was assigned using the Bayesian classifier within DADA2 (Quast et al. 2013). All sequences classified as *Clostridiodes* were identified, however only the reads that identified the genus difficile were considered as *C. difficile* reads. Relative abundance was calculated based on the number of *C. difficile* sequences divided by the total DNA sequence count of the microbiota found in each sample. Relative abundances were obtained for each room location and time point for comparison. To compare the relative abundances between room locations and time point, a two-way analysis of variance (ANOVA)

was performed and tested for interaction effect. The two-way ANOVA was used to determine how the mean of relative *C. difficile* abundance changes according to sample location and time point of collection.

Results

Data Analysis and Figures

A two-way ANOVA was performed to compare the relative abundance group means between the sample locations and the three time points. There was no significance found between the sample locations ($F=1.000$, $p= 0.436$) or the time points ($F= 0.383$, $p= 0.683$). Additionally, the interaction effect of sample location and time points was not significant ($F=0.738$, $p= 0.711$).

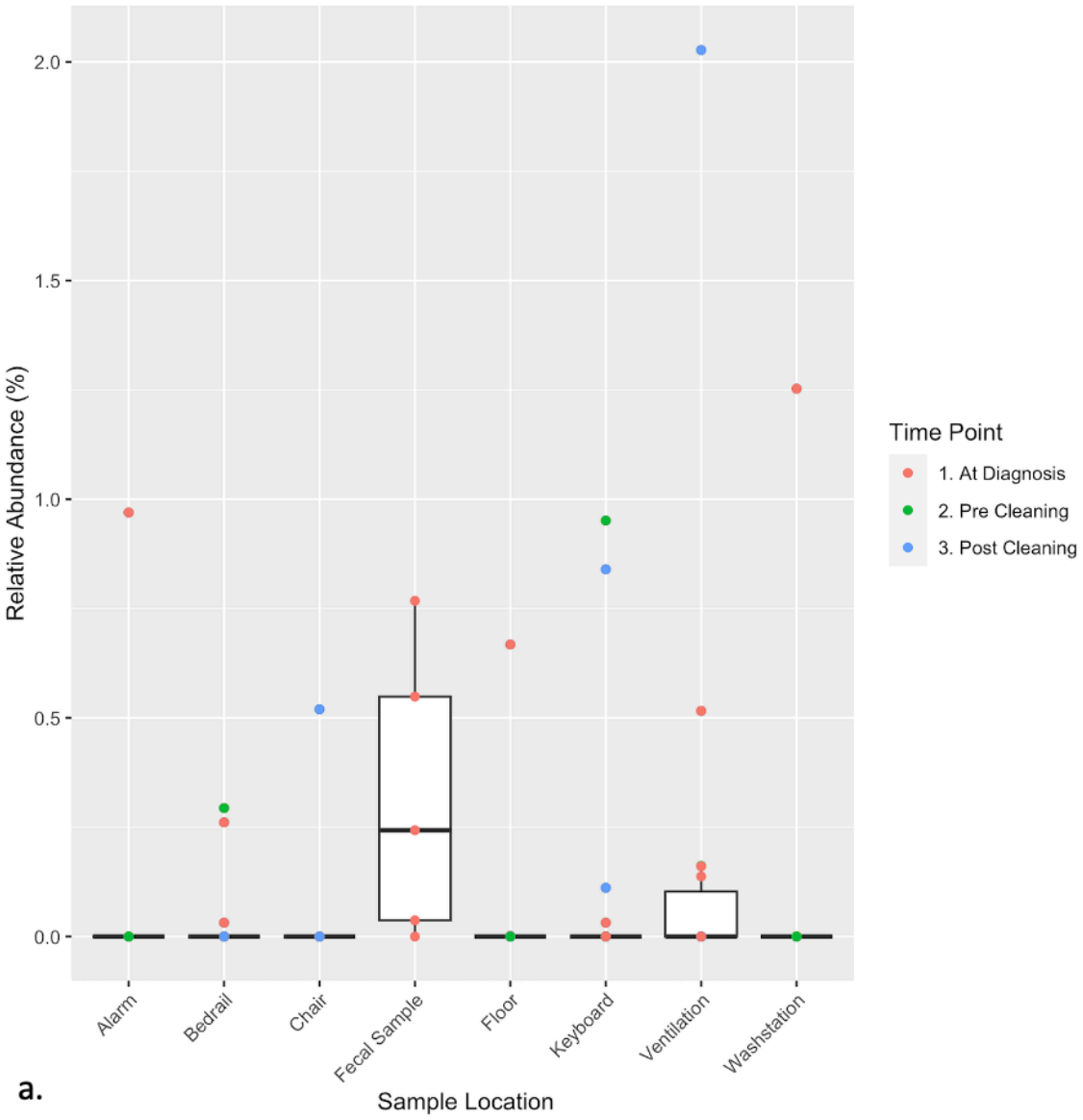

Figure 2. The relative abundance of C. difficile found in the sample locations at the three time points. A) demonstrates the boxplot of the relative abundances of all the samples collected.

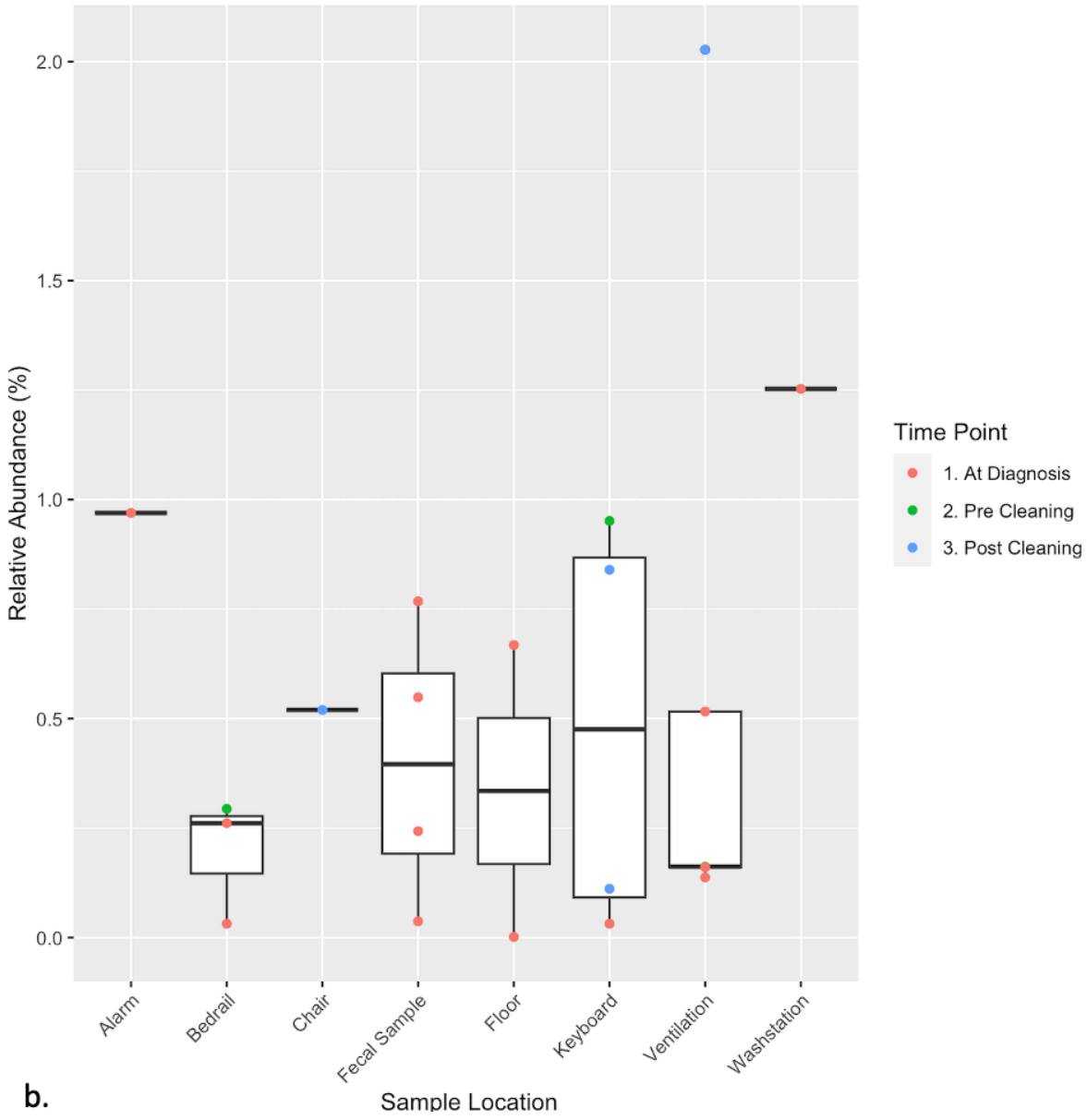

Figure 2. The relative abundance of *C. difficile* found in the sample locations at the three time points. B) illustrates the boxplot of the relative abundances of samples that had a *C. difficile* relative abundance greater than 0.

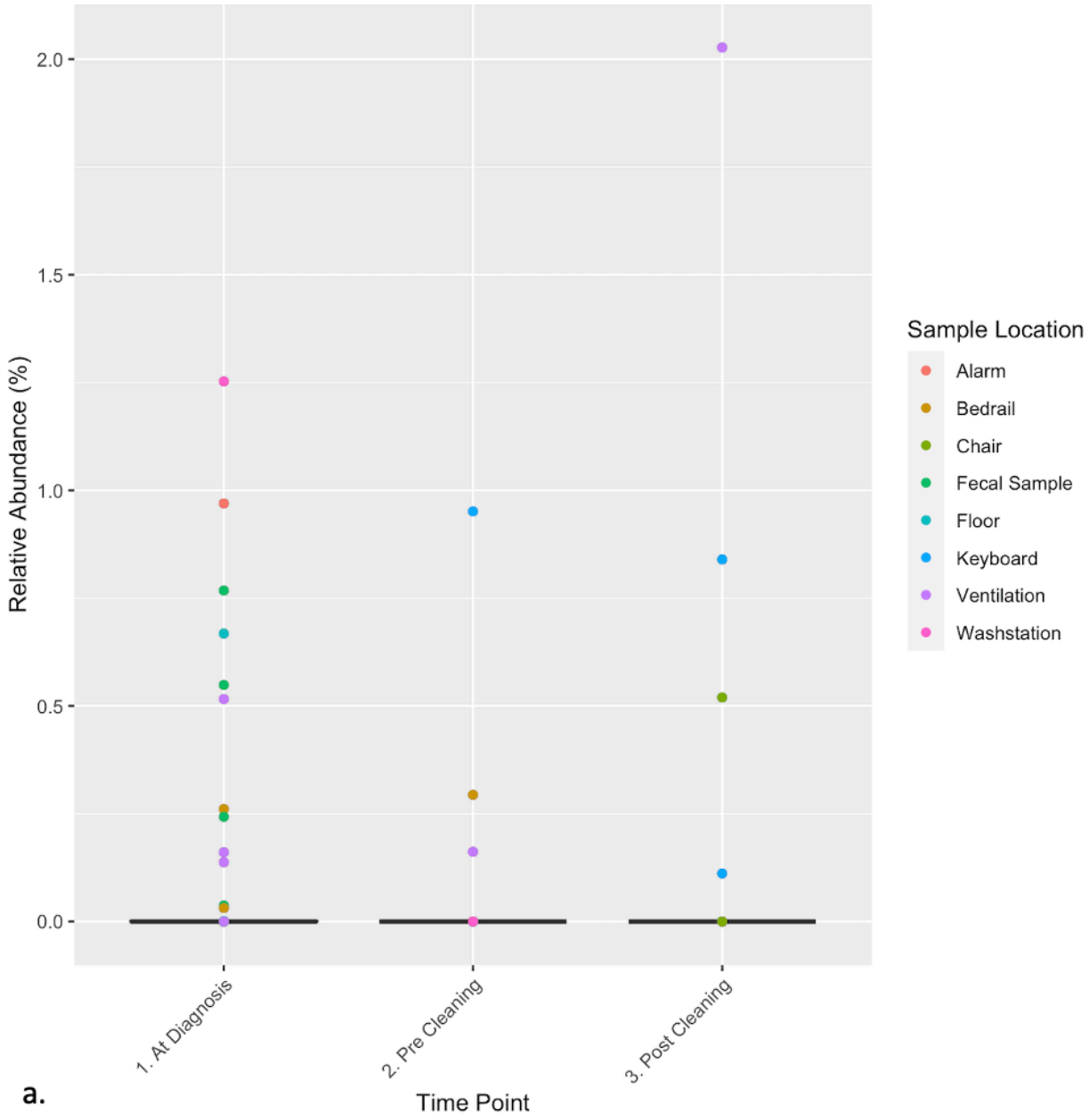

Figure 3. The relative abundance of *C. difficile* found at the three time points: 1. At diagnosis, 2. Pre-Cleaning, 3. Post Cleaning in the sampled locations. A) displays the boxplot of the relative abundance of all samples at the three time points.

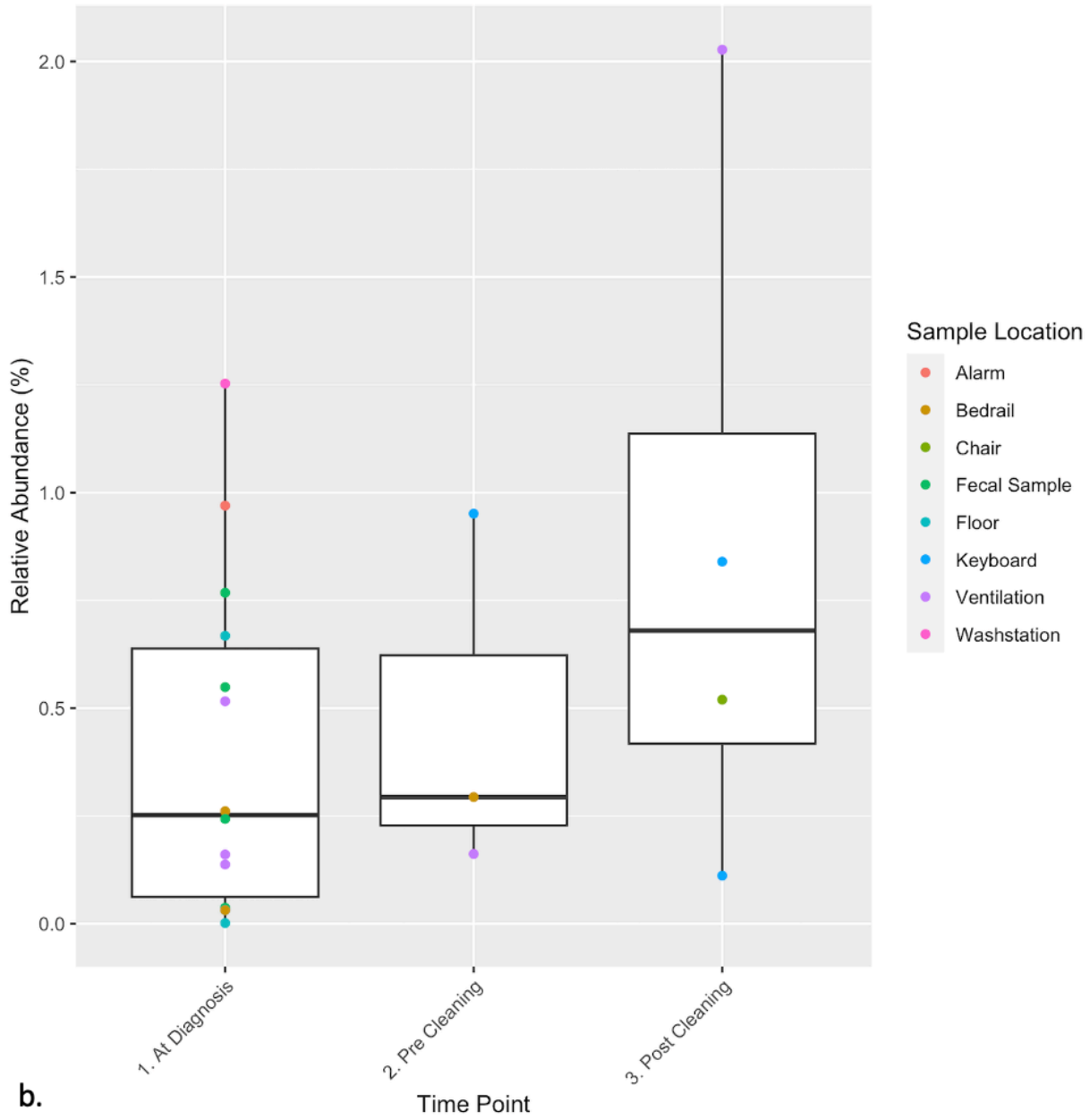

Figure 3. The relative abundance of *C. difficile* found at the three time points: 1. At diagnosis, 2. Pre-Cleaning, 3. Post Cleaning in the sampled locations. B) represents the boxplot for relative abundance of the samples at the three time points for the locations that had an abundance of *C. difficile* greater than 0.

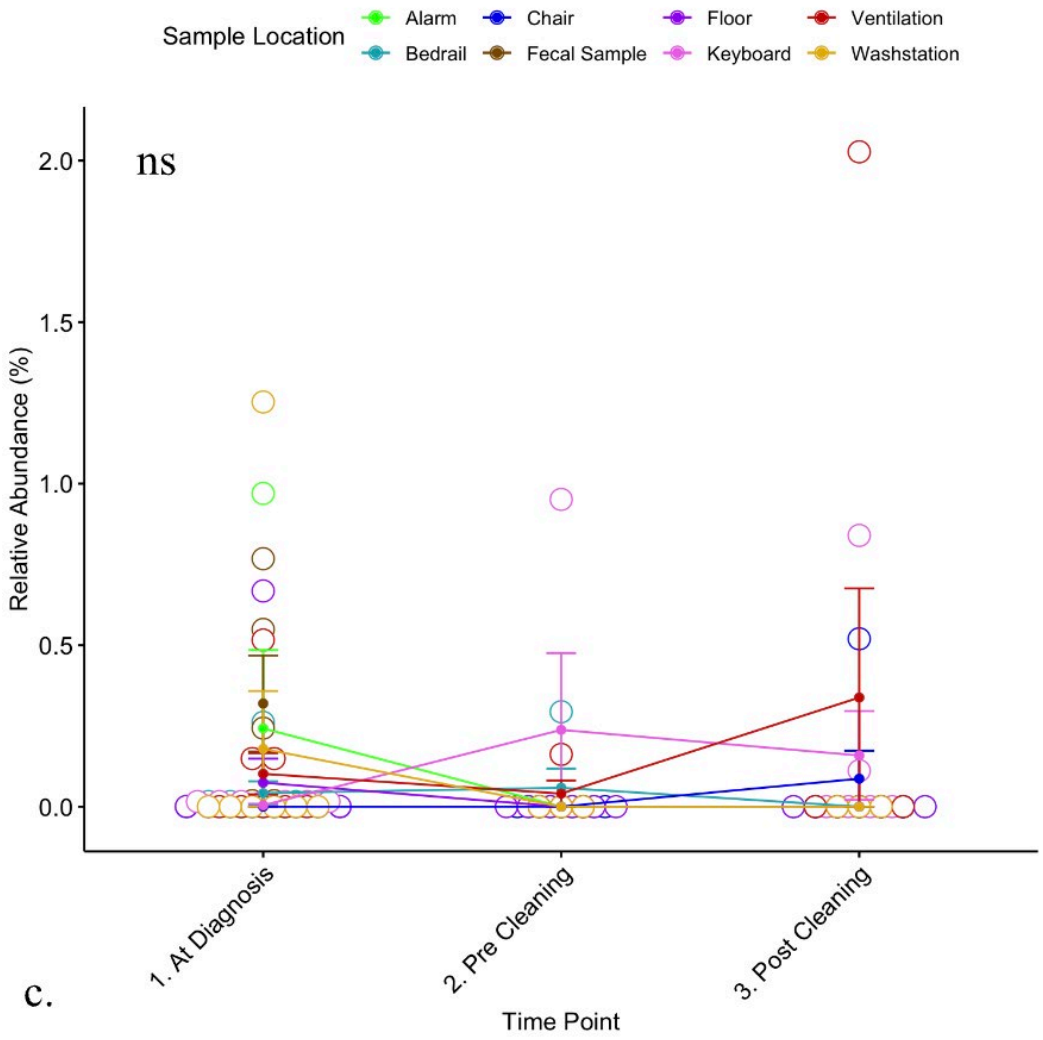

Figure 3. The relative abundance of *C. difficile* found at the three time points: 1. At diagnosis, 2. Pre-Cleaning, 3. Post Cleaning in the sampled locations. C) The relationship between the time of collection and relative abundance for the sample locations, no significant difference between means were found.

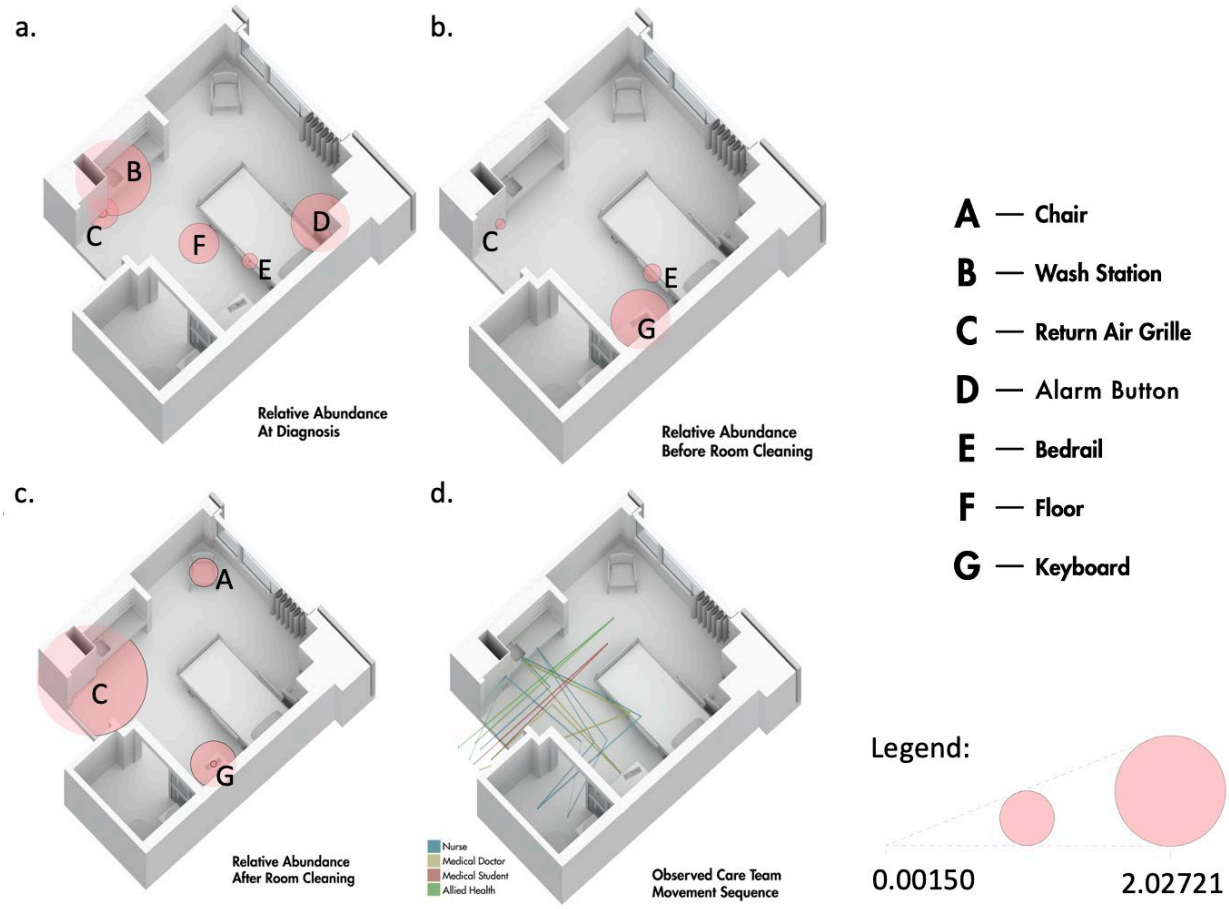

Figure 4. The spatial distribution of the relative abundance of *C. difficile* at diagnosis (a), pre cleaning (b), and post cleaning (c) of samples with a relative abundance of *C. difficile* greater than 0. The radius of the circle corresponds to the relative abundance (0-2%), circle quantity in one location indicates the number of samples collected from that area. The observed staff movements during patient stay are shown in (d).

Results of Culture

Culturing the *C. difficile* samples proved to be unsuccessful. The strain of the environmental samples and the patient stool was not identified.

Discussion

Our study is one of the first to investigate *C. difficile* deposition within the room of a patient diagnosed with CDI over the course of their hospitalization. This study contributes to the understanding of the potential for environmentally mediated mechanisms of hospital-associated *C. difficile* infection transmission. Through the use of quantitative and qualitative assessment of environmental samples and stool samples, this study aimed to (1) identify the *C. difficile* strain of the stool and the environment, (2) assess the environmental relative abundance of *C. difficile* at diagnosis as well as before and after cleaning within the room, and (3) identify potential reservoirs of *C. difficile* in frequently touched and traveled areas. Due to the difficulty to replicate anaerobic and nutrient conditions for *C. difficile* culturing, we were unable to successfully identify the strain found in stool samples and the environment. For the majority of our samples at all three time points and locations, the relative abundance of *C. difficile* was zero (Figure 2a). This may be due to the difficulty of extracting DNA from spores and therefore a lack of detection in some samples, or because there was indeed no *C. difficile* present in the sample.

Despite that there was no significant difference between group means in relative abundance found between sample locations or time points, we observed there to be persistence of *C. difficile* in some samples after cleaning the patient room. *C. difficile* was detected in samples taken from the chair, the staff keyboard, and on the room air return grille located near the floor (figures 3a, 4c). Furthermore, certain locations of the room tended to harbor reservoirs of *C. difficile* regardless of the time point of sample collection. Many samples from the exhaust ventilation had a persistent detectable abundance of *C. difficile* at diagnosis, before and after cleaning (figures 4a,b,c). When comparing this result to the observed staff movements, we see an ample amount of foot traffic near the ground room air return grille (figure 4d). This indicates that

C. difficile found on the floor (figure 4a) or other sources may resuspend, deposit, and collect in the room air return grille through air currents or foot traffic (Aithinne et al. 2019). Furthermore, the CDC mandates that only gowns and gloves must be used as personal protective equipment for patients with CDI (CDC, 2019). Because we have found *C. diff.* on the floor (figure 4a), it is possible that spores could be transferred through healthcare personnel shoes within the room and ward. These results offer insight on where healthcare personnel should focus when cleaning CDI patient rooms, with an emphasis on the ventilation, staff keyboards, and the visitor chair in addition to standard cleaning protocols.

Other studies have investigated persistence of *C. difficile* within the bathroom, as well as the air and surfaces in the presence of symptomatic patients. One study found that a commercial toilet bowl remains contaminated with the bacteria after more than 24 flushes and is capable of temporarily aerosolizing *C. difficile* during this time (Aithinne et al. 2019). This same study also found that the aerosolized *C. difficile* from the toilet flushes can travel through air currents and settle on further surfaces, thereby creating more reservoirs within the bathroom and patient room. Because the bathroom is an area of high traffic for the hospital staff and the patient (figure 4d) and has high exposure to fecal matter, it may serve as an additional *C. difficile* reservoir within the patient room, and facilitate its deposition through brief aerosolization, however further investigation is needed.

Another study collected air samples in the room of asymptomatic and symptomatic CDI patients throughout the duration of their hospitalization. The researchers found that 12% of CDI subjects' samples (10% symptomatic) contained *C. diff.* spores that were collected in the air samples (Emma L. Best et al. 2010). Undiagnosed asymptomatic patients can therefore provide another source for potential HAI transmission as *C. difficile* cleaning and PPE protocols are not

enforced in patients without a CDI diagnosis. This study also found an epidemiological link between the spores in the air and the *C. difficile* found in the environmental samples. The findings from these studies combined with our results suggest strong implications in *C. difficile* transmission as evidence suggests that hospital personnel can contaminate their hands or gloves from environmental spore deposits just as easily as having direct contact with the CDI patient (Weber et al. 2013).Therefore, identifying frequent *C. difficile* reservoirs, such as the exhaust ventilation sources, keyboards, and chairs, can aid hospital staff in the prevention of hospital-associated infections, as persisting environmental spores (lasting up to five months) can infect future patients either through direct contact or through contact with hospital staff.

A limitation of this study is that we only enrolled 13 subjects for participation. This small enrollment number led to fewer samples, and decreased the strength of our results, due to the difficulty to enroll subjects enduring hospitalization, coordinating our research team with hospital staff, and ensuring proper sample collection during time sensitive periods. Moreover, there were sample groups for which we did not collect all three time point series due to miscommunications or staffing issues. The room turnover once a patient was discharged was short, and some samples were not obtained due to the occupancy of a new patient. Furthermore, some patients were transferred between rooms, or moved for surgery, therefore there is a lack of data on the effect of the movement of the CDI patients outside their own room during the study period. Lastly, a main goal of this study was to identify the strain of *C. difficile* that infected the patient and the strain found in the room. Because *C. difficile* is an anaerobic bacterium, to germinate the spores, anaerobic conditions must be replicated (Gerding, Muto, and Owens 2008; Jump, Pultz, and Donskey 2007). The conditions necessary to culture the bacteria are difficult to emulate, we faced difficulties with oxygen leakage, condensation within the anaerobic chamber

and lack of selectivity for *C. diff.* growth in the media we prepared. Because of these issues, culturing *C. difficile* from the stool samples of the patients was unsuccessful.

Despite we were unable to identify the strain of the *C. difficile* in the stool sample and within the room, this study serves as a steppingstone for further investigation. The origin of CDI as hospital-associated infections is still widely argued, as mathematical models indicate that declaring a CDI as a HAI two days after admission is an overestimation (McLure et al. 2018). Our study establishes that *C. difficile* can still be prevalent after cleaning in certain areas, which may contribute to future *C. difficile* infections. A longitudinal study that identifies the strain-level of the *C. difficile* that infects a patient and the strain found in the hospital room and the ward, will provide more insight on the origin of *C. difficile* infections.

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