

Endoscopic Sampling to Determine *Clostridium difficile* Prevalence and Quality of Endoscopic Processing

by

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University of Pittsburgh, 2023

Clostridium difficile is a Gram-positive anaerobic bacterium that is the cause of one of the most significant healthcare-acquired infections. Within the last 20 years, the prevalence of pathogenic strains of *C. diff* as well as antibiotic resistant strains have increased drastically, and several epidemics have occurred. While previously thought to be a healthcare-acquired illness, there has been a drastic increase in community-acquired *C. diff*. 22 million gastrointestinal endoscopies happen each year making it one of the most important outpatient surgeries completed. Endoscopes are reusable and because of this, there are major cleaning protocols put into place to ensure pathogens are not transferring from one patient to another. There has been some speculation that *C. diff* may be spreading among otherwise healthy individuals. Our project wanted to ensure that endoscope reprocessing procedures were adequate in eliminating any possible threat of *C. diff* transmission. In this project, we also measured the prevalence of *C. diff* within the community by measuring patients who were colonized with *C. diff* at the time of their endoscopy. In this study, we discovered that the current cleaning processes were efficient in eliminating *C. diff* bacteria that were present after surgery. We also were able to determine that around 23% of patients were colonized with *C. diff* before their surgery.

Table of Contents

| | |
|--|-----------|
| Preface..... | ix |
| 1.0 Introduction..... | 1 |
| 1.1 <i>Clostridium difficile</i> | 1 |
| 1.1.1 Healthcare-Acquired <i>Clostridium difficile</i> | 4 |
| 1.1.2 Community-Acquired <i>Clostridium difficile</i> | 5 |
| 1.2 Endoscopies | 6 |
| 1.2.1 Current Endoscopic Cleaning Procedures | 8 |
| 1.2.2 Infections Related to Endoscopic Procedures | 9 |
| 1.3 Project Aims | 10 |
| 2.0 Methods and Materials..... | 12 |
| 2.1 Patient Collection..... | 12 |
| 2.2 Collection of <i>Clostridium difficile</i> Samples from Endoscopes..... | 12 |
| 2.3 <i>Clostridium difficile</i> Culturing..... | 13 |
| 2.4 DNA Extraction | 14 |
| 2.5 DNA Quantification and PCR..... | 15 |
| 3.0 Results | 17 |
| 3.1 Patient Data and Endoscope Inspection Data..... | 17 |
| 3.2 <i>C. diff</i> Culturing Data..... | 19 |
| 3.3 DNA Extraction Quantification Data | 22 |
| 3.4 PCR Data..... | 24 |
| 3.5 Patient Status Data | 26 |

| | |
|---|-----------|
| 4.0 Discussion..... | 27 |
| 4.1 Conclusions | 27 |
| 4.2 Limitations | 28 |
| 4.3 Future Directions..... | 30 |
| 4.4 Public Health Significance | 31 |
| Appendix A Appendices and Supplemental Content | 32 |
| Bibliography | 33 |

List of Tables

| | |
|--|-----------|
| Table 1 List of Primers/Sequences Used for qPCR | 16 |
| Appendix Table 1 Make-Up of Master Mix for qPCR..... | 32 |
| Appendix Table 2 Cycling Protocol for qPCR on CFX Connect | 32 |

List of Figures

| | |
|--|-----------|
| Figure 1 <i>C. diff</i> Toxins Result in Tissue Damage (adapted from Poutanen 2004) | 2 |
| Figure 2 Internal Components of a Typical Colonoscope (adapted from Kohli 2019) | 8 |
| Figure 3 Patient Age and Time Under Surgery | 17 |
| Figure 4 Internal Channels of Colonoscope Where Damage and Contamination has Occured | 18 |
| Figure 5 Banana Broth Confirmation | 20 |
| Figure 6 CCFA Plate Confirmation | 20 |
| Figure 7 Banana Broth Pre/Post Disinfection | 21 |
| Figure 8 DNA Extraction for Before and After Disinfection | 23 |
| Figure 9 dsDNA from 4 Different Kinds of Conditions | 24 |
| Figure 10 Patient Groups and CDI Status | 26 |

Preface

I would first like to thank all of the members of UPMC Mercy's Infection Control team that worked hard on this project, as well as the patients whose samples we were able to collect for this project. Thank you, Dr. Mohamed Yassin, Taylor Keck, and Amy Kinzler, for helping collect samples and mentoring me throughout the last year, allowing me to gain the necessary skills to complete this project. Thank you UPMC Mercy for allowing me to work within your facility to help gain and build my current skills and show me what it would be like to work within an Infection Control program. I want to thank Dr. Robbie Mailliard for helping introduce me to Dr. Yassin and his team and helping me to grow as a person over the last 2 years within this program. I also would like to thank Dr. Jeremy Martinson and Joshua Mattila for helping me through my project when I would get stuck and pointing me in the right direction on how to complete parts of my project. Thank you for agreeing to be a part of my committee, showing me I can accomplish much with limited resources.

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1.0 Introduction

1.1 *Clostridium difficile*

Clostridium difficile or *C. diff* is a Gram-positive anaerobic bacterium that is the cause of *Clostridium difficile* infections or CDI (1). CDI is one of the most significant healthcare-acquired infections and has an impact in every hospital throughout the world (1). Clinical manifestations of *C. diff* have a wide range of symptoms, from asymptomatic patients to patients with life-threatening ulcerative colitis (1). Most people who develop CDI have mild diarrhea for an average of 7 days, where they eventually recover (1). Mild symptoms of CDI include abdominal pain, fever, nausea and vomiting, general weakness, and loss of appetite (1). While mild cases of CDI mimic viral gastroenteritis, severe case symptoms can be life-threatening and can include significant dehydration, toxic megacolon, colon perforation, intestinal paralysis, kidney failure, septicemia, circulatory shock, and even death (1). *C. diff* can be prevented by practicing good hand hygiene, especially after coming in contact with fecal matter (1).

There are more than 400 strains of *C. diff*, but only toxin-producing strains can cause CDI (2). Pathogenicity of *C. diff* is most notably caused by the clostridial toxins TcdA (Toxin A) and TcdB (Toxin B) (3). Toxin A is known to disrupt the mucosal cell adherence to the basement membrane of the colon where damage eventually occurs (2). Toxin B enters cells within the colon via a change in pore formation, where it causes degradation of the actin cytoskeleton inducing apoptosis (3). Figure 1 shows how *C. diff* enters through the digestive tract, and once inside the colon toxins A and B are shed where damage occurs (**Figure 1**). One study showed that tissues from mice that were infected with non-toxin-producing strains of *C. diff* had no resulting tissue

damage, similar to the control mice (4). The effect that Toxin B has on cells within the colon is 1000 times more cytotoxic than the effect that Toxin A has (2). Within the last few years, it has been shown that many of the *C. diff* epidemics that have happened have been due to an increased prevalence of *C. diff* strains that only express Toxin B (4).

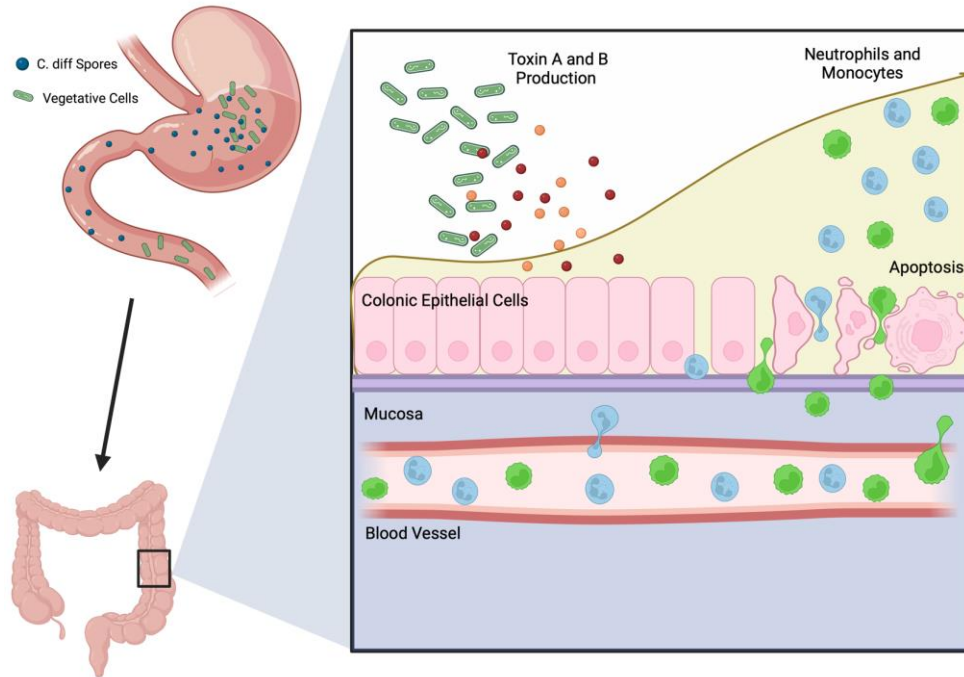


Figure 1 *C. diff* Toxins Result in Tissue Damage (adapted from Poutanen 2004)

While anyone can develop *C. diff* certain groups of people are more at risk for developing CDI than others. These people include those who have been exposed to a broad spectrum of antibiotics, those who are 65 years of age or older, the immunocompromised, and those who are in a healthcare environment for extended periods (1). With the increase in *C. diff* infections within recent years, it has been estimated that *C. diff* causes half a million new infections among patients in the United States each year (5). Approximately 29,000 patients who have been diagnosed with their first occurrence of CDI will have died within 30 days (5). An estimated half of those deaths

were directly caused by *C. diff*, making this a very important infectious disease within the United States (5).

There is a wide array of treatments for *C. diff* including different antibiotics as well as a form of fecal matter transplantations. Before 2018, the standard protocol for *C. diff* treatment was to treat the patient with the antibiotic metronidazole (6). In February 2018, the Infectious Disease Society of America (IDSA) published new clinical practice guidelines that moved away from the usage of metronidazole as the first line of defense against initial CDI (7). It was decided that the use of antibiotics such as fidaxomicin or vancomycin had a better response in treating someone with their first *C. diff* infection (6). In a more recent study comparing vancomycin to fidaxomicin, it was shown that fidaxomicin was the better antibiotic because it is a bactericidal drug, where it kills *C. diff*, whereas vancomycin is a bacteriostatic drug, where it only inhibits the growth of *C. diff* (7). Although antibiotics are used to treat *C. diff*, many healthcare providers avoid their usage due to the potential development of antibiotic-resistant *C. diff* and other bacteria. This has been the pushing factor for alternative treatments, including the development of fecal matter transplants (FMT) (6). FMT is when someone who is infected with *C. diff* has their microbiome replaced by a transplant from a single individual or a pool of donors (6). A person's natural microbiota is a defense mechanism, helping to stop invading bacteria from taking over and causing disease. When the microbiota is disrupted by antibiotics it allows for invading pathogens, such as *C. diff*, to take over (6). The use of FMT helps to provide the patient with recovery by allowing for the growth of commensal bacteria; helping to rid the body of *C. diff* (6).

1.1.1 Healthcare-Acquired *Clostridium difficile*

As mentioned previously one of the risks for developing *C. diff* is through increased exposure within a healthcare setting. Hospital environments play a significant role in the spread of different kinds of infections, and *C. diff* is no exception. *C. diff* spores can survive for long periods outside of the body, and because they are in spore form it has been proven to be more difficult at controlling in a hospital environment (8). *C. diff* is resistant to drying, temperature, and many different kinds of chemical disinfectants, which results in continuous difficulty in destroying spores before they can spread to healthy individuals (8). Due to this, *C. diff* spores can survive anywhere between a few weeks to more than 5 months (8). With the increasing difficulty of trying to remove *C. diff* from environments, every hospital in the world has the potential for an outbreak. The rate of healthcare-associated CDI (HA-CDI) has been increasing each year, and as of 2018, the estimated cost of hospitalization for a patient who has been diagnosed with CDI has raised by more than 54% (9). Since 1993 hospital stays for CDI have increased more than 5 times and mortality rates have more than tripled since this time (10).

To reduce the possibility of a hospital outbreak, infection prevention for healthcare spaces has been recommended as well as debated. One possible infection prevention method that is highly controversial is the use of early diagnosis procedures for *C. diff*. As of right now, healthcare facilities do not outright test everyone for *C. diff*. Instead, they recommend that patients be tested only if they have an onset of new and unexplainable bouts of diarrhea (10). If someone has had more than 3 unformed stools within a 24-hour period that does not have an explanation, then they will have the patient tested for *C. diff* (10). The reason for this is if a patient tests positive for *C. diff*, then they are to be placed in isolation to help prevent the spread of *C. diff* to other patients and healthcare workers (8). Another large factor is since the beginning of the SARS-CoV-2

pandemic, hospital occupancy has averaged around 85% full with an increased average length stayed at an emergency department (11). If someone were to walk into the emergency department with a broken arm and happened to screen positive for *C. diff*, they would have to be placed into isolation and treated for *C. diff*. This takes a room that could have been used for a patient that may have suffered from a stroke. Due to limited beds and rooms in hospitals, it is next to impossible to accommodate all of the possible CDI cases that would need isolation just from an average emergency visit, especially from a community-acquired *C. diff* patient. This is the main reason why most healthcare facilities do not want to screen immediately for *C. diff* without reason.

1.1.2 Community-Acquired *Clostridium difficile*

Until the most recent decade, *C. diff* was mostly thought of as a nosocomial infection, spreading only to high-risk patients on antibiotics or patients hospitalized for long periods. Within the last decade more data has shown that almost 41% of CDI cases happen to be acquired from the community, a fourfold increase from 2005 (12). While most people who have been infected with community-acquired *C. diff* (CA-CDI) have mild symptoms, a large portion has had severe complications. In a recent study, 40% of people who have CA-CDI have required hospitalizations, 20% have had a severe infection, 4.4% had a severe complicated infection, and 28% had recurrent CDI (13). This data shows that patients who have community-acquired *C. diff* are just as at risk as patients who have healthcare-acquired *C. diff* for developing severe complications.

Asymptomatic carriers have been thought to be a main source of community-acquired *C. diff*. Asymptomatic patients can shed the bacteria through feces, and as mentioned previously it is hard to destroy, so if someone did not know they had the bacteria they would not take extra precautions in making sure it was not spread to others. A recent study showed that asymptomatic

carriers outnumbered CDI patients seven to one (13). This helps provide evidence that a large proportion of people within the community are asymptomatic and have the possibility of spreading *C. diff* to healthy individuals. One main difference between patients who have CA-CDI vs HA-CDI is age. People who have developed CA-CDI tend to be patients who are younger and lack the traditional risk factors such as antibiotic exposure or hospitalizations (13). This is a cause for concern because recently, patients who were at high risk of developing CDI were elder patients or patients who have been hospitalized. Knowing that younger people who were not previously at risk are now at risk through community exposure has an impact on how *C. diff* should be handled.

1.2 Endoscopies

As of 2022, an estimated 22 million gastrointestinal endoscopies happen in the United States each year, making it one of the most basic outpatient surgeries in the United States (14). Gastrointestinal or GI endoscopies are used to help diagnose and treat patients with GI diseases as well as help screen otherwise healthy people who have an increased risk of developing GI-related diseases (15). They help to play a major role in the prevention and diagnosis of colorectal cancer which is the third most common cancer worldwide (16). Other GI-related cancers that can be screened and monitored through endoscopies are esophageal, stomach, and small intestine (17). When a possible precursor to colorectal cancer is found, either a lesion or a polyp in the colon, endoscopes are then again used in another procedure to remove and treat them (17). It is recommended that people that are 45 and older be screened regularly for the possibility of the development of polyps by undergoing a colonoscopy (18). It is also recommended that people be screened at an earlier age if they have an inflammatory bowel disease such as Crohn's disease, a

personal family history of colorectal cancers, or a genetic predisposition that may lead to colorectal cancers (18).

The endoscope that is used to perform these upper and lower GI endoscopic procedures are complex structures that are reusable once cleaned and disinfected. Endoscopes are flexible instruments that usually have a combination of cameras and internal tools used to look within someone's body cavity (19). There are multiple different kinds of endoscopes including gastroscopes used for the gastrointestinal tract, colonoscopes used for the colon, and bronchoscopes used for the lungs (19). Scopes help to provide high-definition pictures of the mucosa that the healthcare professional wants to examine, helping to identify and diagnose complications within people (19). Scopes have several different channels that vary in diameter and purposes (19). Important channels seen in almost every type of scope include water, air, optics/cameras, and channels where biopsy tools are inserted (19). Figure 2 shows an illustrated example of the internal structure of a general colonoscope, including all channels and extra protective gear needed for procedures (**Figure 2**).

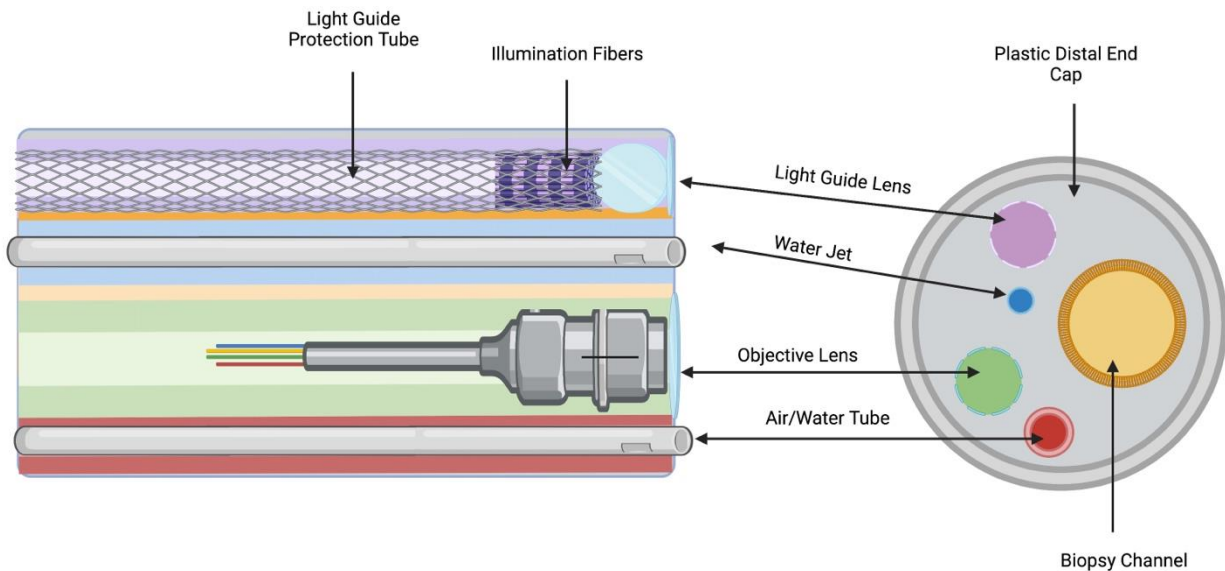


Figure 2 Internal Components of a Typical Colonoscope (adapted from Kohli 2019)

1.2.1 Current Endoscopic Cleaning Procedures

Since the endoscopes are reusable and can be used anywhere from 3,000 to 4,000 times before retiring, there are standard disinfection protocols in place. Endoscopes are categorized as semi-critical devices when it comes to cleaning procedures because they come in contact with mucous membranes and not sterile body cavities (15). The overall expectation for this grade of cleaning is that all microorganisms are to be removed but a small number of bacterial spores are permissible (20). The overall reason for this is that intact mucous membranes such as the lungs and the GI tract are generally resistant to bacterial infections, but contamination is still possible (20). These items, such as endoscopes and laryngoscopes require a high-level disinfection that includes a chemical disinfectant and a meticulous cleaning procedure (20).

Within the last few years, these guidelines for endoscope processing have been revised and they are constantly changing to help ensure the safety of patients undergoing basic gastrointestinal

procedures (21). Processing starts with precleaning after immediate use with an enzymatic flush to prevent the biofilm within the endoscope, where it then goes for a leak test (21). From there the scope then undergoes a manual cleaning cycle where brushes are used throughout the internal chambers of the scope to gently scrape away any possible debris from the side of the scope, and then they soak in the enzymatic cleaners (21). The scope is then placed into an automated endoscope reprocessor (AER) where it goes through a cycle of enzymatic cleaning with a high-level chemical disinfection and rinsing (21). The last step to processing and disinfecting an endoscope is when air and alcohol are flushed through the scope to make sure all possible liquid is out to prevent bacteria and mold from forming in a damp environment (21). Once the endoscope is dried it is carefully hung in a special cabinet until it is used again (21).

1.2.2 Infections Related to Endoscopic Procedures

Inadequate cleaning of complex endoscopes can lead to infection outbreaks within healthcare settings. There have been several reports throughout the last few decades of bacterial and viral infection outbreaks due to contaminated endoscopes (15). While the risk of transmission during endoscopy is low, there is still a 1 in 1.8 million chance of it happening (22). For the past 20 years, small outbreaks of Hepatitis C, Hepatitis B, Avian influenza A virus, *Salmonella*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, and a few other miscellaneous bacteria have been reported due to the improper cleaning and handling of endoscopes (23). The risk of infection due to cross-contamination from not properly processed endoscopes is greater than the risk of infection with any other medical devices. This is partly due to endoscopes coming in contact with mucous membranes during their procedures, increasing the risk of infection due to cross-contamination (15).

1.3 Project Aims

With endoscopies being the most frequently performed surgeries throughout the world there is no surprise that there have been previous outbreaks of pathogens between patients. Proper cleaning protocols have been put in place to prevent viruses such as Hepatitis and HIV from being transferred from patient to patient. Early reports of endoscope-related infections were due to faulty designs and improper processing, which has led to the thought of replacing reusable endoscopes with disposable ones. A major problem with this is that disposable endoscopes have a lower resolution in imaging and that it is very expensive to do since more than 20 million procedures are being completed each year. Healthcare professionals have since moved on to improving endoscope processing quality to ensure scopes are cleaned properly to prevent the transmission of pathogens.

With that being said, studies on whether or not *C. diff* has spread via improperly processed endoscopes have never been completed. Since *C. diff* resides in people's large intestines, it is possible that while doing a colonoscopy that the scope becomes infected. Our hypothesis for this study is to see if there is a possibility that *C. diff* is spreading from one patient to another due to contaminated endoscopes? It is completely possible that although cleaning is being completed, a difficult pathogen such as *C. diff* may be slipping through the cracks, causing otherwise healthy individuals to become colonized with *C. diff*.

There were two main aims of this study. The first aim was to identify the presence of *C. diff* spores after endoscope processing. We took endoscopes after the medical procedure was completed and sampled them; we then took a borescope to look for any damage within the interior of the scope. We then sampled the scopes after the cleaning procedure was completed and examined both sets of samples for *C. diff*. This will help tell us if the cleaning procedure that is currently in place is efficient in making sure that *C. diff* is being eradicated if there. The second

aim was to measure the prevalence of *C. diff* in patients who were not known previously to have CDI. Patients were chosen because their *C. diff* status was previously unknown. This is important because if a scope comes back positive for *C. diff* this means that the patient the scope came from is colonized with *C. diff* and may be spreading it to others who were previously uninfected. This is important because it will give us some kind of measurement of the number of people who are colonized with community-acquired *C. diff*.

2.0 Methods and Materials

2.1 Patient Collection

Patients were chosen from the gastro-intestinal endoscopy unit and were chosen at random. Patients that were selected were determined to have a higher risk of developing *C. diff* after the endoscopy was completed. These patients underwent colonoscopy examination for colorectal screening and other gastrointestinal bleeding, and signs of potential *C. diff* exposure. Patients were recruited from October 2021 until April 2022. Of the more than 600 colonoscopies that were completed during this time period, one hundred and ten patients were included and examined for this study. Samples were collected daily and performed by the Infection Prevention team.

The Electronic Health Record (EHR) was reviewed by a medical professional, and we were able to determine the endoscope model, serial number, indication of the procedure, any possible complications the patient may have endured, findings as well as the duration of the procedure. Additional information about the patient including age, gender, and inpatient vs outpatient procedures was also noted.

2.2 Collection of *Clostridium difficile* Samples from Endoscopes

Samples were collected from used endoscopes using the flush brush flush method. 20mL of DI water was flushed through the distal end of the endoscope and collected within a 50mL collection cup. The cup was clearly labeled before disinfection. A small wire brush on a length of

wire was then used to pull through the length of the internal channel to dislodge any possible bacteria or debris that may have been stuck to the side of the tubing. The brush was cut off from the end of the wire and placed into the collection cup. Once the brushing of the internal channels was completed another 25mL of DI water was used to flush any bacteria or debris that may have been dislodged by the wire brush and was collected in the same collection cup. Samples were kept in a fridge at less than 4 °C degrees for up to 24 hours maximum. Once the endoscope was processed at the treatment facility samples were then again collected from the newly cleaned endoscopes using the same flush brush flush method. 2mL of each sample flushed directly from the scopes were placed into empty microtubes where they were placed into a -80° C freezer for further molecular testing.

***2.3 Clostridium difficile* Culturing**

Five hundred microliters of the fluid obtained from the collection process were aspirated via a sterile pipet and placed into a media that is used for the culturing and recovery of *C. diff* from environmental samples called *C. diff* Banana Broth™ (24). *C. diff* Banana Broth™ is an anaerobic media that helps to promote *C. diff* spore germination from the environment, and it is not used for diagnosis (24). When growth is seen within the media, a change in the pH has occurred changing the neutral red color, which indicates a negative sample, to a yellow color. Any kind of yellow seen within the Banana Broth™ means that an environmental sample is positive for *C. diff* growth. The samples collected were incubated at 36 °C degrees for 72 hours. After 72 hours, the Banana Broth™ was examined to see if they had any yellow coloring, indicating a positive sample.

After samples were cultured within *C. diff* Banana Broth™ they were next plated on Cycloserine Cefoxitin Fructose Agar (CCFA) plates. These plates are used for the isolation and identification of *C. diff*, and when grown will exhibit a characteristic yellow, ground-glass colonial morphology (25). Similar to Banana broth the plates are red indicating a neutral pH and with the presence of *C. diff* will change the red to orange or yellow increasing the pH (25). Samples were grown on CCFA plates in an anaerobic chamber at 36 °C degrees for 72 hours. After 72 hours, all positive growth from CCFA was stored in 20% glycerol cryotubes and stored in a -80° C freezer for further molecular testing.

2.4 DNA Extraction

DNA was extracted from the samples collected from the endoscopes through the flush brush flush method using the QIAGEN DNeasy Blood and Tissue Kit. Samples, both straight from the endoscope and bacterial growth from the CCFA plates, were thawed to room temperature. 1mL was aliquoted into a clean 1.5mL graduated microtube labeled with their corresponding number and location. The color of each sample was noted, which helped show us how dirty the endoscope was before and after cleaning procedures. The tubes were then centrifuged in an Eppendorf MiniSpin plus at 7500 rpm for 10 mins. After 10 minutes each sample was examined to see if a pellet formed at the bottom of the tube, if a pellet was not formed then the centrifugation was repeated. Once the centrifugation was completed the supernatant was poured off with the pellet remaining intact at the bottom of the tube. The pellet was resuspended in 180µL of ATL buffer from the QIAGEN DNeasy Blood and Tissue Kit. 20µL of proteinase K was added to the pellet suspension where it was then mixed thoroughly by vortexing. The tubes were then incubated at

56°C in an incubator for 3 hours. After this incubation period samples were vortexed for 10 seconds. 200µL of 95% ethanol and 200µL of AL buffer were then added to each tube where they were vortexed immediately for another 10 seconds. The mixture was then pipetted into a DNeasy mini spin column with a 2mL collection tube placed underneath. This mixture was centrifuged at 8000 rpm for 1 minute. The collection tube containing the liquid flow tube was discarded and replaced with a clean 2mL collection tube. 500µL Buffer AW1 was added to the spin column and centrifuged at 8000 rpm for 1 minute. Again, the collection tube containing the liquid flow through was discarded and replaced with another clean 2mL collection tube. After this 500µL of Buffer AW2 was added to the spin column where it was centrifuged for 3 minutes at 14500 rpm. The collection tube containing the liquid flow tube was discarded and replaced this time with a clean 1.5mL microtubule, where 200µL of Buffer AE was added to the spin column. After sitting at room temperature for 1 minute the samples were centrifuged at 8000 rpm for 1 minute. The liquid resulting from this last centrifugation was kept and labeled with their corresponding sample number and then stored in -80°C conditions.

2.5 DNA Quantification and PCR

Quantification as well as the quality of the DNA from each sample was performed using Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometers. Samples were taken from the -80°C freezer where they were thawed for 10 minutes at room temperature and then placed into an ice bath to keep cool. Once thawed 1µL of the sample was placed onto the pedestal where quantification of dsDNA was read.

Once quantification was completed samples were immediately subjected to qPCR. Integrated DNA Technologies (IDT) PrimeTime Gene Expression Master Mix was used as well as 3 separate probes, CD16SrRNA, tcdA, and tcdB. These specific primers were used because CD16SrRNA is seen within all strains of *C. diff*, while tcdA and tcdB are specific to pathogenic strains of *C. diff* (**Table 1**). This would have allowed us to look for both commensal strains of *C. diff* as well as pathogenic strains. TaqMan probe was used to help with the detection of the multiple primers that were used when completing qPCR. A 96-well plate was used for the qPCR of each sample where samples were run in triplicates. Positive controls were taken from patients who were PCR-confirmed positive within the hospital, while negative controls were nuclease-free water. Appendix Table 1 shows the make-up of the master mix that was used for each well. qPCR was run on a BIORAD CFX Connect Real—Time PCR Detection System. Appendix Table 2 shows the cycling protocol that was used for this specific test.

Table 1 List of Primers/Sequences Used for qPCR

| Primer | Sequence | Use |
|---------------|----------------------------------|--------------------------------|
| CD15SrRNA-F | GCAAGTTGAGCGATTTACTTCGGT | Confirmation of <i>C. diff</i> |
| CD15SrRNA-R | GTACTGCACCTTTGATATTYAAGAG | Confirmation of <i>C. diff</i> |
| tcdA-F | CAGTCGGATTGCAAGTAATTGACAAT | Toxin A Confirmation |
| tcdA-R | AGTAGTATCTACTACCATTAACAGTCTGC | Toxin A Confirmation |
| tcdB-F | TACAAACAGGTGTATTTAGTACAGAAGATGGA | Toxin B Confirmation |
| tcdB-R | CACCTATTTGATTTAGMCCTTTAAAAGC | Toxin B Confirmation |

3.0 Results

3.1 Patient Data and Endoscope Inspection Data

This study was performed in a single academic medical center between December 2021 and April 2022. Patients were chosen because they were thought to have been previously negative for *C. diff* bacteria and had no previous increased risk for the development of *C. diff* before their procedure. The majority of procedures were performed on female-identified patients. 57% of patients were female-identified while 43% of patients were male-identified. The average age and standard deviation for patients undergoing colonoscopy procedure during this study was 55 ± 15 years (**Figure 3A**). The mean time and standard deviation for the colonoscopy procedure for patients was 19 ± 13 minutes (**Figure 3B**).

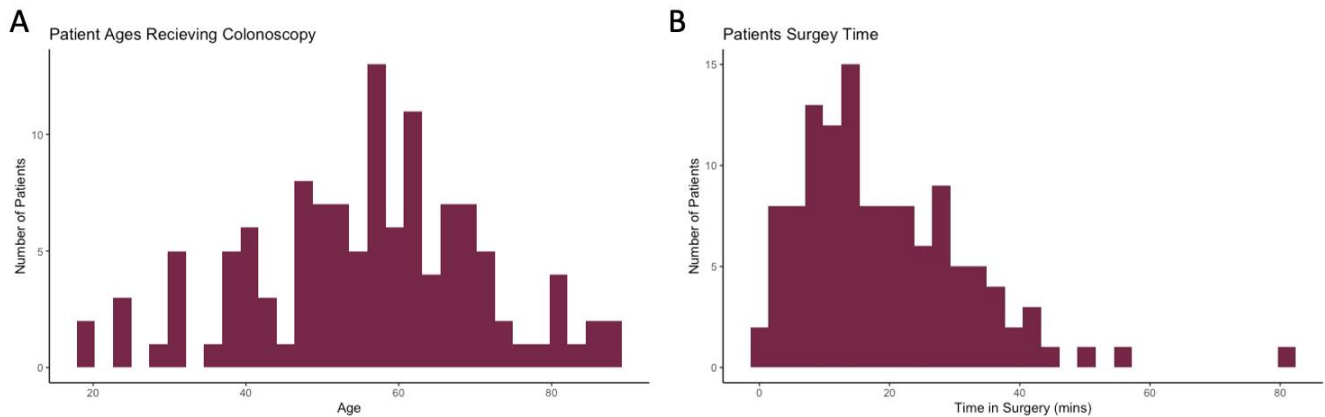


Figure 3 Patient Age and Time Under Surgery

To determine if there is *C. diff* within endoscopes after processing procedures, we selected 120 patients who had undergone an endoscopy that day and flushed out the endoscopes for any possible bacterial contamination. When the endoscope was cleaned and processed, we took the endoscope again and flush it again to look for any possible contamination that may have been left after cleaning. We also used a borescope to inspect the inside of the distal channel for any possible contamination, water droplets, or damage to the internal channels that possible *C. diff* could continue to live in. Figure 3 shows pictures from the borescope within a few different endoscopes where water droplets and internal damage were noted, with a red arrow indicating where the damage had occurred (**Figure 4**). A majority of the scopes we inspected before cleaning had contamination along the walls of the channels.

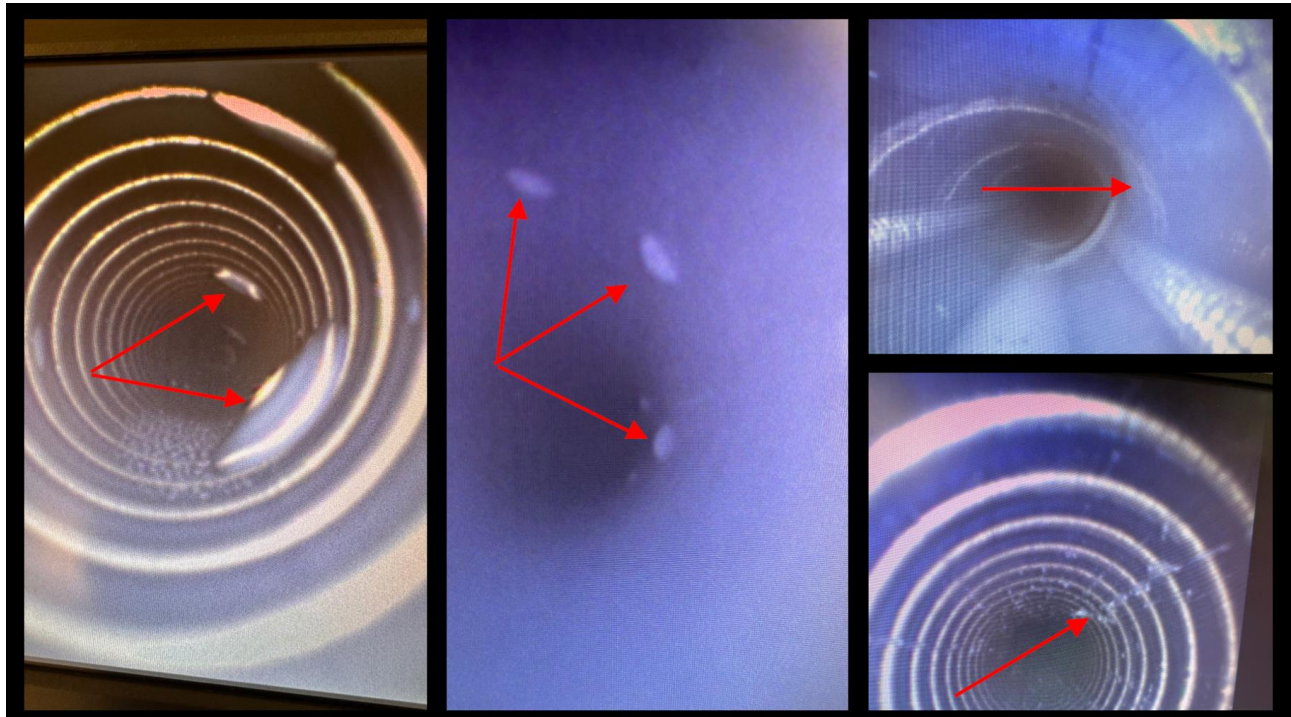


Figure 4 Internal Channels of Colonoscope Where Damage and Contamination has Occurred

3.2 *C. diff* Culturing Data

After the collection of the samples was completed, we cultured the samples on two forms of media, *C. diff* Banana Broth™ and Cycloserine Cefoxitin Fructose Agar (CCFA) plates. 92 endoscopes from pre-disinfection were tested for *C. diff* using Banana Broth™. Post-disinfection the original 92 as well as another 28 scopes were tested for *C. diff*. We then cultured 109 of the samples, including all of the samples that were positive within the Banana Broth™, on CCFA plates to confirm the growth of *C. diff* colonies. Figure 5 shows a good example of the transformation process within Banana Broth™. This figure shows a positive in the form of a yellow coloring, a negative in the form of neutral pH red coloring, and a set of spores growing where the media is in the process of changing colors (**Figure 5**). Figure 6 shows a confirmation growth of *C. diff* within a CCFA plate for the same sample from the Banana Broth™ (**Figure 6**).



Figure 5 Banana Broth Confirmation

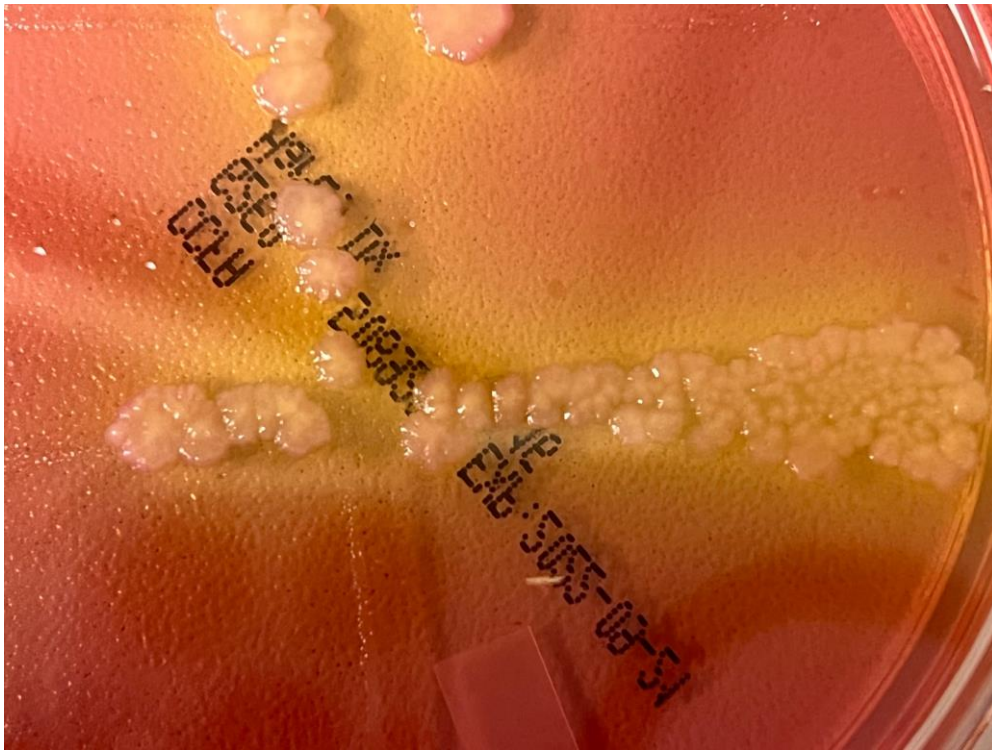


Figure 6 CCFA Plate Confirmation

From the Banana Broth™ culturing alone, we were able to determine that 23% of patients were colonized with *C. diff* at the time of their colonoscopy. Before disinfection a total number of 28 scopes had tested positive for *C. diff* through the use of Banana Broth™ (**Figure 7**). After disinfection it was shown that all 120 endoscopes, we tested through the use of Banana Broth™ were negative for any kind of *C. diff* contamination (**Figure 7**).

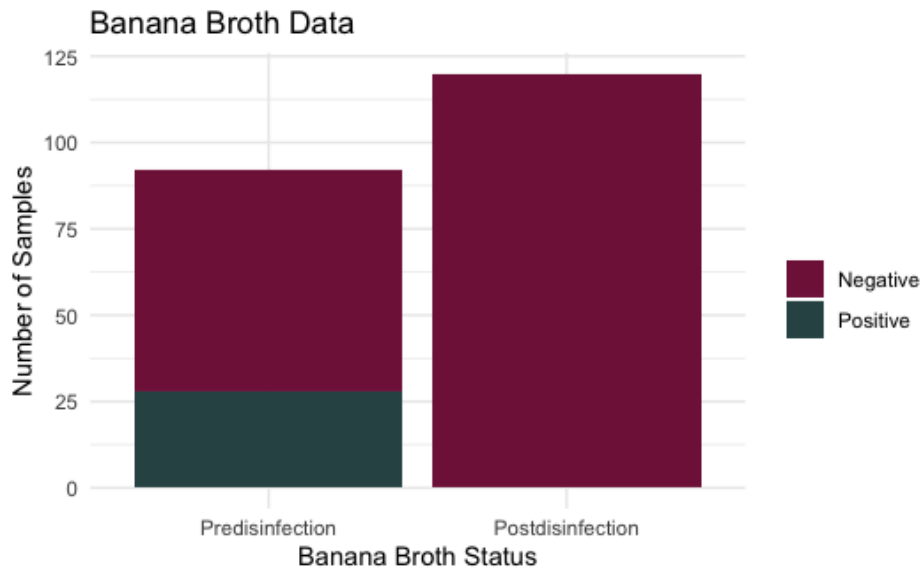


Figure 7 Banana Broth Pre/Post Disinfection

Out of the 109 CCFA cultures we determined that 27, or 24%, of them, were positive for *C. diff* colony growth. This included both before and after disinfection samples. When looking at post disinfection all but 1 sample was negative for *C. diff* growth when plated on CCFA. When this sample was plated a second time no growth was seen, meaning possible contamination during the first plating session. When comparing samples cultured from CCFA plates to their original Banana Broth™ data, 83.5% of the cases matched their original screening data. 16.5% of cases were either negative culture within Banana Broth™ with a positive CCFA plate or vice versa.

3.3 DNA Extraction Quantification Data

Our next step after culturing on the two forms of media was to check the amount of DNA that we obtained during extraction to ensure when doing PCR, we had ample amounts for amplification. Before completing extraction, the colors of each tube were noted. Figures 8A and 8B showed the difference in colors between a before-disinfection endoscope and an after-disinfection endoscope. The dark orange coloring before disinfection shows that there was some kind of contamination as well as debris from this patient (**Figure 8A**). The purple coloring of the after-disinfection tube was one of the very few that had coloring, but when centrifuged did not contain a pellet of debris at the bottom (**Figure 8B**). Only a few of the samples taken from clean tubes had a gray to purple coloring, the rest were clear in color, and all of them had no pellet form when centrifuged.

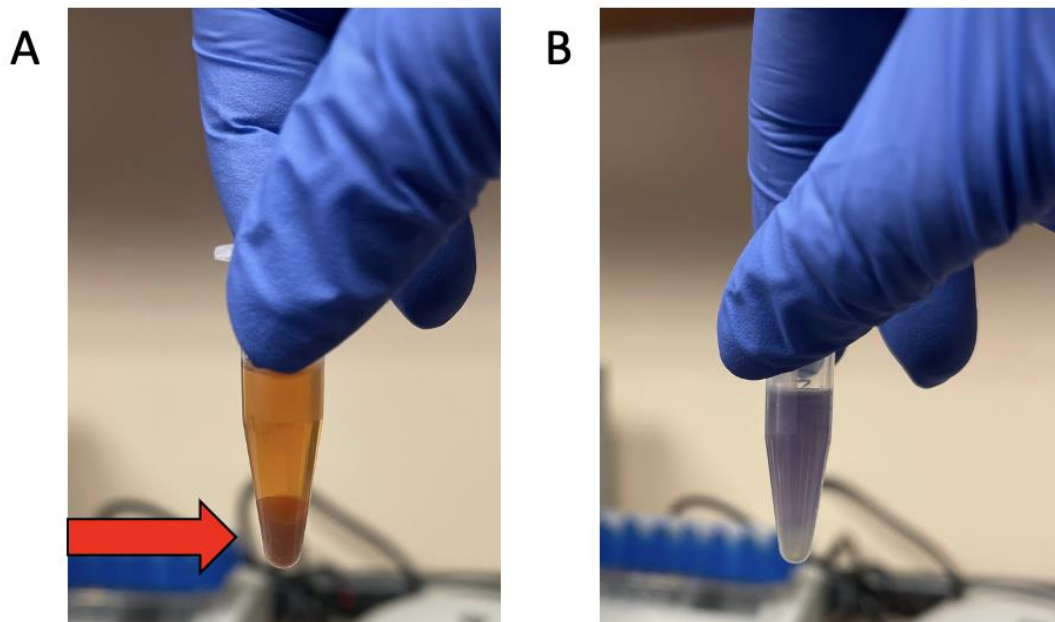


Figure 8 DNA Extraction for Before and After Disinfection

Once DNA extraction was completed, we quantified their dsDNA to make sure we had an ample amount of DNA for qPCR. Using the Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer, we were able to get a quantified number of dsDNA for each sample. We measured 4 different kinds of media in order to see the difference in DNA. We measured dsDNA concentrations in CCFA, Banana Broth, and then straight from the collected samples before and after disinfection. The data shows that after disinfection there was a considerable decrease in DNA collected from the scopes compared to before disinfection (**Figure 9**). This figure also showed that most DNA that was collected was collected from the pure colonies that were grown on the CCFA plates (**Figure 9**). There was also a large clustering of data among all four conditions that showed little to no DNA extracted at all (**Figure 9**).

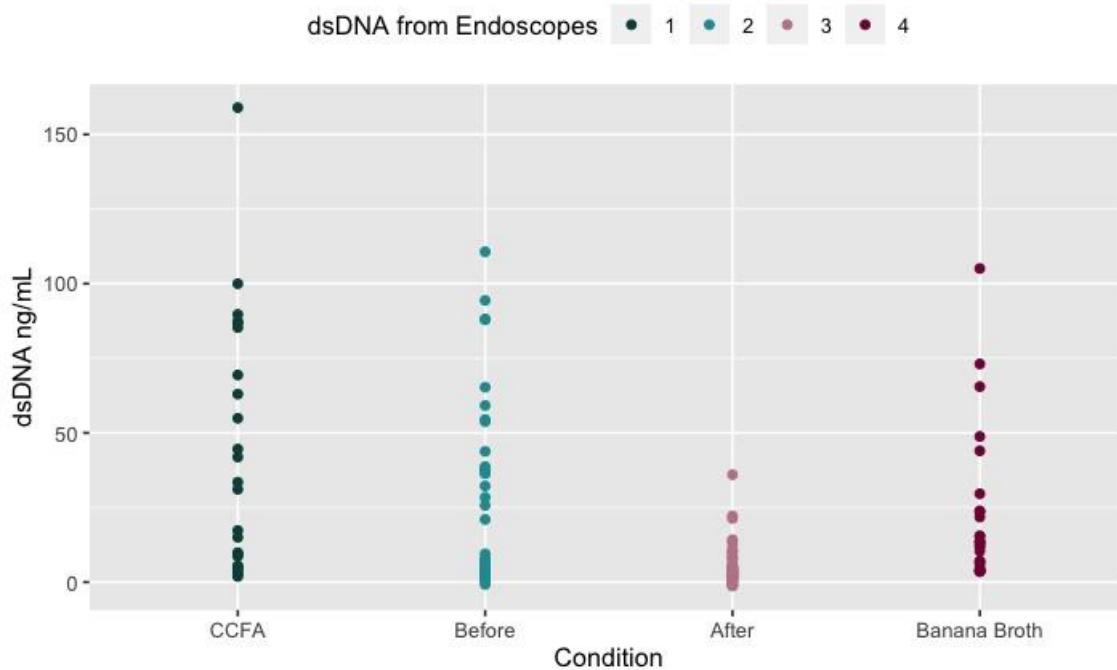


Figure 9 dsDNA from 4 Different Kinds of Conditions

3.4 PCR Data

Our last step after culturing the samples and DNA quantification was to confirm these samples using qPCR. While running our first batch of *C. diff* on our brand new BIORAD CFX Connect Real—Time PCR Detection System, we ran into some issues. When looking at the amplification data, we noticed that there was no amplification at all, with a single horizontal line at 0. Our positive controls we used for this project were positive cultures from patients who were in the hospital with confirmed cases of *C. diff*. We took their positive stool samples and cultured and isolated positive *C. diff* colonies. When our positive controls also did not show any amplification, we knew there was a problem. We believed that maybe there was some kind of

amplification inhibitor within our samples or that there was a possible contamination problem with our reagents.

We next tried to troubleshoot the problem of no amplification. The first thing we tried was replacing our TaqMan probe with a brand-new vial. We had received a few bottles of TaqMan probes of the same lot number and tried replacing the probe with a new bottle. This did not have any change; amplification was still not seen. The second thing we did was replacing all of the reagents for the MasterMix. The original bottle had been opened for a few months and had gone through multiple freeze/thaw cycles, which we thought might have been the problem. We replaced all of the MasterMix bottles with fresh bottles to eliminate any possible problems that may be due to old MasterMix or multiple freeze/thaw cycles. Again, amplification was still not seen with our samples or positive controls. The last thing we tried was to replace all of our primers with fresh bottles of primers as well as trying each primer individually instead of trying to multiplex. Our original set of primers had gone through multiple freeze/thaw cycles similar to our MasterMix, and we believed that maybe this might be the problem. With fresh new primers as well as MasterMix and probes, we tried another run of samples. We used 2 positive Banana Broth samples, with one negative and two positive controls for each primer and pipetted them each into a 96 well plate. Again, our samples and controls showed no amplification for this run.

After we had run out of options, we still wanted to make sure that there was some kind of amplification possible with our extracted DNA. We wanted to make sure that we did not have a possible PCR inhibitor within our extracted samples as well as making sure our new machine was not the reason, we were having issues. We decided to send out our samples to another lab to see if they could get any kind of PCR amplification. As of April 18, 2023, we still have not received our results back from this reference lab.

3.5 Patient Status Data

When looking at patients that were colonized with *C. diff*, we were able to get a sense of what groups were affected more rather than others. When comparing age groups, we saw that the majority of people who underwent surgery were younger than 65 years of age. This group of people is at less risk for developing *C. diff*. When comparing the age groups, 22 people from the younger age group tested positive for *C. diff*. This means that 78% of the total positive cases came from the 64 and younger age group (**Figure 10A**). This coincides with our presumption that people who have been developing *C. diff* through the community are younger than the at-risk age. When comparing men to women, it was shown that more women were colonized with *C. diff* than men (**Figure 10B**). Of all of the positive samples, 16 of them were female, or 58% (**Figure 10B**).

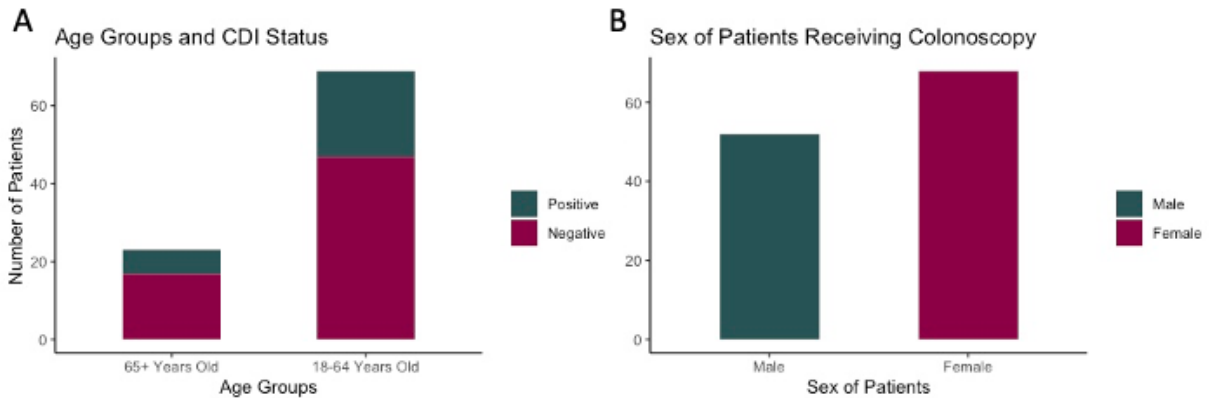


Figure 10 Patient Groups and CDI Status

4.0 Discussion

4.1 Conclusions

This study was used to determine if the current processing and disinfection procedures for reusable endoscopes are in line with current national guidelines. *C. diff* was the chosen pathogen to look for because within the last few decades the prevalence of *C. diff* has increased, both within a hospital environment and within the community. We wanted to ensure that *C. diff* was not spreading within hospitals due to improperly disinfected endoscopes. Through two types of culturing, we were able to determine that endoscopes are being processed properly by national guidelines that are put in place. Looking at pre- and post-disinfection we were able to see that there were traces of *C. diff* in about 30 scopes pre-disinfection, and post-disinfection 0 scopes tested positive for *C. diff*. This gives us an ample amount of data to conclude that *C. diff* is not spreading between patients through used endoscopes.

In this study, we also wanted to determine if there is a prevalence of community-acquired *C. diff* within our patients. After choosing patients that had no previous increased risk of developing *C. diff*, we examined their used scopes for any kind of *C. diff* that could be isolated. We found that around 30 people who were thought to be previously negative for *C. diff* happened to be colonized with *C. diff*. This means that around 23% of our patients were colonized with *C. diff* before their surgery but were asymptomatic. This helps us to show that around ¼ of our otherwise healthy individuals happened to have *C. diff* which was most likely acquired through community means. When comparing men vs. women it was shown that 58% of the positive CDI samples came from women. Age was also a factor we decided to look at because ages 65 and older

are an increased risk factor for the development of *C. diff*. When looking at age groups we noticed that people who were younger than 65 had the most *C. diff* colonization's rather than 65 years or older. This data helps to conclude that people who are developing community-acquired *C. diff* are among the age group of 64 and younger.

4.2 Limitations

While this project was successful in showing that disinfection of endoscopes is being completed to standard, there were a few limitations to this study. One limitation of this study was when collecting samples at the beginning of the study we did not get a pre-disinfection sample from almost 30 samples. This was $\frac{1}{4}$ of our samples that we collected throughout the study. This can have an effect on the number of patients that we initially thought had community-acquired *C. diff*. If this study were to be repeated or done at a separate location it is important to remember to whatever scopes are used in the study should have pre- and post-disinfection sampling done. This will give an accurate measurement of how many patients come in with *C. diff* and whether or not all of the scopes are being cleaned properly.

A second limitation of this study is that we do not know if the *C. diff* that we are isolating from our patients is alive or in a vegetative state. While 23% of our patients were colonized with *C. diff* there was no way for us to confirm if these bacteria were alive and replicating within these patients or if they were in a dormant state. This also has a small effect on us being able to interpret if these patients for sure received *C. diff* through the community or not. At the beginning of the study these patients are thought to have been negative for *C. diff*, they are also thought to have had no prior increased risk for the development of *C. diff*. This means these patients did not have any

course of antibiotics or extended stays within a healthcare environment within 30 days of their procedure. This does not mean that before these 30 days, these patients were exposed to risk factors. If this study were to be completed again, a baseline 30 days before their procedure should be completed to make sure everyone is negative and then measure during their procedure for the possible development of *C. diff*.

A third limitation of this study would be the use of not culturing all samples. While the overall ideal for this study was to culture every sample in both Banana Broth™ and CCFA plates, time as well as resources got the best of us. Culturing each sample took almost a week to do, in our lab, our anaerobic chamber only held 10 samples, and with almost 200 samples it would have taken almost 20 weeks to get through all of our samples. With this time limitation, we began to prioritize the samples we believed were necessary to get accurate results. We measured all positive Banana Broth™ samples on CCFA plates and then made our way through the remaining samples until we could no longer keep plating. We also did not replace any samples that did not coincide in their original Banana Broth™ culture. The next time this study is to be completed, samples that were positive in Banana Broth™ but negative on CCFA should be plated one more time for confirmation. This leads to another limitation, when completing the Banana Broth™ culturing we used fresh samples, but when we cultured on CCFA samples had been frozen at -80° C for prolonged periods. These samples underwent a few freeze/thaw cycles which may have had an effect on the CCFA plates.

The last limitation of this study is our qPCR machine not working properly. Our project ended with troubleshooting what went wrong because none of our samples were amplifying. It was mentioned in the result section that we replaced every reagent, probe, and primers to see if that would fix the problem. We even tried to run each primer individually. We took samples from

positive diagnosed cases within the hospital and grew them over CCFA plates to get fresh colonies, these samples did not have any amplification as well. Our CFX Connect was a brand-new machine at the time of testing, with these sets of samples being the very first runs on it. It is unclear whether or not this may have had an effect on why our samples were not amplifying properly but it would be a good idea to have all calibrations and tests completed on a machine before a study like this were to occur again.

4.3 Future Directions

There are a few different directions this idea can take for future projects. A future direction that this project could take would be to look at other pathogens that can have an impact on the GI tract. This includes *H. pylori*, *E. coli*, and viruses such as HIV. While there have been a few previous studies that showed that there was a possibility of the spread of pathogens from improperly processed endoscopes, there has not been a study completed in almost 20 years. It would be important to look at this data to see if new and improved processing techniques are working to kill these pathogens and to make sure cross-contamination between patients was not happening. It could also help to identify the prevalence of antibiotic-resistant bacteria colonized within people's GI tract.

Another direction this study could benefit from would be to compare patients from different kinds of groups. In this study, the only data we focused on was whether the patients were male or female, their ages, and their time under surgery. We did find data that showed that certain age groups as well as identified sex were more likely to be colonized by *C. diff* than the other groups. This is helpful, but it would be good to also look at other factors such as social economic status as

well as race. It would be interesting to see if other groups of people are more affected by *C. diff* than others as most pathogens do.

4.4 Public Health Significance

This project is important to public health because it helps show whether current disinfection procedures are helping keep pathogens from infecting generally healthy people. Endoscopies are a very common procedure, with almost 20 million being completed each year. With endoscopies being such a common procedure, there is an increased risk for developing a healthcare acquired illness with improperly cleaned scopes. We chose to monitor for *C. diff* because of the affect it has on public health. *C. diff* is a very significant healthcare acquired illness and within the last 20 years pathogenic strains of *C. diff* have increased significantly. Knowing that *C. diff* is spread mostly through the healthcare setting, we wanted to ensure that colonoscopies are not a primary source of transmission. With our data we were able to show that a deadly bacterium is not being spread via endoscopies and can be ruled out as a possible source of transmission.

Appendix A Appendices and Supplemental Content

Appendix Table 1 Make-Up of Master Mix for qPCR

| Component | Final Concentration or Amount | Volume per 20 microliter reaction |
|--|-------------------------------|-----------------------------------|
| PrimeTime Gene Expression Master Mix (2x) | 1X | 10 μ L |
| Forward and reverse primers | 250 – 1000 nM each | 0.4 μ L |
| Probe(s) | 150 – 250 nM each | 0.4 μ L |
| DNA template | 3 pg to 100 ng | 3 μ L |
| Nuclease-Free Water | | 4.2 μ L |

Appendix Table 2 Cycling Protocol for qPCR on CFX Connect

| Step | Cycle | Temperature | Fast Cycling (min:sec) | Standard cycling (min:sec) |
|----------------------------|-------|-------------|------------------------|----------------------------|
| Polymerase Reaction | 1 | 95°C | 3:00 | 3:00 |
| Amplification: | 35-45 | 95°C | 0:05 | 0:15 |
| Denaturation | | 60°C | 0:30 | 1:00 |
| Annealing/Extension | | | | |
| Hold if needed | 1 | 4°C | Up to 24 hrs | Up to 24 hrs |

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