ISOLATION AND ANTIBIOTIC SUSCEPTIBILITY OF *CLOSTRIDIUM DIFFICLE*

BY

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18/4841

A PROJECT SUBMITTED TO THE DEPARTMENT OF BIOLOGICAL SCIENCES AND BIOTECHNOLOGY, COLLEGE OF PURE AND APPLIED SCIENCES, CALEB UNIVERSITY, IMOTA, LAGOS STATE, NIGERIA

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCE (B.SC) IN MICROBIOLOGY AND INDUSTRIAL BIOTECHNOLOGY FROM THE DEPARTMENT OF BIOLOGICAL SCIENCES AND BIOTECHNOLOGY, CALEB UNIVERSITY,

IMOTA, LAGOS

JULY, 2022.

DECLARATION

I, AINA OLUWADAMILOLA YETUNDE, hereby declare that the project work titled **ISOLATION AND ANTIBIOTIC SUSCERPTIBILITY OF *CLOSTRIDIUM DIFFICILE*** is a record of an original work done by me, as a result of my research effort carried out in the Department of Biological Sciences and Biotechnology, Caleb University, Imota, Lagos state.

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CERTIFICATION

This is to certify that this project titled **ISOLATION AND ANTIBIOTIC SUSCEPTIBILITY OF *CLOSTRIDIUM DIFFICLE*** was carried out by AINA OLUWADAMILOLA YETUNDE with the matric number 18/4841 in the Department of Biological Sciences and Biotechnology, College of Pure and Applied Sciences, Caleb University, Imota Lagos, Nigeria.

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DEDICATION

I dedicate this project to God Almighty my creator and my strong pillar. He has been the source of my strength throughout this program. I also dedicate this work to my hardworking mother Mrs Aina and my grandmother, Mrs Odusanya. for their enduring support and contributions in my life and whose constant encouragement has made sure that I give it all it takes to finish that which I have started. Thank you. My love for you both can never be quantified. God bless you.

ACKNOWLEDGEMENT

I am so grateful to God for his mercy, love, wisdom, knowledge and endless grace bestowed upon me and for guiding me till this very moment.

A very big appreciation goes to my hardworking supervisor, Dr. E. A. Ademola. Your effort, support and care brought me this far. Thank you for motivating and scolding me when I lost hope, may the good lord reward you.

To my Head Of Department, Dr.C.C.Ezeanya Bakpa, and my lecturers Dr. B.C. Kotun, Dr. T.C. Bayo-Olajide, Dr. O.A. Folorunsho, Dr.O.S. Ashaka, Dr. Amos Olajide, Mr. O. Onoja, Mr J. Ayedun, thank you all for your moral teachings and great impact in this phase of my life. I am really grateful.

My acknowledgement also goes to the best mother, Mrs Aina, my grandmother, my loving siblings; Ayomide and Tunmise, my big sisters and uncle; Mrs Nejo Oluwaseun, Miss Akintunde Esther, Miss Oyenola Toyosi, Miss Awoyemi Deborah and Mr Afolabi for their financial support, prayers and encouragement always.

To my loved ones, Amb Taiwo, JohnDodo Oluwafemi, Oladeji Kenny, Olorunfunmi Mariam, Aluko Pelumi, Fajimeye Emmanuela, Anosike Angel, Olanrewaju Subomi, Opeaye Damilohun, Olukolu Gabriel, Onojah Blessing and my coursemates. Words fail my sincere expression. I really thank God for making you all part of this journey, thank you all for everything, God bless you all.

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ABSTRACT

Clostridium difficile is a gram-positive rod bacillus that forms spores found in the normal gut microbiota of 1-3 percent of healthy individuals and 15-20 percent of babies. *Clostridium difficile* is a common cause of infections in hospitals, and it is transferred mostly by the fecal-oral route. *Clostridium difficile* infection (CDI) is an emerging health problem in hospital setting. The ability of the spores to persist in the environment is a key factor in rates of infection. *Clostridium difficile* has also been described as one of the leading cause of nosocomial diarrhea. It is responsible for an increase in hospital stay with high healthcare and economic repercussions. It causes a serious toxic-mediated enteric diseases in humans. This study aimed to investigate the isolation and antibiotic susceptibility of *Clostridium difficile* from clinical samples. A total of 49 samples comprising of sea food (5), wastewater (7), soil (16) and stool (21) samples were collected for this study. Isolation was carried out using *C. difficile* base CHROMagar in an anaerobic condition, while identification was done using morphological and biochemical tests. Antibiotic *Clostridium difficile* and was tested for their susceptibility to eight antimicrobials via disc diffusion method. The occurrence of *C. difficile* in the samples was 30.61% (15/49), with 3, 5, 2 and 5 isolates from stool, sea food, wastewater and soil samples, respectively. All the isolates have the characteristics morphological features of *C. difficile* on Chromogenic agar. The highest antibiotic susceptibility test of the isolates was recorded with tetracycline (73.3%), followed by Chloramphenicol (73%), Erythromycin (67%), Ciprofloxacin (67%), Ampicillin (67%), Vancomycin (66.7%), Metronidazole (53%) and Gentamicin (47%).

CHAPTER ONE

INTRODUCTION

1.1 Background of Study

The Gram-positive, obligate anaerobic rod *Clostridium difficile*, also known as *Clostridioides difficile*, was originally discovered in 1935 in the stool of healthy newborns. It was determined as common cause of pseudomembranous colitis associated with antibiotics in the 1970s. It is known to be a common origin of antibiotic-associated diarrhea. The pathogen *C. difficile* is extensively circulated in the environment and spreads through the fecal-oral path. Asymptomatic carriers, sick people, contaminated environments, and animal intestinal tracts are all possible *C. difficile* reservoirs. It is common to refer to the significant condition brought on by this bacterium as “C. *difficile* infection" (CDI) or "*C. difficile*-associated diarrhea". In nursing homes and clinics where patients are routinely administered antimicrobial drugs, CDI is one of the utmost public health problems (Viswanathan *et al.,* 2010).

Community-acquired CDI is on the increase, with frightening growths being stated in some regions of North America and in inhabitants formally presumed to be at low risk. Hospitalized patients, particularly those receiving antibiotics prophylactically or curatively, are at increased risk for CDI (Pituch. 2009). Antibiotic exposure, which changes the natural flora of the intestines, is a major cause of CDI. *C. difficile* in the digestive tract multiplies and produces toxins. The major causes of the onset of CDI are *C. difficile* toxins A and B. These toxins cause colitis and mucosal damage (Voth and Ballard. 2005).

Clostridium difficile spores can live for weeks or months and are resistant to ordinary cleaning chemicals like hand sanitizers. Patients at higher risk of *Clostridium difficile* infection include those on antibiotics, advanced age, those who have had gastrointestinal surgery, and those who have a major underlying condition, Conditions that compromise the immune system, as well as a prolonged stay in a healthcare facility.

C. difficile colonizes 5 percent of adults and 15 to 70 percent of infants, with the prevalence of colonization being numerous periods higher in hospital patients and nursing home residents. Despite the widespread knowledge of *Clostridium difficile* infection in industrialized nations and its well-established association with antibiotic usage, Nigeria has a low rate of infection. Similar to this, a developing issue for *C. difficile* infections treatment is the incidence and increase of *C. difficile* isolates that are resistant to a number of antibiotics, predominantly between the extremely virulent *C. difficile* ribotype 027 strains (Mohammad *et al.,* 2020).

Wrong usage of broad-spectrum antibiotics like cephalosporins, clindamycin, metronidazole, vancomycin, tetracycline, and fluoroquinolones as well as bacterial adaptations that promote resistance have been related to reports of antimicrobial resistance in *C. difficile*. It has also been noted that these processes are accompanied by the acquisition of mobile genetic elements that render medications that go into a cell inactive by enzymatically degrading or changing them into a non-functional form (Dilnessa *et al*., 2022). As a result, it is important to ascertain this organism's prevalence and susceptibility to antibiotics in our environment.

1.2 Statement of Problem

The prevalence of CDI in the community and the severity of the disease are both continuously rising, and this poses a serious public health risk. Significant morbidity, death, and financial impact

on people and the healthcare system are linked to it. The high correlation between illnesses and antibiotic therapy demands for greater vigilance while using antibiotics, especially in Nigeria where everyone has unfettered access to drugs.

1.3 Aim and Objectives

This study aims at the isolation and antibiotic susceptibility of *Clostridium difficile* from environmental and stool samples.

The objectives of this study are:

1) To isolate *Clostridium difficile* from environmental and stool samples
2) To characterize and identify *Clostridium difficile* organism
3) To determine the antibiotic susceptibility of *Clostridium difficile* isolate to commonly used antibiotics.
4) To compare the resistance of isolates from human and environmental sources

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 General Overview of *Clostridium Difficile*

The Greek word "Kloster," which means "spindle," is whence *Clostridium difficile* is derived from. The name *"Bacillus difficilis*" was given to the bacterium in 1935 as a result of the strain's challenges in isolation and need for anaerobic growth conditions. The name was improved to *Clostridium difficile* in the 1970s as it was learned that these bacteria could create toxins. It wasn't until 2016 that molecular evidence led to its reclassification as *Clostridioides difficile*, which had previously been known as *Clostridium difficile.* (Lawson *et al*., 2016).

Clostridium difficile is a Gram-positive bacillus that forms spores {which are usually oval, sub terminal or terminal in position} and is found in the gut microbiome of 1-3 percent of healthy individuals and about 15-20 percent of babies. *C. difficile* is abundant and broadly circulated in nature. They are frequently found in river, sand, soil, lake, sewage, feces of most mammals including human, hospitals and veterinary clinics surroundings, marine sediments, and raw vegetables. It is commonly found in the gastrointestinal (GI) tract and has the potential to cause toxin-mediated C. difficile infections (CDI), which can range in severity from mild diarrhea to pseudomembranous colitis and can lead to death. (George *et al*., 1978).

2.2 Life Cycle of *Clostridium Difficile*

There are two divisions in the *Clostridium difficile* life cycle: vegetative (living forms) and spore forms.

C. difficile, an anaerobic bacteria, uses spore formation to survive in the oxygen-rich environment

outside the gut. Feces are a source of spore shed. They can contaminate water, food, or surfaces if adequate hygiene is not practiced (such as hand washing), where they may then unintentionally spread from one person to the next. Despite the fact that *C. difficile* spores are present everywhere, and individuals are prone to contract them in CDI hotspots like nursing homes or hospitals.

Spores, like seed of plants, are composed of multiple membranes and protein-rich shells that protect the DNA and enzymes that make up mature *C. difficile* cells. The spores develop into vegetative cells after ingestion and successful entry into the host's intestine. They rehydrate throughout this process, and their metabolism begins to function. However, relations amongst the spores and tiny particles, known as germinants, inside the gut determine whether or not germination takes place. These germinants for *C. difficile* are primary bile acids derived from the host (Paredes-Sabaja *et al*., 2014).

The digestive system receives primary bile acids that are formed in the liver from cholesterol. Bile acids, which are like detergent molecules and aid in the breakdown of lipids in the gut, cut through grease like soap. Bile acids come in a variety of forms with various molecular structures. As a result, not all bile acids promote the germination of *C. difficile*. Taurocholate, for example, is a potent C. difficile germinant that works by attaching itself to a receptor on the spore's outer membrane and kicking off the process of germination. The *C. difficile* spores receive the signals from bile acids and co-germinants that they are in the gut and that there will be enough food to support their growth into metabolically active, free-living cells. This is because the spores are already in the gut.

2.2.1 Growth *of C. difficile*: The Vegetative Phase

The hungry phase of *C. difficile* begins after germination has taken place. The microbiota of the host typically mounts an attack against *C. difficile* colonization, in part by obstructing the pathogen's access to nutrients and in part by secreting metabolic chemicals that are suppressive, such as secondary bile acids. However, if the bacterial community is disturbed, *C. difficile* has access to a wide range of carbon sources. Because *C. difficile* is not picky about what it eats, the nutrients constantly present at any given time in its environment can change how it functions metabolically. However, some nutrients, such as the amino acids proline, leucine, and glycine, are essential for effective growth. By transferring electrons from one amino acid to another, Stickland metabolism, a collection of amino acid fermentation reactions typical of *Clostridium* species, produces adenosine triphosphate (ATP) and other essential molecules for life (Johanesen. *et al*., 2015).

Intestinal destruction is a characteristic of the vegetative cell phase of *C. difficile*. Up to three toxins are released by the infection, which damage intestinal barrier integrity and increase inflammation. It's interesting to note that the absence of certain nutritional or metabolic cues actually promotes the development of toxins. According to a number of in vitro studies, *C. difficile* discharges toxins in reaction to stress, including nutrient deprivation. In actuality, the same regulatory proteins that control the hunger response also control the release of toxins. In other words, *C. difficile's* "hungry" response is the production of toxin. According to recent studies, bacterial growth that might compete with *C. difficile* for the same nutrients is inhibited while toxin-mediated inflammation releases nutrients from host cells that *C. difficile* can consume, like collagen. This tactic is comparable to that used by other gut bacterial pathogens, such as *Vibrio cholerae, Typhimurium*, *Salmonella*, and *Enterica serovar*, which feed on the inflammatory

response of the host. Therefore, *C. difficile* toxins might enable the pathogen to occupy a nutritional niche in the gut (Voth and Ballard. 2005).

2.2.2 *C. difficile* Sporulation

The free-living cell *C. difficile* eventually returns to its spore form. Although the precise causes of spore development are still unknown, there is evidence that the process is related to nutritional availability, just like the production of toxins. In fact, the same gene regulators implicated in nutritional starvation and toxin synthesis are also implicated in sporulation, and toxin production and sporulation may occur concurrently. Regardless, after the procedure is through, the fresh spores are released into the environment, where they wait until a different host appears.

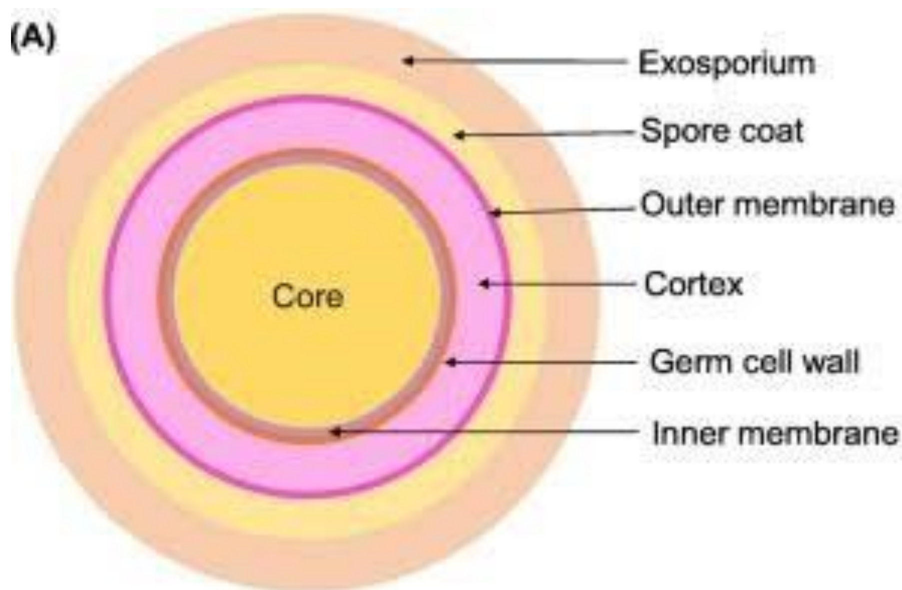

Figure 1: Diagram of *Clostridium difficile* spore. Source: (Amelia J. Lawer *et al*., 2020)

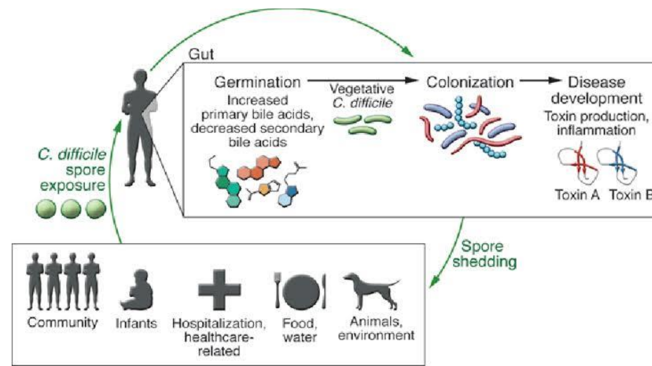

Figure 2.2: Life cycle of *Clostridium difficile*. *Source:* *(Journal of Clinical Invest.2014).*

2.3 Toxins from *Clostridium difficile* and Pathogenicity

There are non-toxigenic and toxigenic strains of *C. difficile*. The Pathogenecity Locus, a 19.6 kb genomic island, is present in toxic strains (PaLoc). Some strains of *C. difficile* have the potential to create a binary toxin that is called *C. difficile* transferase (CDT). This toxin is closely related to the binary toxin that is produced by *Clostridium perfringens.* The principal toxins that are produced by the main stream of *C. difficile* strains are TcdA and TcdB. These toxins are generated by the genes tcdA and tcdB that are located within the Pathogenicity loci (PaLoc) of the organism. Clostridial glucosylating toxins are another name for the family of large clostridial glucosylating toxins (LCGT), which all of these toxins belong to.

The sequenced genes for TcdA and TcdB are discovered in single exposed analysis frames inside a region with a pathogenicity locus of approximately 19.6 kb. Both open reading frames are lengthy, as expected; tcdA is located in a region containing 8,133 nucleotides, and tcdB includes 7,098 nucleotides. The G+C content of the *C. difficile* genome is equivalent to the Low-G+C (>28 percent) gene content of tcdA and tcdB, and the toxins share a high degree of similarity. Furthermore, the G+C composition of the *C. difficile* genome is comparable to that of the tcdB genome (66 percent). The similarity in the biochemical action of TcdA and TcdB, which both use

a highly conserved N-terminal domain to modify the same substrates, is more support for the gene duplication idea. Utilizing the same region, both proteins modify the substrates. Enzymatic receptor-binding domains of both toxins contain the sections of TcdA and TcdB that are most similar to one another. TcdA and TcdB show comparable substrate selectivity, which may be traced back to their highly similar N-terminal domains, which have 74% sequence identity. TcdA is responsible for encoding five distinct sets of connected repetitive oligopeptides. These oligopeptides can be repeated throughout the protein's C termini and range in length from 21 to 50 amino acids. In addition, TcdB codes for five groups of concatenated repeating oligopeptides, four of which are identical to those encoded by TcdA. In contrast, the paired repeating oligopeptides found in TcdB are more distinctive and occur less frequently than those found in TcdA. In addition to the similarities between TcdA and TcdB, both of these toxins have a significant degree of similarity with the vast majority of big clostridial toxins (Kuehne *et al*., 2010).

2.3.1 Toxin A and B Structure and Activity

The major clostridial glucosylating toxin family also includes the lethal toxin (TcsL) and the hemorrhagic toxin (TcsH) from *Clostridium sordellii,* the alpha toxin (TcnA) from *Clostridium novyi,* and the TpeL from *Clostridium perfringens.* The toxin sequences are highly similar and range in size from 250 to 308 kDa. Both of their routines are remarkably similar. Toxins A and B each have three core domains: a catalytically active N-terminal domain, a translocation domain positioned in the center, and a receptor-binding domain located at the C-terminus. These monoglucosyltransferases have comparable substrate selectivity, and they are the sort of toxins that catalyze the transfer of a glucose moiety onto Rho family GTPases (Rho, Rac, and Cdc42) within the target cells. Rho family GTPases regulate a variety of cellular processes, including the organization of the actin cytoskeleton. They play a regulatory role in immune cell signaling and

motility, as well as the epithelial barrier's function. Toxins A and B glucosylate Rho GTPases, rendering these proteins inactive and preventing the activation of further signaling pathways in the cell. Intoxicated cells eventually undergo apoptosis and die, resulting in breakdown of the actin cytoskeleton and cell rounding. (Glen *et al*., 2010).

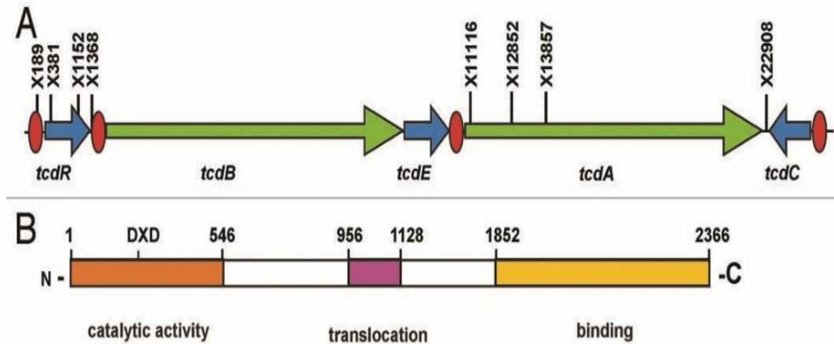

Figure 3.0: Genetic organization of PaLoc and toxin structure. (A) Gene structure of PaLoc obtained from the genomic organization of *C. difficile*. The Xbai sites (X) and the PaLoc location are displayed. Furthermore, red ovals depict gene-specific promoters, and arrows indicate the direction in which transcription will occur. (B) Structural organization of toxin B (toxin A is similar). **Source:** Gerding *et al.,* 2014.

2.4 *Clostridium difficile* Infections

There are two types of *Clostridium difficile* infections: exogenous and endogenous. Endogenous infection is propagated by carrier strains, whereas exogenous infection is spread through infected people, contaminated settings, contaminated medical personnel, and nosocomial sources (Vaishnavi. 2010).

The oral-fecal channel is how *Clostridium difficile* is transmitted from one person to another. Consuming spores that are resistant to the environment and tolerable of stomach acid is how it is

transmitted. In the small intestine, ingested spores germinate into the vegetative form. Despite the use of antimicrobial medications and the disruption of normal colonic bacteria, *Clostridium difficile* colonization happens in the large intestine (Owens *et al.,* 2008).

Two toxins produced by *Clostridium difficile*, Toxin A and Toxin B, leads to intestinal inflammation. A range of bacteria (microbiota) that are generally present in the gastrointestinal tract inhibit *Clostridium difficile* colonization and damage to intestinal cells. Hospital infections frequently originates from *Clostridium difficile*, which is spread mostly through the fecal-oral route. Being a spore-forming bacillus, *Clostridium difficile* can easily spread through an infected person's stool, contaminating water, food, and other nearby objects or surfaces. The most common way that *C. difficile* infects its victims is through the ingestion of spores, which germinate in the intestine into vegetative cells and go on to produce toxins when their growth is stationary. The entry of the disease-causing phase coincides to the cell's transition into stationary phase, when toxin gene expression increases.

The clinical presentations of *Clostridium difficile* includes;

2.4.1 Carrier Stage: Carriers may be *C. difficile* reservoirs depending on their health since they have *C. difficile* in their stools but do not have diarrhea. Numerous investigations have shown that the carrier stage is present in around 3% of healthy individuals, 20% - 30% of hospitalized patients, and 50% of patients with prolonged hospital stays. A s a result, the carriers help the spores spread into the environment in less amounts than do individuals who have diarrhea or other symptoms.

2.4.2 *C. difficile*-Associated Diarrhea (CDAD): *C. difficile* is responsible for around 25–30% of all diarrhea cases associated with antibiotic use (AAD). It is characterized by unexplained diarrhea that commonly begins two hours to two months after taking an antibiotic and is followed

by abdominal discomfort and pain. The illness is known as diarrhea associated with antibiotics. A patient was diagnosed with diarrhea if they passed at least three loose stools over a minimum of two days. In addition, CDAD is diagnosed when toxin A is present in the stool, regardless of whether *C. difficile* was successfully identified. At one time, it was thought that hospitalization was almost invariably associated with CDAD. Alternatively, there are two distinct possibilities regarding how CDAD is acquired and its role in disease. According to the first school of thought, a patient who gets *C. difficle* while being treated in a hospital stands the danger of developing CDAD whenever antimicrobial medications are administered. In the other scenario, a hospitalized patient acquires *C. difficile*, but does not become at high risk for infection until they begin getting antibiotics. *C. difficile* may only be spread from one person to another through feces or stool.

2.4.3 *C. difficile*-Associated Colitis (CDAC): The most prevalent clinical manifestation of CDI is colitis that does not develop to pseudo membrane formation. In addition to increased morbidity and high healthcare costs, CDAC is associated with longer hospital stays and longer stays overall. Among the symptoms include abdominal cramping, nausea, weakness, loss of appetite, watery diarrhea, and the presence of trace amounts of blood in the feces. Additional undesirable side effects include pyrexia, leukocytosis, dehydration, and fever of a lesser intensity. Even in the absence of diarrhea, patients receiving antimicrobials should have a high white blood cell count (WBC) regularly monitored for potential symptoms of *C. difficile* infection (CDI).

2.4.4 Pseudomembranous Colitis (PMC): The term "Pseudomembranous Colitis" (PMC) refers to a specific type of colitis that was originally identified following the performance of a gastrojejunostomy to treat an obstructive peptic ulcer. This method led to the identification of the condition. Antimicrobial medication that altered the patient's normal flora has been blamed for the rise in occurrences of pseudomembranous colitis during the past several years. Antibiotics

clindamycin and lincomycin are considered to be the root cause of the great majority of PMC cases. In contrast, a large number of other antibacterial substances with similar capabilities have been found. PMC symptoms include abdominal pain, dehydration, hypoalbuminemia (less than 30 mg/dL), watery diarrhea, and a rise in inflammatory cells, serum proteins, and mucus. In addition, the best PMC detection markers are yellowish plaques ranging in size from 2 to 10 millimeters that are visible during a sigmoidoscopic examination in the mucosa of the gastrointestinal tract and occasionally in the terminal ileum.

2.4.5 Fulminant Colitis: The majority of unfavorable outcomes, including as perforation, delayed ileus, megacolon, and mortality, are the result of fulminant colitis, which affects roughly 3 percent of CDI patients. In recent years, there has been an increase in the occurrence of fulminant colitis, which causes symptoms, organ failure, and an increased risk of death. This increase has been linked to a particularly severe strain of *C. difficile*.

2.4.6 Recurrent CDI: Recurrent CDI, which may be caused by relapse or reinfection, is one of the condition's most unpleasant aspects. Within four weeks of the conclusion of their antibiotic therapy, 25% of individuals treated with metronidazole or vancomycin will experience a recurrence of their symptoms. On the other hand, it appears that the disruption of normal gut flora and an inadequate immune response to *C. difficile* and/or its toxins play an important role in the development of recurrent *C. difficile* infection (CDI). The major cause of recurrent CDI has not been identified as of yet.

2.4.7 Extra colonic Infections: Recent research indicates that CDI is not exclusive to the colon alone. Small intestine disease, which can result in the formation of pseudo membranes on the ileal mucosa, bacteremia, reactive arthritis, visceral abscess, appendicitis, intra-abdominal abscess, osteomyelitis, and empyema are all clinical manifestations of *C. difficile* infections. The most

prevalent causes of extracolonic *C. difficile* infections are underlying diseases such as digestive problems, *C. difficile* colitis, or a surgical or anatomical malfunction of the colon.

2.5 Modes of *Clostridium difficile* Transmission

The environment is heavily populated with *C. difficile*. It produces spores that can withstand environmental elements like temperature and humidity as well as common cleaning products for longer periods of time. It transforms into a spore to defend itself from unfavorable environmental circumstances. Transmission of *C. difficile* occurs via the fecal-oral route. Hospitals and the general public both have the potential to harbor *C. difficile*. Patients may contract *C. difficile* through contact with contaminated surfaces (including vegetative cells and spores), poor environmental cleaning, and careless handling of bedpans and toilets.

2.6 Epidemiology of *Clostridium difficile* Infections

Several studies conducted in the United States, Europe, and Canada have suggested that between 20 and 27 percent of all CDI infections may be community-related. Up to 3 million instances of diarrhea and colitis may result from *C. difficile* infection (CDI), which mostly affects hospitalized patients in the United States. Healthcare-related diarrhea associated with CDI has a substantial impact on cancer patients. The frequency of reported CDI infections keeps rising. According to McFarland *et al*., report 's from the 1980s, the organism was present in the cultures of 7% of patients admitted to hospitals and 28% of patients who were hospitalized. The prevalence of *C. difficile* in hospitalized patients increased to 30–40 per 100,000 people in the 1990s, and to 84 per 100,000 people in 2005. (McFarland *et al*., 2008).

In fact, from 2000 to 2009, the incidence rates of other nosocomial infections decreased, although the number of hospitalized patients with any CDI as their discharge diagnosis increased by more

than twice that amount, from about 139,000 to 336,600. Additionally, between 33,000 to 111,000 individuals had CDI as their primary diagnosis (Czepiel *et al.,* 2015).

Studies have refuted the idea that *C. difficile* is primarily a hospital infection as new CDI populations have emerged and as more cases are being reported in the community. These cases include those involving inflammatory bowel illness, pregnant women, and people with community-acquired infections who hadn't previously used antibiotics (IBD).

According to the findings of a population-based study that was carried out in Olmsted County, Minnesota between the years 1991 and 2005, the incidence of *C. difficile* infections that were acquired either in the community or in a hospital considerably rose. In the cases of *C. difficile* infection, the community was responsible for the acquisition of 41% of the cases.

An increase in the incidence, severity, mortality, and recurrence of *C. difficile* infection has been linked to a new strain of *C. difficile* known as North American pulsed-field gel electrophoresis type 1 (NAP1), polymerase chain reaction (PCR) ribotype 027, toxinotype III, and restriction endonuclease analysis type BI (i.e., BI/NAP1/027). (Marsh *et al*., 2012).

Community-acquired *C. difficile* infection rates are lower than nosocomial *C. difficile* infection rates; nonetheless, up to forty percent of patients with community-acquired infection require hospitalization, and recurrence rates are comparable between the two populations. Two factors that contribute to the reduced prevalence of community-acquired *C. difficile* infection in the non-hospitalized population are their younger age and less underlying illnesses. A *C. difficile* infection is linked to serious illness, a 5-percent infection-related mortality rate, and a 15–20 percent all-cause mortality rate. 3,32 A white-cell count of more than 15,000 cells per cubic millimeter, hypoalbuminemia, and acute kidney injury are all indicators of severe *C. difficile* infection and are independent predictors of need for an urgent colectomy and death (Kufelnicka. 2011).

2.7 Methods of Detection *Clostridium difficile* Infection

The clinical diagnosis of *Clostridium difficile* infection is backed by test results. Due to their lengthy turnaround times, the historical *C. difficile* detection methods were not practical for diagnostic testing. As a result, several laboratories quickly embraced enzyme immunoassays (EIAs) for the detection of *Clostridium difficile* due to their efficiency, practicality, and low cost. The laboratory diagnosis of *C. difficile* is simple and frequently performed on patients with illnesses that compromise their immune systems. Several diagnostic procedures are presently used to identify CDI, including:

2.7.1 Enzyme immunoassay (EIA) for glutamate dehydrogenase (GDH): EIA for *C. difficile* glutamate dehydrogenase (GDH) or DNA-based tests that locate the *Clostridium difficile* toxin genes in the stool sample. This test looks for the GDH antigen, an enzyme present in both toxic and nontoxic strains that enables the bacteria to reduce oxidative stress.

2.7.2 EIA for toxins A and B: This test is insensitive (sensitivity range: 63%–94%) and has a high false-negative rate. Toxins that are produced directly by the toxigenic strain are identified by this test, making it especially specific for active infections. Patients who have just undergone chemotherapy may not qualify for this test. A change in fecal proteases or temperature that inhibits toxin stability and produces false-negative results is an additional factor that influences the sensitivity of the test. The modification may provide false-negative results.

2.7.3 Polymerase chain reaction 'PCR' testing: This test has a high false-negative rate and is not sensitive (or temperature that alter toxin stability might also affect test sensitivity and cause false-negative results. Sensitivity: 63 percent to 94 percent). It is highly selective for infections that are actually active since it only picks up toxins that the toxigenic strain itself directly produces.

Patients who have recently undergone chemotherapy might not be suitable for this test. (Khan *et al*., 2012).

2.7.4 Stool culture: This is also known as the **gold standard**. Cyloserine-cefoxitin-fructose-agar (CCFA) is a common medium for stool culture, where the first two ingredients prevent the growth of other bacteria and fructose serves as a nutrition (Martinez-Melendez A *et al*., 2017). The majority of the culture media available may produce results with excellent specificity and sensitivity. The test takes a long time and cannot distinguish between strains that are toxic and those that are not (Khan *et al*., 2012). Stool culture tests demand skilled personnel and particular laboratory procedures. Anaerobic culture is necessary for *C. difficile* stool culture but is not commonly available (Leffler and Lamount, 2015).

Another diagnostic method is the endoscopy, which is not always necessary but may be helpful if there is any doubt about CDI in patients with inflammatory bowel disease or if there are clinical indicators present along with all negative results from the laboratory tests.

2.7.5 Toxigenic culture: When it comes to *Clostridium difficile*, toxicogenic culture involves isolating the organism from fecal specimens and determining whether the recovered isolate is a toxin-producing strain. This is done in order to determine whether the organism can produce toxins. In order to accomplish this goal, a variety of different approaches have been suggested. The utilization of broth culture or anaerobic agar with selective and differential agents is the central component of each method. These agents are used to inhibit the expansion of other fecal bacteria while simultaneously facilitating the recovery of *C. difficile*. The method that has been shown to be the most successful in culture for recovering the organism is still up for dispute. Before administering the inoculation, certain researchers have tried to boost the number of *C. difficile* spores and inhibit the growth of other organisms by subjecting the culture to a heat shock or an

alcohol shock. There have been many various formulations of medium described, and a significant number of them take advantage of the capability of *C. difficile* to ferment fructose. The most common type of medium is called cycloserine, cefoxitin, and fructose agar (CCFA). However, several variations on the theme of CCFA have been recorded. These variations include cefoxitin cycloserine egg yolk agar (CCEY), which is also known as Brazier's medium, and cycloserine cefoxitin egg yolk agar with lysozyme. Both of these mediums were developed by (CCEYL). In order to encourage spore vegetation, a number of different medium compositions have been supplemented with various chemicals, some of which include lysozyme and taurocholate. It would appear that improving the broth's nutritional profile before plating it over solid agar results in better recovery. It is essential to pre-reduce the medium before inoculating the specimen in order to maximize organism recovery, and this is true regardless of the medium or the method used. It is standard practice to keep culture results in storage for at least 48 hours and frequently for as long as seven days before reporting them as negative. In most cases, toxicogenic culture is considered to be more of a reference method than a diagnostic tool.

2.8 Treatment of *Clostridium Difficile* Infection

Whether a CDI is considered to be a first episode, recurring, severe, or complicated CDI will affect how it is treated. Metronidazole is frequently the recommended treatment for CDI with a first episode. Rifaximin and Teicoplanin, among other substances, can be used. The non-absorbable oral antibiotic rifaximin is effective against initial and recurrent CDI infections. In cases of recurrent CDI, vancomycin administration is advised. Because of its possible neurotoxicity, metronidazole is not prescribed for recurring episodes or as a long-term treatment (Gardner. *et al.,* 2011).

Due to its low cost and lack of selection for vancomycin-resistant enterococci, metronidazole is utilized as a first-line therapy. Another advantage of metronidazole is that it can be administered intravenously to those who are unable to take oral medications. The preferred initial course of treatment is 10 days of oral or intravenous (IV) metronidazole administration (250 mg four times day or 500 mg every eight hours).

Vancomycin is only administered orally because the IV version is useless for treating gut infections. A typical dose is 125 mg administered four times each day for ten to fourteen days (McDonald *et al*., 2017). The 2018 National Comprehensive Cancer Network (NCCN) guidelines for treating cancer patients suggested beginning with oral vancomycin. Vancomycin has the added benefit of having fewer gastrointestinal side effects than metronidazole, such as nausea, which are frequent in cancer patients (Neeman and Freifeld. 2017). Other options for cancer patients include metronidazole and fidaxomicin.

Fidaxomicin is a clostridia-specific macrolide antibiotic that works locally (Neeman and Freifeld. 2017). In 2011, this drug has been approved by the Food and Medication Administration (FDA) for the treatment of *Clostridium difficile* infection (Leffler and Lamont, 2015). Fidaxomicin treatment was not found to be lower to vancomycin treatment in a study of non-cancerous patients. This antibiotic has the advantage of maintaining normal intestinal flora, which lowers the rate of infection (Mathur *et al*., 2014).

Nitrothiazolide is another class of antibiotics such as nitazoxanide and amixicle that work by inhibiting pyruvate ferredoxin oxidoreductase (PFOR), which is an essential enzyme in metabolism (Warren. *et al*., 2012). Protozoal infections and helminth infections are frequently treated with nitazoxanide. It has also been linked to effective resistance to *Clostridium difficile*,

though. On the other hand, Amixicle, a water-soluble Nitazoxanide derivative, is also efficient against *Clostridium difficile* infection.

Monoclonal antibodies, fecal microbiota transplantation (FMT), and probiotics are examples of recent treatments. The effectiveness of intravenous monoclonal antibodies in preventing CDI recurrence has been investigated by certain researchers. In a double-blind study, patients who first got metronidazole or vancomycin were then given treatment with antibodies against toxins A (CDA1) and B (CDB1), respectively. In comparison to control patients, those receiving antibody treatment displayed a decreased rate of CDI recurrence over the course of the 12-week research, according to the study's findings (McCollum and Rodríguez. 2012).

Usually, FMT is advised following the third CDI episode. In a 2012 randomized trial, C. P. Kelly chose adults who had three or more recorded CDI recurrences. This procedure involves giving a patient a suspension of feces from a healthy donor in an effort to restore their "normal" microbiome. The patient receives the donor material through a rectal enema, a nasoduodenal tube, or a colonoscopy. Five days prior to the start of the treatment and a few weeks afterward, the faecal microbiota of patients and donors was examined. According to the initial research, patients had less firmicutes and Bacteroidetes and more gamma- and beta-proteobacteria than donors. They came to the conclusion that fresh FMT from a donor supplied by colonoscopy to patients who had previously received a course of vancomycin was effective in avoiding additional CDI episodes. Since the researchers also found that the treatment's effectiveness varied depending on the area of the intestine where the infection developed, some patients may not benefit from FMT (Debast *et al.*, 2014).

Probiotics give the host a health advantage when given in sufficient numbers. Probiotics work in three different ways: (i) altering the host's defenses (ii) impacts on other microorganisms due to

probiotic bacteria adhering to epithelial cells and preventing pathogen adherence. Therefore, if pathogens are unable to stick to epithelium, enter cells, and (iii) have an impact on microbial products such toxins or host products. By creating more toxins, some microbes can suppress the generation of poisons. *Saccharomyces boulardii*, for instance, offers some defense against *C. difficile* toxin A (Ofosu. 2016).

Table 2.1 Treatment of *Clostridium difficile* Infections

SEVERITY	**CLINICAL MANIFESTATION**	**TREATMENT**
Asymptomatic carrier	No signs or symptoms	No treatment indicated
Mild	Abdominal pain or tenderness, mild diarrhea, no visible laboratory abnormalities	Hydration, monitoring of clinical status, administration of metronidazole(500mg three times per day)

Moderate	Moderate non bloody diarrhea, moderate abdominal discomfort, occasional vomiting with nausea, dehydration, blood urea creatinine levels above baseline	Cessation of predisposing antibiotics, administration of metronidazole(500mg three times per day) or first line therapy with oral vancomycin(125mg four times per day), Consideration of hospitalization, monitoring of clinical status
Severe	Bloody or severe diarrhea, pseudomembranous colitis, ileus, vomiting, temperature> 38.9^{o}c, white blood cell count>20,000/mm^{3}, acute kidney injury	Hospitalization, oral vancomycin(500mg four times per day) with or without metronidazole(500mg three times per day) if the risk recurrence is high instead of vancomycin
Complicated	Respiratory distress, toxic megacolon, peritonitis, hemodynamic instability	Antibiotics, surgical consultation, fecal microbial transplantation

Source: Daniel Leffler and Thomas Lamont. (2015). "*Clostridium difficile infection", New England Journal of Medicine* 372; 1539-1548.

2.9 Antimicrobial Resistance of *Clostridium Difficile*

In addition to helping to cause or cause a recurrence of CDI, *C. difficile's* resistance to commonly prescribed antibiotics for bacterial infections is a significant factor in driving epidemiological modifications and the emergence of new strain types. Additionally, *C. difficile* antibiotic resistance results in less than ideal clinical outcomes and may even result in CDI treatment failures. Collateral damage to the microbiota may happen when unusual antibiotics are used to treat CDI, and this should not be disregarded. The first line of antibiotics used to treat CDI is still metronidazole and vancomycin (Leffler and Lamont. 2015) (Cohen *et al.,* 2015). The majority of CDI cases can still be treated with these antibiotics, but some *C. difficile* isolates have been found to be significantly

less susceptible to them, particularly those that are resistant to metronidazole (Goudarzi *et al.,*2013) (Adler *et al.,*2015). Over the past ten years, there has been a notable increase in the number of CDI cases that have failed to respond to metronidazole therapy (Leffler and Lamont. 2015). There have been reports of *C. difficile* that is resistant to metronidazole in various parts of the world. According to the CLSI breakpoint (susceptible 8 g/ml), 0.11 percent of the strains examined were resistant to metronidazole in a Pan-European longitudinal study of antibiotic resistance among common *C. difficile* ribotypes (Freeman *et al.,* 2015).

The proportion of *C. difficile* strains with reduced metronidazole susceptibility has been gradually increasing (Spigaglia. 2016). According to some studies, *C. difficile's* metronidazole resistance is variable (Peláez *et al.,* 2008). According to Moura *et al.,* 2013, metronidazole heteroresistance should be a concern in clinics because the use of sub inhibitory concentrations of the drug contributed to the selection and maintenance of colonies with elevated minor inhibitory concentrations.

Vancomycin resistance in *C. difficile* has also been recognized. Vancomycin resistance in clinical isolates of *C. difficile* was 8.0% in a study by Goudarzi *et al.* (2013).

C. difficile can become resistant not only to metronidazole and vancomycin, but also to rifamycins, fidaxomicin, tetracyclines, and chloramphenicol. Only one *C. difficile* isolate with a minimum inhibitory concentration (MIC) of 16 g/ml was found in a clinical experiment with fidaxomicin. This isolate came from a person who got sick again after getting better. Even though the percentage of Tetracycline-resistant *C. difficile* isolates in different countries ranged from 2.4% to 41.6%, it is still a potentially big problem that should be thought about along with CDI. This is because tigecycline is now suggested as an alternative antibiotic for treating people with severe or complex severe CDI. *Clostridium difficile* is only sometimes resistant to the drug chloramphenicol. In

Europe, only 3.7% of *Clostridium difficile* isolates with a MIC of more than 32 g/ml have been found to be resistant to chloramphenicol (Freeman *et al*., 2015).

It is common knowledge that *C. difficile* is resistant to numerous antibiotics, including aminoglycosides, clindamycin, tetracyclines, lincomycin, cephalosporins, erythromycin, penicillins, and fluoroquinolones. Most of the time, doctors use these antibiotics to treat bacterial infections (Johannesen *et al*., 2015). (Spigaglia. 2016). Recent statistics based on 30 antimicrobial susceptibility studies of *C. difficile* clinical isolates published between 2012 and 2015 show that resistance to clindamycin ranges from 8.3% to 100%, resistance to cephalosporin ranges from 51.3% to 100%, resistance to erythromycin ranges from 13.3% to 100%, and resistance to fluoroquinolone ranges from 47.3% to 100%. CLSI or EUCAST cutoffs are used to figure out these percentages (Spigaglia. 2016).

C. difficile can become resistant to antibiotics if it has genes for resistance on its chromosome, if it has mobile genetic elements (MGEs), if antibiotics change their targets or if *C. difficile's* metabolic pathways change, or if it makes biofilms. Mobile Genetic Elements (MGEs), specifically transposons between *C. difficile* strains and/or between *C. difficile* and other bacterial species, must be conjugated, transduced, and/or transformed for *C. difficile* to get antimicrobial resistance genes (Peláez *et al*., 2008). This is the only way for *C. diff* to get genes that make it resistant to antibiotics. *C. difficile* is thought to be resistant to tetracycline because of transposons called Tn5397, Tn916 or Tn916-like. Research done by (Spigaglia, 2016) and (Tsutsumi et al., 2014) shows that these elements can move tet-class genes. Some of these genes are called tet(M), tet(44), and tet (W). This makes tetracycline less effective against *C. difficile*.

Most of the time, fluoroquinolone resistance in bacteria is caused by two main things: (1) a change in the drug target caused by a mutation in the encoding genes, which makes the drug less attractive

to the target, and (2) either an increase in the active efflux of the drug or a decrease in the permeability of the cell. When these two treatments are used together, they reduce a person's desire for the drug. The binding of RIF is made possible by a change in the beta subunit of DNA-dependent RNA polymerase. This interaction makes a stable complex that limits the ability of DNA polymerase (RpoB) to copy DNA (Tsutsumi e*t al*., 2014).

A multilayered biofilm is produced by a dense matrix of proteins, DNA, and polysaccharides that is composed of many biofilm components. It has been suggested that this biofilm's multilayered structure contributes to *C. difficile's* drug resistance (Dapa *et al*., 2013). Alterations in the metabolic pathways and/or antibiotic targets of *C. difficile* are an additional mechanism that leads to the emergence of antibiotic resistance in this bacterium. Even if the exact process is not fully known, the fact that it is believed to be the mechanism that mediates *C. difficile's* resistance to metronidazole and vancomycin is crucial (Spigaglia. 2016). According to a study conducted by Chong *et al*., in 2014, metronidazole resistance is likely produced by alterations in undefined metabolic pathways, such as those affecting the activity of nitroreductases, iron absorption, and DNA repair. On the other hand, vancomycin resistance may be the result of amino acid substitutions in proteins involved in peptidoglycan production, such as MurG.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials and Media Used

3..1.1 Equipment and Apparatus

The following equipment and apparatus were used for this experiment: Autoclave, Weighing test tubes, tube-tube racks, balance, microscope Petri-dishes, needle and syringes, incubator,

measuring cylinder, cotton wool, nose mask, glass slides, spirit lamp, , , marker, wire loop spatula, conical flask, beaker, cryovial tubes swab stick, and foil paper.

3.1.2 Culture Media and Reagents

The culture media used in this study include: CHROMagar *C.difficile* base agar, Nutrient Agar peptone water, nutrient broth, Urea agar, Triple Sugar Iron Agar, Urea agar base and blood agar. All the culture media were prepared conferring to the manufacturer's guidelines. In a conical flask that was lined with non-absorbent cotton wool, the media were prepared. Aluminum foil was wrapped around the flask's blocked neck and secured with paper tape before sterilization in an autoclave at 121°C for 15 minutes and aseptic dispensing into sterile petri dishes.

Decolorizer, iodine, lactose, safranin, glucose, crystal violet, hydrogen peroxide, sucrose, and KOVAC's reagent are among the other chemicals employed in this study project.

3.2 Methods

3.2.1 Sample Collection

A total of 49 samples were collected for this study. The stool samples were collected from Solid Rock Hospital Ojodu, Lagos. Fresh stool samples were collected in a sterile universal container. The stool sample was free from urine. The samples were transported to the Microbiology Laboratory of the Department of Biological Sciences and Biotechnology, Caleb University, Imota. The stool samples were further kept in the freezer until analysis was carried out.

The sea food samples (4 crabs, 1 crayfish) were purchased at Ikorodu Garage Market, Lagos State. The samples were kept in a Ziploc bag to avoid further contamination until the samples were inoculated from their mouth.

The environmental samples (wastewater and soil samples) were collected from Caleb University Imota Lagos. The waste water samples were collected in a well labelled and sterile universal bottles from different drainage of the Girls and Boys hostel. The soil samples were collected from different spots of the dumpsite area. This was collected from 1cm depth of the soil surface with the use of non-reusable plastic spoons and placed into sterile bags which were labelled properly.

3.2.2 Isolation of *C. difficile*

Initial pre-enrichment of the samples was carried out by inoculating a portion of each of the samples in sterile Clostridial broth and pretreated at $80^{O}C$ for 10 minutes in water bath. The pretreated samples were then incubated in anaerobic jar with CO_2 generating kit at $37^{O}C$ for 24 hours**.** After this, the samples were plated out by streaking on *C. difficile* Chromogenic Agar plate, and incubated under anaerobic condition in anaerobic jar with CO_2 generating kit at $37^{o}C$ for 48 hours. After 48 hours, growth was observed on each of the petri dishes and colonies characteristics of *C. difficile* with fluorescent when observed against UV light were subcultured until pure isolates were obtained.

3.2.3. Morphological Characteristics of the isolates

The colonial morphological characteristics of the isolates such as color, elevation, edge, texture, optical characteristic were determined via the method described by Sapkota (2021).

3.2.4 Biochemical Characterization and Identification of the Isolates

To aid the identification of the organism the following biochemical test were carried out: Gram staining, sugar fermentation test, catalase, potassium hydroxide, motility test, indole, hydrogen sulphide production test, lecithinase test, salicin fermentation test, caseinase test, hydrolysis test**,** and urea hydrolysis test.

Gram Staining Techniques

A young culture of 18–24 hours was selected using a sterile wire loop after a drop of distilled water was deposited on a clean, grease-free slide. On a drop of distilled water, this was utilized to create an emulsion, which was then given time to dry in the air before being heated. The principal stain, crystal violet, was applied to the smear for 60 seconds, and any excess was gently washed off with running water. The slide was then stained with iodine for 60 seconds before being washed off. Following the application of the acetone/decolorizer, the area was stained with the secondary stain safranin for 60 seconds before being promptly washed with running water. After adding immersion oil, the slides were allowed to dry naturally before being inspected under a microscope with a 100 objective lens.

Catalase Test

The test was employed in the identification of the microorganism which produces the enzyme catalase. A bubble formation will be observed which indicates that the catalase enzyme produced by the micro-organism is able to neutralize the hydrogen peroxide to indicate a positive result.

The test was prepared by slide method placing a little amount of the colony on the slide then adding few drops of 3% H_2O_2 on the slide to observe formation of bubbles within 10seconds.

Sugar Fermentation

Lactose, glucose, sucrose, and salicin sugars were measured out properly into each beaker containing 100ml of peptone water (which serves as the nutrient base). Each of the sugars was placed in beakers with indicator (Phenol red), which was then added. The tubes containing the sugars were then sterilized for 15 minutes at 121°C. After cooling, the sugars were infused with the isolates, and they were then incubated at 37°C for 48 hours. A transition from red to yellow in color indicates a successful outcome. This assay was used to determine each isolate's capacity to ferment sucrose.

Motility Test

Nutrient agar was made aseptically, dispensed at half strength, and allowed to harden upright in cryovial tubes. Using a sterile syringe, a single stab was made by inserting the inoculums vertically into the tube. This was studied 48 hours after being cultured at room temperature in an anaerobic jar. A motile creature begins to expand or swarm widely along the stab line zone, which is a sign that the experiment was successful.

Urea hydrolysis Test

As directed by the Manufacturer, the isolates were inoculated by gently streaking into a prepared urease agar that was in a slant form.

This test aids in identifying the microorganisms that can manufacture the urease enzyme. The urea is hydrolyzed by this enzyme into NH_3 and CO_2. On the slant, which may even reach the butt,

there is a vivid pink tint that denotes urease production. Pink to any extent is regarded as a successful outcome.

Caseinase Test

1g of caseine agar was added after peptone water had been made. After cooling, the preparation was plated out after being sterilized for 15 minutes. After being inoculated, the samples were kept in the anaerobic jar for 48 hours.

Indole Production Test

This examination was done to find bacteria that can generate the tryptophanase enzyme. When the enzyme is able to change the tryptophane amino acid, indole gas is created.

Prepared peptone water was placed within tubes and sterilized at 121°C for 15 minutes. The isolates were introduced into the peptone water, and they were then incubated for 48 hours at 37 °C. The Kocav's reagent was used following incubation. A pink or crimson ring that forms after a few drops of Kovac's reagent have been dispensed into the tubes signifies a successful outcome.

Hydrogen Sulphide Production

A small amount of the 24-hour culture would be put into the triple sugar iron agar tubes that were already prepared, and would then be incubated at 37°C for 48 hours. Following incubation, the tube is checked for the production of hydrogen sulfide (a gas with a black color).

Salicin Test

A 48-hour anaerobic incubation period followed the aseptic transfer of an inoculum from a *C.difficile* culture plate to a sterile tube of phenol red salicin broth. A positive test results in a switch in hue from red to yellow, indicating an acidic pH. This test was performed to determine whether the bacterium can ferment the carbohydrate salicin as a source of carbon.

Lecithinase Test

The media utilized in this test was egg yolk agar, which is an enriched, non-selective, and differential agar. Lecithin, a naturally occurring substance in egg yolks, is a component of lecithinases, which are enzymes secreted by bacteria and capable of destroying animal tissues and contributing to pathogenicity. The isolated organism was streaked in a straight line on an egg yolk agar plate and anaerobically incubated for 48 hours at 37 °C. Plate examination followed incubation. An affirmative test result is shown by the formation of a white, opaque, diffuse zone that extends into the medium around the colonies. On the same plate, a lipase test was also carried out. A lipase positive test is identified by the immediate appearance of an iridescent oil sheen (oil on water), which is visible when the plate is held at an angle to a light source, immediately around the colonies.

Gelatinase Test

An exoenzyme called gelatinase breaks down the gelatin protein into amino acids and short chain peptides. By making peptone water and adding 1g of gelatin to the solution, a gelatinase test was conducted. After cooling, the preparation was plated out after being sterilized for 15 minutes. After being inoculated, the samples were kept in the anaerobic jar for 48 hours.

Spore Staining

A young culture of 48 hours was selected using a sterile wire loop after a drop of distilled water had been placed on a grease-free and clean slide. On a drop of distilled water, this was utilized to create an emulsion, which was then given time to dry in the air before being heated. The organism was squared off using blotting paper and heat-fixed onto a glass slide. Malachite green stain

solution was applied to the blotting paper, which was then steamed for five minutes while additional dye was added as needed. The slide was counter stained for 30 seconds with 0.5 percent safranin after being rinsed under running water. The slide was washed with water (and no blotting was done), and then it was viewed under a microscope to check for endospores. Endospores are a vivid green color, and vegetative cells range in color from brown to pink.

3.2.5 Antibiotic Susceptibility Test

On Blood agar plates, this test was conducted using the Kirby-Bauer Disc diffusion method. Two to three colonies of the organism were aseptically added to sterile tubes of normal saline to create a suspension of the isolates, which was then tested for turbidity using the 0.5 McFarland standard. Antibiotic disks (Liofilchem Diagnostic, Italy) were used to seed the culture suspension into blood agar plates, which were then incubated anaerobically for 48 hours at 37 °C in an anaerobic jar with a CO2 producing kit. Vancomycin, Metronidazole, Ampicillin, Ciprofloxacin, Tetracycline, Chloramphenicol, Gentamicin, and Erythromycin were the antibiotics that were used. Zone of inhibition (mm) was measured and the findings were recorded after 48 hours.

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CHAPTER FOUR

RESULTS

Of the of 49 samples used in this study, occurrence of *Clostridium difficile* was observed in 15 samples (30.61%). The highest occurrence was observed in soil and sea food with 5 (33.33%) each, while stool and waste water samples had occurrence of 3 (20.00%) and 2 (13.33%), respectively (Table 4.1).

However, all the isolates showed the characteristics circular, grey-white, creamy colony with elevation ranging from flat to raised, and all 15 isolates were gram positive rod (Table 4.2).

All the isolates were motile, catalase negative, salicin positive, glucose positive, 14 were urease positive while 1 was urease negative, 10 were indole negative while 5 were indole positive,14 were lactose positive while 1 was lactose negative, 12 were H_2s positive while 3 were H_2s negative, 13 were lecithinase positive while 2 were lecithinase negative, the table also shows that all isolates, were identified under anaerobic conditions (Table 4.3).

All the *Clostridium difficile* were 66.7% susceptible to Vancomycin, 53% were susceptible to Metronidazole, 73% of the isolates were susceptible to Chloramphenicol, Tetracycline, Ampicillin, 67% of the isolates were also susceptible to Ciprofloxacin and Erythromycin and all the isolates were 47% susceptible to Gentamicin (Table 4.4).

Stool samples had the highest resistance of 95.80% and Wastewater had the lowest resistance of 6.25% to all the antibiotics used (Table 4.5).

Antibiotic resistance phenotypes of the *Clostridium difficile* isolate which shows the number of isolates, the source of isolates and their percentage with a total of 103% antibiotic phenotype

resistance. Gentamicin had high phenotypic resistance. The table also shows two stool (B & C) samples and one sea food (E) samples were resistance to all the antibiotics with 27% (Table 4.6).

Highest resistance to Erythromycin (100%), Gentamicin (100%), Ciprofloxacin (100%), Tetracycline (100%), Vancomycin (100%), Metronidazole (100%), Chloramphenicol (100%) while the lowest resistance to Ampicillin (20%), Ciprofloxacin (20%), and Metronidazole (20%) was recorded from soil samples (Table 4.7).

Table 4.1: THE OCCURRENCE OF *CLOSTRIDIUM DIFFICILE* IN THE SAMPLES

Sample	Number of Samples (%)	Occurrence of *Clostridium difficile* (%)
Stool	21(42.86)	3(14.28)
Sea Food	5(10.20)	5(100)
Waste Water	7(14.29)	2(29)
Soil	16(32.65)	5(31.25)
Total	49(100)	15(30.61)

Table 4.2: **THE MORPHOLOGICAL CHARACTERISTICS OF THE ISOLATES**

Sample code	Form	Color	Elevation	Edge	Optical characteristics	Texture surface	Smell
A	Circular	Grey-white	Flat	Entire	Opaque	Creamy	Foul
B	Circular	Grey-white	Flat	Entire	Opaque	Creamy	Foul
C	Circular	Grey-white	Raised	Entire	Opaque	Creamy	Foul
D	Circular	Grey-white	Flat	Undulate	Opaque	Creamy	Foul
E	Circular	Grey-white	Flat	Undulate	Opaque	Creamy	Foul
F	Circular	Grey-white	Raised	Entire	Opaque	Creamy	Foul
G	Circular	Grey-white	Flat	Undulate	Opaque	Creamy	Foul
H	Circular	Grey-white	Raised	Undulate	Opaque	Creamy	Foul
I	Circular	Grey-white	Raised	Undulate	Opaque	Creamy	Foul
J	Circular	Grey-white	Flat	Entire	Opaque	Creamy	Foul
K	Circular	Grey-white	Raised	Undulate	Opaque	Creamy	Foul
L	Circular	Grey-white	Raised	Entire	Opaque	Creamy	Foul
M	Circular	Grey-white	Flat	Undulate	Opaque	Creamy	Foul
N	Circular	Grey-white	Raised	Undulate	Opaque	Creamy	Foul
O	Circular	Grey-white	Raised	Entire	Opaque	Creamy	Foul

Table 4.3: BIOCHEMICAL CHARACTERISTICS OF THE ISOLATES

Isolates origin	Sample code	Catalase	Sucrose	Gram reaction	Urease	Glucose	Lactose	Motility	Indole	Hydrogen sulphide	Salicin	Lecithinase	Lipase	Gelatinase	Caseinase	Spore formation	Cell shape	Growth under aerobic	Probable identity
Stool	A	_	_	+	+	+	+	+	_	+	+	+	_	+	_	S.t	R	_	*C. difficile*
Stool	B	_	+	+	+	+	+	+	_	_	+	_	_	+	_	S.t	R	_	*C. difficile*
Stool	C	_	_	+	+	+	+	+	_	+	+	_	_	+	_	S.t	R	_	*C. difficile*
Crab	D	_	+	+	+	+	+	+	_	+	+	+	+	+	_	S.t	R	_	*C. difficile*
Crab	E	_	+	+	+	+	+	+	_	_	+	_	_	+	_	S.t	R	_	*C. difficile*
Crab	F	_	+	+	+	+	+	+	_	_	+	_	_	+	_	S.t	R	_	*C. difficile*
Crab	G	_	+	+	+	+	+	+	_	+	+	_	+	+	_	S.t	R	_	*C. difficile*
Crayfish	H	_	+	+	+	+	+	+	_	+	+	_	+	+	+	S.t	R	_	*C. difficile*
Waste water	I	_	+	+	+	+	+	+	+	+	+	_	_	+	_	S.t	R	_	*C. difficile*
Waste water	J	_	+	+	_	+	+	+	+	+	+	_	_	+	_	S.t	R	_	*C. difficile*
Soil	K	_	+	+	+	+	+	+	+	+	+	_	_	+	+	S.t	R	_	*C. difficile*
Soil	L	_	+	+	+	+	+	+	+	+	+	_	_	+	_	S.t	R	_	*C. difficile*
Soil	M	_	+	+	+	+	+	+	+	+	+	_	_	+	_	S.t	R	_	*C. difficile*
Soil	N	_	+	+	+	+	+	+	+	+	+	_	_	+	_	S.t	R	_	*C. difficile*
Soil	O	_	+	+	+	+	_	+	+	+	+	_	_	+	_	S.t	R	_	*C. difficile*

KEY: POSITIVE +, NEGATIVE _, S.t SUB TERMINAL, R- ROD

Table 4.4: ANTIBIOTIC SUSCEPTIBILITY OF *CLOSTRIDIUM DIFFICILE* IN THE STUDY

ANTIBIOTICS	RESISTANT	SUSCEPTIBLE
VANCOMYCIN	5(33.3)	10(66.7)
METRONIDAZOLE	7(47)	8(53)
CHLORAMPHENICOL	4(27)	11(73)
GENTAMICIN	8(53)	7(47)
CIPROFLOXACIN	5(33)	10(67)
ERYTHROMYCIN	5(33)	10(67)
TETRACYCLINE	4(27)	11(73)
AMPICILLIN	4(27)	11(73)

Table 4.5: PERCENTAGE SUSCEPTIBILITY AND RESISTANCE OF ISOLATES USED IN THE STUDY

Isolates origin	No. Of samples	% resistant	% susceptible
Stool	3	23(95.80)	1(4.16)
Sea food	5	14(35)	26(65.5)
Waste water	2	1(6.25)	15(93.75)
Soil	5	4(10)	36(90)

Table 4.6: ANTIBIOTIC RESISTANCE PHENOTYPES OF *CLOSTRIDIUM DIFFICILE* ISOLATES

RESISTANCE PHENOTYPE	STOOL	WASTE WATER	SOIL	SEA FOOD	NO. OF ISOLATE	%
E	_	1	_	_	1	10
CN	_	_	_	2	2	18
AMP, CIP	__	_	1	_	1	10
CN, MTZ	_	_	1	1	2	18
CN, VA, MTZ	_	_	_	1	1	10
E, CN, CIP, TE, VA, MTZ, C	1	_	_	_	1	10
E, CN, AMP, CIP, TE, VA, MTZ, C	2	_	_	1	3	27
TOTAL					11	103%

KEY: E-ERYTHROMYCIN, CN-GENTAMICIN, AMP-AMPICILLIN VA-VANCOMYCIN, MTZ -METRONIDAZOLE, C-CHLORAMPHENICOL, TE-TETRACYCLINE, CIP-CIPROFLOXACIN

Table 4.7: PERCENTAGE RESISTANCE OF THE SAMPLES USED IN THE STUDY

No. of isolates	Samples	Erythromycin	Gentamicin	Ampicillin	Ciprofloxacin	Tetracycline	Vancomycin	Metronidazole	Chloramphenicol
3	Stool	3(100%)	3(100%)	2(66.7%)	3(100%)	3(100%)	3(100%)	3(100%)	3(100%)
5	Sea food	1(20%)	4(80%)	1(20%)	1(20%)	1(20%)	2(40%)	3(60%)	1(20%)
2	Waste water	1(50%)	0%	0%	0%	0%	0%	0%	0%
5	Soil	0%	0%	1(20%)	1(20%)	0%	0%	1(20%)	0%

CHAPTER FIVE

CONCLUSION DISCUSSION AND RECOMMENDATIONS

5.1 DISCUSSION

CDI is a potentially deadly infection that is growing more prevalent throughout the world. It is responsible for 10–20 percent of instances of antibiotic-associated diarrhea (AAD) and practically all cases of colitis that are linked with antibiotic therapy. In this investigation, the susceptibility profile of 15 different clinical isolates of *C. difficile* was analyzed in relation to 8 different antibiotics that are typically employed in the treatment of patients who are confined to a hospital. Within the scope of this study, *Clostridium difficile* was investigated.

In this study, the occurrence in stool samples were 3(14.28%) which is contrary to 79 (36.7%)reported by Janezic *et al*., 2016 which may be due to the low sample size used or a variation in the type of soil samples used. This study also shows an occurrence of 28.5% isolates from waste water samples which is in agreement with the previously reported *C. difficile* isolation rates (27%-100%) by Romano *et al*., 2012. A study by Gardner *et al*.,2011 showed that only 5.3% of *Clostridium difficile* isolates used were resistant to Metronidazole which is contrary to the result of this study.

High resistance to Metronidazole (47%) was seen in this study, which can be caused by the use of Metronidazole in the treatment of CDI without consideration which alters the ability of bacteria to deactivate the drug. Even though metronidazole resistance is gradually progressing, but the drug is still useful for treatment of *C. difficile*-associated diseases (Wullt and Odenholt. 2004). The resistance rate to erythromycin was 33.3% in this study was lower than those in China (85.3%), Scotland (94.8%) (Huang *et al*.,2009) and higher than those in Hungary (25%) and Sweden (13.8%) (Fang *et al*., 2010). The possible reasons for high resistance rate to Erythromycin maybe related to the use of Erythromycin in the treatment of *C. difficile*

infection, which may increase these isolates' exposure to new macrolide and ribosomal methylation (Huang *et al*.,2009).

5.2 CONCLUSION

In this study *Clostridium difficile* were isolated and their susceptibility to 8 antibiotics were observed and recorded. This study shows an increase of 31.25% in soil samples, in comparison with the reports carried out in Ohio-USA which had a low 6.5% prevalence rate (Tariq *et al.,* 2011), it is contrary to the report of a study carried out in Lagos, Nigeria which had 0% prevalence which was due to the geographical distribution (Adegboyega. 2014). This study confirms that *C. difficile* has become an emerging pathogen in Nigeria. It is crucial that healthcare professionals are made more aware of the transmission, management, treatment and prevention of *Clostridium difficile*. However, despite the fact that some medicines are effective and can be used to treat infections brought on by *C. difficile*, the restrictions of utilizing these antibiotics have become a major cause for concern because this disease is becoming increasingly resistant to them.

5.3 RECOMMENDATION

More research work should be carried out to determine the occurrence and antibiotic susceptibility on *Clostridium difficile* isolates to avoid more health complications especially in diarrheic cases.

In order to provide antimicrobial drugs and prevent *Clostridium difficile* infections, further research is required to understand how *Clostridium difficile* develops drug resistance, notably to Metronidazole.

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APPENDIX

APPENDIX I: ANTIBIOTICS SUSCEPBILITY TEST OF THE ISOLATES COMPARED WITH CLSI AND EUCAST STANDARD

Sample code	Erythromycin	Gentamicin	Ampicillin	Ciprofloxacin	Tetracycline	Vancomycin	Metronidazole	Chloramphenicol
A	R	R	S	R	R	R	R	R
B	R	R	R	R	R	R	R	R
C	R	R	R	R	R	R	R	R
D	S	R	S	S	S	S	S	S
E	R	R	R	R	R	R	R	R
F	S	R	S	S	S	S	R	S
G	S	R	S	S	S	S	S	S
H	S	R	S	S	S	R	R	S
I	R	S	S	S	S	S	S	S
J	S	S	S	S	S	S	S	S
K	S	S	R	R	S	S	S	S
L	S	S	S	S	S	S	S	S
M	S	S	S	S	S	S	S	S
N	S	S	S	S	S	S	S	S
O	S	R	S	S	S	S	R	S

KEY: R- RESISTANCE   S- SUSCEPTIBLE

APPENDIXII: ANTIBIOTICS SUSCEPBILITY TEST OF THE ISOLATES

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SAMPLE CODE	E	CN	AMP	CIP	TE	VA	MTZ	C
A	R	R	9	R	R	R	R	R
B	R	R	R	R	R	R	R	R
C	R	R	R	R	R	R	R	R
D	14	17	38	19	20	21	27	26
E	R	R	R	R	R	R	R	R
F	25.5	R	9.5	36.5	22	9	R	24
G	25	R	28	30	28	20	33	32
H	30	R	11	27	15	R	R	29.5
I	R	12	25	14	21	25	34	30
J	20	10	34	20	16	24	16	13
K	22	11	R	16	19	20	31	20
L	23	14	26	20	23	22	20	25
M	29	15	40	13	24	21	29	29
N	15	14	39	14	14	22	30	26
O	29	R	12	R	10.5	9	R	19.5