EPIDEMIOLOGY AND GENOMIC INVESTIGATION OF *CLOSTRIDIOIDES DIFFICILE* IN HOSPITALIZED PATIENTS WITH NOSOCOMIAL DIARRHOEA

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DECLARATION

I affirm that this thesis is my original work and has been submitted to the University of Nairobi for the degree of Doctor of philosophy in Medical Microbiology and has not been presented for a degree at any other university. All referred published materials by others have been acknowledged.

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PUBLICATIONS AND COMMUNICATIONS

Published articles

- High prevalence of multidrug-resistant *Clostridioides difficile* following extensive use of antimicrobials in hospitalized patients in Kenya. **Mutai WC**, Mureithi MW, Anzala O, Revathi G, Kullin B, Burugu M, Kyany'a C, Odoyo E, Otieno P and Musila L. Frontiers in Cellular and Infection Microbiology, 2021. doi: 10.3389/fcimb.2020.604986.
- Assessment of independent comorbidities and comorbidity measures in predicting healthcare facility-onset *Clostridioides difficile* infection in Kenya. **Mutai WC**, Mureithi M, Anzala O, Kullin B, Ofwete R, Kyany' A C, Odoyo E, Musila L, Revathi G. PLOS Global Public Health, 2022. doi.org/10.1371/journal.pgph.00000902.

Oral and Poster presentations

- Predominance of Toxin B-positive *Clostridium difficile* isolates associated with diarrhea in hospitalized patients in a referral hospital in Kenya. ASM Microbe 2019, June 20 – 24, 2019, San Francisco, CA, USA.
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Genome information

- The genomes were deposited in the DDBJ/ENA/GenBank as part of the Whole Genome Shotgun project under BioProject number PRJNA732612 (Appendix VII).

ACRONYMS AND ABBREVIATIONS

AAD	Antibiotic associated diarrhea
ACG	American College of Gastroenterology
ADP	Adenosine diphosphate
ARVs	Antiretrovirals
ASAs	Acid suppressive agents
BclA	Bacillus collagen-like protein of anthracis
BHI	Brain-heart infusion
CbpA	Collagen binding protein A
CCEY	Cycloserine-cefoxitin egg yolk agar
CCI	Charlson Comorbidity Index
CCNA	cell cytotoxicity neutralization assay
CDAD	*Clostridium difficile associated disease*
CDC	Center for Disease Control and Prevention
CDI	*Clostridium difficile* infections
C-di-GMP	Cyclic diguanylate
CDRN	*Clostridium difficile* Ribotype Network
CDT	*Clostridium difficile* transferase
CE-PCR	Capillary gel electrophoresis
CgMLST	Core genome multilocus sequence typing
CI	Confidence Interval
CKD	Chronic kidney disease
CLSI	Clinical and Laboratory Standards Institute
CROPS	Combined repetitive oligopeptides
CRS	Cumulative resistance score
CSPG4	Chondroitin sulfate proteoglycan 4
Cwp	Cell wall protein
ECDIS-Net	European *Clostridium difficile* Infection Surveillance Network
ECI	Elixhauser Comorbidity Index
ECOFF	Epidemiological cut-off value
EIA	Enzyme immunoassay

eMLVA	Extended Multiple-locus variable-number tandem-repeat analysis
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
EUCAST	European Committee on Antimicrobial Susceptibility Testing
Fbp	Fibronectin binding protein
FDA	Food and Drug Administration
FMT	Faecal microbiota transplantation
GDH	Glutamate dehydrogenase
GERD	Gastroesophageal reflux disease
GTP	Guanosine triphosphate
H2	Histamine 2
HBC	Hyperimmune bovine colostrum
HCWs	Health care workers
HIV/AIDs	Human immunodeficiency virus/ Acquired immunodeficiency syndrome
HMW	High Molecular Weight Proteins
HO-CDI	Healthcare facility-onset *Clostridium difficile* infection
HSP	Heat shock protein
IBD	Inflammatory bowel disease
ICD-10-CM	International Classification of Diseases, Tenth Revision, Clinical Modification
IDSA	Infectious Diseases Society of America
Insp6	Inositol hexakisphosphate
ISR	Intergenic spacer region
ITS	Internal transcribed spacers
KNH	Kenyatta National Hospital
KNH-UON ERC	Kenyatta National Hospital-University of Nairobi Ethics and Research Committee
LMW	Low molecular weight protein
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry
MICs	Minimum inhibitory concentrations
MLST	Multilocus sequence typing
MLVA	Multiple-locus variable-number tandem-repeat analysis

mMLVA	Modified Multiple-locus variable-number tandem-repeat analysis
MRSA	Methicillin-resistant *Staphylococcus aureus*
NAAT	Nucleic acid amplification tests
NAD	Nicotinamide adenine dinucleotide
NGT	Nasogastric feeding
NOX	NADPH oxidase
OR	Odds ratio
PaLoc	Pathogenicity locus
PFGE	Pulsed-field gel electrophoresis
PMC	Pseudomembranous colitis
PPI	Proton pump inhibitors
PVRL3	Poliovirus receptor-like 3
QRDR	Quinolone resistance-determining regions
RBD2	Receptor-binding domain
RCT	Randomized control trials
REA	Restriction endonuclease analysis
RFLP	Restriction fragment length polymorphism
ROS	Reactive oxygen species
SHEA	Society for Healthcare Epidemiology of America
SLP	Surface layer protein
SNVs	Single nucleotide variants
SSA	Sub-Saharan Africa
ST	Sequence types
TB	Tuberculosis
TC	Toxigenic culture
TcdA	Toxin A
TcdB	Toxin B
UDP	Uridine diphosphate
WGS	Whole genome sequencing
WHO	World Health Organization
WSES	World Society of Emergency Surgery

ABSTRACT

Introduction: *Clostridioides difficile* is associated with hospital-acquired diarrhoea with underreported disease burden from African countries. This study aimed to ascertain the prevalence and the predictors of healthcare facility-onset *C. difficile* infection (HO-CDI) in symptomatic hospitalized patients admitted at Kenyatta National Hospital (KNH), as well as to characterize the toxin variants, antibiotic resistance determinants, sequence types, and evolutionary strains of the isolates associated with HO-CDI.

Methods: A cross-sectional study was conducted in 333 hospitalized patients with hospital-onset diarrhoea at KNH. Patients' demographic, admission, and clinical information were extracted from their medical records. Stool samples from study participants who gave their consent were tested for *C. difficile* using anaerobic culture-based methods in selective media. HO-CDI cases were confirmed by a positive real-time PCR assay for *tpi* gene along with one or more toxin genes (*tcdA, tcdB,* or *cdtA/cdtB*). E-test strips were used to detect the susceptibility of confirmed isolates to a panel of antibiotics, including vancomycin, metronidazole, rifampicin, ciprofloxacin, tetracycline, clindamycin, erythromycin, and ceftriaxone. Logistic regression was used to examine potential risk predictors in cases of confirmed HO-CDI. The genetic relatedness of selected isolates was determined using multi-locus sequence typing (MLST). The Oxford nanopore MinION technology was used to sequence the entire genome of nine *C. difficile* strains. PubMLST and MLST (v2.0) were used to perform multilocus sequencing typing on the generated genomes. Various databases, including card, vfdb core, plasmidfinder, resfinder, and virulencefinder, were utilised to detect virulence factors, antimicrobial resistance genes, toxin coding genes, and plasmids replicons in the generated genomes. Phylogeny and metadata overlay were carried out using Phandago to determine the degree of genetic relatedness between the isolates. The sequences were aligned using Roary, and a maximum likelihood tree was constructed using RAXML (v0.9.0).

Results: *C. difficile* was found in 71 (21%) of the patients. One or more toxin genes were present in 69 (97.1%) of the 71 confirmed isolates. An incomplete *tcdA* gene was present in more than half of the toxigenic isolates. All isolates were sensitive to vancomycin, but three (2.1%) were resistant to metronidazole (MIC >32 mg/L). Resistance to rifampicin (65/71, 91.5%), erythromycin (63/71, 88.7%), ciprofloxacin (59/71, 83.1%), clindamycin (57/71, 80.3%), and ceftriaxone (36/71, 50.7.8%) was observed. Among the resistant isolates, 61 (85.9%) were multidrug-resistant.

Significant predictors in the multivariate logistic regression model included chronic obstructive pulmonary disease (odds ratio [OR], 9.51; 95% confidence interval [CI], 1.80-

50.10), diabetes (odds ratio [OR], 3.56; 95% CI, 1.11-11.384), chronic kidney disease (odds ratio [OR], 3.88; 95% CI, 1.57-9.62), iron deficiency anemia (OR, 3.67; 95% CI, 1.61–8.34) and hypertension (OR, 2.47; 95% CI, 1.00–6.07). CCI score of 2 (OR, 6.67; 95% CI, 2.07 – 21.48) and ECI scores of 1 (OR, 4.07; 95% CI, 1.72 – 9.65), 2 (OR, 2.86; 95% CI, 1.03 – 7.89), and 3 (OR, 4.87; 95% CI, 1.40 – 16.92) were significantly associated with increased odds of developing HO-CDI. In addition, age, antibiotic exposure, use of more than one antibiotic, surgical interventions and nasogastric feeding were significantly associated with increased odds of developing HO-CDI.

The analysis of the nine assembled genomes revealed that, with the exception of three genomes lacking resistance genes, the majority of isolates conferred antimicrobial resistance. Some of the antimicrobial resistance genes found in the six genomes included those for lincosamides (*erm(G*) and *erm(B*)), tetracycline (*tet(M*)), macrolides (*msr(C*), *msr(D*) and *msr(A*)), rifamycin *(RpoB)*, fluoroquinolone *(GyrA)*, and aminoglycosides (*ant(6') and aac(6'*)). RepUS43 plasmid was found in six isolates in the PlasmidFinder database. Four previously described sequence types were identified (ST37, ST743, ST40, and ST58), while two were novel. The phylogenetic inference analysis of *C. difficile* isolates' genomes revealed that they belonged to two distinct clades (clades 1 and 4). The Kenya sequences clustered with sequences from Indonesia, the United States of America, and Ghana.

Conclusion: *C. difficile* diarrhoea was identified in the hospitalized population, and the risk was higher for patients with prior exposure to antibiotics, invasive procedures, and co-morbidities. The presence of diverse sequence types and virulence genes among the few sequenced isolates provides novel insights into *C. difficile* isolates from this region, forming a basis for future studies using a larger population to investigate the genetic relationship of these isolates.

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CHAPTER ONE: INTRODUCTION

1.0 Introduction

Clostridium difficile, currently renamed *Clostridioides difficile* was initially identified in the mid-1930s as an endospore-forming bacterial agent that is quite difficult to isolate and was later associated with antibiotic-associated pseudomembranous colitis (Bartlett et al., 1978; Hall & O'toole, 1935; Lawson et al., 2016). *C. difficile* is present in 0-15% of the healthy human population, with significantly higher rates in infants and hospitalized individuals who serve as reservoirs for increased carriage and put vulnerable population at risk of contracting healthcare facility-onset *C. difficile* infection (HO-CDI) (Adlerberth et al., 2014; Furuya-Kanamori, Marquess, et al., 2015). Additionally, disruption of colonic flora following antibiotic use and other medications, such as proton pump inhibitors, allows for progression from asymptomatic colonization to disease due to the overgrowth of toxin-producing strains (Pérez-Cobas et al., 2014).

Because of the serious risks associated with antimicrobial overuse, the Centers for Disease Control and Prevention (CDC) has listed the bacterium as one of the five "urgent threats" that require immediate and decisive action (CDC, 2014). The majority of cases of antibiotic-associated diarrhoea are found in healthcare settings, where *C. difficile* has been identified as the primary cause. The infection is fecal orally transmitted and can cause symptoms ranging from uncomplicated diarrhoea to life-threatening pseudomembranous colitis and death (Eyre, Griffiths, et al., 2013; C. P. Kelly et al., 1994). Although antibiotic use is the main trigger of the infection, other reported risk factors that influence the development of *C. difficile* infection (CDI) include advanced age, immunity of the patient, long hospitalization duration, and underlying diseases.

Health care systems face a substantial financial burden from the global *C. difficile* disease burden of approximately 500,000 infections and 15,000 deaths annually due to the prevalence of prolonged hospital infections, re-hospitalization following recurrence, and the cost of laboratory tests and medication (CDC, 2014; Dubberke & Olsen, 2012; Lessa et al., 2015). In order to allocate sufficient resources to CDI diagnosis, treatment, and prevention efforts and to determine which treatment and prevention strategies are cost-effective, a comprehensive understanding of the impact of CDI on healthcare delivery is essential (Dubberke & Olsen, 2012).

Although the expression of multiple virulence factors is associated with *C. difficile* pathogenesis, the glycosylating toxins (A and B) are the most important in progression of disease. (Awad et al., 2014). These toxins inactivate the small GTPases in the intestinal epithelium resulting in increased fluid secretion and intestinal inflammation, both of which are indicators of *C. difficile* enterocolitis and pseudomembranous colitis (Chandrasekaran & Lacy, 2017). Between 17% and 23% of *C. difficile* strains produce a third ADP ribosylation toxin (binary toxin), however its function in pathogenesis is uncertain (Barbut et al., 2007; Martijn P. Bauer et al., 2011; Popoff et al., 1988).

Recent advances in *C. difficile* molecular epidemiology have been made possible by sequence-based genotypic approaches, such as multilocus sequence typing (MLST), multilocus variable-number tandem-repeat analysis (MLVA), and whole-genome sequencing (WGS). Even though the high costs of these assays limit their routine use, these methods provide unprecedented means of understanding the genetic traits and pathogen evolutionary relationships. While evolutionarily distinct clades of *C. difficile* continue to emerge, the clonal population structure of *C. difficile* in Africa remains largely unknown.

C. difficile is largely a neglected pathogen in Africa, and it is not regarded as a significant contributor of nosocomial diarrheal disease. As a result, the burden of CDI is underestimated, in part due to limited diagnostic capacity and laboratory resources coupled by a lack of awareness among clinicians and microbiologists. Therefore, failure to investigate this pathogen may result in 'silent' transmission underscoring the need for clinical or diagnostic interventions.

1.1 Study rationale and justification

Recent years have seen a dramatic rise in the incidence and morbidity of HO-CDI due to the spread of the hypervirulent strain ribotype 027. Antimicrobial use in humans and animals, other drug-prescribing practices, and non-compliance with infection prevention and control practices have all been linked to shifting patterns in *C. difficile* epidemiology. In addition, because it is technically difficult to isolate *C. difficile* from patient samples, many physicians may not request for its diagnosis and instead rely on syndromic treatment, leading to misdiagnosis and incorrect treatment. Further, the risk factors are under-investigated in resource-limited countries, contributing to the scarcity of data on *C. difficile* in healthcare facility-onset diarrhoea. Thus, the purpose of this study was to ascertain the prevalence of *C.*

difficile infection among a cohort of hospitalized population who presented with diarrhoea and to establish the predictors attributed to higher risk of developing HO-CDI. To address the disease potential pathogenicity, toxin genes were detected in positive phenotypic isolates, and whole-genome sequencing was performed to determine the distinct multilocus sequence types and antibiotic-resistant determinants of the isolates. Subsequently, the genetic traits of the sequenced *C. difficile* isolates from this study were compared to similar sequence types from different geographical locations using phylogenetic analysis to elucidate clonal clusters that will aid in inferring the evolutionary relationship or diversity of clustered lineages.

1.2 Research questions

a. What proportion of patients admitted at Kenyatta National Hospital (KNH) have HO-CDI?
b. Which risk factors during the hospitalization at KNH are the primary predictors of HO-CDI?
c. What are the toxin variants and antimicrobial susceptibility profiles of HO-CDI isolates?
d. What are the genetic determinants of antibiotic resistance in the strains studied, as well as the sequence types based on allelic polymorphisms of the housekeeping genes, and how do they compare to strains from other geographical areas?

1.3 Study objectives

1.3.1 General objective

To investigate the epidemiology and genomic characteristics of *C. difficile* in symptomatic hospitalized patients admitted at Kenyatta National Hospital (KNH)

1.3.2 Specific objectives

1. To determine the prevalence of HO-CDI in symptomatic patients admitted at KNH.
2. To characterize the toxin profile of *C. difficile* isolates recovered from the stool samples and evaluate their antimicrobial susceptibility patterns.
3. To assess the risk factors and stratify significant predictors associated with increased odds of developing HO-CDI.
4. To investigate the antibiotic-resistant determinants and genetic relatedness of the isolates and compare this to strains from various geographical locations.

1.4 Significance of the study

The outcomes of this study will provide insight into the burden of *C. difficile* in Kenyan healthcare facilities and will be used to enhance HO-CDI awareness among healthcare

providers. Due to the lack of well-established anaerobic laboratory facilities to aid in *C. difficile* diagnosis, the findings from this study will highlight the importance of such facilities in strengthening anaerobic pathogen surveillance. The data on risk factors will aid in stratification of patients with significant predictors, allowing the design of prevention strategies and targeted treatment at an early stage of HO-CDI diagnosis. Consequently, this will have a substantial impact on the refinement of hospital infection control guidelines, particularly the antimicrobial use policies in healthcare settings. Furthermore, the identification of distinct virulent strains linked to multidrug resistance, significant outbreaks, and severe infections will compel the recognition of CDI as a notifiable disease in Kenya.

1.5 Thesis structure

This thesis is divided into six chapters. The first chapter provides an overview of the research project, including its context, rationale and justification, as well as the study objectives and benefits. The second chapter delves extensively into various emerging issues surrounding *C. difficile*, including facts, controversies, and gaps identified in numerous studies on the pathogen. The third chapter details step-by-step procedures tailored to the study objectives, which are expanded upon in chapters 4-6, where the study findings are discussed in detail in relation to each themed objective and in the context of the available literature. The thesis then concludes with a discussion of the study limitations, a summary of the findings, and suggestions for additional research in the same field.

CHAPTER TWO: LITERATURE REVIEW

2.1 History and microbiology of *Clostridioides difficile*

Hall and O'Toole isolated *Clostridioides difficile* from neonatal meconium in 1935 and named it *Bacillus difficilis* due to challenges in propagating and isolating the bacteria (Hall & O'toole, 1935). Before it was linked to antibiotic-associated pseudomembranous colitis in 1978, its role in human infections was unknown (Bartlett et al., 1978). *C. difficile* is found in 0 to 15% of the healthy population; however, rates are higher in hospitalized patients and infants. For decades, the gut of a fetus was thought to be sterile until birth, when colonization is initiated by commensals acquired from the mother and the surrounding environment (Hall & O'toole, 1934; Mackie et al., 1999). However, studies have demonstrated that meconium obtained from neonates contains a diverse bacterial community, implying that colonization occurred prior to birth (Collado et al., 2016; Jiménez et al., 2008; Nagpal et al., 2016). In 1935, Hall and O'Toole reported that approximately 37% of the microorganisms found in neonatal meconium were acquired from the mother (Hall & O'toole, 1935). Additionally, the same study identified *C. difficile* as a common microflora in the gastrointestinal tract of healthy infants. Infants carry both toxigenic and non-toxigenic strains of *C. difficile*, but because they lack toxin receptors, they exhibit no disease symptoms. Colonization rates have been shown to be significantly higher in C-section delivered infants and formula-fed infants compared to breastfed infants (Pandey et al., 2012; Penders et al., 2005; Timmerman et al., 2017). As a result, infants may serve as *C. difficile* reservoirs, potentially infecting anyone who comes into contact with them (Adlerberth et al., 2014).

Clostridioides species are classified in the class Clostridia, the order Clostridiales, the family Clostridiaceae, and the genus Clostridioides. *C. difficile* is a member of clostridial cluster XI, along with closely related non-spore producing species *Peptostreptococcus anaerobius* and *Eubacterium tenue* (M. D. Collins et al., 1994). For decades, Clostridia was classified based on phenotypic characteristics, which caused confusion because spore-forming and non-spore-forming genera were grouped together, necessitating taxonomic revision of these genera. With recent advances in ribosomal protein phylogenetic analysis using 16S rRNA sequencing, it is apparent that the genus Clostridioides is heterogeneous. Consequently, *C. difficile* was reclassified to the family Peptostreptococcaceae and the genus Clostridioides (Galperin et al., 2016; Ludwig et al., 2015). Previously, (Yutin & Galperin, 2013) suggested renaming *C. difficile* to *Peptoclostridioides difficile*; however, (Lawson et al., 2016) recently proposed that the name be revised to *Clostridioides difficile* to avoid phonetics and

communication issues. Several databases, including the List of Prokaryotic Names withstanding in Nomenclature (Parte, 2014), the Ribosomal Database Project (Cole et al., 2014), and the National Center for Biotechnology Information Taxonomy database, have adopted the proposed classification (Federhen, 2012).

A gram-stained smear of *C. difficile* bacterium reveals a typical gram-positive straight rod with slightly rounded ends and an oval sub-terminal spore. The bacterium can exist in a vegetative form where it grows and divides, but when environmental conditions become unfavorable, it transforms into a dormant spore form. While in its spore form, the bacterium can survive adverse conditions such as high temperatures, ultraviolet light, disinfectants, an acidic environment, and antibiotics. In this form, it is well-suited for propagation in the human gastrointestinal tract, where it survives harsh conditions of the stomach and eventually go on to germinate and potentially produce toxins in the colon (Rineh et al., 2014). When released into the environment, the spore can withstand desiccation and high oxygen levels for a long time before reverting to its vegetative state when conditions are favorable. *C. difficile* is a strict anaerobe that moves in a tumbling motion via peritrichous flagella. In some strains, the flagellin proteins (flagellar cap [FliD] and flagellin [FliC]) produce glycan, which facilitates its attachment to the intestinal wall, thereby initiating the disease process (Baban et al., 2013; Tasteyre, Barc, et al., 2001; Twine et al., 2009).

According to the Sanger Institute's entire genome sequence of reference strain 630, C. difficile has a large complex genome composed of a circular chromosome aligned with 4,290,252 bp and a circular plasmid of 7,881 bp with a 27.9% G+C content (Sebaihia et al., 2006). Additionally, 11% of the genome is made up of mobile genetic elements, including putative conjugative transposons, mobilizable transposons, prophages, and many others that contribute to *C. difficile* virulence. Recent genomic sequencing of four clinical isolates from New York, USA revealed a single circular chromosome with a length of 4,075,361 to 4,190,038 base pairs (Yin et al., 2018). Figure 2.1 summarizes how the historical aspects of *C. difficile* have changed over time.

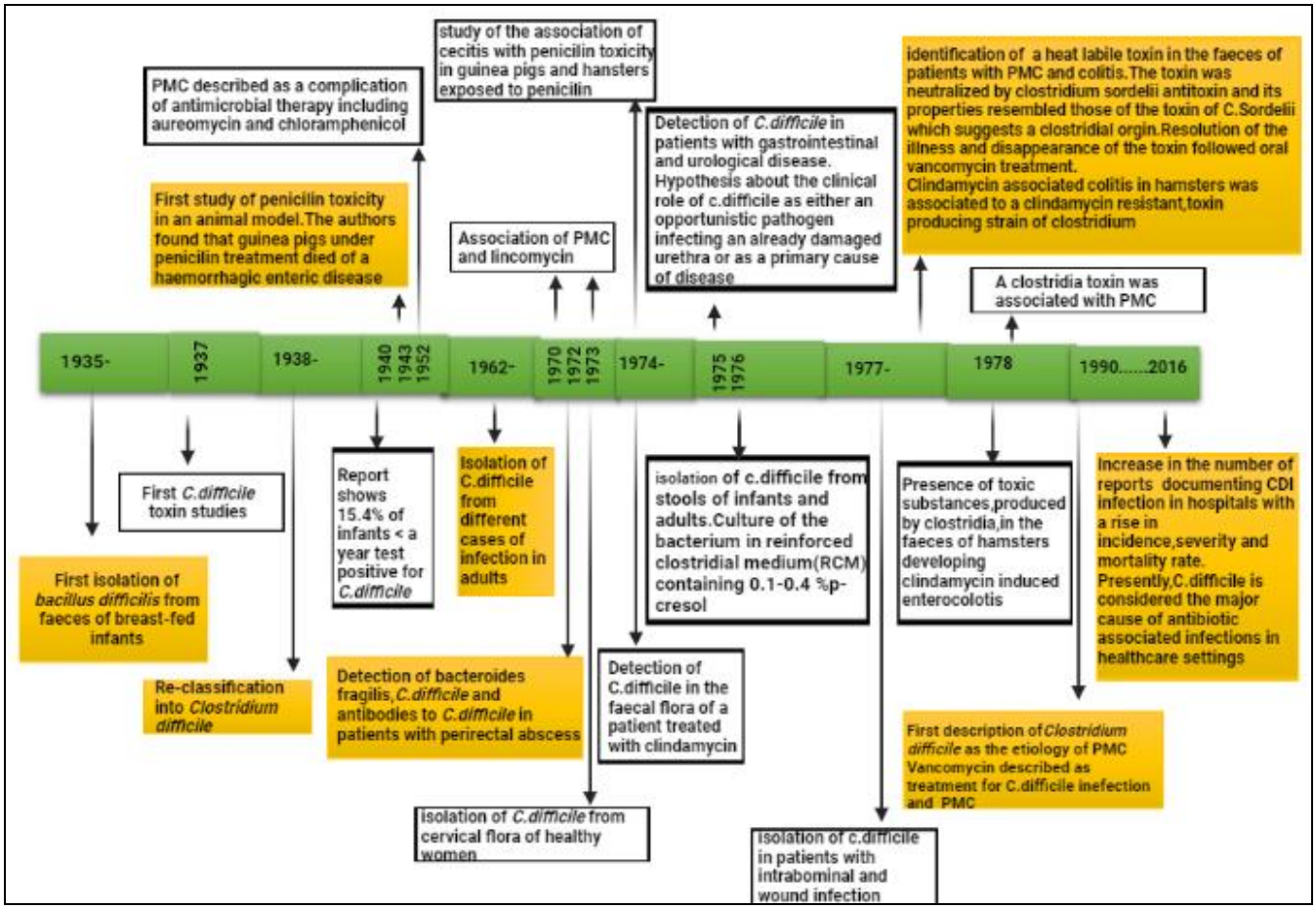


Figure 2.1: *C. difficile* historical events. The progression of *C. difficile* during the past 80 years, from its initial description in 1935 to its current epidemiological situation, is shown in this figure (Adapted from (Rodriguez et al., 2016).

2.2 Disease burden

Clostridioides difficile infection (CDI) rates and mortality have risen considerably in high- and upper-middle-income economies while remaining remarkably low in low- and middle-income countries. Multiple factors, including shifting population composition, expanded use of broad-spectrum antibiotics, and the emergence of hypervirulent *C. difficile* strains, have been implicated in the alarming increase in disease prevalence. In this section, the burden of CDI in developed countries as well as developing countries, with a particular emphasis on African countries, will be discussed. In addition, the direct impact of the disease on the health-care system in terms of expenses incurred in disease management will be evaluated.

2.2.1 Burden of CDI in high and upper-middle-income economies

Nosocomial infections caused by *C. difficile* are common in developed countries. Over the last decade, the United States, Canada, and Europe have all seen significant increase in the number of cases and deaths from CDI (Freeman et al., 2010). Despite the strengthening of

infection and control programs in these nations, the incidences continue to rise. According to recent studies, *C. difficile* is now the leading cause of healthcare-associated infections, surpassing methicillin-resistant *Staphylococcus aureus* (MRSA) (Miller et al., 2011). A study conducted in German hospitals revealed that the incidence density of CDI was more than two-fold higher than the incidence density of MRSA (0.47 vs 0.20) (Meyer et al., 2012).

In 2011 Lessa and colleagues conducted a population and laboratory survey in 34 states across the US. Based on this survey, they estimated 453,000 cases of CDI, with the majority being hospital-onset (107,600) versus community-onset (81,300) (Lessa et al., 2015). Using a decision-analytic model that consolidated various parameters such as age, comorbidities, facility setting and disease epidemiology, Desai et al., estimated an incidence of 439,237 primary cases which was slightly lower than the previous estimate by Lessa et al., (Desai et al., 2016; Lessa et al., 2015). Furthermore, death rates following severe CDI are high and have risen significantly in the United States. According to Redelings et al., the mortality rate in the US increased from 5.7 deaths per million population in 1999 to 23.7 deaths per million population in 2004 (Redelings et al., 2007). Similarly, Revel et al. also reported an increase in morality from 6.6% in 2001 to 7.2% in 2010 (Reveles et al., 2014). According to a survey conducted by the European *C. difficile* Infection Surveillance Network (ECDIS-Net) in 2011 through 2012, an estimated 123,997 patients acquired hospital-associated CDI. In this report, the incidence rate of primary cases ranged from 4.2 to 131.8 per 10,000 hospital discharges, while the incidence rate of the recurrent infection ranged from 0 to 118.6 per 10,000 hospital discharges (van Dorp et al., 2016). Using a prevalence-based burden of illness model, Levy et al estimated that there were 37,932 cases of CDI in Canada in 2012, the majority of which were hospital-onset cases (20,002) (Levy et al., 2015).

CDI data from Asian countries are scarce. According to a systematic review and meta-analysis of studies conducted in 16 Asian countries, *C. difficile* positive cases were reported in 14.8% of all patients tested (37,663), corresponding to an incidence rate of 5.3 per 10,000 patient days and a pooled death rate of 8.9% (Borren et al., 2017). According to this review, this may not be a true estimate due to significant variation in study setting (hospital/community), sample size and the study period; consequently, individual studies end up reporting more elevated cases than the pooled analysis. For instance, a study in China reported an incidence of 15.41 cases/100,000 persons in 2006, which increased to 36.31

cases/100,000 persons in 2014; 91.4% of the cases were healthcare-related and the overall mortality rate was 22.5% (Ho et al., 2017).

CDI results in prolonged hospital stay following primary diagnosis or as a result of recurrent infection. According to the data from Healthcare Cost and Utilization Project (HCUP), CDI accounts for 1% of the entire hospital stay, with primary diagnosis accounting for one-third of the total (Lucado et al., 2006). With such high incidence rates, most facilities are compelled to develop and implement infection control guidelines and programs in order to reduce CDI rates, which requires enormous additional resources, directly impacting the cost of health care systems. Additionally, the majority of patients endure the burden of recurring infections, which can be multiple, adding to the treatment and management costs associated with CDI. The estimated cost of managing recurring cases is discussed in Section 2.2.3.

2.2.2 The burden of CDI in Sub-Saharan Africa (SSA)

According to studies conducted in Africa, the prevalence of CDI is lower than in high and upper-middle-income economies, even though populations in these economies are exposed to similar risk factors (Forrester et al., 2017). CDI is rarely reported in most African countries, owing to the fact that the majority of laboratories in this region do not perform routine *C. difficile* diagnosis. However, given the pathogen's recent attention, a few countries are now diagnosing CDI using either a rapid test for glutamate dehydrogenase, geneXpert, or an enzyme immunoassay (EIA) for toxin A/B (Keeley et al., 2016). According to published data, *C. difficile* prevalence in Africa appears to range between 0% and 93.3%: Kenya 0% and 93.3 % (Mwachari et al., 1998; Oyaro et al., 2018), Zimbabwe 8.6 % (Simango & Uladi, 2014), Zambia (Nehanda et al., 2020), Ghana 4.9 % (Janssen et al., 2016), South Africa 16 % (Brian Kullin, Meggersee, D'Alton, Galvao, Rajabally, Whitelaw, Bamford, Reid, & Abratt, 2015), Tanzania (6.7 %) (M Seugendo et al., 2015), Malawi 13.6 % (Beadsworth et al., 2014), and Nigeria 43% (Onwueme et al., 2011). A summary of these studies is provided in Table 2.

Since antibiotic use has been shown to increase the risk of CDI, it is possible that the incidence rate of CDI is higher in developing countries where antibiotic use is unregulated and over-the-counter antibiotic purchase without a prescription is common (Bebell & Muiru, 2014). The uncontrolled antibiotic use intensifies the spread of *C. difficile*, enhancing the morbidity and mortality of infections in settings with a low prevalence. Two SSA studies found an association between antibiotic use and CDI development ($p=0.0001$) (Rajabally et al., 2013; M Seugendo et al., 2015).

The burden of human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) is greatly felt in Africa and is known to likely increase the development of other health conditions (Grabovac et al., 2020). In line with this, studies that have assessed CDI in HIV/AIDS patients have shown that CDI incidences are high among this population and in most cases accounts for the common cause of bacterial diarrhea (Anastasi & Capili, 2000; Imlay et al., 2016b; Sanchez et al., 2005). The link between CDI and HIV are likely related to modifications in the fecal microbiota, changes in the intestinal mucosa barrier function, and impairment of humoral and cell-mediated immunity (Di Bella et al., 2015). HIV/AIDS has been hypothesised to alter the mucosal layer of the gut and affect patients' humoral and cellular immunity, interfering with their body's ability to produce antibodies in response to *C. difficile* toxins and predisposing them to CDI. (Di Bella et al., 2015; Haines et al., 2013; Lorraine Kyne et al., 2000). The burden of CDI among HIV patients in Africa varies and according to a weight pooled systematic review a prevalence of 7.4% infection rate was reported among HIV patients (Forrester et al., 2017). A pilot study in Nigeria documented a CDI prevalence of 43.5% among HIV-positive in-patients and 14% among HIV-positive out-patients (Onwueme et al., 2011). In South Africa, a prevalence of 11.4% among HIV-positive patients was documented (Samie et al., 2008) which was slightly lower than that reported in Tanzania (12.7%) (M Seugendo et al., 2015). The study in Malawi found that patients with severe immunosuppression (CD4 count of <50) had a higher carriage (18.1%) of toxigenic strains of *C. difficile* than those with CD4 count of >50(7.9%), despite the fact that the association (0.058) was not statistically significant due to the low number of patients investigated. Interestingly with the high prevalence of CDI among HIV patients, most of these studies did not show an association except for the study in Nigeria (p=0.001) and Tanzania (p= 0.004) (Onwueme et al., 2011; M Seugendo et al., 2015). However, with the post-antiretroviral therapy, the CD4 count among this population increases leading to an improved immunological response to opportunistic infections. Additionally, these patients may not require antibiotics for prophylaxis therefore reducing the exposure to CDI (Sivapalasingam & Blaser, 2005).

Although data on the toxigenic strains and ribotypes is limited in studies conducted in Africa, it is essential to note that the toxigenic strains of *C. difficile* were highly isolated from diarrheal cases than from non-diarrhea cases. For example, in South Africa toxigenic strains were characterized from 92.4% of the *C. difficile* isolates (B. R. Kullin et al., 2018) while in

Tanzania 57% of *C. difficile* isolated from the study participants with symptoms of diarrhea were toxigenic (Mwanaisha Seugendo et al., 2018).

Table 2.1: Summary of published studies in SSA

Country	Year	Sample size/study design	Participants	Parameter	Associated risks	Test	Antibiotic resistance	Toxin profile	Ribotype	Reference
Tanzania	2014	Cross-sectional/ 250(141 diarrheal and 110 non-diarrheal)	All patients with diarrhea+ 109 controls	Prevalence (6.40%)	Hospital duration (p=0.036), HIV status (p=0.004)	Culture Rapid test for glutamate dehydrogenase PCR for toxin genes	Clarithromycin (3) Rifampicin (1)	A+B+CDT+ (2) A+B+ (2)	038 (3 nontoxigenic) 045 (2 toxigenic)	(Seugendo et al., 2015)
Malawi	2004-2005	Case-control/206	Adult inpatients with diarrhoea	Prevalence (13.6%) Association with diarrhea (22), HIV status (21) and immunosuppression (13)	None	ELISA for toxin detection	N/A	N/A	N/A	(Beadsworth MBJ, Keeley AJ, Roberts P, Watson A, 2014)
Nigeria		Pilot/140	140	Prevalence (43% for in-patients and 14%out-patients)	N/A	EIA for toxin detection	N/A	N/A	N/A	(Onwueme et al., 2011)
South Africa	November 2004-May 2005	Cross sectional/322(255 from hospital, 67 from schools)	all age, outpatient	prevalence, toxin profile, pathogenicity and association	Diarrhoea (p=0.001), occult blood- (p=0.001), lactoferrin>20yrs(p=0.041)	N/A	N/A	A+B+CDT− (9) A+B+CDT+ (9) A-B-CDT+ (3) A-B+CDT- (2)	N/A	(Samie et al., 2008)
South Africa	2015	Pilot/34	CD positive isolates	Ribotypes	Auto-aggregation, biofilm formation (014 p=0.001, 017 p=0.0019)	MLVA	Moxifloxacin (69%), erythromycin (74%)	A+B+	17,001,015,056	(Brian Kullin, Meggersee, D'Alton, Galvao, Rajabally, Whitelaw, Bamford, Reid, Abratt, Valerie RoseKullin, et al., 2015)

Zimbabwe	2014	Cross sectional/268	Outpatients >2yeras	Prevalence (8.6%)	N/A	Culture PCR for toxin genes	Clindamycin, ciprofloxacin, gentamicin, cefotaxime, cotrimoxazole		N/A	(Simango & Uladi, 2014)
Kenya	1998	prospective cross-sectional study 75	HIV positive adults with chronic diarrhoea 75	Prevalence-0%	*C. parvum* (p =0.007), EAggEC (p=0.007)		Cytotoxicity assay	N/A	N/A	(Mwachari et al., 1998)
Ghana	Nov2013-sep2014	Cross sectional	All ages 176 hospitalized with diarrhea 131 asymptomatic non-hospitalized	Prevalence (4.9%)	Age <5years (p=0.004), antibiotic use-ceftriaxone (p=0.023) current plasmodium infection(p=0.042) recent rashes (p<0.0001) age 0-5(p=0.004)		Erythromycin (46.6%) Ciprofloxacin (100%)	Toxigenic Nontoxigenic (75%) 3-A+B+ (toxinotype O) 1-A-B-CDT+ (toxinotype XIb)	084	(Janssen et al., 2016)
Zambia	2000	Cross sectional 68	HIV seropositive non diarrheal volunteers from community	Prevalence (0%)	N/A	N/A	N/A	N/A	Culture Toxin assay-ELISA	(I Zulu et al., n.d.)

2.2.3 Health care economic burden

CDI is among the most expensive infections to manage, and as such it places a significant economic burden on infected individuals and the health care systems (Heimann et al., 2018). Extensive hospitalization, re-hospitalization due to recurrence, and the cost of laboratory tests and therapy all contribute to the economic burden of CDI. (Cançado et al., 2021; Heimann et al., 2018). In 2013, CDC reported that approximately 250,000 CDI occur every year in the US this resulted in more than $1 billion in excess of medical costs per year and the cost was even higher following the emergence of hypervirulent strain (CDC, 2014). Between 2005 and 2015, Zhang et al. critically reviewed and analyzed 42 published studies that determined the impact of direct medical costs on CDI management in the US, where they. estimated that $6.3 billion was attributed to CDI (S. Zhang et al., 2016). This figure is significantly higher than the previous estimate of $4.8 billion reported by (Dubberke & Olsen, 2012), $3.2 billion (O'Brien et al., 2007), and 1.1 billion (L. Kyne et al., 2002). The cost could also escalate significantly in patients with co-morbidities. According to Ghantoji *et al*., (2010), a patient diagnosed with CDI with underlying inflammatory bowel diseases will incur approximately $22,873 in management costs (Ghantoji et al., 2010). Aside from the studies conducted in the US, other countries have also reported on the high costs attributed to CDI management: Korea $2.4–$15.8 million (H.-Y. Choi et al., 2015), Europe €3billion/year (Jones et al., 2013), Rome €14,023/patient/year (Asensio et al., 2015), Italy €3,270.52/ patient/year (Poli et al., 2015) and Canada $281 365 million (primary and recurrent infections) (Levy et al., 2015).

Recurrent CDI also contributes to the high cost of management of CDI for example in the US, the estimated cost of managing a recurrent CDI is $45,148 (Shah et al., 2016), and in Germany, the treatment cost is approximately €73,898 per patient (Heimann et al., 2015), resulting in a €464 million annual cost burden to the German healthcare system (Grube et al., 2015). Metronidazole and vancomycin are the first-line drugs recommended for the treatment of CDI. Although these antibiotics are less expensive, they are associated with a high recurrence rate, which raises the cost of managing recurrent infection, and the alternative drug fidaxomicin is equally expensive (Burton et al., 2017; Heimann et al., 2018).

An economic computational simulation model previously developed to determine the cost of CDI on the health care system, estimated that it would cost a hospital >$496 million per year to manage cases of CDI (McGlone et al., 2012). Although this model was developed to calculate the cost of hospital duration it can however be adopted to estimate the cost of other

parameters on the healthcare system including the cost of interventions aimed at decreasing the severity of CDI. However, another study in the US applied a decision-analytic model that factored in other parameters including primary and recurrent infections, natural history of the disease, economic and epidemiological outcomes where they estimated that approximately $5.4 billion was annually spent in managing both hospital and community-acquired CDI cases (Desai et al., 2016).

The cost variation in these studies may be due to the participants' different age groups, healthcare setting (hospital, community, or long-term facility), the study design, and the comparable parameters analyzed. However, it is evident from the highlighted trends that the cost of managing CDI is increasing, which may be attributed to the increasing severity of the disease as a result of the emergence of hypervirulent strains. In addition, based on the limitations from the earlier studies conducted on the cost-effectiveness of CDI, the recent analytical and modelling approaches have established some of the direct and indirect costs that could significantly impact the economic burden of CDI on the healthcare systems (Desai et al., 2016; S. Zhang et al., 2016).

2.3 *C. difficile* transmission, colonization and infection

This section provides an in-depth description of the events preceding CDI, beginning with how *C. difficile* establishes itself in the gut and progresses to symptomatic presentations of mild or severe disease, based on Durovic et al. model, which is depicted in figure 2.2 below (Durovic et al., 2018).

2.3.1 Transmission

C. difficile infection is transmitted faecal-orally through ingestion of spores or vegetative cells in contaminated hands or materials. Direct contact with symptomatic CDI patients is the primary transmission pathway, accounting for approximately 35% of patient to patient transmission of both primary and recurrent infections (Eyre, Cule, et al., 2013; Kumar et al., 2016). Asymptomatic colonization and the hospital environment, including inanimate objects/surfaces in both inpatient and outpatient settings, pose a risk to patients because they can serve as infection reservoirs (Jury et al., 2013; L. Y. Kong et al., 2019; Simecka et al., 2019). According to a mathematical model, the probability of contracting *C. difficile* in a hospitalized setting is 2.3% per day, compared to 0.12% in a community setting (Durham et al., 2016). However, use of whole-genome sequencing (WGS) to study disease transmission dynamics have revealed that both symptomatic adults and pediatric patients transmit low rates of CDI, implying the importance of other sources in disease transmission (Eyre, Cule, et

al., 2013; Kociolek, Gerding, et al., 2018). Further Eyre et al also reported that over 45% of *C. difficile* isolates analyzed in their study were genetically diverse, suggesting that other possible sources exist (Eyre, Cule, et al., 2013).

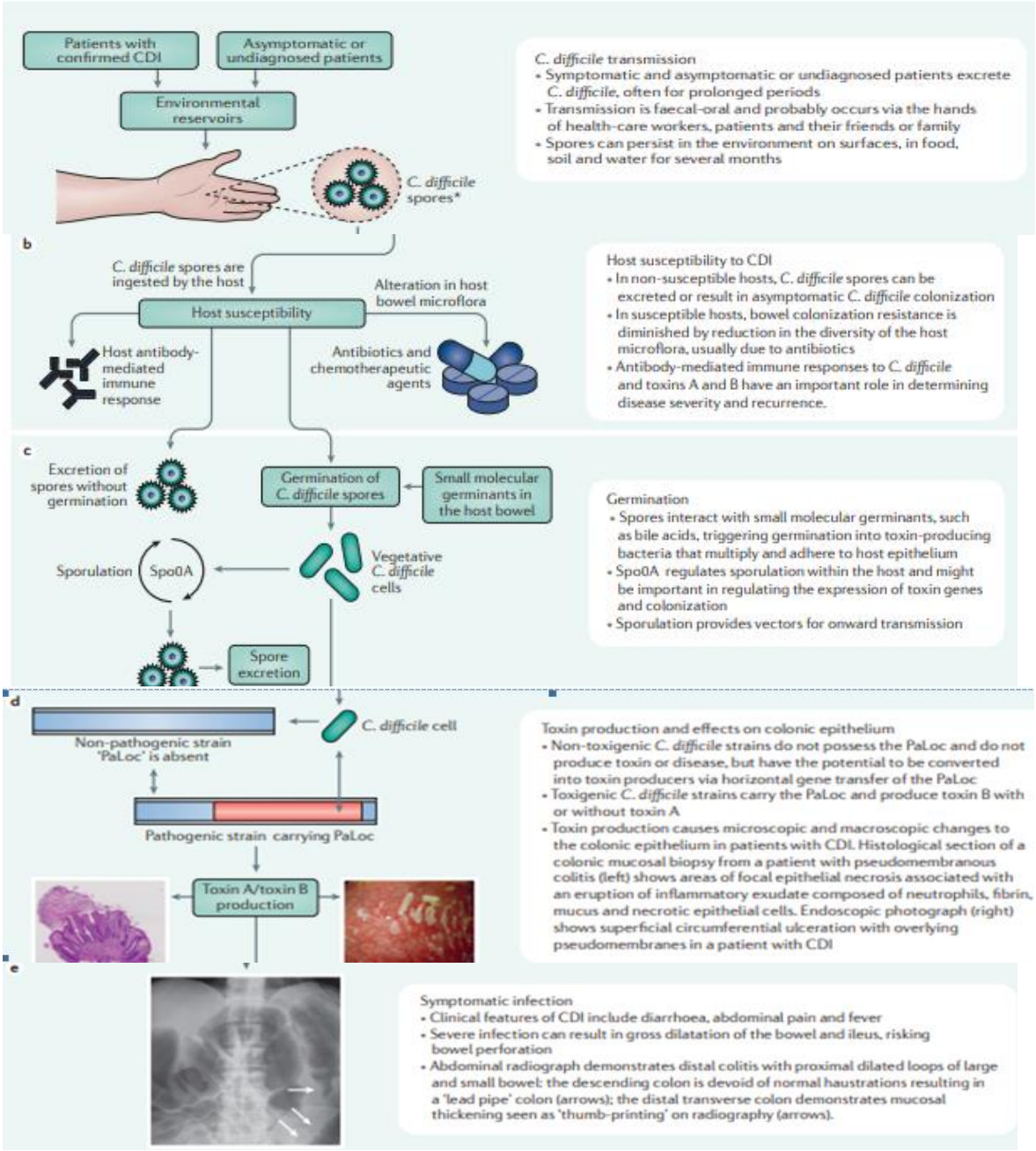


Figure 2.2: *C. difficile* infection. (a): This diagram depicts various ways in which *C. difficile* can be acquired. (b-c): The sequence of events that occurs after the spores are ingested, including factors that promote germination and excretion. (d-e): Pathological and clinical effects of toxigenic strains of *C. difficile*. Image adapted from (J. Martin et al., 2016).

Animal sources, contaminated food, community and natural environment have all been associated with community acquired CDI, providing supporting for the One Health paradigm (Alam et al., 2017; A. W. W. Brown & Wilson, 2018; Janezic et al., 2016; Knetsch et al.,

2018; Knight & Riley, 2019; Moono et al., 2017; Muñoz-Price et al., 2020; Rodriguez Diaz et al., 2018; Warriner et al., 2017). Evidence of zoonotic transmission has been found through the recovery of ribotype 078, which has been linked to sporadic outbreaks of CDI in humans (Abraham Goorhuis et al., 2008; Knetsch et al., 2018).

C. difficile spores were previously isolated from the air within a health facility. As a result, aerial dissemination of the spores may be to blame for the contamination of surfaces and the environment reported in hospitals (Best et al., 2010). Spores have also been isolated from the HCWs hands and hospital equipment (Landelle et al., 2014). Guerrero et al. recovered *C. difficile* in HCWs gloved hands after contact with environmental surfaces and CDI patients (Guerrero et al., 2012). Since the spores are resistant to the bactericidal and sporicidal effects of most disinfectants used in healthcare facilities, they can spread and survive in hospitals for up to 5 months (K. H. Kim et al., 1981; Uwamahoro et al., 2018). According to one study, the spores remained viable after being exposed to sodium dichloroisocyanurate at the recommended concentration and exposure time to kill the spores (Dyer et al., 2019). Furthermore, poor hand hygiene, including improper glove removal technique, contributes to contamination of not only the hands but also the skin of the HCWs (Tomas et al., 2015).

Antibiotic use indirectly alters the gut environment by increasing spore shedding into the environment, which increases the risk of acquisition to patients who will occupy the same environment in future. This was investigated by Freedberg et al who found that admission into a bed previously occupied by a patient who received antibiotics was strongly correlated with subsequent development of CDI (Freedberg et al., 2016). Additionally, Shaughnessy et al reported that household contamination, including vacuum cleaners and bathroom areas, due to inadequate cleaning, could also contribute to predisposition of primary or recurrent CDI (Shaughnessy et al., 2016).

2.3.2 Asymptomatic colonization

Asymptomatic colonization occurs when a stool sample tests positive for *C. difficile* or *C. difficile* toxins, but no symptoms of the disease are present (Furuya-Kanamori, Marquess, et al., 2015). Asymptomatic colonization varies between groups, with children under one year of age having a high colonization rate ranging from 1% to 84% (Al-Jumaili et al., 1984; Hall & O'toole, 1935; Clotilde Rousseau et al., 2012; Snyder, 1940). The high rate of colonization in this age group is influenced by absence of mucosal receptors due to immature bowel,

intestinal microbiota composition, and presence of immunoglobulins in the breast milk, which protects against *C. difficile* infection establishment (Cooperstock et al., 1983; Tullus et al., 1989). The rate of colonization after infancy however decreases with age from 15% to 5% by age 2 and above (E. A. Lees et al., 2016). Further, colonization rates in adults vary between healthy adults (4-15%), health care workers (4.2%) and hospitalized patients (3-21%) (M. J. T. T. Crobach et al., 2018; Miyajima et al., 2011; Säll et al., 2015; Elisabeth M. Terveer et al., 2017; Zacharioudakis et al., 2015). Prior hospitalization, exposure to antibiotics, underlying disease and corticosteroids use are all risk factors that facilitate colonization, as illustrated in figure 2.3 (Furuya-Kanamori, Marquess, et al., 2015; L. Y. Kong et al., 2015).

Given that colonized individuals can mount an immune response against CDI, colonization with toxigenic strains is however a prerequisite to infection; thus, colonization can progress to infection when combined with other underlying risk factors (Mulligan et al., 1993; Schäffler & Breitrück, 2018; Zacharioudakis et al., 2015). Blixt and colleagues recently demonstrated this in a large cohort study in Denmark, where they discovered that individuals colonized with toxigenic strains of *C. difficile* were five times more likely to develop infection than the non-colonized group (Blixt et al., 2017).

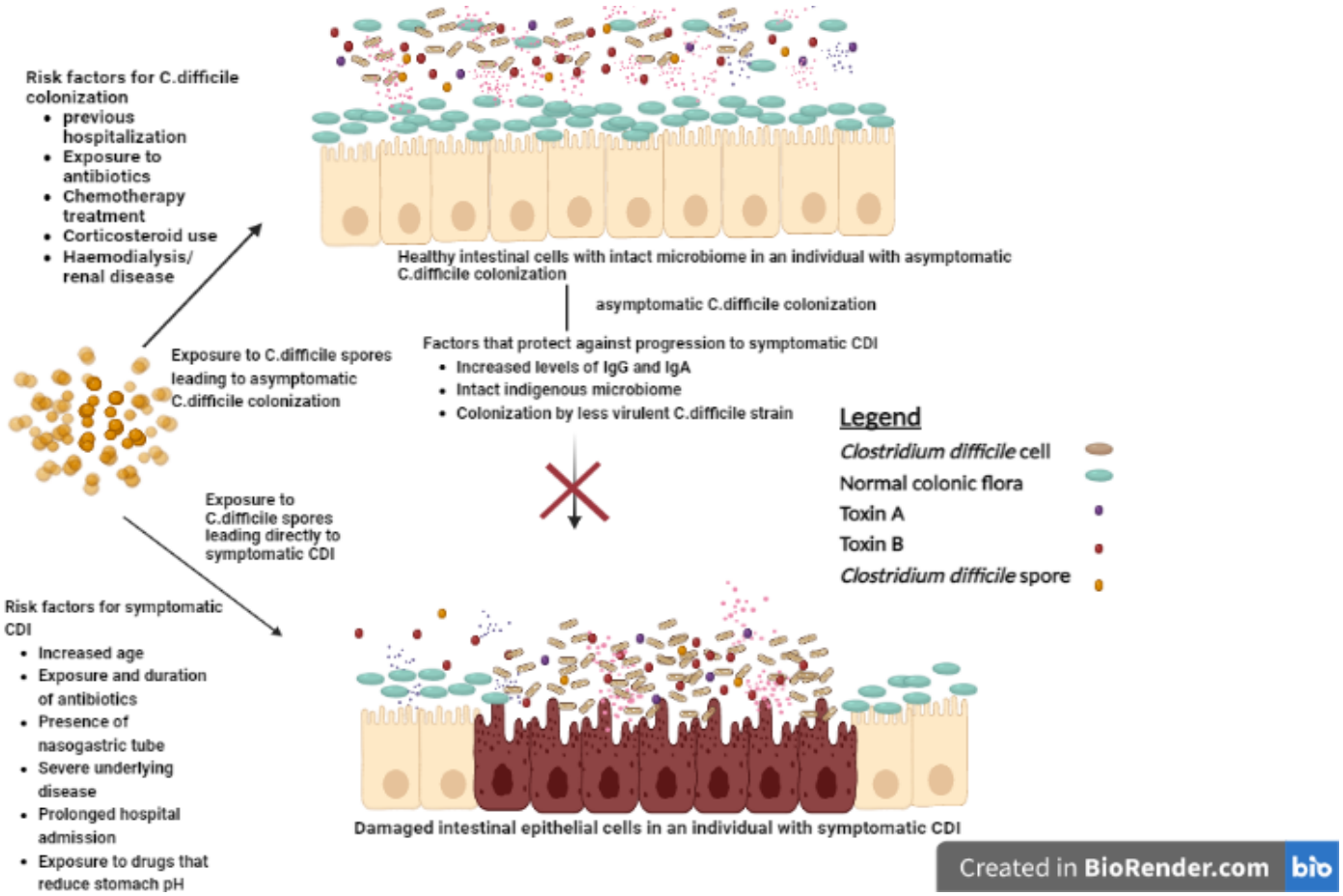


Figure 2.3: Asymptomatic colonization to infection. Schematic diagram adapted from (Furuya-Kanamori, Marquess, et al., 2015) showing the outcome of exposure to *C. difficile* spores. Highlighting the risk factors that promote asymptomatic colonization or symptomatic presentation. In some cases, the host and pathogen factors can work together to protect against CDI.

2.3.3 Infection

The CDI incubation period is yet to be ascertained; however, some studies have estimated a period of 2 to 3 days following exposure (Cohen et al., 2010). The disease begins with uncomplicated diarrhoea accompanied by fever, abdominal pain, and can progress to chronic diarrhoea, pseudomembranous colitis (PMC), fulminant colitis, and in rare cases, death (Bartlett, 2002; Dallal et al., 2002; C. P. Kelly et al., 1994). In pseudomembranous colitis, a pseudomembrane forms on the surface of the colon, characterized by yellow-white plaques which are classified as type 1, 2 or 3 based on their histological appearance (Price & Davies, 1977). In addition to the pseudomembranes, patients also present with profuse watery diarrhoea, abdominal distension, and leukocytosis (Gebhard et al., 1985). Although other broad-spectrum antibiotics have been identified, PMC is significantly associated with long-term clindamycin use (Tedesco et al., 1974). Although there are other causes of PMC, *C.*

difficile is responsible for approximately 90–99% of PMC cases (Farooq et al., 2015; C. Surawicz & McFarland, 1999).

Fulminant colitis and toxic megacolon with subsequent colonic perforation, peritonitis, and septic shock can complicate PMC cases that do not respond to treatment (Sartelli et al., 2019). Fulminant colitis affects approximately 3-8% of CDI patients, with a mortality rate ranging from 34% to 80% (Adams & Mercer, 2007; Dallal et al., 2002). Diarrhoea is absent in the majority of fulminant colitis cases, making diagnosis difficult. As a result, systemic signs such as fever, hypoalbuminemia, hypertension and renal failure as well as a computerized tomography **(**CT) scan and endoscopy are important when evaluating fulminant CDI (Adams & Mercer, 2007; Martijn P Bauer et al., 2012; Girotra et al., 2012). In-case of severe and complicated cases of CDI, surgical interventions such as abdominal colectomy or loop ileostomy can be performed (Juo et al., 2019). A case study also reported that a severe PMC patient was successfully managed with a combination of fecal microbiota therapy (FMT) and fidaxomicin (Konturek et al., 2017). Recurrence of CDI as a result of treatment failure is a common outcome reported in the majority of cases and is discussed further in section 2.6.1

2.4 Pathophysiology

C. difficile gut colonization is dictated by the anaerobic environment and the absence of competitor gut microbiome depleted during antibiotic treatment (Britton & Young, 2012; Ferreyra et al., 2014; Pérez-Cobas et al., 2014). Although toxin production is the primary virulence factor required for host tissue damage and disease manifestation, several events must occur prior to toxin production (Heinlen & Ballard, 2010). The first step is the ingestion of *C. difficile* spores where they germinate into metabolically active vegetative form in the small intestine. Following germination, vegetative *C. difficile* descends to the colon, and adheres to the intestinal epithelium using surface structures and adhesins. The interaction with the host tissue resulting in the production and release of toxin A (TcdA) and toxin B (TcdB), as well as a binary toxin called *C. difficile* transferase (CDT) in other strains (Awad et al., 2014; Voth & Ballard, 2005). Once in the colon, the toxins (TcdA and TcdB) primarily affect the intestinal epithelium, causing fluid secretion, inflammation, and tissue apoptosis/necrosis by inactivating small GTPases, whereas the binary toxin ADP - ribosylates actin, resulting in depolymerised actin cytoskeleton (Awad et al., 2014; Chandrasekaran & Lacy, 2017; Gerding et al., 2014). Toxin-induced pathophysiology results in increased adherence, cytopathic effects, and cytotoxicity. Furthermore, the virulence factors trigger an

inflammatory response that leads to neutrophil migration to the infection site, resulting in fluid accumulation, cell damage, and intestinal inflammation, all of which are indicators of *C. difficile* enterocolitis and pseudomembranous colitis (figure 2.4) (Cowardin et al., 2014; Jose & Madan, 2016; Madan et al., 2012; Péchiné & Collignon, 2016; Sun & Hirota, 2015). The mechanisms by which toxin and non-toxin mediated virulence factors contribute to *C. difficile* pathophysiology are discussed further below.

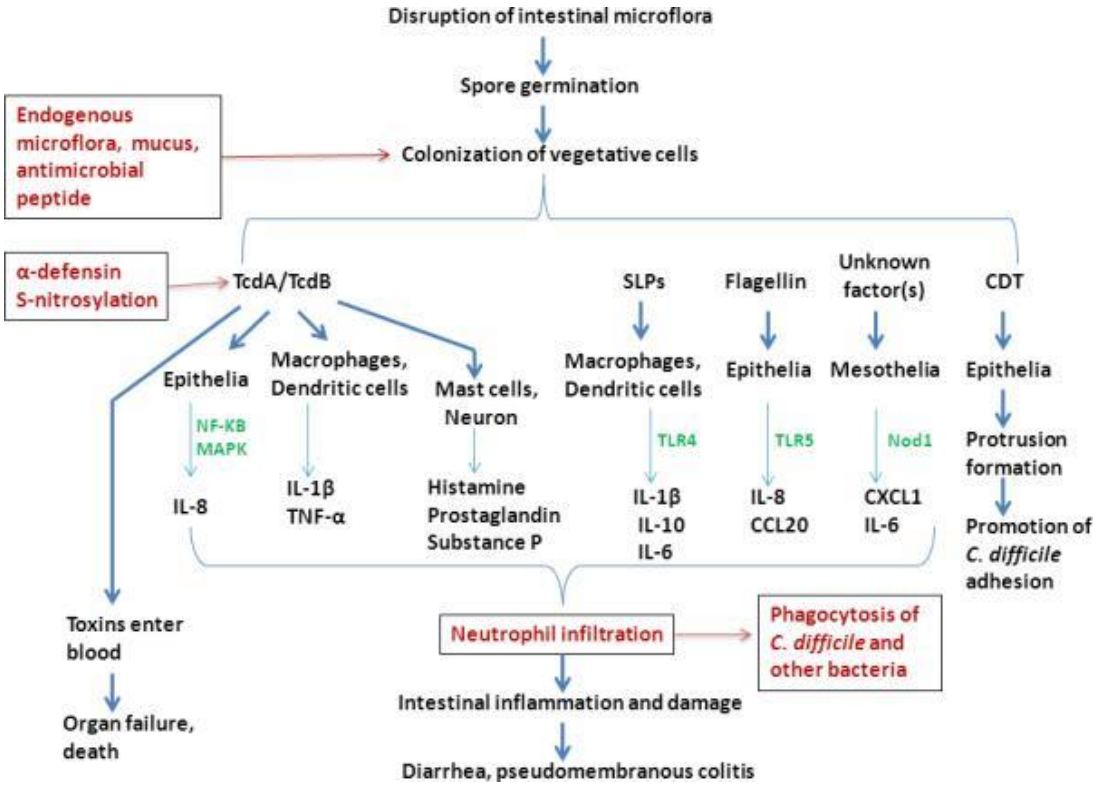


Figure 2.4: Infection cycle. *C. difficile* pathogenesis and host immune response triggered by toxins (TcdA, TcdB, and CDT) and non-toxin virulence factors (flagellin, surface layer proteins and unknown factors). Adapted from (Sun & Hirota, 2015).

2.4.1 Toxin mediated virulence factors

2.4.1.1 Toxin A and Toxin B

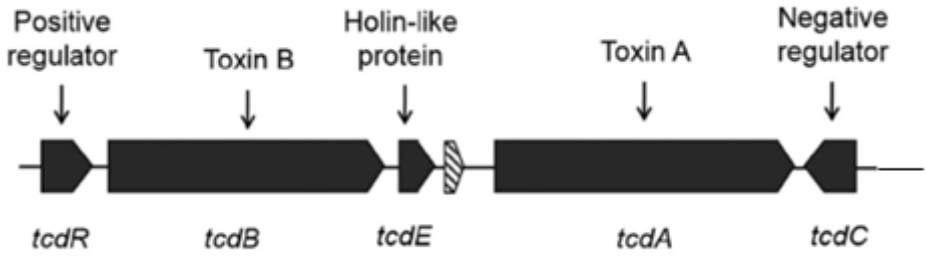
Toxin A (TcdA) and toxin B (TcdB) are both members of the large clostridial glucosylating toxins (LCTs) that play crucial role in the pathogenesis of *C. difficile*. The toxins are structurally similar in size and genetically closely related; TcdA measures 8kb while TcdB measures 7kb, and they both have approximately 63% amino acid relatedness (Christoph von Eichel-Streiber et al., 1992).

Enterotoxin A (TcdA) and cytotoxin B (TcdB) are located in a 19.6 kb region of the chromosome called the pathogenicity locus (PaLoc) (figure 2.5 A). Both toxins have similar

structure consisting of an enzymatic A subunit and a receptor binding B subunit. The A subunit is located at the N-terminal whereas the B subunit contains three domains: the receptor binding domain, the central delivery domain and the cysteine protease domain (figure 2.5 B) (Di Bella et al., 2016; Jank & Aktories, 2008; Manse & Baldwin, 2015; Pruitt et al., 2010).

Furthermore, the PaLoc encodes three other proteins, tcdR, tcdE and tcdC which are involved in toxin regulation and secretion. TcdC negatively regulates the transcription of *tcdA* and *tcdB* genes (Hundsberger et al., 1997; Matamouros et al., 2007), whereas TcdR is a positive regulator activating the transcription of these genes and stimulating self-expression (Mani & Dupuy, 2001; Moncrief et al., 1997). TcdE is a bacteriophage protein that mediates toxin secretion from the cell by forming pores on the cell membrane however, when this protein is released in high concentrations, it can cause cell lysis (Govind et al., 2012, 2015; Wee et al., 2001).

(A)



(B)

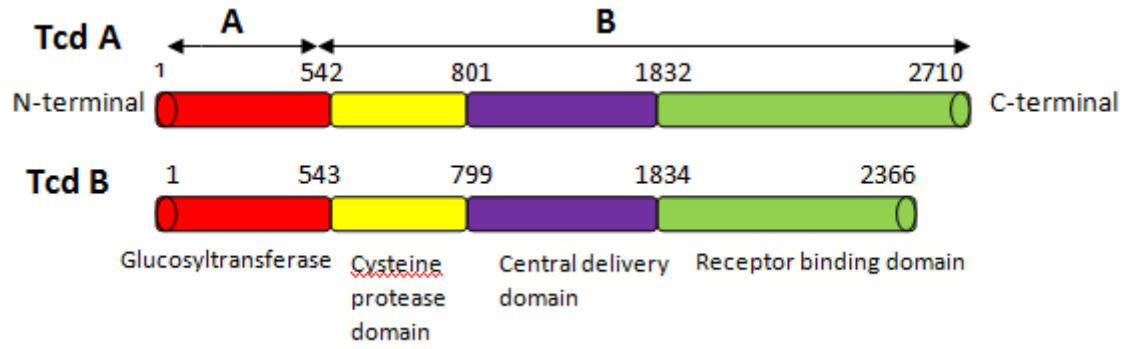


Figure 2.5: Structure of pathogenicity locus, TcdA and TcdB of *C. difficile*. (A) Organization of the pathogenicity locus (PaLoc). (B) The toxin (TcdA/TcdB) is organized into four structural domains (Jank & Aktories, 2008; Pruitt et al., 2010). Glucosyltranferase domain (red) located at N-teminal and is responsible for inactivation of small GTPases (Hofmann et al., 1997); the cysteine protease domain (yellow) which binds to inositol hexakisphosphate (Insp6) inducing autoproteolysis and release of the

enzymatic component of the toxin (Martina Egerer et al., 2007), Central delivery domain (purple) required for pore formation and translocation of the toxin to the cytosol (Genisyuerek et al., 2011); and the receptor binding domain (green) composed of combined repetitive oligopeptides (CROPS) is located at the C-terminal and is responsible for mediating binding of the toxin to the specific receptors on the host cell surface (C von Eichel-Streiber et al., 1992).

The non-toxigenic strains of *C. difficile* are devoid of the Paloc region and thus do not express either of the toxins (Fluit et al., 1991). Studies on the genomic sequences of non-toxigenic strains CD37 and 7322 revealed that the PaLoc region had been replaced by 115 bp element whose function is unknown (Braun et al., 1996; Brouwer et al., 2012). Mathis and colleagues demonstrated that the non-toxigenic strain M90 expressed the PaLoc gene but had low levels of tcdA, tcdB, and tcdE (Mathis et al., 1999). Another important group of strains are those of toxinotype XI, which do not produce toxin A and B but possess a paloc element that expresses *tcdA* gene as well as the binary toxin (Geric Stare & Rupnik, 2010). Previously, these strains were thought to be non-toxigenic because they were prevalent in asymptomatic cases. This strain has however been recovered from severe cases using more advanced diagnostic tools in the recent years, emphasizing their role in clinical disease (Geric et al., 2003, 2006). This occurrence can be explained by horizontal gene transfer of the *Paloc* gene from toxigenic strains to non-toxigenic strains, rendering the non-toxigenic strains pathogenic (K. E. Dingle et al., 2014). Brouwer et al., demonstrated this by showing that the PaLoc of *C. difficile* strain 630Δ*erm* was transferred by conjugative transposons via conjugation-like mechanism to the non-toxigenic strain CD37 (Brouwer et al., 2013; Mullany et al., 2015). Studies on the epidemic strain 027 have confirmed the existence of another locus *agr* locus (*agrACDB*), that regulates quorum signaling and, as a result, facilitates the synthesis of *C. difficile* toxins while also contributing to its colonization (Darkoh et al., 2016; M. J. Martin et al., 2013).

There has been a lot of conflicting information about the potency levels of toxins A and B. Previous research has shown that toxin B is more potent than toxin A, and as a result, strains producing toxin B (A^-B^+) are responsible for CDI clinical manifestations, as well as nosocomial outbreaks of the disease (Alfa et al., 2000; D Drudy et al., 2007; Kuijper et al., 2001; Sambol et al., 2000; Sato et al., 2004). This strain was found to either lack a complete *tcdA* gene or to possess a truncated portion of it (Janezic et al., 2015; Sambol et al., 2000; C von Eichel-Streiber et al., 1999). In-vivo experiments on animal models revealed that toxin A and not toxin B initiates the infection; however, if toxin A is present, toxin B may cause a characteristic symptom (Lyerly et al., 1985; Mitchell et al., 1986).

The availability of molecular techniques has enabled the genetic analysis of these two toxins concluding that *C. difficile* strains that produce both toxins or toxin B and/or the binary toxin, should be considered pathogenic (S A Kuehne et al., 2010; Sarah A Kuehne et al., 2014; L. Lemee et al., 2004).

2.4.1.2 Mode of action of toxin A and B

TcdA and TcdB are glucosyltransferases that inactivate the small GTPases proteins responsible for regulating cellular functions. These two toxins have an identical mode of action, which consists of the four stages as described in figure 2.6; binding and endocytosis, pore formation and tranlocation of glucosyltransferase domain (GTD) across the membrane, autoproteolysis and release of GTD to the cytosol and inactivation of the GTPases (Chandrasekaran & Lacy, 2017; Pruitt & Lacy, 2012).

In the presence of a susceptible host, CDI is initiated by binding of the combined repetitive oligopeptides (CROPs) located at the carboxyl-termini (C- termini) of the toxin to the specific glycoproteins or carbohydrates expressed on the outermost layer of the host colon epithelial cells (Frisch et al., 2003; Greco et al., 2006; Krivan et al., 1986; Olling et al., 2011; Tucker & Wilkins, 1991). It is important to note that receptor binding is not restricted to CROPS, as was noted by Lambert & Baldwin, where they identified a self-reliant receptor-binding domain (RBD2) in TcdA (residues 1361–1874) that works as a second binding region enhancing cellular toxicity (Lambert & Baldwin, 2016). In support of this, other studies have observed that a truncated toxin A ($TcdA_{1–1874}$) and Toxin B ($TcdB_{1501–1753}$ and $TcdB_{1–1550}$) devoid of CROPS possessed other receptor binding domains that could still bind to the host cell promoting cytotoxic effects (Genisyuerek et al., 2011; Olling et al., 2011).

Chondroitin sulfate proteoglycan 4 (CSPG4) expressed on human intestinal subepithelial myofibroblasts (Terada et al., 2006; Yuan et al., 2015), frizzled proteins and poliovirus receptor-like 3 (PVRL3), both displayed on the human colonic epithelial cells have so far been identified as human cell receptors for TcdB (LaFrance et al., 2015; Tao et al., 2016). TcdA on the other hand binds to plasma membrane protein gp96 (Na et al., 2008), carbohydrate blood antigens I, X, and Y expressed on human intestinal epithelial cells (Tucker & Wilkins, 1991), sucrase-isomaltase (SI), located in the ileal microvilli (Pothoulakis et al., 1996) and trisaccharide carbohydrate (Galα1-3Galβ1-4GlcNac) (Greco et al., 2006;

Krivan et al., 1986; Tucker & Wilkins, 1991). Once bound, the receptor binding complex then triggers internalization of the toxins through endocytic pathway. The acidic environment of the endosome causes pore formation in the host membrane, through which the toxins are translocated to the cytoplasm. This process is mediated by the central delivery domain of the toxin (Barth et al., 2001; Chumbler, Rutherford, et al., 2016; Z. Zhang et al., 2014). Cysteine protease domain of the toxin then cleaves to the inositol hexakisphosphate ($Insp_6$) facilitating autoproteolysis which results in the release of GTD, the enzymatic subunit of the toxin, to the cytosol of host target cell (M Egerer et al., 2009; Pruitt et al., 2009; Reineke et al., 2007). GTD catalyses the inactivation of small GTPases including Rho, Rac, cdc42, Rap, Ral and R-Ras. Using uridine diphosphate (UDP)-glucose as the co-substrate, GTD of the toxin can glycosylate the GTPases where active guanosine triphosphate (GTP) is hydrolysed to inactive guanosine diphosphate (GDP) by transferring a glucosyl group from UDP- glucose and attaching it to the threonine residue in the GTPase. This subsequently results in a modified theronine (Just, Selzer, von Eichel-Streiber, et al., 1995). Glycosylation of Rho GTPase prompts cytopathic and cytotoxic effects of the host cells (Chandrasekaran & Lacy, 2017; Chen et al., 2015).

Rho protein works by regulating cellular functions, including organization of actin filament, regulation of cytoskeleton, and permeability of tight junction (Nusrat et al., 1995). Their inactivation results in cytopathic effects including detachment of actin filaments, disruption of epithelial tight junction and increased cellular permeability all of which promote fluid accumulation in the bowel, which is secreted as characteristic watery diarrhea, a hallmark feature of CDI infection (Hecht et al., 1988; Just, Selzer, Wilm, et al., 1995).

Monoglucosylation of Rho GTPase activates the pro-apoptotic Rho proteins which induce cytotoxic effects or apoptosis on intoxicated colonic epithelial cells through caspase 9 and 3 regulated pathway (Gerhard et al., 2008; Hippenstiel et al., 2002), however caspase-independent apoptosis can occur in toxin B intoxicated cells though at a slower rate than the caspase-dependent pathway (Qa'Dan et al., 2002). This was previously demonstrated by Brito and colleagues where they hypothesized that the inactivation of Rho protein by toxin A-induced apoptosis on cell-cultured T84 cells (Brito et al., 2002). However, the different mechanisms of apoptosis produced by toxin A and B could be dependent on the concentration of the toxins, the varying host receptors that the toxins bind to, the substrate proteins and the strain variants (Chumbler, Farrow, et al., 2016; Huelsenbeck et al., 2007). Notably, TcdB

induces both apoptotic mechanism of cell death as well as necrotic cell death, however the switching from one mechanisms to another is also dependent on the concentration of the toxin such that at high concentration TcdB induces necrosis independent of glucosyltransferase inactivation while at low concentration it triggers apoptosis that is dependent on glucosyltransferase activity (Chumbler et al., 2012; Chumbler, Farrow, et al., 2016; Wohlan et al., 2014). Farrow and colleagues observed that TcdB causes cell necrosis by activating NADPH oxidase (NOX) which triggers the production of reactive oxygen species (ROS) including superoxide that reduces the supply of oxygen to cells consequently leading to ATP depletion and direct DNA damage (Farrow et al., 2013).

Following cytotoxicity effects, the host cell responds by releasing cytokines and chemokine mediators including IL-12, IL-1α, IL-1β, IL-6, IL-22, IL-17A, IL-16, MIP-1α, MIP-2, tumor necrosis factor-α, leptin, IFN-γ and CXC chemokine (Fang et al., 2014; Flegel et al., 1991; Madan et al., 2012; Mykoniatis et al., 2003; Solomon, 2013). Activating these inflammatory mediators stimulates the influx of neutrophils to the infection site, provoking the inflammation of the intestinal mucosa characterized by pseudomembranous colitis. The neutrophils can also opsonize the pathogen enhancing *C. difficile* clearance from the body (Jose & Madan, 2016). Some of these cytokines for instance, IL-2, IL-15, and IL-16 represent a hallmark of severe CDI (Cowardin et al., 2014; Yu et al., 2017) Further, when toxin gains entry into the lamina propria it activates macrophages, dendritic cells and mast cells into the host cells. Mast cell degranulation causes the release of inflammatory mediators like histamine, which increases mucosal permeability and ultimately causes fluid leak into the intestinal lumen and copious watery diarrhea (Solomon, 2013).

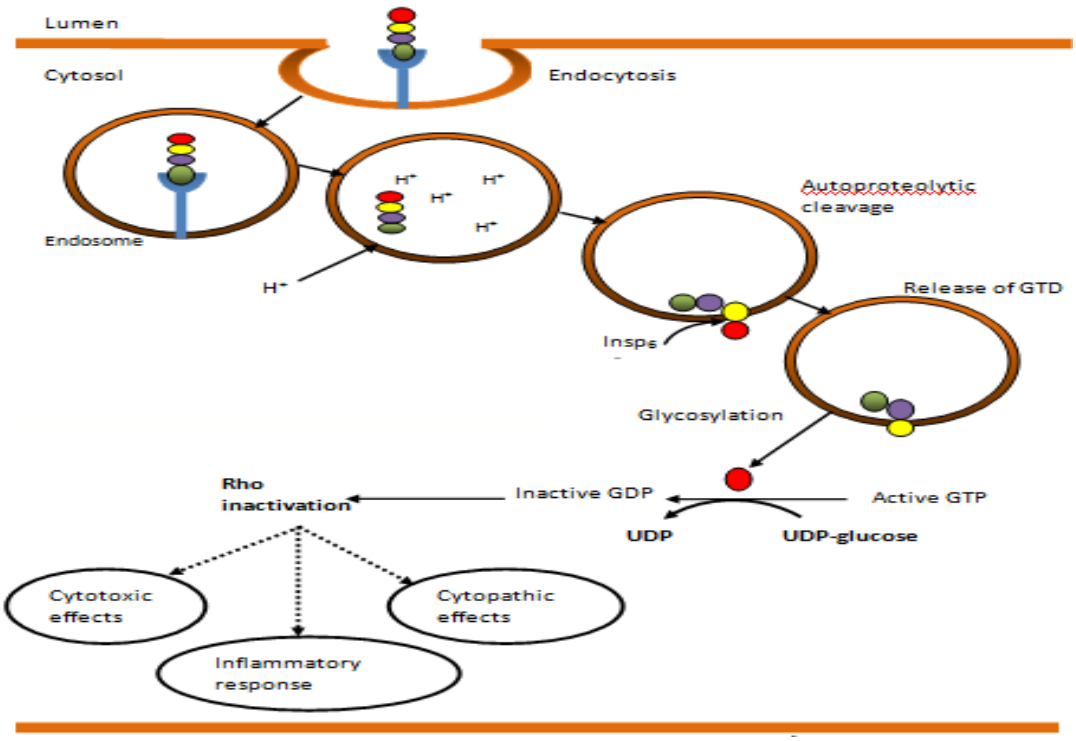


Figure 2.6: Mechanism of action of Toxin A/B. CROPS initiates host-pathogen interaction by binding to specific receptors present on the host cell. The toxin is then internalized to the cytosol through endocytosis. The low pH of the endosome triggers pore formation through which GTD is translocated. Inositol hexakisphosphate (Insp6) cleaves to the cysteine protease domain inducing autoproteolysis and release of the GTD. Once released, GTD then catalyses the modification of active GTP to inactive GDP, which inhibits cellular functions, resulting in cytotoxic and cytopathic effects. The host cell responds by stimulating the release of inflammatory mediators that triggers an inflammatory response. Refer to figure 2.5 (B) for the color codes of the toxin domain. Modified from (Awad et al., 2014).

2.4.1.3 Binary toxin

Popoff and coworkers first described binary toxin or *Clostridium difficile* transferase designated as CDT in the year 1987 from a *C. difficile* strain isolated from a patient diagnosed with pseudomembranous colitis (Popoff et al., 1988). Since the emergence of hypervirulent strains (BI/NAP1/027 and RT078), several studies have reported the presence of CDT together with toxin A and B in severe cases from hospital facilities and in community settings (Barbut et al., 2005). Previous work eluded that CDT cannot act alone unless in the presence of toxin A or B (Geric et al., 2006). Evidence from a study in hamsters reported high level of cytotoxicity in cells exposed to toxin A and CDT ($A^+B^-CDT^+$) and toxin B and CDT ($A^-B^+CDT^+$) as opposed to those that lacked toxins A and B ($A^-B^-CDT^+$) (Sarah A Kuehne et al., 2014). Studies in US, France and Australia have isolated strains of toxinotype type XI expressing CDT but negative for toxin A and B ($A^-B^-CDT^+$) from symptomatic and

asymptomatic patients, however the prevalence was low (2%, 0.5% and 4.7 % respectively) (Androga et al., 2015; Eckert et al., 2015; Geric et al., 2003; McGovern et al., 2017). $A^{-}B^{-}CDT^{+}$ strains have been described more in animals than in humans, suggesting possibility of zoonotic or foodborne transmission of this strain (Knight et al., 2013; Knight, Squire, et al., 2015; M. Rupnik, 2007; Schneeberg et al., 2013).

CDT belongs to the iota family of toxins known for ADP ribosylation and is produced by other pathogenic species of *Clostridioides* and Bacillus. CDT structurally composed of two components, CDTa and CDTb encoded by *cdtA* and *cdtB* genes respectively (Perelle et al., 1993; Weber et al., 2013). The genes are located on a 6.2 kb Cdt locus (cdtLoc) encoding a regulatory gene *cdtR*, which positively activates CDT production (Carter et al., 2007; Kaiser et al., 2011). CDTa component of the toxin induces enzymatic activity of the toxin while CDTb facilitates binding and transportation of the toxin to the host cell (Gülke et al., 2001).

The role of CDT in the pathogenesis of *C. difficile* is not well defined however, there is a growing evidence suggesting possible role in adherence and colonization of host cells (Schwan et al., 2009, 2014). The mode of action of CDT (illustrated in figure 2.7) is initiated by activation of the binding domain, CDTb by serine-type protease. Once activated the C-terminal (receptor binding domain) of CDTb binds to immunoglobulin(Ig)-like domain of lipolysis stimulated lipoprotein receptor (LSR) expressed in the gut of the host cell (Hemmasi et al., 2015; Mesli et al., 2004; Papatheodorou et al., 2011). Once bound, CDTb then triggers the accumulation of LSR to detergent-resistant membrane/lipid rafts and facilitates the formation of oligomers (Hale et al., 2004; Nagahama et al., 2004; Papatheodorou et al., 2013). Previously, Wigelsworth and colleagues demonstrated that another surface protein CD44 cleaves to the iota toxin's transport component, suggesting that other than LSR, this protein may also act as a receptor for the toxin (Wigelsworth et al., 2012). With the discovery of these two receptors, it remained unclear whether LSR and CD44 interact or if one receptor affects the cleavage of the toxin to the other.

The cognate complex formed is then internalized into the endosome. The acidic pH of the endosome promotes the insertion of the membrane and generates a pore in the membrane of the endosome that allows for translocation of the enzymatic component of the toxin through the ionic channels into the host cystol (Kaiser et al., 2011). Once in the cystol, the enzymatic component of CDTa catalyses the transfer of ADP-ribose from nicotinamide adenine dinucleotide (NAD+) to arginine 117 therefore blocking polymerization of G-actin and

stimulating the depolymerization of F-actin filaments (Gülke et al., 2001). The ADP-ribosylated actin is unable to polymerize hence it attaches to the barbed end of the actin filament and in the process blocks polymerization of unmodified actin. This event destroys the microfilament network of actin and allows for protrusion of microtubules to the cell surface facilitating adherence of the pathogens (Aktories et al., 2011; Schwan et al., 2009; Wegner & Aktories, 1988).

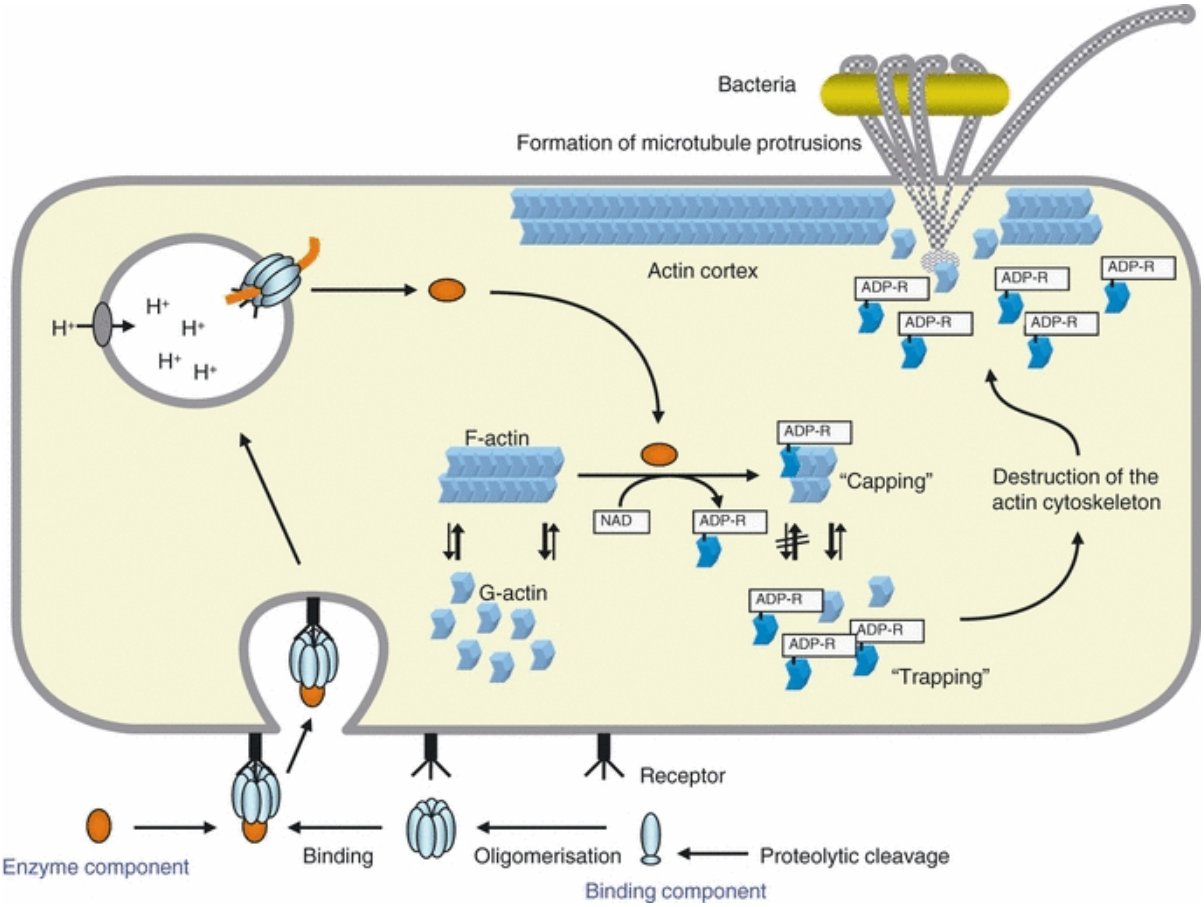


Figure 2.7: Mechanism of action of binary toxin. Adapted from (Gerding et al., 2014).

2.4.2 Non-toxin mediated virulence factors

2.4.2.1 Cell surface proteins

The cell surface of *C. difficile* is composed of numerous proteins that establish an interaction with the host. The proteins including the surface layer protein, cell wall proteins, heat shock protein, fibronectin-binding protein and collagen-binding protein are involved in adherence, colonization and immunoregulatory activities (Bradshaw et al., 2018; Fagan et al., 2011; Kirk, Banerji, et al., 2017a). The functions of these proteins are explored in depth below.

2.4.2.1.1 Surface layer proteins and other cell wall proteins

The outer cell surface of *C. difficile* is composed of complex heterodimer crystalline layer that is made up of two proteins a High Molecular Weight Proteins (HMW) and low molecular weight protein (LMW) (E Calabi et al., 2001; Cerquetti et al., 2000). The two proteins are derived from post-translational cleavage of single precursor Surface-Layer Protein (SLP) A encoded by *slpA* gene located on the *slpA* locus (E Calabi et al., 2001; Cerquetti et al., 2000; Karjalainen et al., 2002). The LMW is located on the exterior surface and exhibits genetic variability therefore providing a basis for typing of *C. difficile* strains (K. E. Dingle et al., 2013; Karjalainen et al., 2002). Further, LMW is thought to facilitate adhesion of *C. difficile* to host intestinal epithelial cells (Merrigan et al., 2013). This process occurs in two stages: first, SLP prevents adhesion of *C. difficile* to the microvilli of the host intestinal epithelial cells, resulting in deposition of the toxins to the enterocytes. Consequently, partially degraded extracellular matrix molecules will be exposed during cell damage, allowing for SLP to adhere to them and contribute to more tissue damage (Emanuela Calabi et al., 2002). The HMW on the other hand reconstitutes the inner layer and is highly conserved whose role is to anchor the cell wall to the S-layer (Willing et al., 2015). A recent study by Kirk et al., demonstrated that the S-layer protein may also contribute to sporulation, lysozyme resistance, and toxin production (Kirk, Gebhart, et al., 2017).

The S-layer is immunogenic and is recognized by antigen presenting cells via the toll-like receptor 4. This interaction modulates activation of innate and adaptive immune response by inducing production of pro-inflammatory cytokines (TNF-α, interleukin (IL)-12, IL-23, IL-1β and IL-6) and an anti-inflammatory or regulatory cytokine IL-10 which contributes to bacterial clearance (Ausiello et al., 2006; Bianco et al., 2011; Bradshaw et al., 2018; Ryan et al., 2011). However, the sequence variation in SLP among the different ribotypes suggests a possible variation in the modulation of immune response that eventually defines the severity of the disease. Lynch et al. (2017) revealed that SLP isolated and purified from hypervirulent strains (027 and 078) produced equivalent levels of pro-inflammatory cytokines and cell surface indicators as non-hypervirulent strains (Lynch et al., 2017). Interestingly, they noted a low internalization rate for the hypervirulent strains. Therefore, according to the aforementioned study, despite the upregulation of cytokines by SLP of hypervirulent strains, macrophage activation to phagocytose the bacteria is modest, hence prolonging the duration of infection (Lynch et al., 2017).

In addition to SLP other cell proteins detected in *C. difficile* include Cwp84, Cwp66, CwpV and Cwp2 (Fagan et al., 2011). Cwp84 protein is a cysteine protease involved in degradation of extracellular matrix protein of the host facilitating tissue damage and dissemination of the infection (Janoir et al., 2007). Cwp66 and Cwp2 act as adhesins. Notably, the expression of Cwp66 is increased in response to heat-shock. CwpV promotes cell anchoring and induces *C. difficile* aggregation resulting in biofilm formation in the gut (Bradshaw et al., 2017; Reynolds et al., 2011; Waligora et al., 2001).

2.4.2.1.2 Other cell surface proteins

Other important cell surface proteins involved in host-pathogen interaction include; (i) Fibronectin binding protein (Fbp68 in strain 79-685 and FbpA in strain 630) that binds to soluble and immobilized fibronectin and fibrinogen (Barketi-Klai et al., 2011; C. Hennequin et al., 2003), (ii) a solute-binding lipoprotein CD0873 that adheres to enterocyte-like Caco-2 cell lines (Cerquetti et al., 2002; Kovacs-Simon et al., 2014) and (iii) Collagen binding protein A (CbpA) with high affinity to collagens I and V (Tulli et al., 2013). Further, hair-like projections seen on the exosporium layer of strain R20291 spore play a role in binding to the intestinal mucosa (Mora-Uribe et al., 2016). However, *C. difficile* strain 630, which lacks these hair-like projections, possesses bacillus collagen-like protein of anthracis (BclA) on its exosporium, which also promotes the initiation of *C. difficile* colonization (Díaz-González et al., 2015; Phetcharaburanin et al., 2014). (iv) Heat shock protein (HSP); Extraordinary environmental conditions, such as antibiotics, heat, acidic pH, and inadequacy of iron, promotes *C. difficile* adhesion to the host (Waligora et al., 1999). Consequently, these external stimuli induce the up-regulation of HSPs such as DnaK and GroEL to reflect these conditions and facilitate host-pathogen interaction (Claire Hennequin et al., 2001; Jain et al., 2017). In support of this, a recent study demonstrated that mice inoculated with GroEL caused low levels of adherence, as well as high level of specific GroEL antibodies, indicating that, in addition to acting as adhesins, GroEL also elicit an immunological response, providing a first line of protection (Péchiné et al., 2013).

2.4.2.4 Flagella

Flagellin proteins are expressed on the surfaces of most *C. difficile* strains. Notably, their distribution on the cell surface varies between strains; for example, R20291 produces a single flagellum, whereas 630erm produces multiple flagella. While flagella are primarily

responsible for *C. difficile* motility, it does not always contribute to its pathogenesis (Twine et al., 2009). An earlier study concluded that the flagellum in *C. difficile* is more of an accessory virulence factor, promoting adherence to colonic epithelial cells at a 10-fold higher level than non-flagellated strains dependent or independent on motility (Tasteyre, Barc, et al., 2001). Two flagellin proteins, the structural protein FliC and the capping protein FliD, are primarily responsible for adherence to the intestinal mucosal layer. The cap flagellin protein adheres strongly because it is highly conserved and specific to mucosal receptors (Tasteyre, Barc, et al., 2001; Tasteyre, Karjalainen, et al., 2001). Contrary to these findings, an in vitro study conducted on hamsters established that the two flagellin proteins do not contribute to *C. difficile* adhesion to the mucosal layer as earlier indicated (T. C. Dingle et al., 2011). Additionally, some non-flagellated strains of *C. difficile* express a cryptic flagellin gene FliC that is silent and is immobilized by gene rearrangement or deletion, making its expression dependent on inducing biological factors or growth conditions (Tasteyre et al., 2000). Along with other surface structures, the flagella of *C. difficile* also contributes to the formation of late-stage biofilms (Đapa et al., 2013).

Flagella gene regulation is needed for the expression and transcription of the TcdA and TcdB toxins. Aubry et al., observed that activation of genes located in the third region of the flagellar operon (F3) upregulated toxin transcription, resulting in increased toxin levels during the early logarithmic phase of *C. difficile* growth. In contrast, the same study observed an increase in TcdR, TcdB, TcdE, and TcdA toxin levels when structural genes (FliC) encoding late-stage flagella regulon were silenced (Aubry et al., 2012). Taken together, these findings suggest that activating or inactivating genes in the flagellum locus can either upregulate or downregulate toxin gene expression depending on the inducing factor (Baban et al., 2013; Martin-Verstraete et al., 2016; Stevenson et al., 2015).

2.4.2.5 Fimbriae and pili

The pilin proteins cover the entire surface of *C. difficile* cell, though epidemic strains have a higher level of pilin protein expression than non-epidemic strains (Bergeron & Sgourakis, 2015; Purcell et al., 2016). While it has long been recognized that fimbriae contribute to bacteria' adhesion to biotic and abiotic surfaces (Piepenbrink & Sundberg, 2016), recent research has revealed that they also contribute to biofilm formation, gliding and twitching motility, as well as horizontal gene transfer (Maldarelli, Piepenbrink, et al., 2016; Purcell et al., 2012; Varga et al., 2006). On the cell surface of *C. difficile*, nine pilin proteins of Type IV

Pili (T4P) have been identified, with PilA1 and PilJ classified as the major and minor pilins, respectively (Maldarelli et al., 2014; Melville & Craig, 2013; Piepenbrink et al., 2015). Additionally, animal studies have shown that pilin proteins are immunogenic and, thus, present an important unit for vaccine target; nevertheless, antibody response to pilin proteins vary. Maldarelli and colleagues demonstrated that immunization with PilW and PilJ proteins elicits a more robust and specific antibody response than immunization with major pilin PilA1 (Maldarelli et al., 2014; Maldarelli, Matz, et al., 2016).

2.4.2.6 Biofilm formation

Persistent infections can occur as a result of ability of the bacteria to form biofilms, which enable them to resist antibiotic treatment and immune response (Crowther et al., 2014). The formation of biofilms in *C. difficile* is a complex process regulated by multiple factors. These factors include surface layer proteins (Cwp84 and CwpV), which contribute to the maturation of the S-layer and biofilm (Reynolds et al., 2011), cyclic diguanylate (c-di-GMP) a second messenger that regulates cellular processes (Purcell et al., 2012), type IV pili (Purcell et al., 2016), flagella and finally Spo0A, which regulates sporulation and stimulates aggregation of *C. difficile* colonies during biofilm formation (Đapa et al., 2013; Dawson et al., 2012). In-vitro studies show that *C. difficile* forms a more robust biofilm in a mucosal polymicrobial community than in a monobacterial environment where it coexists with other biofilm-forming bacteria such as Bacteroidetes and Firmicutes (Donelli et al., 2012; Semenyuk et al., 2015). This was confirmed in a recent in-vivo study in which researchers discovered that *C. difficile* formed a mono-aggregate biofilm in a mouse model, but the size of the biofilm was smaller than previously thought (Soavelomandroso et al., 2017). Despite these efforts, it appears that in-vivo biofilm formation studies are constrained by uncontrollable internal environmental factors such as peristalsis, whereas in-vitro biofilm formation studies may not accurately reflect what occurs in-vivo (Bjarnsholt et al., 2013; Roberts et al., 2015).

2.4.2.7 Polysaccharides

The vegetative form of both toxigenic and non-toxigenic *C. difficile* possesses a capsular like polysaccharide on its cell surface, but the adhesive properties of this polymer have not been fully elucidated. Nevertheless, it has been shown to provide protection against host phagocytic cells (Baldassarri et al., 1991; H. A. Davies & Borriello, 1990). Aside from the polysaccharide capsules, some strains have three cell wall-linked polysaccharides on their cell surfaces designated PS-I, PS-II and PS-III that aid in the surface anchoring of the toxins

(Ganeshapillai et al., 2008; Willing et al., 2015). The Majority of *C. difficile* ribotypes including hypervirulent strain 027, express elevated PS-II levels in their spore and vegetative forms. PS-II is immunogenic and has been proposed as a carbohydrate-based vaccine target against *C. difficile* toxin and the surface polysaccharides (Kirk, Banerji, et al., 2017b; Monteiro, 2016; Monteiro et al., 2013). Humans produce anti- PS-II antibodies when exposed to *C. difficile*, which recognizes PS-II on the cell surface of *C. difficile*. This anti-PS-II specific IgA antibodies were detected in the stool supernatant of patients infected with *C. difficile;* this finding highlights the immunogenic potential of this polysaccharide (Oberli et al., 2011). Furthermore, PS-II conjugated to toxins A and B fragments induced the production of anti-polysaccharide IgG antibodies in a mouse model experiment (Romano et al., 2014), and recently, PS-II conjugated to an immunostimulatory protein keyhole limpet hemocyanin (KLH) provided 90% protection to experimental mice challenged with *C. difficile* spores (Monteiro, 2016).

2.4.2.8 Spore formation

C. difficile forms spores to survive when growth conditions are unfavorable. The spore is highly resistant to heat, aerobic conditions, antibiotics, and disinfectants such as ethanol-based products, which are often used in hospital settings, thereby facilitating *C. difficile* transmission and persistence within the hospital environment, as well as contributing to infection relapse/recurrence (Paredes-Sabja et al., 2014). Strains expressing the protein A (*Spo0A*) gene are capable of sporulation, whereas those lacking this gene can only remain in a vegetative state. The presence of bile acid, amino acids and calcium in the small intestines promotes the vegetative growth of ingested spore, whereas other bile salts such as deoxycholate inhibit vegetative growth and chenodeoxycholate inhibits spore germination (Calderón-Romero et al., 2018; Gil et al., 2017; Rosenbusch et al., 2012; Underwood et al., 2009). In addition to activating sporulation, Spo0A has been shown to regulate biofilm formation, motility, toxin production, growth and metabolism, and infection persistence in *C. difficile* (Đapa et al., 2013; Dawson et al., 2012; Deakin et al., 2012; Mackin et al., 2013; Pettit et al., 2014).

2.5 Risk factors

Multiple variables are driving the global epidemiological evolution and disease severity of *C. difficile*. It is important to identify the primary risk factors that predispose to CDI and mitigate ways of eliminating or altering the causality to reduce the disease outcome. This

section will critically review some of the risk factors linked to the development of CDI, such as antibiotic use, age, the use of acid suppressive agents, prolonged hospitalization, and comorbidities.

2.5.1 Antibiotics

Antibiotics are intended to eliminate disease-causing pathogens, but their use destroys the indigenous gut microbiota (Britton & Young, 2014; Peterfreund et al., 2012). The transformation of primary bile salts to secondary bile acids is aided by the gut bacteria. Therefore, impairment of the gut microbiota results in a high concentration of primary bile salts which promotes the germination of *C. difficile* spores into toxin-producing vegetative cells (Giel et al., 2010; Hopkins & Wilson, 2018). Additionally, antibiotics generate a selective pressure that permits potential pathogens to overgrow, resulting in severe and fatal infections like diarrhea and pseudomembranous colitis (Jernberg et al., 2010; Vincent et al., 2016). Antibiotic use accounts for 5-25% of cases of antibiotic associated diarrhea (AAD) in individuals receiving antibiotics, with *C. difficile* contributing to 25–33% of AAD cases. Other possible etiologies of AAD include *Clostridioides perfringens, Staphylococcus aureus, Campylobacter jejuni*, *Klebsiella oxytoca* some fungi and viruses (L. V McFarland, 2008).

Prior antibiotic exposure not only promotes the growth and colonization of spore-forming *C. difficile*, but also makes it easier for it to produce toxins in the long run, contributing to a 60% risk of CDI (Pultz & Donskey, 2005; Slimings & Riley, 2014). Broad spectrum antibiotics such as ampicillin, amoxicillin, third generation cephalosporins, clindamycin, ceftriaxone, and piperacillin-tazobactam have been implicated in severe cases of AAD (Gerding, 2004; Leffler & Lamont, 2015; Pultz & Donskey, 2005; Slimings & Riley, 2014). Even when other risk factors are taken into consideration, the use of multiple antibiotics to treat underlying conditions increases the risk of CDI (K. A. Brown et al., 2013). It is estimated that taking two antibiotics triples the risk of CDI (Stevens et al., 2011).

Antibiotics that are effective against *C. difficile* lessen the chance of colonization; nevertheless, the risk increases when the strain is resistant to the antibiotic. (Gerding, 2004; Owens et al., 2008). According to several research, *C. difficile* is becoming increasingly resistant to commonly used antibiotics such as clindamycin, cephalosporins, erythromycin, and fluoroquinolones (Peng et al., 2017; Spigaglia, 2016). Resistance to these antibiotics

consequently results in treatment failure, recurrence of CDI, and global emergence of fluoroquinolone resistant hypervirulent strain (*C. difficile* BI/NAP1/027) (He et al., 2013; Hopkins & Wilson, 2018; Spigaglia, 2016; Wieczorkiewicz et al., 2016).

2.5.2 Acid suppressive agents

Because gastric acidity kills 99.9% of ingested vegetative microbes, low gastric acidity makes it easier for microbes to colonize the gastrointestinal tract. Proton pump inhibitors (PPI) and histamine 2 (H2) blockers, known to reduce gastric acid secretion provides a favorable environment for spore germination, growth, and survival of vegetative cells (Barletta & Sclar, 2014). PPI and H2 blockers are used in treatment of peptic ulcers, dyspepsia and gastroesophageal reflux disease (GERD). Omeprazole, lansoprazole, pantoprazole, rabeprazole and esomeprazole are the most commonly prescribed PPIs, whereas H2 receptor blockers include nizatidine, famotidine, cimetidine and ranitidine.

Although the mechanism by which PPIs or H2 blockers predispose to CDI is unknown, the association has been established based on the fact that PPIs damage the colonic mucosa and destroy the intestinal flora, allowing for colonization and the growth of vegetative forms of oral and fecal bacteria. (Bavishi & DuPont, 2011; Fried et al., 1994; Jump et al., 2007; Thorens et al., 1996). Seto and colleagues studied the gut microbiota of PPI users and discovered a reduction in the diversity of the gut microbiota among them (Seto et al., 2014). Another intriguing study profiled the gut microbiota of PPI users and found that there was a decrease in gut flora and an increase in commensals of the upper respiratory tract, particularly those of the genera *Rothia* and *Streptococcus* (Jackson et al., 2016). Further, the presence of pathogens such as *Escherichia coli, Shigella sonnei* and *Streptococcus pneumoniae* increases CDI susceptibility (Jurburg et al., 2019; Schubert et al., 2015; Theriot & Young, 2015). The ingested spore, on the other hand, is acid resistant and can only germinate to vegetative form when triggered by a combination of amino acids (glycine), bile salts (taurocholate) and minerals; otherwise, the spore will remain dormant and serve as a reservoir in recurrent/relapse CDI (Howerton et al., 2013; Nerandzic et al., 2009; Sorg & Sonenshein, 2008; Wombwell et al., 2018). Another possible mechanism is that PPI exposure directly induces the expression of genes coding for toxin synthesis; however, the precise dosage required for this effect has not been discovered. (Biswal, 2014; Stewart & Hegarty, 2013).

Furthermore, PPIs suppress the expression of colocyte gene, which is important for cell junction, toxin target, and mucosal protection in human cells (Hegarty et al., 2014).

Gastric acidity (pH <4) limits bacterial pathogen colonization, and therefore any decrease in gastric acid secretion increases the risk of gastrointestinal infections (Bavishi & DuPont, 2011; Howden & Hunt, 1987). When Wang and co-workers investigated gastrointestinal infections in individuals on H2-receptor antagonist or PPI, they discovered that patients taking these agents experienced intra-gastric bacterial infections than those not exposed (K. Wang et al., 2004). Kaur and colleagues reported histopathological damage in the colon of BALB/c mice pretreated with PPI (lansoprazole).Additionally, culture of cecal contents revealed significantly high levels of *C. difficile* toxins compared to mice not given PPI (Kaur et al., 2007).

According to systematic reviews and meta-analyses of published data, there is insufficient evidence to suggest a causal relationship between PPI use and an increased risk of CDI (Novack et al., 2014). In a review, Villafuerte-Gálvez and Kelly stated that demonstrating causality may be difficult due to numerous comorbidities and many uncontrolled confounding variables that are prone to bias, making it difficult to correlate PPI and CDI (Villafuerte-Gálvez & Kelly, 2018). However, PPIs should be used with caution in those at high risk of CDI, such as hospitalized patients receiving antibiotics (Tleyjeh et al., 2013). Despite the contrary reports, the majority of studies and meta-analytical analyses support the concept that PPI usage increases the risk of hospital and community-acquired CDI in both adults and children, including infants, with a computed risk of 1.4 to 2.75 times greater in the exposed group compared to the non-exposed group (Arriola et al., 2016; Cao et al., 2018; Freedberg et al., 2015; Gandra et al., 2016; Jimenez et al., 2015; Oshima et al., 2018; Safe et al., 2016; Trifan et al., 2017; Wombwell et al., 2018). The risk of recurrent CDI is much higher in people who take PPI on a regular basis, with a reported hazard ratio of up to 1.5 (E. G. McDonald et al., 2015). A recent meta-analysis based on observational studies reported an increased risk (odds ratio (OR) = 1.52) of recurrent CDI in patients on gastric acid suppressive medication (Tariq, Singh, et al., 2017a). Because these medications, like antibiotics, are available over-the-counter, the extent of the problem, particularly at the community level, may be underestimated (Heidelbaugh et al., 2012; Lu et al., 2013). After controlling all other factors, including antibiotic use, a community-based case-control study found a relative risk of 2.9 and 2.0 for PPI and H2 blockers usage respectively (Dial et al., 2005).

Due to the possible association between CDI and the use of acid-suppressing medications, studies are recommending minimal use of these agents by adhering to treatment criteria to reduce the risks associated with their misuse and for CDI patients, reevaluating the need for PPIs (England, 2013; Tosetti & Nanni, 2017). The Food and Drug Administration (FDA) published a safety announcement informing health care providers and patients about use of acid-suppressive agents for short duration and at low doses (US FDA, 2012). Other considerations include establishing and executing a hospital antacid policy, using low doses of PPIs, and minimizing long-term PPI use (Bavishi & DuPont, 2011; Heidelbaugh et al., 2012; Metz, 2008; Thachil, 2008).

2.5.3 Age

C. difficile affects persons of all ages; however, the severity of the infection increases with age, with the majority of cases affecting adults over the age of 65. (Lessa et al., 2015; Lucado et al., 2006). Frequent and long-term hospitalization, frailty, decreased immunity, and functional status owing to underlying conditions make the elderly more susceptible to CDI (Asempa & Nicolau, 2017; Balsells et al., 2019; Lorraine Kyne et al., 2002; Olsen et al., 2018; Rao et al., 2013; Ticinesi et al., 2015).

The frequency of both hospital-acquired and community acquired CDI among the younger population, particularly in children, is increasing (Jason Kim et al., 2008; Julia Shaklee Sammons, Toltzis, et al., 2013). From 1997 to 2006, a cross-sectional data analysis research in the US found that the number of children hospitalized with CDI increased from 7.24 to 12.80 per 10,000 hospitalizations (Zilberberg et al., 2010). Other existing risk factors including antibiotic use, underlying medical conditions such as malignancies or children undergoing hematopoietic stem cell transplantation, inflammatory bowel diseases, prolonged hospitalization, nasogastric tube feeding, co-infection with other gastrointestinal pathogens, and use of acid suppressants are mainly contributing to the observed rates in both hospitalized and non-hospitalized children (Crews et al., 2015; Migriauli et al., 2018; Nylund et al., 2014; Salamonowicz et al., 2018; Samady et al., 2014; Julia Shaklee Sammons, Localio, et al., 2013; Sandora et al., 2011; Turco et al., 2009). Despite these findings, controversies on how to classify *C. difficile* in the pediatrics population exist because this group has a high *C. difficile* carrier rate and a milder form of the disease than adults, making it difficult to quantify the disease burden in this age group (Y. M. Lee et al., 2016; L. V. McFarland et al., 2016; Julia S. Sammons & Toltzis, 2015).

Recent investigations have found a shared transmission of toxigenic/non-toxigenic *C. difficile* in infants and adults, implying that *C. difficile* colonization in healthy infants contribute considerably to infections seen in adults (Adlerberth et al., 2014; C. Rousseau et al., 2011; Clotilde Rousseau et al., 2012; Stoesser et al., 2017). A study that examined CDI among asymptomatic and symptomatic individuals observed that even with substantial colonization of toxigenic *C. difficile* in these two categories, there was a statistical difference between the positivity rates (Leibowitz et al., 2015). In other research, CDI in infants has been linked to an increase in incidence and rate of hospitalization (Zilberberg et al., 2008, 2010). CDI affects young infants and newborns to a previously unrecognized degree. In Minnesota, a population-based study reported a 43.6 per 100,000 person-years age-specific incidence linked to CDI in infants (Khanna et al., 2013). Based on observations from a cohort of 4,895 hospitalized children with *C. difficile*-associated disease, Kim and colleagues determined that *C. difficile* may affect children younger than one year, with 26% being infants less than one year and 5% being infants younger than one month (Jason Kim et al., 2008). Current published guidelines, however, advice against testing CDI in children under the age of one year who present with diarrhea (Antonara & Leber, 2016; L. C. McDonald et al., 2018; Schutze et al., 2013). If this is necessary, all other causes of diarrhea must first be explored and eliminated (Sandora et al., 2018). With the current gaps in the burden of *C. difficile* in this age group, additional research is needed to ascertain the age-specific and true disease outcome (Balsells et al., 2019; Y. M. Lee et al., 2016).

2.5.4 Prolonged hospital stay

There is evidence to show that prolonged hospitalization increases the likelihood of acquiring CDI. A population-based surveillance conducted by Centers for Disease Control and Prevention (CDC) in the US linked increased cases of CDI among hospitalized patient to health care settings (Centers for Disease Control and Prevention (CDC), 2012). The association has been attributed to frequent antibiotic exposure, given that these patients maybe receiving therapy for underlying diseases (Carignan et al., 2008; Garey et al., 2008; Kuntz et al., 2016). Hospital-based studies have shown a high prevalence of *C. difficile* contamination in healthcare facilities and on the hands of healthcare workers, with results showing a positive correlation between patient isolates and those found on healthcare workers' hands and in the environment (W N Fawley & Wilcox, 2001; Samore et al., 1996).

2.5.5 Comorbidities

Recent systematic reviews have demonstrated that comorbid conditions such as inflammatory bowel disease (IBD), haematological malignancies**,** diabetes mellitus, acute myeloid leukemia, neutropenia, chronic kidney disease (CKD), organ transplant, and other immunodeficiency disorders increase the risk of CDI, particularly in the elderly population (Furuya-Kanamori, Stone, et al., 2015; Harris et al., 2018; Ticinesi et al., 2015). The attributable risks of the aforementioned comorbidities vary significantly, and the risk is particularly high in patients with multiple chronic conditions. Multimorbidity presents a treatment problem due to increased polypharmacy, which leads to drug misuse and medication non-adherence. Furthermore, the severity of the underlying diseases impairs immune responses, allowing *C. difficile* to colonize more easily (Lorraine Kyne et al., 2002; Predrag, 2016).

2.6 Prevention and treatment strategies of CDI

The treatment and management strategies for CDI are determined by the severity of the disease and the likelihood of a primary or recurrent infection. In most cases, if an antibiotic causes diarrhea, the antibiotic should be discontinued and the patient should be monitored for clinical signs (C. M. Surawicz et al., 2013). Stopping the respective antibiotic favors the restoration of the normal gut flora. Antibiotic treatment of CDI has proven to be difficult in recent years due to metronidazole-resistant strains, a high risk of infection recurrence following antibiotic therapy, and the introduction of hypervirulent strains that display poor response to antibiotics used in CDI management. With this in mind, new antibiotic targets and non-antibiotics therapeutic approaches have been developed and continue to be evaluated (Dieterle et al., 2018; Kociolek & Gerding, 2016). This section discusses currently approved CDI treatment and management options, as well as any alternative treatment strategies developed/under research for future consideration. These strategies are summarized in figure 2.8 below.

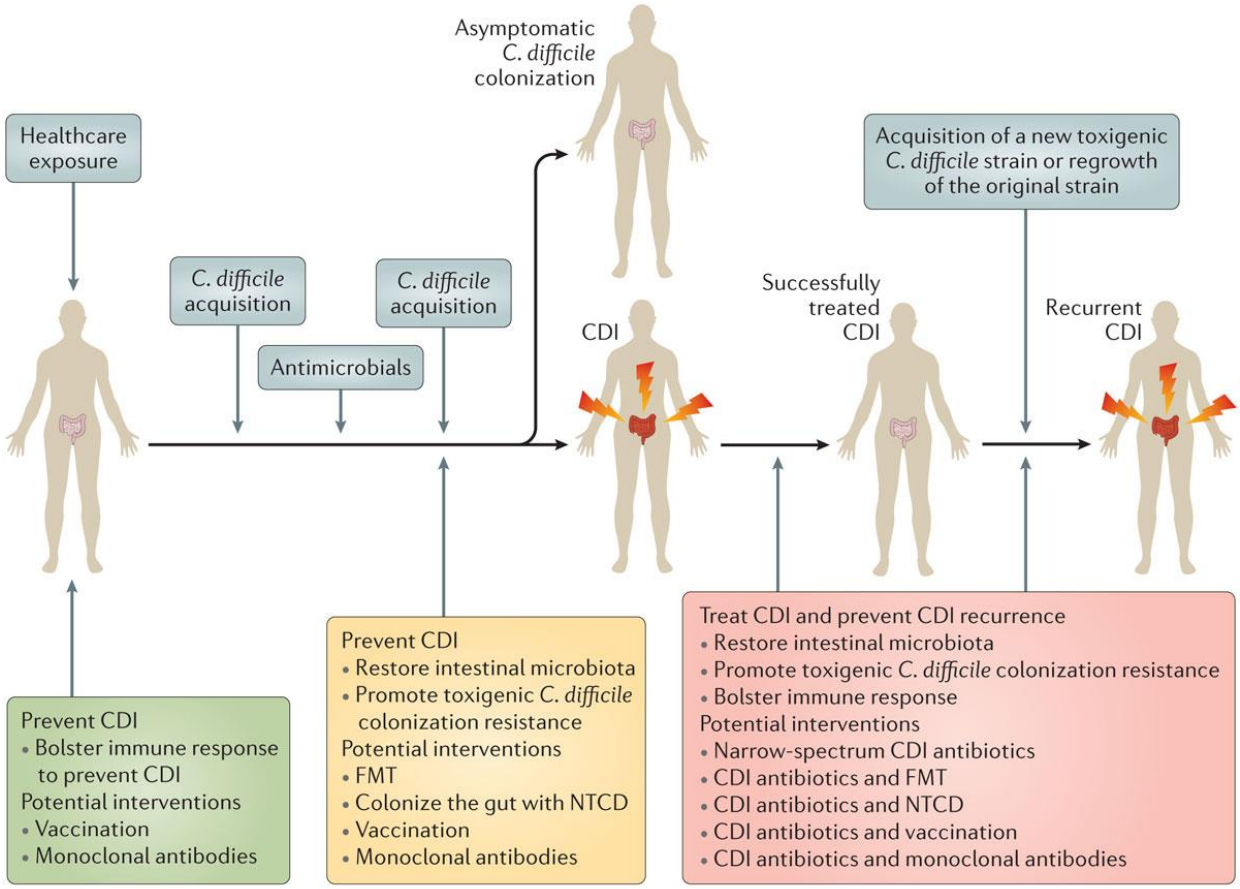


Figure 2.8: Prevention and treatment strategies. A schematic diagram summarizing potential approaches that can be adopted in treatment and prevention of CDI. Adapted from (Kociolek & Gerding, 2016).

2.6.1 Current antibiotic treatment regimes

For decades, metronidazole has been used as the first-line treatment for mild to moderate primary CDI as well as the first recurrence of the disease (Cohen et al., 2010). Furthermore, despite its association to neurotoxicity from long-term use, metronidazole is a preferred choice for most clinicians due to its low cost (Kapoor et al., 1999; Yamamoto et al., 2012). Metronidazole treatment for CDI has been linked to a significant failure rate, attributed to a number of circumstances including emergence of a resistant hypervirulent strain ribotype 027, low albumin levels of less than 2.5g/l, and prolonged hospitalization in an intensive care unit (Fernandez et al., 2004; Musher et al., 2005).

Vancomycin is the antibiotic of choice in severe instances or when metronidazole cannot be prescribed due to intolerable side effects (M.P. Bauer et al., 2009; Li et al., 2015). Initial randomized control trial (RCT) studies comparing the treatment outcomes of metronidazole and vancomycin found that the cure rate for both interventions/antibiotics were proportional (Teasley et al., 1983; Wenisch et al., 1996), however a subsequent Cochrane review of evidenced-based research suggest that vancomycin is superior to metronidazole (Nelson et al., 2017). The high cost of vancomycin may however reduce full-dose compliance, allowing resistance to arise, in addition its routine usage may increase the risk of colonization and shedding of Vancomycin Resistance *Enterococcus* (VRE) and overgrowth of *Candida* species (Al-Nassir et al., 2008; Nerandzic et al., 2012; Sethi et al., 2010).

Recurrent CDI is defined as the "re-occurrence of the infection <8 weeks after the onset of a previous episode, provided the symptoms from the previous episode resolved after completion of initial treatment" (Debast et al., 2014). Relapse with the same endogenous strain of *C. difficile* persisting in the gut or a re-infection with a different strain of extrinsic origin might result in re-establishment of the infection following treatment (Barbut et al., 2000; Johnson et al., 1989). The rate of first recurrence after initial treatment is estimated at 15%-30%, but it can escalate to 65% after second and other subsequent treatments (L. V. McFarland et al., 1999, 2002). The majority of RCT reports on recurrence rates of CDI in patients subjected to initial monotherapy of either metronidazole or vancomycin show equal recurrence rates considering that both antibiotics are not sporicidal, allowing for the persistence of the spore and re-establishment of the infection after initial treatment (Chilton et al., 2016). For the first episode of CDI, the revised 2017 Society for Healthcare Epidemiology of America (SHEA) treatment guidelines recommend using pulsed-tapered vancomycin or fidaxomicin antibiotics; however, in settings where these drugs are not available, metronidazole should be prescribed (Cornely, Miller, et al., 2012; L. C. McDonald et al., 2018). Additionally, multiple recurrences are managed by fecal microbiota transplantation while high dosages of vancomycin or a combination of metronidazole and vancomycin are recommended for complicated or fulminant CDI (Gerding et al., 2016; L. C. McDonald et al., 2018). Colectomy or loop ileostomy is done to preserve the colon especially in critically ill patients with fulminant CDI who do not respond to vancomycin or metronidazole treatments (Lamontagne et al., 2007; L. C. McDonald et al., 2018; Neal et al., 2011).

Fidaxomicin was licensed by FDA in 2011 for treatment of CDI. In a randomized controlled trial comparing the efficacy and safety of fidaxomicin and vancomycin, it was determined that fidaxomicin is superior to vancomycin and is associated with a 7.8% reduced CDI recurrence rate compared to vancomycin (25.5%). Fidaxomicin is also applicable in maintaining of the gut microbiota. However, both fidaxomicin and vancomycin compare equally in achieving the clinical response and adverse events (Cornely et al., 2014; Cornely, Crook, et al., 2012; Louie et al., 2011; Tannock et al., 2010). Despite the high cost of using fidaxomicin as a first-line treatment, a cost analysis report indicate that using fidaxomicin to treat mild to moderate CDI is cost-effective since the overall cost of treating CDI will reduce due to the low likelihood of a recurrent infection requiring readmission of the patient (Stranges et al., 2013; Watt et al., 2017). The economic effectiveness of treating severe and initial CDI recurrence with fidaxomicin was examined using a modeling approach, and similar results were observed (Nathwani et al., 2014). Ridinilazole (Vickers et al., 2015), rifaximin (Major et al., 2019; Ng et al., 2019), fusidic acid, bacitracin (L. C. McDonald et al., 2018) and cadazolid (Gerding et al., 2019) are all alternatives to metronidazole and vancomycin that are undergoing clinical trials.

2.6.2 Non antibiotic therapy

Alternative therapies for managing *C. difficile* are available if the above-mentioned standard CDI remedial treatments fail. Because of limited evidence-based studies supporting their use in humans and lack of standardized formulations, the FDA and other national guidelines regulatory agencies have not approved most of these strategies. These strategies include fecal microbiota transplantation (FMT), immunological therapies (monoclonal antibodies and vaccines), probiotics and phage therapy.

2.6.2.1 Emerging immunological therapies

Immunological therapies have been used to provide passive and active protection against *C. difficile* antigens such as toxins, surface proteins and spores. This has been accomplished through the use of monoclonal antibodies and vaccines. Despite the availability of these agents, the American College of Gastroenterology (ACG) and the World Society of Emergency Surgery (WSES) are the only organizations that advocate immunological therapy only in cases of hypogammaglobulinemia and multiple CDI recurrences or fulminant CDI, respectively (Fehér & Mensa, 2016).

2.6.2.2 Monoclonal antibodies

Monoclonal antibodies against *C. difficile* act by binding and neutralizing the toxins produced by *C. difficile*. Many monoclonal antibodies have been developed to date; however, the most common are actoxumab and bezlotoxumab, which neutralize toxin A and B, respectively. In 2016, the FDA approved bezlotoxumab for the treatment of patients at risk of recurrent CDI (FDA, 2016b). Because both monoclonal antibodies target separate toxins, a monoclonal antibody that is effective against strains that produce both toxins was needed. Actoxumab–bezlotoxumab (MK-3415A) combination is one such product that is still undergoing clinical trials. Evidence from animal models have shown that the combined monoclonal antibody offered protection of the gut epithelium from toxin-induced damage and restoration of the lost gut microbiota in the event of antibiotic treatment (Džunková et al., 2016; Qiu et al., 2016; Zhiyong Yang et al., 2015).

Hyperimmune bovine colostrum (HBC) and whey protein isolate are two more innovative immunological techniques that have been investigated and shown promising outcomes (Heidebrecht et al., 2019; Steele et al., 2013). These products have been tested against other gastrointestinal pathogens, including Enterotoxigenic *Escherichia coli* (ETEC), and have shown to be significantly effective (Otto et al., 2011).

2.6.2.3 Vaccines

One of the current options being studied for CDI prevention is administration of vaccines. Many toxoid-based or recombinant antigen vaccines are being evaluated to determine their ability to effectively elicit an active immune response by neutralizing toxin activity and eventually offering protection against primary or recurrent CDI. TcdA and TcdB inactivated with formalin- was among the first potential toxoid-based vaccine tested in humans (Kotloff et al., 2001). According to this study, the patient's serum had higher levels of antitoxin A and B IgG antibodies after administration of the third dose of this vaccine. Subsequent investigations raised safety concerns about this vaccine, especially its cytotoxic effects resulting in improved vaccine preparations over time (de Bruyn et al., 2016; Sheldon et al., 2016).

Clinical studies on several of these vaccine promising products have however been halted because of lack of substantial evidence, as was the case of Sanofi's toxin A and B vaccine (ACAM-CDIFF), which was discontinued due to interim analysis (Dieterle et al., 2018;

Sanofi, 2017). Furthermore, the availability of recombinant technology has prompted the development of advanced recombinant toxoid vaccines made up of genetically modified non-toxin TcdA and TcdB fragments (Donald et al., 2013). Pfizer's ongoing Phase 2 trial of a recombinant vaccine (PF-06425090) in healthy US individuals aged 65 to 85 years revealed elevated neutralizing antitoxin A and B antibodies (Kitchin et al., 2020). VLA84 and IC84, which consist of truncated fragments of TcdA and TcdB, and the DNA vaccine are two more immunogenic vaccines under investigation (Bézay et al., 2016; B. Z. Zhang et al., 2016). Susan and colleagues recently developed a tetravalent vaccine that protects against binary toxin-producing strains by combining a binary toxin antigen with a bivalent recombinant attenuated TcdA and TcdB (Secore et al., 2017).

Antibodies to *C. difficile* adhesins, such as Heat shock protein (GroEL), flagella antigens (FliC & FliD) and surface layer protein (Cwp84), as well as spore proteins, have been detected in patients' serum. These antibodies have been linked to enhanced protection and reduced disease recurrence, suggesting that they could be used as potential vaccine candidates (Kirk, Banerji, et al., 2017a; Leuzzi et al., 2014; Péchiné et al., 2018, 2005).

2.6.2.4 Probiotics

Probiotics are live bacteria that are administered to help restore the gut microbiota after an imbalance caused by antibiotic treatment or other risk factors. Probiotics such as *Lactobacillus* spp, *Bifidobacterium* spp, *Saccharomyces boulardii* and *Streptococcus* used alone or in combination have been tested in both human and animal models, with varying outcomes in terms of preventing initial and recurrent CDI episodes (Barker et al., 2017). Recent meta-analysis findings based on published studies and RCTs focusing on hospitalized patients demonstrate that probiotics given within two days of commencing antibiotics reduced the incidence of CDI by more than half (B. C. Johnston et al., 2018; Lau & Chamberlain, 2016; Shen et al., 2017; Simpson & Lyon, 2019). However, these findings excluded a number of patients including the immunocompromised and those with a history of gastrointestinal surgery, who, according to literature, are a high-risk group. Further, because the RCT studies did not record specific adverse events that occurred during the intervention, further research on the safety and efficacy of probiotics in the prevention of CDI is needed. Despite the growing evidence supporting the use of probiotics as an alternative approach in preventing *Clostridium difficile* associated disease (CDAD), particularly in patients on antibiotic therapy, probiotics are neither FDA-approved nor recommended by national

guidelines as part of CDI prevention strategies (L. C. McDonald et al., 2018; C. M. Surawicz et al., 2013). However, the World society of emergency surgery (WSES) advises their usage as a supplemental therapy in cases of recurrent infection (Fehér & Mensa, 2016).

2.6.2.5 Fecal microbiota transplantation

Fecal microbiota transplantation (FMT) involves the introduction of pre-screened feces from a healthy human donor to a recipient by either endoscopy or enema to restore the gut microbiota to its premorbid state. Apart from re-establishing the gut microbiota, this approach also triggers innate immune response via toll-like receptors (TLR), which protects against chronic gastrointestinal infections (Buonomo & Petri, 2016; Hasegawa et al., 2011). FMT was first demonstrated in 1958 by Eiseman and colleagues, who first utilized fecal enema to successfully treat pseudomembranous colitis in four patients (Eiseman et al., 1958). Since then, FMT has emerged as a promising therapeutic option for chronic gastrointestinal infections such as CDI, inflammatory bowel diseases, irritable bowel syndrome, ulcerative colitis and crohn's disease (Borody et al., 2003; H. H. Choi & Cho, 2016; Smits et al., 2013). SHEA recommended FMT in 2017 for the treatment of severe CDI and multiple recurrences after unsuccessful standard antibiotic treatment. FMT has shown to have better therapeutic outcomes relative to vancomycin treatment, with cure rates of approximately 90% (Quraishi et al., 2017). A case report of a patient with a history of recurrent transplant pyelonephritis established that FMT was sufficient in eliminating Extended spectrum Beta-lactamase (ESBL) producing *Enterobacteriaceae* linked to the condition (Singh et al., 2014). Following successful FMT, studies have shown a reduction in antibiotic-resistant organisms, primarily multidrug-resistant organisms like VRE, Carbapenem-resistant *Enterobacteriaceae* (CRE), and Methicillin-resistant *Staphylococcus aureus* (MRSA), as well as elimination of recurrent infections such as urinary tract infections (Dubberke et al., 2016; García-Fernández et al., 2016; Tariq, Pardi, et al., 2017; T. Wang et al., 2018).

FMT is administered through a variety of techniques, including oral capsules, colonoscopy, rectal enemas and a nasogastric tube (Chehri et al., 2018; Staley et al., 2017). Each of these delivery modalities has its own set of benefits and drawbacks (Drekonja et al., 2015; C. . Kelly et al., 2016). Successful innovative approaches, such as delivery via oral capsule formulations, have been devised to counter some of these downsides, such as the risks and delayed invasive procedures, cost implications and patients' unwillingness to adopt some of the delivery methods (Chehri et al., 2018; Staley et al., 2017; Youngster et al., 2016).

However, Jiang and colleagues revealed that both oral and rectal enema formulations of frozen and lyophilized products show corresponding efficacy in a RCT that compared delivery via oral capsule and colonoscopy (Jiang et al., 2018; Kao et al., 2017). Regardless, changes in microbiome before and after capsule delivery should be monitored to guarantee the viability of the bacteria preserved in the preparation (Staley et al., 2017).

Due to limited availability of fresh samples, stool banks have been established to store and freeze extensively screened donor samples and make them readily available to recipients (E. M. Terveer et al., 2017). This ensures quality of the procedure while also reducing the turnaround time it takes to set up the procedure. Donor feces can also be frozen and thawed without losing their efficacy, allowing for long-term banking. Furthermore, storage of frozen material allows donors to be re-tested for possible incubating of viral infections prior to administration. Due to safety concerns and recent occurrences of multidrug-resistant pathogens transmitted through FMT, the FDA has nonetheless imposed a discretionary requirement that these facilities (stool banks) submit an investigation new product application (FDA, 2016a). In addition, some facilities providing these services are not regulated and therefore administer stool samples that have not been tested.

Other possible non-antibiotic treatments include CamSA, a bile salt antispore germinant (Howerton et al., 2018), and chicken IgY antibodies engineered to target the spore (Pizarro-Guajardo et al., 2017). Taurocholate can also be used since it forms conjugates with the toxins (TcdA & TcdB) and shields the colonic epithelial cells from their toxicity (Darkoh et al., 2013). Alternatively, accessory gene regulator molecules can be used to block the quorum signaling pathway, which induces toxin production (Darkoh et al., 2016).

2.7 Detection of CD

Appropriate management and implementation of CDI prevention and control strategies rely heavily on accurate diagnosis of *C. difficile*. The primary diagnosis is made after a patient is evaluated for the specific clinical signs and symptoms as well as the detection of *C. difficile* toxins in the stool samples. Many *C. difficile* testing assays have been developed over the years and are currently available for use. Of note, are the rapid and simple tests for detecting the markers of infection directly from the fecal samples. However, the performance of these tests varies in their specificity, sensitivity, cost of the test and the turnaround time required to obtain the results. Stool culture, toxigenic culture (TC) assay, cell cytotoxicity neutralization assay (CCNA), enzyme immunoassays (EIAs) for detection of toxins A and B, or glutamate

dehydrogenase (GDH) antigen, and nucleic acid amplification tests (NAATs) for detection of toxin genes are some of the diagnostic tests available (Martínez-Meléndez et al., 2017a). For many years, TC and CCNA have been recognized as gold standard methods for diagnosing CDI (Planche & Wilcox, 2011).

International guidelines from SHEA, the Infectious Diseases Society of America (IDSA), the American College of Gastroenterology (ACG), and the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) recommend using a multi-step algorithm approach involving a combination of these tests to enhance CDI diagnosis because the vast majority of these tests are not optimized. (Cohen et al., 2010; Schmidt & Gilligan, 2009; Wilcox et al., 2010). A two- or three-step algorithm where a positive result in the screening step is confirmed with one or two confirmatory assays are cost effective (Larson et al., 2010; M. Qutub et al., 2019). The positive predictive value of a two- or three-step algorithm is higher than one-step testing. The choice of the tests to combine should take into consideration their specificity and sensitivity, as well as the turnaround time (L. C. McDonald et al., 2018; M. O. Qutub et al., 2011). For example, the ESCMID diagnosis guidelines recommends a two-step algorithm in which NAAT or GDH test is performed first, and then, if positive, a toxin A/B EIA test if performed (M. J. T. Crobach et al., 2016).

2.7.1 Toxigenic culture (TC)

When combined with a highly sensitive and specific test, TC is the gold standard method for detecting *C. difficile.* The method is highly sensitive but has a low specificity because it cannot distinguish between toxin-producing strains and non-toxin-producing strains. In this instance, a two-stage technique of diagnosis is appropriate, with the organism propagated in the first step and a toxin assay conducted in the second step (Martínez-Meléndez et al., 2017). The ability to perform epidemiologic investigations, monitor antibiotic susceptibility, and strain typing of the isolates are all advantages of stool culture. *C. difficile* growth is accomplished by plating the sample on a selective agar plate such as Cycloserine-cefoxitin egg yolk agar (CCEY) or chromogenic medium (chromID CD) (Lister et al., 2014; J. J. Yang et al., 2014). Pretreatment of the stool sample with either alcohol shock or heat shock improves spore recovery (Borriello & Honour, 1981). The plates are then incubated anaerobically for 24-48 hours. Subsequently, *C. difficile* colonies are identified phenotypically based on their characteristic “horse barn” odor, gram-positive bacilli and spot indole (Wren, 2010). Automated techniques including Matrix-Assisted Laser Desorption

Ionization –Time of Flight Mass Spectrometry (MALDI-TOF MS) can however be used for rapid identification (Dierig et al., 2015). To distinguish toxin-producing strains, typical *C. difficile* colonies are further tested for toxin production using either the CCNA, EIA, or NAAT tests.

2.7.2 Cell Cytotoxic Neutralization Assay (CCNA)

The gold standard technique for confirming CDI is the CCNA test, which detects the toxins biological activity (Planche & Wilcox, 2011). The stool filtrate from a fresh stool sample is inoculated onto cell lines (Vero or McCoy cells) during the experiment (Maniar et al., 1987). The presence or absence of characteristics cytopathic effect (CPE) is noticed after 24 to 48 hours of incubation and confirmed by neutralization with a specific antitoxin (Planche & Wilcox, 2011). This test is time-consuming and hence it is not suitable for routine diagnosis (Strachan et al., 2013). Although the test is highly sensitive and specific, fresh stool samples must be used, to guarantee that the toxin's activity is not lost during the test (Freeman & Wilcox, 2003; Strachan et al., 2013). The test is also technically demanding, therefore the capacity to perform tissue cultures limits its utility in clinical settings. Finally, because this method is not standardized, the findings will differ depending on the type of cells utilized, the stool dilution, and the incubation period (Burnham & Carroll, 2013).

2.7.3 Glutamate dehydrogenase (GDH) enzyme detection

Immunoassays for glutamate dehydrogenase (GDH) enzyme detection are more sensitive test than toxin assays. However, due to its low specificity and high negative predictive value, it cannot be used as a standalone test and is thus recommended for preliminary screening in a multistep algorithm (Ticehurst et al., 2006). The test is performed directly on the stool and has a quick turnaround time. GDH is an enzyme found in the cell wall of *C. difficile* that is produced by both toxigenic and non-toxigenic strains of *C. difficile.* As such, a positive culture result is not indicative of an infection and must be confirmed by other complementary tests such as CCNA or TC. Several chemiluminescent immunoassays and EIAs panel kits that combine detection of GDH and the toxins have been developed in recent years and are now commercially available for diagnostic use (Blaich et al., 2017; Makristathis et al., 2017; Yoo et al., 2019). Despite the diverse diagnostic performance, these assays are rapid and cost effective.

The C. Diff Quik Chek Complete assay, for example, consists of a lateral flow kit that detects both GDH and the toxins A and B directly from stool samples (Quinn et al., 2010). When compared to performing each of the individual tests separately, this test is extremely sensitive. Samples that are negative for GDH and toxins are eliminated at this stage, but those with contradictory results (GDH positive/toxin negative or GDH negative/toxin positive) are subjected to NAAT, preferably GeneXpert *C. difficile* PCR assay, for confirmation (Sharp et al., 2010). Because of the quick results that support timely patient management, many hospitals globally have adopted this algorithm for routine diagnosis (Chung & Lee, 2017; M. Qutub et al., 2019). GDH performed before a confirmatory test is cost-effective because only positive tests are examined for toxins, lowering the number of PCR tests to be conducted (Sharp et al., 2010).

2.7.4. Nucleic acid amplification tests (NAAT)

The use of NAAT in the diagnosis of CDI is becoming increasingly common in many facilities. When compared to the reference methods discussed above, these tests have a higher sensitivity and specificity; however, the high cost of these tests, as well as the requirement of molecular diagnostics infrastructure, limits their widespread use, particularly in resource-limited settings (Martínez-Meléndez et al., 2017; Schroeder et al., 2014). Genes coding for toxin A, toxin B, binary toxin, 16S ribosomal RNA (rRNA) or other accessory genes are detected using either a singleplex or multiplex assays (Chen et al., 2017). However, while these tests can be used as stand-alone tests, they are expensive for routine diagnosis; therefore, a multi-step algorithm is required, in which only specimens positive for *C. difficile* by GDH are confirmed by NAAT (Schroeder et al., 2014). The FDA has approved a range of PCR based assays (e.g. Cepheid GeneXpert, BD GeneOhm, Prodesse ProGastro Cd) and isothermal or*illumi* gene-based assays (e.g. AmpliVue, or Portrait Toxigenic *C. difficile* Assay [helicase-dependent amplification]) that are currently available for commercial use (Martínez-Meléndez et al., 2017). Aside from the fact that these tests provide results in a short period of time, they also have a high sensitivity and specificity. However, with the widespread use of NAAT to detect *C. difficile,* there has been a tremendous increase in the CDI incidences. This is due to the fact that the test yields a positive result even in cases of asymptomatic carriers, resulting in overdiagnosis and overtreatment in colonized individuals (Koo et al., 2014; Polage et al., 2015).

2.8 *C. difficile* molecular typing methods

Strain typing is fundamental in describing the epidemiological dynamics of a diseases, investigating and monitoring disease outbreaks, and guiding in the designing of infection control interventions. Various methods have been developed and have evolved from phenotypic to genotypic based approaches. These methods are designed to detect genetic polymorphism occurring within the genome and cluster homologous isolates in relation to their toxin profile, antibiotic resistance pattern and epidemiological relationship (Huber et al., 2013; Killgore et al., 2008). In the 1980s and 1990s, methods such as surface-layer protein A-encoding gene (*slpA*) typing, restriction typing, restriction endonuclease analysis (REA), pulsed-field gel electrophoresis (PFGE), antibiogram, toxinotyping, PCR ribotyping, multilocus sequence typing (MLST), and Multiple-locus variable-number tandem-repeat analysis (MLVA) were used in *C. difficile* typing. However, recent advances in molecular technology have resulted in modifications to some of these techniques, including modified multiple-locus variable number tandem repeat analysis (mMLVA) and capillary PCR-ribotyping. Additionally, following the first sequencing of whole genome of *C. difficile* in 2006, Whole genome sequencing (WGS) appears to be the current most promising typing method globally (Didelot et al., 2012; Sebaihia et al., 2006). Utilization of these methods is dependent on their discriminatory power and gene expression stability as a consequence more techniques or ultimate ways of utilizing the available approaches with improved discriminatory ability are being developed. A research in Germany combined PCR ribotyping, MLVA, and WGS to identify an outbreak of ribotype 018, and from this study they proposed a two-step typing algorithm consisting of an initial step of capillary electrophoresis ribotyping and a subsequent WGS or MLVA for identical ribotypes (M Krutova et al., 2019). Furthermore, the hypervirulent strain BI/NAP1/027 is classified according to the three genotypic methods (REA group BI, North American PFGE type 1, and PCR ribotype 027) (L. C. McDonald et al., 2005). Some of the methods used in the typing of *C. difficile* will be briefly described in this section.

2.8.1 Toxinotyping

Toxinotypes of *C. difficile* were defined by Rupkin et al as strains with variable coding regions due to insertions and deletions in restriction sites of toxins A and B coding regions (PaLoc) in comparison to the VPI 10463 reference strain. Such that strains with similar changes in these regions are classified in the same toxinotype group, while those identical to

the reference strain are classified as non-variants and belong to toxinotype 0 (Maja Rupnik et al., 1998; Maja Rupnik & Janezic, 2016). As described in section 2.4, the PaLoc is primarily composed of three fragments: A1 and B1 (catalytic domain), A2 and B2 (putative translocation domain), and A3 and B3 (repetitive domain), but toxinotyping is only based on changes occurring on A3 (for *tcdA*) and B1 (for *tcdB*) as shown in figure 2.9 (Maja Rupnik et al., 1997). *C. difficile* variant strains have to date been classified into 34 toxinotypes assigned roman I to XXXIV. The toxinotypes are broadly classified into two categories: minor toxinotypes, which show changes in only one part of the toxin gene fragment. and major toxinotypes, which show changes in almost the entire toxin gene fragment (M Rupnik, 2008; Maja Rupnik & Janezic, 2016).

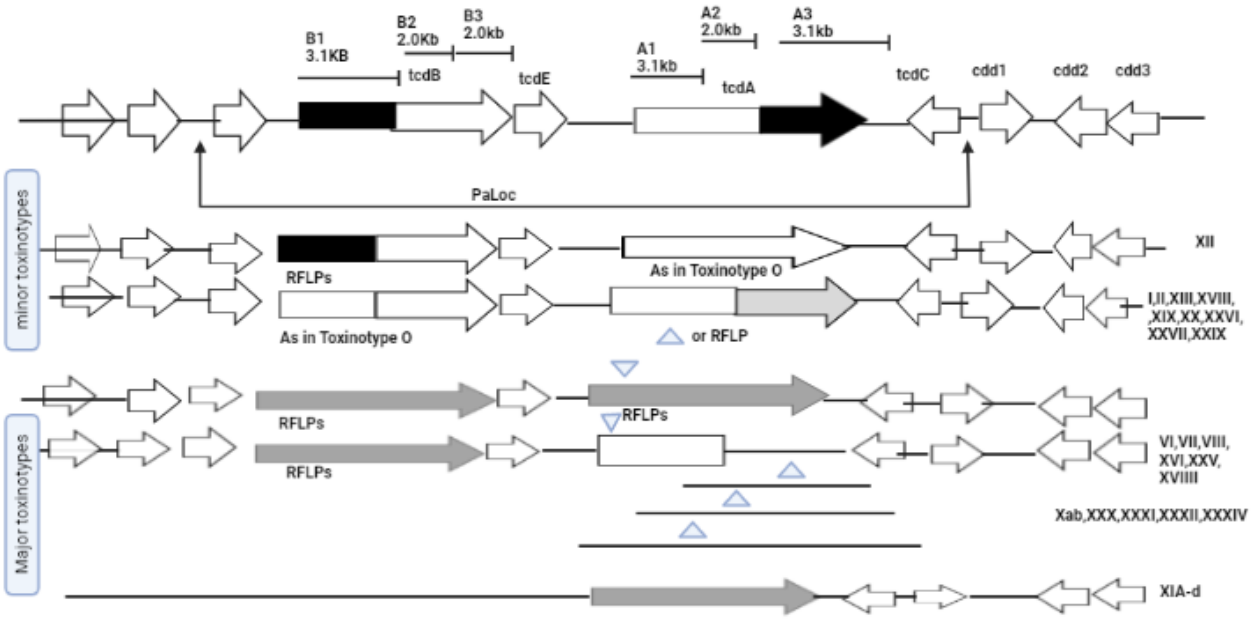


Figure 2.9: PaLoc fragment and toxinotypes. An overview of the PaLoc region showing the changes in B1 and A3 of the various toxinotypes, classified as minor and major toxinotypes. It is worth noting that toxinotypes correlate with toxin profiles (toxin A, toxin B, and binary toxin), such that different toxinotypes can have the same toxin profiles as shown in table 2.2, with the exception of the recently identified A+B− which does not react with standard primers used for toxinotyping (Monot et al., 2015; Maja Rupnik & Janezic, 2016).

Toxinotyping employs a PCR based restriction fragment length polymorphism (RFLP) method in which PCR fragments B1and A3 are amplified and then subjected to restriction enzyme digestions to identify single nucleotide polymorphisms (Maja Rupnik et al., 1997). The restriction profiles of the variant isolates are then generated based on insertions, deletions, or

point mutations seen in the PaLoc and compared to that of the reference strain before being assigned to the respective toxinotypes. Insertions/deletions are common in the *tcdA* gene, whereas point mutations are typically more common in the *tcdB* gene (Maja Rupnik & Janezic, 2016).

The method has been widely used in describing toxinotypes that are widespread in humans, such as toxinotypes III of PCR ribotypes 027, 034, 075, and 080, toxinotype IV of PCR ribotype 023, toxinotype V of PCR ribotypes 078/126, and toxinotype VIII of PCR ribotypes 017 and 047 (M. Rupnik et al., 2001). Additionally, toxinotype XI, a novel toxinotype that produces the binary toxin but lacks genes encoding both toxins A and B and is associated with clinical diseases, has recently been identified (Eckert et al., 2015).

Although this method does not provide a high level of strain discrimination, it is still useful in epidemiologic studies in investigating the genetic changes and evolution of the PaLoc and can be utilized as a baseline in the development of new/advanced diagnostic tools for strain typing in the near future.

Table 2.2: Updated Toxinotypes with corresponding toxin profiles

Toxin profiles	**Toxinotype**
A+B+CDT−	0, I, II, XII, XIII, XVIII, XIX, XX, XXIXX, XXVIXXI, XXVI, XXVII, XXIX, XXXIII
A+B+CDT+	0/V, IIIa, IIIb, IIIc, IIId, IIIe, IV, V, VI, VII, IXa, IXb, IXc, IXd, XIVa, XIVb, XXIIXIVb, XXII, XXV, XXVIII
A−B+CDT−	VIII, XXXII, XXXIV
A−B+CDT+	XVI, Xa, Xb, XXXI, XXX
A−B−CDT+	XIa, XIb, XIc, XId

Drawn from the updated toxin types review by (Maja Rupnik & Janezic, 2016)

2.8.2 PCR ribotyping

PCR ribotyping is a method of determining polymorphisms occurring in the variable intergenic spacer region (ISR) or internal transcribed spacers (ITS) between the 16S and 23S rRNA genes (Bidet et al., 1999). The prokaryotic ribosomal RNA (rrn) operon is made up of three highly conserved regions: 16S, 23S, and 5S. ISR/ITS are found between the 16S and 23S of the rRNA operon. The ribotyping phylogeny is based on flank nucleotide sequences found in the ISR/ITS, resulting in short oligonucleotide fragments of varying lengths that cause variation between closely related species (Gurtler, 1993). The copy number of the rrn

operon varies between *C. difficile* strains and ranges from 9 to 15, with *C. difficile* strain 630 having 11 copies (Gürtler & Grando, 2013; Sebaihia et al., 2006). Various methods can be used to determine the number and size of the fragments. Previously, the ribotypes were identified on a conventional agarose gel-electrophoresis using southern hybridization. With this method, it was extremely difficult to distinguish between closely related ribotypes. The current recommended method is capillary gel electrophoresis (CE) PCR ribotyping, which has been shown to have a high resolution (Warren N Fawley et al., 2015). Using this method, a single pair of primer set, each containing a fluorescent labeled 16S specific primer and a 23S specific primer is used to amplify the ISR/ITS. The amplicons are then subjected to fragment analysis on a genetic analyser. A software program is used to compare the major peaks, and the data is then loaded into a web-based database (http://webribo.ages.at) in order to determine the specific PCR ribotypes (Warren N Fawley et al., 2015). Most countries in Europe have adopted this method as a standard typing method for tracking the spread and outbreaks of *C. difficile* over time (M. Krutova et al., 2018)

By 1999, Stubbs et al (1999) had constructed a C. *difficile* library containing 116 distinct *C. difficile* PCR ribotypes (S. L. Stubbs et al., 1999). However, approximately 200 distinct *C. difficile* PCR ribotypes have been identified to date (Tenover et al., 2011) and over 600 exist in the *C. difficile* Ribotype Network (CDRN) database, but only a few are known to be pathogenic to humans (Warren N Fawley et al., 2015). Studies have demonstrated R027, R017 and R078 PCR ribotypes have emerged in outbreaks and have distinct clonal lineages, highlighting the pathogen role in the ongoing strain evolution. Table 2.3 lists some of the ribotypes described to date with their corresponding strain types and toxin profiles. The most important and widely discussed PCR ribotype 027 was first reported in Quebec, Canada (Pepin, 2004), and has since been linked to significant outbreaks and severe CDI in the United States and Europe (Barbut et al., 2007; Bidet et al., 1999; Kuijper et al., 2008). Comparative phylogenetic studies have revealed that PCR-ribotype 027 is constantly evolving and shares similar characteristics with other ribotypes. An additional significant ribotype is the PCR-ribotype 176, which has been shown to closely related to ribotype 027 (Marcela Krutova et al., 2014). Capillary gel electrophoresis PCR ribotyping has thus provided an ultimate scheme in *C. difficile* strain discrimination when compared to other typing methods (Janezic & Rupnik, 2019; Kociolek, Perdue, et al., 2018). In order to integrate ongoing *C. difficile* surveillance data, laboratories should implement the standardized protocol developed by Fawley and colleagues (Warren N Fawley et al., 2015).

Table 2.3: Reference list of *C. difficile* ribotypes with related strain types and toxinA/B profiles

PCR ribotype	Type strain	Toxin A/B Profile	PCR ribotype	Type strain	Toxin A/B Profile	PCR ribotype	Type strain	Toxin A/B Profile
001	R8366	+/+	044	R10976	+/+	089	R8603	−/−
002	R8375	+/+	045	R10842	+/+	090	R10737	+/+
003	R8384	+/+	046	R10991	+/+	091	R8643	−/−
004	R8386	+/+	047	R10541	−/+	092	R10630	+/+
005	R8373	+/+	048	R10069	+/+	093	R8853	+/+
006	R8268	+/+	049	R6320	+/+	094	R10078	+/+
007	R8264	+/+	050	R9414	+/+	095	R8858	+/+
008	R10568	+/+	051	R9549	−/−	096	R9759	+/+
009	R8269	−/−	052	R6155	+/+	097	R8914	+/+
010	R8270	−/−	053	IS21	+/+	098	R9116	−/−
011	R7619	+/+	054	IS22	+/+	099	R7425	−/−
012	R6187	+/+	055	R11652	+/+	100	R12104	−/−
013	R5252	+/+	056	IS25	+/+	101	R10836	+/+
014	R11446	+/+	057	IS27	+/+	104	R9180	+/+
015	R6685	+/+	058	R10456	+/+	106	R10459	+/+
016	R10424	+/+	059	R9304	+/+	107	R9313	+/+
017	R7404	−/+	060	IS40	−/−	110	R7771	−/+
018	R6184	+/+	061	R12099	+/+	111	R10870	+/+
019	R8637	+/+	062	R11382	+/+	112	R8631	−/−
020	R10079	+/+	063	IS47	+/+	114	R11212	−/−
021	R8763	+/+	064	IS48	+/+	115	R11244	+/+
022	R4262	+/+	065	IS49	−/−	116	R11347	+/+
023	R6928	+/+	066	IS51	−/−	117	R10071	+/+
024	R6321	+/+	067	IS52	−/−	118	R11394	+/+
025	R7276	+/+	068	IS56	−/−	119	R11805	−/−
026	R10118	+/+	069	IS59	−/−	120	R11830	+/+
027	R12087	+/+	070	R9367	+/+	121	R9378	−/−
028	R9300	−/−	071	IS64	−/−	122	R9385	+/+
029	R8438	+/+	072	R12095	+/+	123	R11907	−/−
030	R11004	−/−	074	IS72	−/−	124	R11919	−/−
031	R11631	−/−	075	IS93	+/+			
032	R6598	−/−	076	R11548	+/+			
033	IS58	−/−	077	R10955	+/+			
034	IS81	+/+	078	R7605	+/+			
035	R11812	−/−	079	R7606	−/−			
036	CCUG20309	−/+	081	R9764	+/+			
037	R6641	+/+	082	R7638	−/−			
038	NCTC11206	−/−	083	R10566	+/+			
039	R10738	−/−	084	R8768	−/−			
040	R10917	−/−	085	R12098	−/−			
041	R10920	−/−	086	R1880	+/+			
042	R11817	+/+	087	R11840	+/+			
043	NCTC11382	+/+	088	R10855	−/−			

2.8.3 Multiple-locus variable-number tandem-repeat analysis (MLVA)

MLVA is a highly discriminative PCR-based approach for clustering isolates with comparable PCR ribotypes (Van Den Berg et al., 2007). The variable region of the *C. difficile* genome, which contains approximately seven short tandem repeats *(*A6, B7, C6, E7, F3, G8, and H9), is amplified and the size of the resulting fragment is determined using capillary electrophoresis (Sebaihia et al., 2006). The copy numbers are then analyzed, and isolates with a total tandem repeat difference of ≤2 are determined to be genetically identical (Van Den Berg et al., 2007). Various MLVA schemes have been established. For instance, in 2011, Manzoor and colleagues devised an extended MLVA (eMLVA) scheme which showed a strong correlation with PCR ribotyping. With this scheme, the researchers discovered additional MLVA loci, bringing the total number of target sites to 15. However, the increased number of target sites to be amplified renders the technique labor-intensive and expensive (Manzoor et al., 2011). Additionally, because MLVA was unable to detect variability in F3 and H9, a modified MLVA (mMLVA) scheme was designed to enable detection of toxin genes and deletions (Broukhanski et al., 2011).

2.8.4 Whole genome sequencing (WGS)

When compared to the above-mentioned typing schemes, WGS has a higher discriminatory power for distinguishing between closely related *C. difficile* strains. Genetic relationship of strains is determined by detecting single nucleotide variants (SNVs) or utilizing a gene-by-gene approach to construct allelic profiles of core genome genes designated core genome multilocus sequence typing (cgMLST) (Maiden et al., 2013). Bletz and coworkers recently developed a cgMLST platform for *C. difficile* that contains 2,270 targets in comparison to 3,756 genes in the reference strain genome (*C. difficile* strain 630) (Bletz et al., 2018; Riedel et al., 2016). Seven housekeeping gene loci including *adenylate kinase (adk), ATP synthase subunit alpha (atpA), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (dxr), serine hydroxymethyltransferase (glyA), recombinase A (recA), superoxide dismutase (sodA)* and *triose phosphate isomerase (tpi)* are analyzed in classical MLST and categorized into sequence types (ST) but with the introduction of next-generation sequencing, classical MLST is expanded to whole genome MLST (wgMLST) where more genes including repetitive and non-repetitive genes are compared (Griffiths et al., 2010; Janezic & Rupnik, 2019; Quainoo et al., 2017).

Lemee and colleagues were the first to establish the clonal population structure of *C. difficile*, and they distinguished three distinct evolutionary lineages in their findings (Lemee et al., 2004). Additional lineages have been discovered as a result of comparative phylogenomic studies of isolates from various continents. To date, eight distinct phylogenetic lineages (Five clades [clades 1-5] and three cryptic clades [C-I-III]) linked to specific PCR ribotypes and sequence types have been described (Knight et al., 2 015, 2021). Clade 1 is the most diverse group, with around 200 toxigenic and non-toxigenic sequence types (Knight, Elliott, et al., 2015). CDI cases in Europe have previously been associated with PCR RT014 and RT018 belonging to clade 1 (K. A. Davies et al., 2016). Clade 2 has the predominant hypervirulent ST1 strain corresponding to PCR RT027 (Knight, Elliott, et al., 2015). Clade 3 is the least well-characterized of the five clades. This lineage, however, is dominated by common *C. difficile* strains, including the completely sequenced reference strain CD305 and the dominant toxigenic ST5(RT023) strain (Shaw et al., 2020). With exception of ST37, which corresponds to PCR RT017 that expresses deletions at the 3′ end of *tcdA* and the complete *tcdB* toxin gene (A^-B^+), Clade 4 is composed primarily of nontoxigenic variants (D. A. Collins et al., 2013; Liu et al., 2018). This variant is extremely resistant to clindamycin and fluroquinolones and has been associated with disease epidemics in China and Asia (D. A. Collins et al., 2013; Imwattana, Knight, et al., 2019). Additionally, a recent study in Cape Town, South Africa, similarly found a high prevalence of multidrug resistant PCR RT017 in tuberculosis patients (B. Kullin et al., 2017). Lastly, clade 5 is mostly composed of variants of animal origin, with around three sequence types and ten ribotypes, including the most common PCR RT078 (Knight & Riley, 2019).

2.9 Gaps in literature

According to the aforementioned review, it is evident that the pathogenicity, transmission potential, disease progression, management, and genotypic relatedness of *C. difficile* strains isolated from diverse regions in developed nations have been extensively investigated. However, in resource-constrained countries, information in these research areas is limited. Existing literature from Kenyan studies did not capture the status of CDI in hospitalized population, and as previously stated hospitalization, along with other risk factors such as antibiotic use, are the main precursors for CDI development and progression. Due to the high prevalence of asymptomatic colonization, children under the age of two years are excluded from *C. difficile* surveillance studies discussed above. This population, however, was

included in this study due to their potential to serve as reservoirs for clinically relevant strains of *C. difficile.*

As earlier stated, the virulence of *C. difficile* is attributed to the production of toxins A and or B and occasionally binary toxin. While much of the symptomatic disease, severe cases and nosocomial outbreaks have been linked to variants that produce both toxin A and B (A^+B^+), new variants are constantly being discovered. Recent reports indicate that A^-B^+ variant with a truncation in the 3′-region of toxin A gene (*tcdA*) that predominated several years ago is re-emerging in the population. In addition, because this variant cannot be detected by enzyme immunoassays or culture cytotoxin assays, it is frequently omitted during toxigenic typing resulting in a TcdA negative phenotype. As a result, Lemee et al., developed an assay using primers designed to amplify the partially deleted *tcdA* fragment, which allowed for correct characterization of A^-B^+ variant strains (Ludovic Lemee, Dhalluin, Testelin, et al., 2004). The protocol devised by Lemee et al., was employed in this study, with a few modifications (explained in Chaper three) to characterize the toxin profile of the isolates recovered.

As mentioned in section 2.5.1 CDI is triggered by antibiotic exposure and the development of resistance to these antibiotics. This trait has resulted in the global spread of emerging epidemic strains like *C. difficile* RT027, as well as the persistence of specific strains, particularly in health-care settings. Despite these epidemiological shifts, Kenyan clinical microbiology laboratories do not routinely culture and test *C. difficile* isolates for antimicrobial susceptibility. In this regard, there is a scarcity of information on the phenotypic and genotypic resistant strains of *C. difficile* isolates from this region. Understanding the mechanisms that underlie antimicrobial resistance in *C. difficile* is crucial especially when designing disease prevention strategies. As a result, the Clinical and Laboratory Standards Institute (CLSI) guidelines were followed in order estimate the minimum inhibitory concentrations (MICs) of selected antibiotics, and the resistant isolates were detected using the CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints.

A number of key risk factors for HO-CDI were not explored in the studies conducted in Kenya; as a result, in an effort to build on current information and generate data from developing countries, we assessed some of the potential predictors. The risk factors (mentioned in section 2.5) were derived from published HO-CDI studies. A further classification and assessment of the main groups of medication and comorbidity disorders were performed. The *International Classification of Diseases, Tenth Revision, Clinical*

Modification (ICD-10-CM) was used to stratify the comorbidities. The findings generated from this analysis may aid in stratifying patients exposed to certain antibiotics and those with significant comorbidities at an early stage of HO-CDI diagnosis, allowing for monitoring of disease progression and designing of targeted therapy and preventive initiatives.

While numerous research studies in developed counties have used the MLST scheme to relate/discriminate a diverse collection of *C. difficile* isolates, there is a significant gap in the documentation of genetic information of *C. difficile* strains isolated from various settings and regions in Africa. Only two sequenced isolates from Africa are accessible in the public databases for molecular typing and microbial genome diversity (https://pubmlst.org/), one from Zimbabwe and the other from South Africa. The isolate from Zimbabwe was collected from environmental sources, whereas the South African isolate was derived from human clinical samples. New perspectives regarding clustering of *C. difficile* diverse phylogenetic types continue to evolve. Thus, understanding how certain evolutionary variations affect CDI outcomes may impact the development of variant specific diagnostic tests and targeted treatment regimens. As such, this research employed the WGS technique to investigate the genotypic traits linked with virulence and antibiotic resistance and compared the results with phenotypic data. Additionally, the WGS data was evaluated for phylogenetic relationships and evolutionary clades based on the consequent allelic combinations generated as a result of allelic polymorphisms discussed in section 2.8.4.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Overview

This chapter illustrates the details of appropriate methodological approaches applied in assessing the objectives. This was a cross sectional study in which patient were recruitment from inpatient's wards of Kenyatta National Hospital (KNH). Ethical research approval was acquired, and study participants were approached consecutively for consent/assent. Patient recruitment and sample collection occurred over a two-year period to attain the study's computed sample size of 384. Prior to initiating experimental procedures, standard operating procedures (SOPs) (Appendix I) were designed based on published protocols to guide in anaerobic culture methods for initial isolation of *C. difficile* from stool samples, molecular assays for toxin characterization, and the analysis of the genetic relationship of selected isolates using WGS. The culture-based technique was performed at the Department of Medical Microbiology, University of Nairobi, whereas toxin profiling and WGS were carried out at the Center of Medical Microbiology, Kenya Medical Research Institute (KEMRI).

3.2 Study site

This study was conducted in KNH, the largest referral hospital, located in Nairobi County. Aside from providing medical services to the local metropolitan population, the majority of hospital admissions in this facility are referrals from other hospitals/health care facilities across the country. The hospital has approximately a total bed capacity of 1800 and nine inpatient wards. An estimated 50,000 people are admitted each year on average.

Considering that the hospital is solemnly funded by the government, its provision of low-cost specialized health care services has had a significant impact on overcrowding and overstretching of available resources. Furthermore, bed capacity compares less favorably to the large number of hospital admissions. This situation influences the high incidence and transmission of nosocomial infections among the hospitalized population. Overall, these were the factors that determined the choice of this site, in addition to the feasibility dynamics of the study.

3.3 Study design

This study adopted a cross sectional design where the patients were recruited prospectively to establish the epidemiological dynamics of HO-CDI in patients presenting with nosocomial diarrhea and characterize isolates based on their toxin profiles and genetic traits.

3.4 Ethical considerations

The study was reviewed and approved by the Kenyatta National Hospital-University of Nairobi Ethics and Research Committee (KNH-UON ERC) (Reference number P8/01/2014) (Appendix II). The study participants gave written informed consent/assent (Appendix III). The information gathered from the study participants was treated with confidentiality. Permission to undertake the study was granted by KNH research and programs department.

3.5 Sample size calculation

At the time of this study, there was lack of information on nosocomial diarrhea linked to *C. difficile* in Kenya. The sample size was therefore calculated using Cochran's formula (Cochran, 1977), assuming a 5% level of precision and that 50% would present with the symptomatic characteristic of interest (nosocomial diarrhea):

$$n = \left(\frac{z}{m}\right)^2 p(1-p)$$

Where,

n = sample size

z is the critical value based on the desired confidence level at 5% often 1.96

m is the margin of error or precision of the estimate in this case 5% (0.05)

p is the estimated proportion of the study subjects to present with the outcome of interest where we assumed a proportion of 50%

Thus = 1.96^2 X 0.5 X 0.5 / 0.05^2 = 384 participants were recruited

3.6 Study population and participants selection

Study participants were recruited on the basis of presenting with diarrhea 48hrs post admission at KNH. Participants of all age groups were eligible to participate in the study. The study used purposive sampling approach, in which all accessible and consenting participants were recruited into the study until the desired sample size of 384 was attained. In order to recruit the requisite number of participants, two approaches were used. The first approach was to identify the wards with patients who met the study's eligibility criteria. Once these wards were identified, the participants files were reviewed to check if they matched the inclusion criteria, following which the patients were approached for consent.

The second approach involved tracing the participants from the laboratory. Every characteristic stool sample received in the laboratory from inpatient wards was noted and traced back to the specific admission ward to ascertain if the patients met inclusion criteria and to obtain consent. Participation in this study was entirely voluntary.

3.6.1 Inclusion criteria

- Patients of all age groups with a history of admission for more than 48 hours.
- Symptomatic patients presenting with diarrhoea.
- Patients experiencing at least three episodes of unformed or watery stool over 24 hours.
- Written informed consent for the adult patients and assent for the children.

3.6.2 Exclusion criteria

- Adults with poor cognitive function because of inability to make decision.
- Vulnerable group of patients i.e prisoners due to fear on of undue influence.

3.7 Study variables

3.7 1 Outcome variable

The outcome variable in this study was HO-CDI. *C. difficile* infection was defined as presence of unexplained clinical diarrhoeal symptoms (≥3 loose stools in 24 hours) plus a positive NAAT for *C. difficile* toxin A or toxin B or both or binary toxin. A case was considered HO-CDI if the patient had a positive test for *C. difficile* infection and presented with symptoms >2 days after admission.

3.7.2 Independent variables

The demographic and clinical variables included age, gender, date of admission, admission ward, reason for admission, duration of hospitalization, history of admission in the last three months, invasive procedures (surgery, colonoscopy/endoscopy/sigmoidoscopy and nasogastric tube feeding [NGT]) and diarrhea-related symptoms such as fever, dehydration, adnominal pain or distension, and vomiting. Pharmacological agents were categorized into antibiotics, acid suppressive agents (ASAs), antiretrovirals (ARVs), chemotherapy, laxatives, and analgesics while comorbid conditions were categorized as per ICD-10-CM as documented in appendix IV. Comorbidity was defined as the pre-existence of one or more medical conditions coexisting with the primary condition. A total of 22 specific comorbidities were considered: congestive heart failure, cardiac arrhythmias, chronic obstructive

pulmonary disease (COPD), peripheral vascular disease, hypertension, peptic ulcer disease, diabetes, hemiplegia, hypothyroidism, pulmonary circulation disorders, chronic kidney disease (CKD), liver disease, leukemia, solid tumor without metastasis, metastatic solid tumor, HIV/AIDS, lymphoma, tuberculosis, rickets, inflammatory bowel disease, weight loss (malnutrition), iron deficiency anemia, and depression.

3.8 Study procedures

3.8.1 Data collection process

A structured questionnaire (Appendix V) was designed to aid in collection of demographic and clinical data from the study participants. Potential questions were derived from the European surveillance of *Clostridioides difficile* infections Surveillance protocol version 2.1, developed in 2015 (Kola et al., 2015), as well as from relevant information gathered from literature. Each consenting participant was assigned a unique identifier. This number was recorded on the questionnaire and on the stool sample collection container, and the key information was retrieved in accordance with the questionnaire.

3.8.2 Specimen collection and storage

Consenting participants were given a sterile container labelled with their unique identifier and asked to provide a stool sample. The samples were transported in a cool box the same day they were collected. Upon arrival at the laboratory, the stool specimens were first qualified against the Bristol visual stool scale (Kasırga, 2019) (Appendix I). The stool sample types 4 and 5 were then considered for testing. The samples were either processed within 30 minutes of arrival or refrigerated at -20 °C for up to three days before culture.

3.8.3 Pre-treatment and culture of stool samples

Prior to culture, the stool samples were pre-treated by alcohol shock technique to eliminate the vegetative organisms as previously described (Riley et al., 1987). Briefly, in a 1 ml Eppendorf tube, each stool sample was suspended in an equal volume of absolute alcohol. The suspension was mixed by vortexing and allowed to stand for 1 hour at room temperature. The mixture was then centrifuged at 4000rpm for 1 minute and 50 µl of the deposit was streaked on Cycloserine-cefoxitin egg yolk agar (CCEY) supplemented with egg yolk cycloserine/cefoxitin (LabM, United Kingdom) and lysed sheep blood. The culture plates were subsequently incubated anaerobically at 37 °C for 48 hours using anaerobic jars and anaerobic gas generating sachets (Oxoid, United Kingdom).

3.8.4 Phenotypic identification and storage of *C. difficile* isolates

Following incubation, the culture plates were examined for *C. difficile* growth. The colonies were identified using typical morphological properties on culture media and gram stain characteristics. On CCEY, *C. difficile* produced grey, opaque, elongated fimbriated edged colonies with a characteristic phenolic odor. The colonies were also examined for the presence of gram positive rods. *Clostridioides difficile* DSMZ 27147 (Leibniz Institute DSMZ, Brunswick, Germany) was used as a positive control (Appendix VI). All positive pure *C. difficile* isolates were then preserved in duplicate. The isolates were preserved at room temperature in brain-heart infusion (BHI) agar slants for short-term storage. Long-term preservation was accomplished by inoculating a loopful of the pure growth at -80 °C in 1 mL of BHI (LabM, United Kingdom) containing 15% glycerol (v/v).

3.8.5 Antibiotic Minimum Inhibitory Concentration (MIC) Assay

Minimum inhibitory concentrations (MIC) were determined by ETEST as described by (Clinical and Laboratory Standards Institute, 2019). Pure culture colonies of *C. difficile* isolates were diluted to a 0.5 McFarland standard and swabbed on Brucella Base agar (Becton & Dickinson, USA) supplemented with 5% sheep blood, vitamin K1 (Becton & Dickinson, USA) and hemin (Becton & Dickinson, USA). E-test strips (BioMérieux, France) for vancomycin, metronidazole, ciprofloxacin, erythromycin, clindamycin, tetracycline, ceftriaxone and rifampicin were then applied on the inoculated culture plates. Following a 48 hour anaerobic incubation period, MICs for each of the antibiotics tested were determined and interpreted as per CLSI (M100, 2020) and EUCAST (version 10) guidelines. The MIC breakpoints were categorized into susceptible (S), intermediate (I), and resistant (R). The MIC breakpoints for all the antimicrobials tested except rifampicin were determined according to CLSI 2020. The EUCAST defined epidemiological cut-off value (ECOFF) of 0.004 mg/L was used to interpret the susceptibility of rifampicin. Both CLSI and EUCAST standards do not specify the breakpoints for ciprofloxacin, so the CLSI breakpoints of moxifloxacin were used as a proxy. *C. difficile* DSM 27147 (R20291) with published MICs (vancomycin [0.2–0.93 mg/L], metronidazole [0.21–0.9 mg/L], clindamycin [18 mg/L], erythromycin [≥256 mg/L], ciprofloxacin [≥32 mg/L], tetracycline [0.22 mg/L]) served as an internal control (M. L. Kelly et al., 2016; Mathur et al., 2013; Stabler et al., 2009).

3.8.6 Molecular assay

3.8.6.1 DNA extraction

Prior to DNA extraction, the isolates were recovered from frozen stocks by plating them on chromogenic agar (CHROMagar *C. difficile*, Paris, France) and validated using the criteria outlined in section 3.8.4 above. DNA extraction was carried out in accordance with instructions provided by Zymo Research Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA, USA). Briefly, a loopful of discrete colonies of each isolate was suspended in 200 µl of nuclease free water in Eppendorf tubes. The tubes were vortexed before being transferred to a bashing bead lysis tube, to which 750 µl of the bashing bead buffer was added. The tubes were then placed in a bead beater for 10 minutes. In a microcentrifuge, the bashing bead lysis tubes were centrifuged at 10,000xg for 1 minute at room temperature. 400 µl of the supernatant was transferred to a Zymo-Spin III-F filter in a collection tube and centrifuged at 8,000 xg for 1 minute. In the collection tube, 1,200 µl of the genomic lysis buffer was added to the filtrate, and 800 µl of the mixture was transferred to Zymo-Spin II-CR column 3 in a collection tube. The mixture was centrifuged for 1 minute at 10,000xg, with the flow-through from the collection tube discarded. In a new collection tube, 200 µl DNA pre-wash buffer was added to the Zymo-Spin II-CR column and centrifuged at 10,000xg for 1 minute. After that, 500 µl g-DNA of the wash buffer was added to the Zymo-Spin II-CR Column and centrifuged at 10,000xg for 1 minute. The Zymo-Spin II-CR Column was then transferred to a clean 1.5 ml microcentrifuge tube where 50 µl of the DNA elution buffer was added, and the DNA was eluted by centrifugation at 10,000xg for 30 seconds. The extracted DNA was then stored at 2–8 °C.

3.8.6.2 Quantification of DNA

The concentration of double-stranded DNA (dsDNA) in the extracted samples was measured using the Qubit dsDNA HS (High Sensitivity) Assay Kit in a Qubit Fluorometer System (Invitrogen, UK). A Qubit working solution was prepared in a plastic tube for each batch of samples to be measured by diluting 1 ml of Qubit dsDNA HS Reagent in 199 ml of the Qubit dsDNA HS Buffer to make a final volume of 200 µl. The mixture was then vortexed, and 190 ml aliquoted into each standard tube, while 199 ml was added into labelled sample tubes. A final volume of 200 µl solution was generated for each labelled assay tube by adding 10 ml of the standards (Qubit dsDNA HS Standard #1 & Qubit dsDNA HS Standard #2) into each standard tube and 1 ml of the gDNA into each sample tubes. The mixture was vortexed for 2–

3 seconds and incubated at room temperature for 2 minutes before loading the tubes into the Qubit Fluorometer device to obtain gDNA concentrations in the samples.

3.8.6.3 Real time PCR (qPCR) for the detection of *tpi* gene

A qPCR assay using primers targeting the *tpi* house keeping gene was performed to confirm the culture positive *C. difficile* isolates. In accordance with a protocol published by Lemee et al, the tpi-F AAAGAAGCTACTAAGGGTACAAA and the tpi-R CATAATATTGGGTCTATTCCTAC were used to detect the presence of this gene (Ludovic Lemee, Dhalluin, Testelin, et al., 2004). The qPCR amplification and analysis were performed using a Magnetic Induction Cycler and micPCRv2.4.0 software, respectively (Bio Molecular Systems, Sydney, NSW, Australia). A 20 µl reaction was prepared by mixing 10 µl of 2X Luna universal qPCR mix SYBR Green master mix (New England Biolabs, MA, USA) with 7 µl of nuclease-free water, 0.5 µl of 10 micro molar (µM) of both forward and reverse primers, and 2 µl of template DNA. The real-time PCR profile was set up as follows: a cycle of 95 ˚C for 3 minutes, followed by 40 cycles of 95 ˚C for 30s, 60 ˚C for 30s, and 72 ˚C for 30s. To test for specificity, a post amplification dissociation curve was generated with a ramp from 72 ˚C to 95 ˚C for the melting curve stage.

3.8.6.4 qPCR for the detection of tcdA, *tcdB, cdtA* and *cdtB* genes

All the *tpi* positive isolates were then subjected to a singleplex SYBR Green based qPCR assay to investigate the presence of *C. difficile* toxin A (*tcdA*), toxin B (*tcdB*) as well as the accessory toxins A (*cdtA*) and toxin B (*cdtB*) genes. For all investigations, amplification was carried out using Magnetic Induction cycler with MICv 2.4 (Bio Molecular Systems, Sydney, NSW, Australia). A final volume of 20 µl reaction containing 10 µl of 2X Luna qPCR universal master mix blend (New England Biolabs, MA, USA), 7 µl of PCR water, 0.5 µl of 10 µM forward and reverse primers (primer list used in this assay shown in table 3.1 below) and 2 µl of extracted DNA was prepared. The real-time PCR profile was programmed as follows: a cycle of 95 ˚C for 3 minutes, followed by 40 cycles at 95 ˚C for 30s, 60 ˚C for 30s, and 72 ˚C for 30s. To test for specificity, a post amplification dissociation curve was generated with a ramp from 72 ˚C to 95 ˚C for the melting curve stage. A *C. difficile* toxigenic strain was defined one that expressed either *tcdA*, *tcdB, cdtA/B,* or all of these genes. *Clostridioides difficile* DSMZ-27147 (Leibniz Institute DSMZ, Brunswick, Germany) that expresses all these genes was used as an internal positive control strain to validate the PCR results.

Table 3.1: List of primers used in qPCR assay

Main target	Gene target	Primer	Primer sequence 5'- 3'	Product size (bp)	Reference
Toxin A	*tcd A*	tcd-F tcd-R	AGATTCCTATATTTACATGACAATAT GTATCAGGCATAAAGTAATATACTTT	369/110	(Ludovic Lemee, Dhalluin, Testelin, et al., 2004)
Toxin B	*tcd B*	tcd-F tcd-R	GGAAAAGAGAATGGTTTTATTAA ATCTTTAGTTATAACTTTGACATCTTT	160	(Ludovic Lemee, Dhalluin, Testelin, et al., 2004)
Binary toxin	*cdt A*	cdt-F cdt-R	TGA ACC TGG AAA AGG TGA TG AGG ATT ATT TAC TGG ACC ATT TG	375	(S. Stubbs et al., 2000)
	ctd B	ctd-F ctd-R	CTTAATGCAAGTAAATACTGAG AACGGATCTCTTGCTTCAGTC	510	

3.8.7 Whole-genome sequencing

3.8.7.1 Library construction and sequencing

At the inception of this assay, the DNA template was quantified as described in 3.8.6.2 where samples with DNA concentration of >/=25 ng/ul were selected for sequencing. WGS was performed on 32 selected isolates using oxford nanopore platform in two batches of sixteen samples each. The protocol (version: NBE_9065_v109_revAL_14Aug2019) from Oxford Nanopore Technologies manufacturer was followed (Appendix VII). A starting DNA input of 1 µg of the DNA template adjusted to a final volume of 49 µl with nuclease free water was prepared. The ends of fragmented DNA template (48 µl) were blunted to get rid of damaged bases using 2 µl NEBNext FFPE DNA repair mix, 3.5 µl NEBNext FFPE DNA repair buffer, 3.5 µl of Ultra II End-prep reaction buffer and 3.5 µl Ultra II End-prep enzyme mix in a total volume of 60 µl. The reaction was incubated at 20 °C for 5 minutes and heat inactivated at 65 °C for 5 minutes. The repaired and end prepped DNA was then purified in 60 µl of Agencourt AMPure XP beads and two subsequent clean-up using 70% alcohol. The purified product was then eluted in 25 µl of nuclease free water. Each sample was then tagged with unique barcode adapters such that 22.5 µl of 500 ng end prepared samples (two samples from batch 1 and 5 from batch two omitted from further processing because of low DNA concentration) were tagged with 2.5 µl of the native barcodes (1-12 [EXP-NBD 104] and 13-24 [EXP-NBD 114]) in 25 µl of blunt/TA ligase master mix to make a final volume of 50 µl. This reaction was incubated for 10 minutes at room temperature following which purification using 50 µl of Agencourt AMPure XP beads and two step clean-up using 70% alcohol. The purified products were then eluted in 26 µl of nuclease free water. The barcoded samples were then pooled together into one tube. Each barcode adapter also has a cohesive end, and this is used as a hook to ligate to the supplied sequencing adapters. Approximately15 ng of the pooled library was then loaded onto R10.3 flow cell and sequencing performed on MinION device for 30 hours.

3.9 Data analysis

Descriptive and inferential statistics were applied to generate associations between study variables. The data obtained was summarized and presented in form of tables and figures. The statistical analysis and visualization were performed using STATA version 13.1. To investigate the distribution of HO-CDI within the different variables including clinical information, pharmacological agents and stratified comorbidities, data were summarized into frequencies (n) and proportions (p) of positive and negative patients, with the outputs

presented in tables. The Z-test for proportion was then used to assess for significant differences between the two proportions. Fisher's exact test was used to analyze distributions that contained less than six patients in at least one group, regardless of whether positive or negative for *C. difficile*. Additionally, chi-square test for independence was calculated for variables with more than one category to ascertain an association between the grouping variable and HO-CDI outcome. In all cases, a p value of < 0.05 and confidence intervals that did not include the null value (1 for OR and 0 for log OR) justified statistical significance. For the comorbidities, each individual comorbidity was explored separately, and for the construction of the comorbidity scores, each comorbid condition was allocated a weight based on its relative mortality risk for risk adjustment (Appendix IV). The indexes were then combined to provide the overall scores and categories prior to examining their relationship with HO-CDI.

A binary logistic regression analysis was conducted in a sequential approach to identify significant predictors of HO-CDI and the results were presented in a forest plot. To begin, variables with p values of ≤ 0.2 in Tables 4.2, 4.3 and 4.4 were selected for the model development stage along with variables known to be clinically relevant based on the literature. To obtain crude odds ratios of the risk factors associated with the likelihood of developing HO-CDI, the variables were individually fitted into bivariate binary logistic regression models. Secondly, variables with p values ≤ 0.05 in the bivariate analyses were considered in final multiple binary logistic regression models that adjusted for potential confounders. Significant variables related with the probability of developing HO-CDI were identified using the adjusted odds ratio (AOR), corresponding p value and the 95% confidence interval (CI). Variables with $p \leq 0.05$ were considered statistically significant. The goodness of fit test was used to assess whether adding more parameters to the models had significant impact in predicting HO-CDI outcome. Here, the likelihood ratio test (assumed to follow chi-square distribution) was constructed by calculating the difference between the log-likelihoods of the simple and complex models, as well as the degrees of freedom represented by additional parameters in the complex model. Finally, the models fit were evaluated using a confusion matrix to test their predictive ability for HO-CDI outcome.

3.10 Bionformatics analysis of WGS data

The reads were base called in real-time using Guppy v3.22 base caller embedded in the MinKNOW software (Oxford Nanopore Technologies, Oxford, UK) with the quality score of

>6. Porechop (https://github.com/rrwick/Porechop), a python command-line tool for demultiplexing Oxford Nanopore reads from FASTQ files, was used to do the demultiplexing and adaptor trimming to a minimum coverage of 30x. Unicycler (https://github.com/rrwick/Unicycler), a bacterial genome assembly workflow that generates draft assemblies/genomes, was used to perform de-novo genome assembly. Bandage (https://rrwick.github.io/Bandage/), was used to visualize the genomes/draft assemblies. Prokka (https://github.com/tseemann/prokka), was used to annotate the draft assembly genomes. The comprehensive antibiotic resistance database (https://card.mcmaster.ca/) was used for identifying acquired antimicrobial resistance genes while ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/), was used to conduct a comprehensive analysis of antimicrobial resistance genes established to date in the draft assemblies generated. Virulence genes and plasmids were detected using VirulenceFinder (v.2.0) and PlasmidFinder server (v.2.1) respectively. PubMLST (https://pubmlst.org/) was then interrogated for multilocus sequencing typing on the generated genomes. The whole-genome sequences of the isolates were deposited in the DDBJ/ENA/GenBank databases under BioProject number PRJNA732612.

3.11 Phylogenetic comparisons

Five genomes with similar subtypes as those from this study were selected from GenBank (Appendix VIII). *C. difficile* core genome alignment was done using Roary (https://sanger-pathogens.github.io/Roary/) and the results were compared and presented in a phylogenetic tree. Maximum-likelihood phylogenetic tree was generated using RaxML (v0.9.0).

CHAPTER FOUR: RESULTS

4.1 Respondance rate

Figure 4.1 illustrates the study participants recruitment process. At the inception of this study, we aimed to prospectively recruit 384 patients. Between the year 2016 and 2018, 356 patients qualified to take part in the study. In the end, only 333 consented to participate in the study, completed the questionnaires, and provided stool samples for microbiological analysis. The twenty-three patients fell out for the following reasons: five patients did not return the consent form, three failed to present stool samples for microbiological analysis, three patients provided inappropriate samples, six others provided duplicate samples, and four questionnaires were incomplete, while two patients withdrew their consent prior to sample collection, yielding an overall response rate of 93.5%.

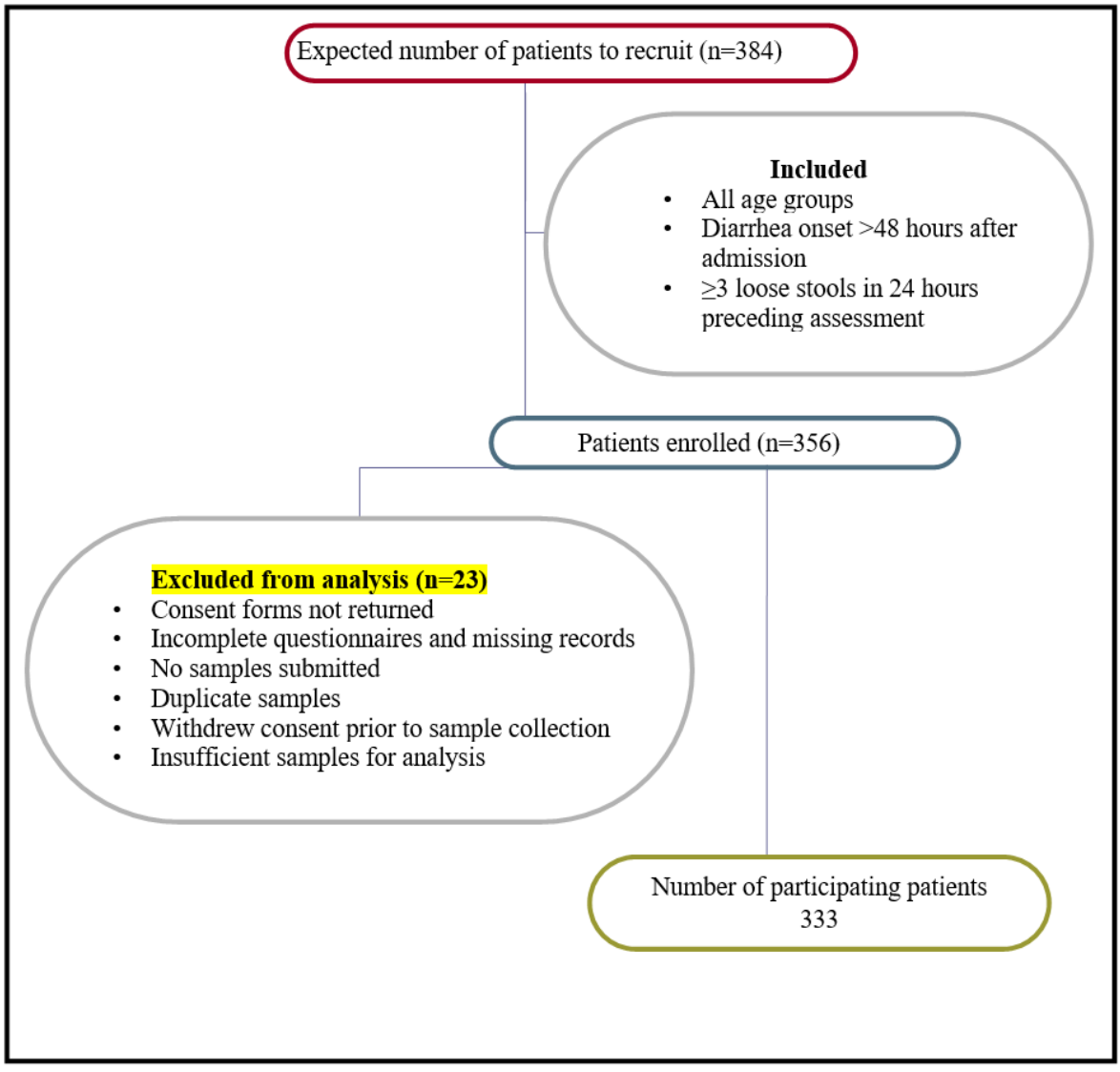


Figure 4.1: Patient recruitment flowchart

4.2 Demographic, clinical characteristics of the participants and the prevalence of HO-CDI

The ages of the study participants ranged from one month to 83 years (mean 23.25, standard deviation (SD) = 21.17), with the majority, 108 (32.4%), falling between the ages of 26 and 45. There were 101 (30.3%) participants under the age of two years. Over half of the study participants 170 (51.1%) were female. The average length of stay in the hospital was 6.74 weeks (SD = 18.69), with 48 (14.4%) reporting previous hospital admission in the last three months. Patients reported vomiting (37.2%), abdominal pain (11.7%) and dehydration (18.1%) during diarrhoeal episodes, with some presenting with bloody diarrhoea (6.3%). Antibiotic use was documented in 297 (89.2%) of patients, with the majority receiving more than two antibiotics. In total, more than half of the 230 patients (69.1%) had underlying conditions. Sixty-five patients (19.5%) had a history of surgery, while 26 (7.8%) had received nasogastric feeding, and an additional 9 (2.7%) had undergone colonoscopy/sigmoidoscopy/endoscopy procedures prior to the diarrhea episodes. Nearly 71 patients were diagnosed with HO-CDI resulting in a prevalence of 21.3%. A summary of the baseline characteristics of the 333 study participants is provided in Table 4.1.

Table 4.1: Demographic, clinical characteristics of the study participants and the prevalence of HO-CDI

Variables	Total (*n*=333)	%
Age in years (Mean ± SD)	23.25 ± 21.7 (IQR, 1month-83years)	
≤2	101	30.3
3-15	44	13.2
16-25	31	9.3
26-45	108	32.4
45-59	30	9.0
≥60	19	5.7
Gender		
Female	170	51.1
Male	163	48.9
Admission ward		
Internal medicine	118	35.4
Paediatrics	116	34.8
Surgery	59	17.7
Other	40	12.0
Admission duration in weeks (Mean ± SD)	6.74 ± 18.69	
≤ 1 week	175	52.6
2 weeks	36	10.8
3 weeks	27	8.1
≥ 4 weeks	95	28.5
Antibiotic use	297	89.2
Number of antibiotics used		
None	36	10.8
One	59	17.7
Two	97	29.1
Three	68	20.4
≥ Four	73	21.9
Duration of antibiotic exposure		
1 week	114	34.2
2 weeks	63	18.9
3 weeks	30	9.0
≥ 4 weeks	90	27.0
History of invasive procedures		
Surgery	65	19.5
NGT	26	7.8
Colonoscopy/sigmoidoscopy/endoscopy	9	2.7
Clinical symptoms		
Abdominal pain	39	11.7
Bloody stool	21	6.3
Dehydration	27	8.1
Vomiting	124	37.2
Duration of diarrhea		
<1 week	283	85.0
1-3 weeks	43	12.9
≥3 weeks	7	2.1

Variables	Total (*n*=333)	%
Previous hospital admission	48	14.4
Comorbidity	230	69.1
Prevalence of HO-CDI	71	21.3

Abbreviations: Interquartile range, IQR; SD, Standard deviation; HO-CDI, Healthcare facility-onset *C. difficile* infection; NGT, Nasogastric tube feeding.

4.3 qPCR for detection of *tpi* housekeeping gene

The 71 *C. difficile* isolates that had been presumptively identified by culture were confirmed by detecting species-specific internal fragment of the *tpi* house keeping gene. The primer pair for *tpi* gene yielded an amplicon of 230bp for each of the 71 *C. difficile* isolates tested as shown in a representative amplification curve and corresponding gel electrophoresis image in figure 4.2 visualized on 1% agarose gel.

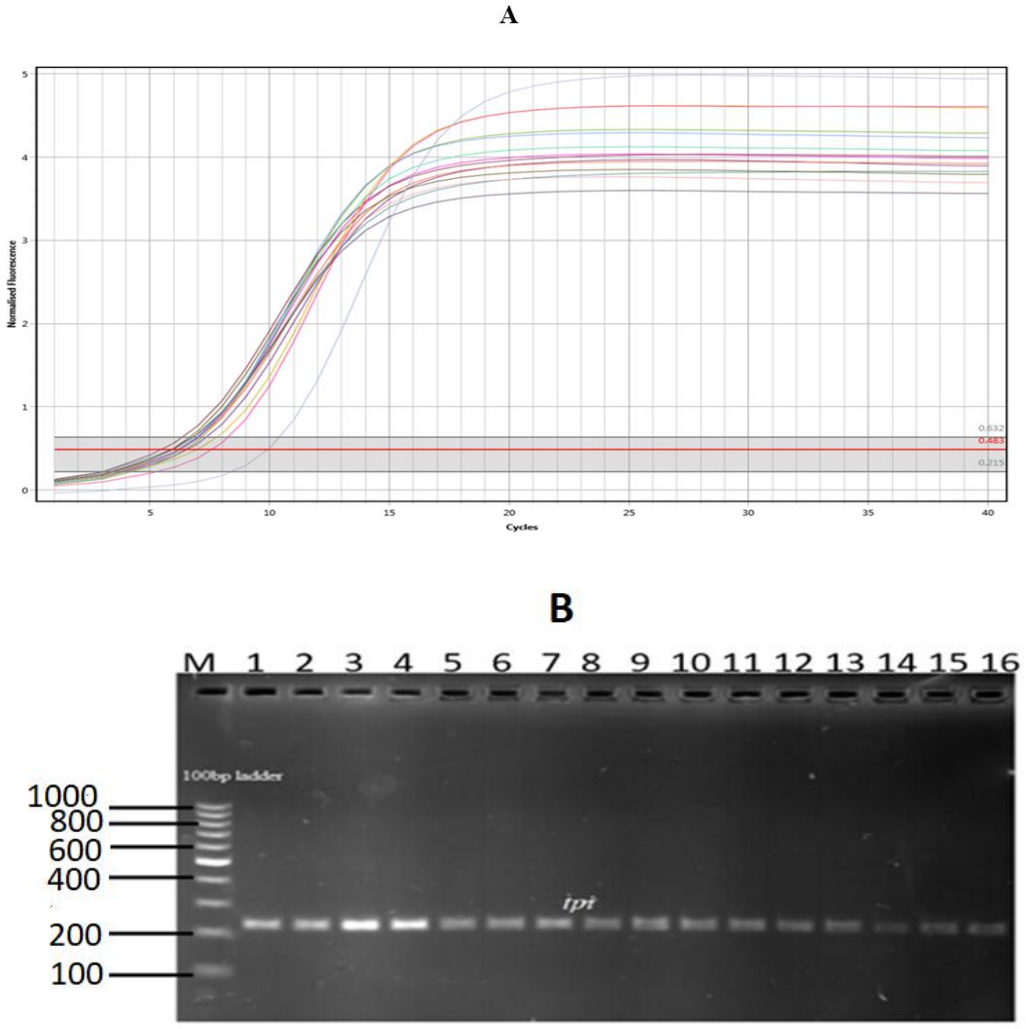


Figure 4.2: qPCR amplification curve and gel electrophoresis image of *tpi* gene. A shows an amplification curve of the *tpi* gene and B a gel electrophoresis image showing the 230bp amplicon.

4.4 Variable characteristics of patients with and without HO-CDI

The data were presented in the form of three tables comparing patients with and without HO-CDI based on demographic, clinical, and admission information (Table 4.2), pharmacological agents exposure (Table 4.3), and comorbidities (Table 4.4). The data were presented in proportions, and Pearson's Chi-square analysis was used to ascertain the differences between variable categories.

The prevalence of HO-CDI is shown in Table 4.2 for the various epidemiological baseline characteristics of the patients. The results indicate that almost all HO-CDI patients (98.6%; $p < 0.001$) received antibiotics, and there is strong evidence that outcome of HO-CDI is associated with number of antibiotics taken ($X^2 = 42.272$, df = 4, $p < 0.001$). Nonetheless, significance was not observed with duration of antibiotic use. Additionally, the prevalence of HO-CDI was significantly higher in patients with a history of previous hospital admission in the preceding three months when compared to non-HO-CDI cases (28.2% vs 10.7%, $p < 0.001$). Comorbid conditions were significantly more prevalent in HO-CDI cases than non-HO-CDI cases, with both groups reporting high prevalence of comorbidities (88.7% vs 63.7%). Although both groups had a low prevalence of NGT feeding (14.1% vs 6.1%, $p = 0.026$), HO-CDI patients was significantly more common in patients with than in those without HO-CDI. Nevertheless, no statistically significant differences were found between the categories of other variables. However, the majority of HO-CDI cases were male 38 (53.5%) and the prevalence was relatively higher in the patients aged 60 years or older (31.6%), as was those who underwent a surgical procedure (25.4%).

Table 4.2: Comparison of demographic, clinical, and admission information between patients with HO-CDI and patients without HO-CDI

Variables	HO-CDI		
	Negative, *n* (%)	Positive, *n* (%)	*p* value[a]
Age group in years			0.183
≤2	87 (33.2)	14 (19.5)	
3-15	31 (11.8)	13 (18.3)	
16-25	26 (9.9)	5 (7.0)	
26-45	83 (31.7)	25 (35.2)	
46-59	22 (8.4)	8 (11.3)	
≥60	13 (5.0)	6 (8.5)	
Gender			0.385
Female	137 (52.3)	33 (46.5)	
Male	125 (47.7)	38 (53.5)	
Admission duration (before diarrhoea onset)			0.107
≤ 1 week	144 (55.0)	31 (43.7)	
2 weeks	23 (8.8)	13 (18.3)	
3 weeks	21 (8.0)	61 (8.5)	
≥ 4 weeks	74 (28.2)	21 (29.6)	
Antibiotic exposure			0.004
No	35 (13.4)	1 (1.4)	
Yes	227 (86.6)	70 (98.6)	
Number of antibiotics exposed to			< 0.001
None	35 (13.4)	1 (1.4)	
One	55 (21.0)	4 (5.6)	
Two	83 (31.7)	14 (19.7)	
Three	48 (18.3)	20 (28.2)	
≥ Four	41 (15.6)	32 (45.1)	
Duration of antibiotic exposure			0.591
1 week	92 (40.5)	22 (31.4)	
2 weeks	47 (20.7)	16 (22.9)	
3 weeks	22 (9.7)	8 (11.4)	
≥ 4 weeks	66 (29.1)	24 (34.3)	
Previous hospital admission hospitalization			< 0.001
No	234 (89.3)	51 (71.8)	
Yes	28 (10.7)	20 (28.2)	
Had surgery			0.162
No	215 (82.1)	53 (74.6)	
Yes	47 (17.9)	18 (25.4)	
NGT			0.026
No	246 (93.9)	61 (85.9)	
Yes	16 (6.1)	10 (14.1)	
Colonoscopy/sigmoidoscopy/endoscopy			0.086
No	257 (98.1)	67 (94.4)	
Yes	5 (1.9)	4 (5.6)	
Comorbidity			< 0.001
No	95 (36.3)	8 (11.3)	
Yes	167 (63.7)	63 (88.7)	

[a], Pearson's χ^2 was used to test the difference between the groups. Abbreviations: HO-CDI, Healthcare facility-onset *C. difficile* infection. NGT, Nasogastric feeding.

The proportion of the participants exposed to different pharmacological agents by HO-CDI is shown in Table 4.3. The number of antibiotics prescribed prior to the diagnosis of HO-CDI was reported and classified into twenty groups. As shown in Table 4.3, additional pharmacological agents included antiretrovirals, acid suppressive agents, laxatives, and analgesics. The most frequently prescribed medications were ceftriaxone 187 (56.2%), proton pump inhibitors 101 (30.3%), a combination of amoxicillin/clavulanic acid 92 (27.6%), analgesics 78 (23.4%), metronidazole 87 (26.1%) and a combination of benzylpenicillin and gentamicin 57 (17.1%). A pairwise comparison of the proportions of HO-CDI positive and HO-CDI negative patients exposed to specific agents revealed significant differences in amoxicillin/clavulanic acid (42.3% vs 23.7%; $p = 0.002$), benzylpenicillin/gentamicin (25.4% vs 14.9% $p = 0.038$), meropenem (21.1% vs 11.5%; $p = 0.034$), ciprofloxacin (21.1% vs 8.4%; $p = 0.002$), clarithromycin (9.9% vs 1.9%; $p = 0.001$), trimethoprim/sulfamethoxazole (22.5% vs 9.5%; $p = 0.003$), clindamycin (14.1% vs 3.1%; $p = 0.001$), ceftriaxone (67.6% vs 53.1%; $p = 0.028$), anti-tuberculosis agents (19.7% vs 9.2%; $p = 0.013$), anti-retroviral agents (23.9% vs 13.0%; $p = 0.023$) and chemotherapy agents (9.9% vs 3.1%; $p = 0.014$). The proportions did not differ between HO-CDI and non-HO-CDI group in patients exposed to metronidazole ($p = 0.097$), amikacin ($p = 0.184$), ceftazidime ($p = 0.793$), flucloxacillin ($p = 0.320$), vancomycin ($p = 0.774$), cefuroxime ($p = 0.448$), proton pump inhibitors ($p = 0.060$), analgesics ($p = 0.090$) and laxatives ($p = 0.820$). The use of azithromycin, tetracycline, erythromycin, linezolid, doxycycline, and histamine-2 receptor antagonists (H2As) was very low, and no proportional significance was observed in HO-CDI outcome.

Table 4.3: Comparison of pharmacological agents exposure between patients with and patients without HO-CDI

Medication	Total *n*	HO-CDI		*p* value
		Negative, *n* (%)	Positive, *n* (%)	
Metronidazole				0.097
No	246	199 (76.0)	47 (66.2)	
Yes	87	63 (24.0)	24 (33.8)	
Amoxicillin/Clavulanic acid				0.002
No	241	200 (76.3)	41 (57.7)	
Yes	92	62 (23.7)	30 (42.3)	
Benzylpenicillin/Gentamicin				0.038
No	276	223 (85.1)	53 (74.6)	
Yes	57	39 (14.9)	18 (25.4)	
Meropenem				0.034
No	288	232 (88.5)	56 (78.9)	
Yes	45	30 (11.5)	15 (21.1)	
Ciprofloxacin				0.002
No	296	240 (91.6)	56 (78.9)	
Yes	37	22 (8.4)	15 (21.1)	
Amikacin				0.184
No	300	239 (91.2)	61 (85.9)	
Yes	33	23 (8.8)	10 (14.4)	
Ceftazidime				0.793
No	312	245 (93.5)	67 (94.4)	
Yes	21	17 (6.5)	4 (5.6)	
Flucloxacillin				0.320
No	317	251 (95.8)	66 (93.0)	
Yes	16	11 (4.2)	5 (7.0)	
Azithromycin				0.201*
No	329	260 (99.2)	69 (97.2)	
Yes	4	2 (0.8)	2 (2.8)	
Clarithromycin				0.001
No	321	257 (98.1)	64 (90.1)	
Yes	12	5 (1.9)	7 (9.9)	
Trimethoprim/sulfamethoxazole				0.003
No	292	237 (90.5)	55 (77.5)	
Yes	41	25 (9.5)	16 (22.5)	
Clindamycin				< 0.001
No	315	254 (96.9)	61 (85.9)	
Yes	18	8 (3.1)	10 (14.1)	
Vancomycin				0.868
No	311	245 (93.5)	66 (93.0)	
Yes	22	17 (6.5)	5 (7.0)	
Tetracycline				0.213*
No	332	262 (100)	70 (98.6)	
Yes	1	0 (0.0)	1 (1.4)	
Erythromycin				0.314
No	322	252 (96.2)	70 (98.6)	
Yes	11	10 (3.8)	1 (1.4)	
Linezolid				0.116*
No	330	261 (99.6)	69 (97.2)	
Yes	3	1 (0.4)	2 (2.8)	

Medication	Total *n*	HO-CDI		
		Negative, *n* (%)	Positive, *n* (%)	*p* value
Doxycycline				1.000*
No	332	261 (99.6)	71 (100)	
Yes	1	1 (0.4)	0 (0.0)	
Cefuroxime				0.448
No	324	254 (96.9)	70 (98.6)	
Yes	9	8 (3.1)	1 (1.4)	
Ceftriaxone				0.028
No	146	123 (46.9)	23 (32.4)	
Yes	187	139 (53.1)	48 (67.6)	
Anti-tuberculosis antibiotics				0.013
No	295	238 (90.8)	57 (80.3)	
Yes	38	24 (9.2)	14 (19.7)	
Acid suppressive agents				
Proton pump inhibitors				0.060
No	232	189 (72.1)	43 (60.6)	
Yes	101	73 (27.9)	28 (39.4)	
H2As				0.381*
No	331	261 (99.6)	70 (98.6)	
Yes	2	1 (0.4)	1 (1.4)	
Anti-retrovirals				
No	282	228 (87.0)	54 (76.1)	0.023
Yes	51	34 (13.0)	17 (23.9)	
Chemotherapeutic agents				
No	318	254 (96.9)	64 (90.1)	0.014
Yes	15	8 (3.1)	7 (9.9)	
Analgesics				0.090
No	255	206 (78.6)	49 (69.0)	
Yes	78	56 (21.4)	22 (31.0)	
Prior treatment with laxatives				0.820
No	316	249 (95.0)	67 (94.4)	
Yes	17	13 (5.0)	4 (5.6)	

p-value; Pearson's χ^2 was used to test the difference between the groups. * *p*-value obtained from Fisher's exact test of independence used. Abbreviation: H2As, histamine-2 receptor antagonists; HO-CDI, Healthcare facility-onset *C. difficile* infection.

The frequency and summary statistics of comorbidities, as well as CCI and ECI scores, are shown in Table 4.4 by HO-CDI outcome. Independent comorbidities that differed significantly between the HO-CDI patients and the comparison group included hypertension (23.5% vs. 9.5%; $p = 0.001$), iron deficiency anemia (22.5% vs. 9.5%; $p = 0.003$), tuberculosis (21.1% vs. 9.5%; $p = 0.008$), diabetes (16.9% vs. 6.9%; $p = 0.009$), chronic kidney disease (15.5% vs. 4.6%; $p = 0.001$) and chronic obstructive pulmonary disease (9.9% vs. 1.5%; $p < 0.001$). Furthermore, the findings revealed that while the HO-CDI group had a higher proportion of patients with HIV/AIDS (25.4% vs. 15.6%) and peptic ulcer disease (11.3% vs. 5.3%), the difference in proportions with non-HO-CDI group was not significant. Patients with HO-CDI were the only ones who had underlying leukemia, metastatic solid

tumors, and lymphoma, all of which had a low prevalence. Congestive heart failure, liver disease, cardiac arrhythmias, hypothyroidism, rickets, and inflammatory bowel disease (IBD) were among the other comorbidities reported in at least 1% of the HO-CDI group; however, no statistical differences were found when comparing the proportions of these comorbidities in the non-HO-CDI group. Both the CCI (n = 230) and the ECI (n = 158) had a majority of participants with a score of zero. However, more than a third of the participants with a CCI score of 1 (33.3%), 2 (44.0%) and ≥ 3 (34.8%) were positive for HO-CDI, compared to only 14.4% with a score of 0. Furthermore, 28.0%, 30.0% and 48.0% of the participants who tested positive for HO-CDI had ECI scores of 1, 2, and ≥ 3, respectively. The differences between the CCI and ECI categories were statistically significant at $p < 0.001$.

Table 4.4: Comparison of categorized comorbidities by HO-CDI outcome

Comorbidity	Total *n*	HO-CDI		
		Negative, *n* (%)	Positive, *n* (%)	*p* value
Congestive heart failure	2	1 (0.4)	1 (1.4)	0.381*
Chronic obstructive pulmonary disease	11	4 (1.5)	7 (9.9)	<0.001
Peptic ulcer disease	22	14 (5.3)	8(11.3)	0.075
Peripheral vascular disease	1	1 (0.4)	0 (0.0)	1.000*
Liver disease	5	3 (1.2)	2(2.82)	0.304*
Diabetes	30	18 (6.9)	12(16.9)	0.009
Hemiplegia or paraplegia	3	3 (1.1)	0(0.0)	1.000*
Chronic kidney Disease	23	12 (4.6)	11 (15.5)	0.001
Leukemia	4	0 (0.0)	4 (5.6)	0.002*
Metastatic Solid Tumor	2	0 (0.0)	2 (2.8)	0.045*
HIV/AIDS	59	41 (15.6)	18 (25.4)	0.058
Cardiac arrhythmias	1	0 (0.0)	1 (1.4)	0.213*
Hypertension	42	25 (9.5)	17 (23.9)	0.001
Hypothyroidism	1	0 (0.0)	1 (1.4)	0.213*
Lymphoma	2	0 (0.0)	2 (2.8)	0.045*
Solid tumor without metastasis	9	5 (1.9)	4 (5.6)	0.086*
Weight loss (Malnutrition)	35	28 (10.7)	7 (9.9)	0.840
Iron deficiency anemia	41	25 (9.5)	16 (22.5)	0.003
Depression	1	1 (0.4)	0 (0.0)	1.000*
Tuberculosis	40	25 (9.5)	15 (21.1)	0.008
Rickets	20	19 (7.3)	1 (1.4)	0.066
Inflammatory bowel disease	8	6 (2.3)	2 (2.8)	0.797*
Charlson comorbidity scores				<0.001
CCI=0	230	197 (75.2)	33 (46.5)	
CCI=1	12	8 (3.1)	4 (5.6)	
CCI=2	25	14 (5.3)	11 (15.5)	
CCI≥3	66	43 (16.4)	23 (32.4)	
Elixhauser comorbidity scores				<0.001
ECI = 0	158	142 (54.2)	16 (22.5)	
ECI = 1	100	72 (27.5)	28 (39.4)	
ECI = 2	50	35 (13.4)	15 (21.1)	
ECI ≥3	25	13 (5.0)	12 (16.9)	

p-value; Pearson's χ^2 was used to test the difference between the groups. * *p*-value obtained from Fisher's exact test of independence used. Abbreviations: CCI, Charlson Comorbidity Index; ECI, Elixhauser Comorbidity Index; HO-CDI, Healthcare facility-onset *C. difficile* infection

4.5 Toxigenic and nontoxigenic *C. difficile* variant types

A *C. difficile* toxigenic variant was defined as an isolate that tested positive for toxin A, toxin B or the binary toxin. Following this definition, toxigenic *C. difficile* isolates accounted for 69 (97.1%) of the total, while non-toxigenic isolates accounted for only 2 (2.8%) of the total isolates tested. The nine toxin variant types detected included: $A^+B^+CDT^+$, $A^+B^+CDT^-$, $A^{-*}B^+CDT^+$, $A^{-*}B^+CDT^+$, $A^{-*}B^+CDT^-$, $A^+B^-CDT^+$, $A^{-*}B^-CDT^+$, $A^+B^+CDTA^+/CDTB^-$ and $A^{-*}B^+$ $CDTA^+/CDTB^-$. The majority of the isolates that tested positive for toxin B 19 (26.8%) had a 110 bp deletion on the *tcdA* gene and were devoid of the binary toxin ($A^{-*}B^+CDT^-$). A further 16 (22.5%) isolates were positive for toxin B and binary toxin with a

110 bp deletion in the *tcdA* gene ($A^{-*}B^{+}CDT^{+}$), 15 (21.1%) isolates expressed both toxin A and toxin B but were devoid of binary toxin ($A^{+}B^{+}CDT^{-}$), and 4 (5.6%) isolates expressed all the toxin profiles ($A^{+}B^{+}CDT^{+}$). *tcdA* and *tcdB* genes ($A^{+}B^{+}CDT^{-}$) were detected in 24 (33.8%) of the isolates. Two isolates did not express *tcdB* gene; one was positive for the full-length *tcdA* gene and *cdt* gene ($A^{+}B^{-}CDT^{+}$), while the other had a 110bp deletion in the *tcdA* gene but expressed the *cdt* gene ($A^{-*}B^{-}CDT^{+}$). A few isolates only expressed the binary toxin *cdtA* gene without *cdtB* gene: 5 (7.0%) $A^{+}B^{+}CDTA^{+}/CDTB^{-}$) and 8 (11.3%) of $A^{-*}B^{+}CDTA^{+}/CDTB^{-}$. The two non-toxigenic variant types included 1 (1.4%) isolate which appeared to harbor a truncated *tcdA* gene but lacked the *tcdB* and the *cdt* genes ($A^{-*}B^{-}CDT^{-}$) and another that completely lacked the PaLoc as well as the *cdt* genes despite multiple attempts to amplify them. Figure 4.3 summarizes the distribution of toxigenic and non-toxigenic *C. difficile* strains with representative qPCR amplification output (Appendix IX) and gel electrophoresis images in figure 4.4 (A, B, C, D).

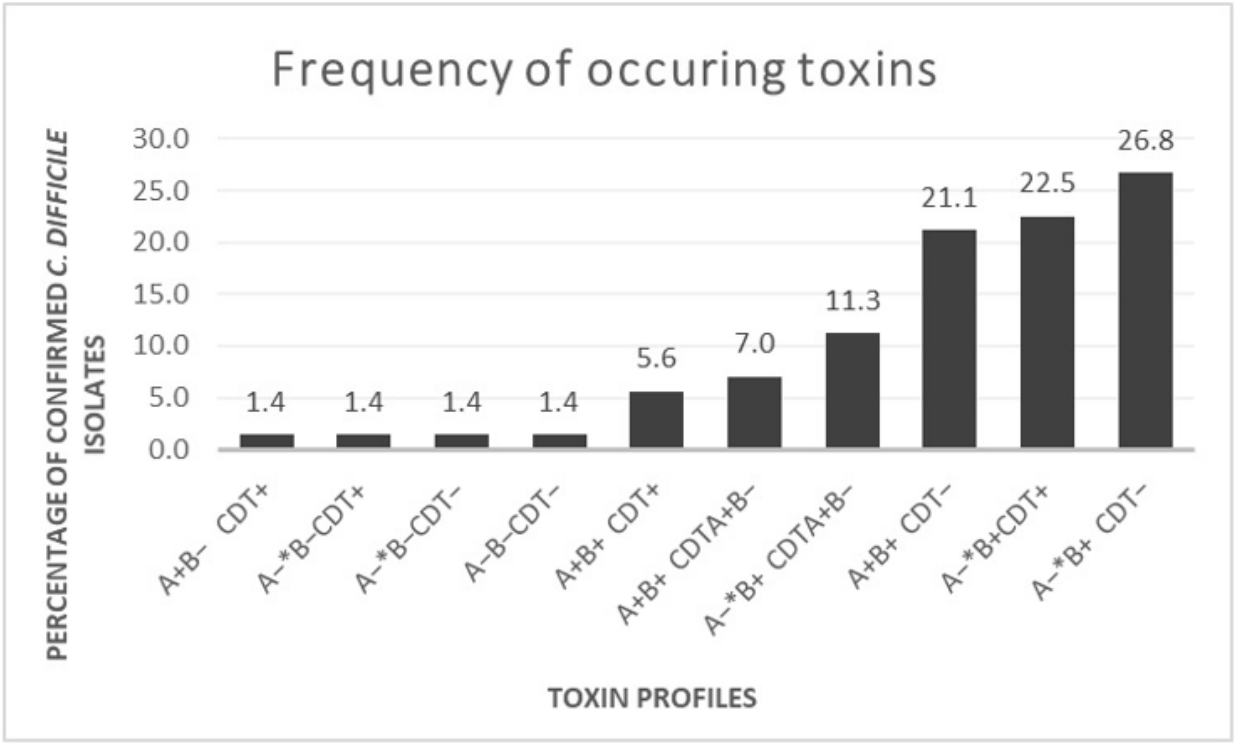


Figure 4.3: Distribution of the toxin variant types of the 71 *C. difficile* isolates. A−*B+CDT−and A−*B−CDT−: isolates in which the *tcdA* gene had a 110bp deletion compared to the wildtype *tcdA* gene. Abbreviations: CDT; *Clostridium difficile* transferase/ Binary toxin.

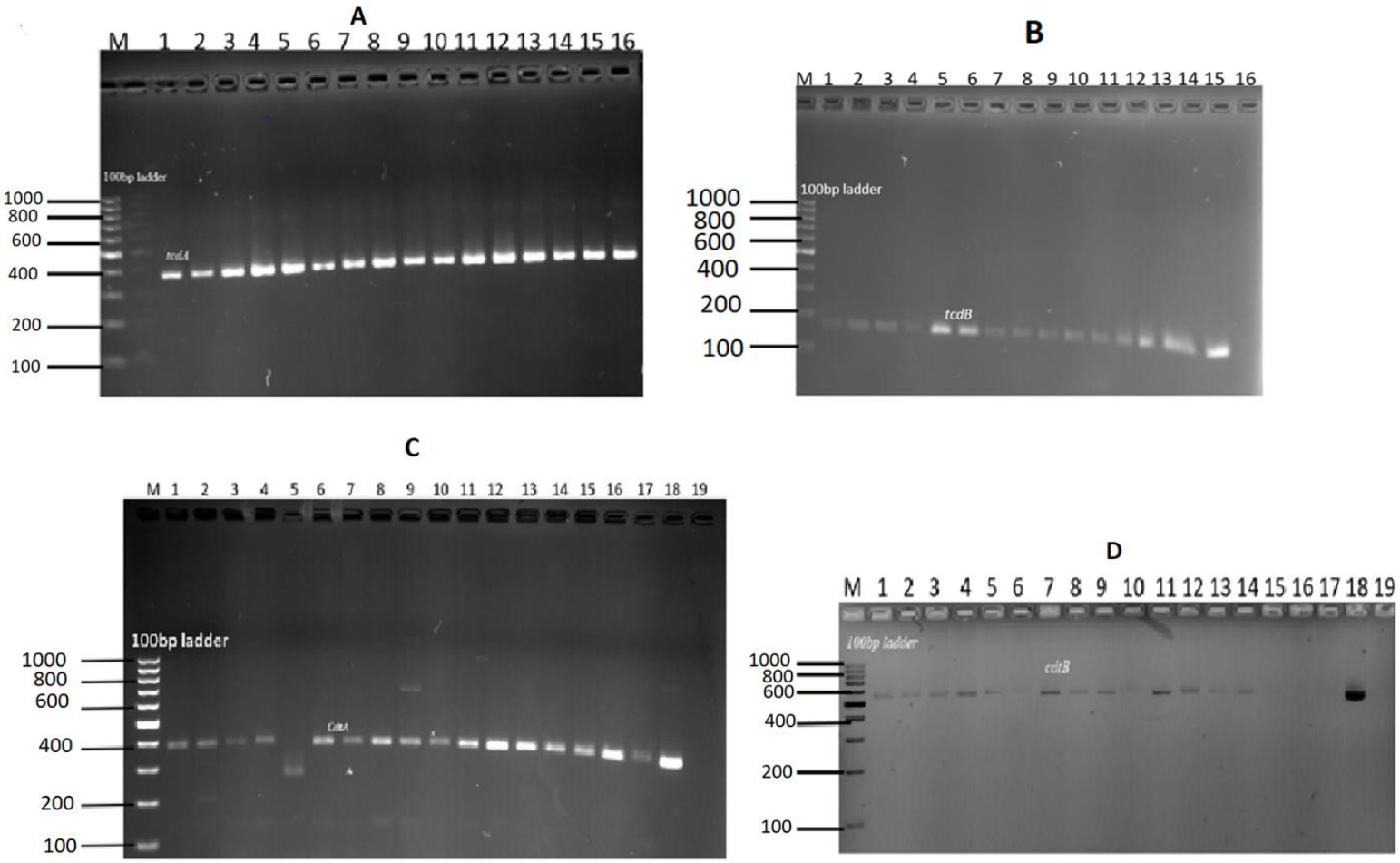


Figure 4.4: Agarose gel electrophoresis of *tcdA, tcdB, cdtA* and *cdtB* qPCR amplicon products. In all images, lane M represents the 100 bp molecular weight marker while lane 1 represents amplicons for the positive control (*C. difficile* DSM 27147). (A) Lane 2-16 full length *tcdA* gene (369 bp). (B) Lanes 2-15 isolates with *tcdB* gene (160 bp) and lane 16 no template control. (C) Lane 2-18 amplicons for *cdtA* gene (375 bp) and lane 19 no template control. (D) Lanes 2-5, 7-9, 11-15, 18 amplicon products of isolates with *cdtB* gene (375 bp) while lanes 6, 10, 16 and 17 represents clinical isolates lacking *cdtB* gene and lane 19 is no template control.

4.6 Antimicrobial susceptibility of the *C. difficile* isolates

The MIC values of the eight antimicrobials tested against 71 *C. difficile* isolates are shown in Table 4.5. The MIC breakpoints were divided into three categories: susceptible (S), intermediate (I), and resistant (R). Except for rifampicin, the MIC breakpoints for all antibiotics tested were determined using CLSI M100 2020. The MIC breakpoint for rifampicin was based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (version 6.0.16) epidemiological cut-off value (ECOFF) of 0.004 mg/L. The MIC range for vancomycin, metronidazole, clindamycin, ceftriaxone, erythromycin, and tetracycline was 0.016 - 256 mg/L, while rifampicin and ciprofloxacin were 0.002 - 0.032 mg/L, as shown in table 4.5. Further, because CLSI and EUCAST guidelines did not define ciprofloxacin breakpoints; therefore, the moxifloxacin breakpoints were used to determine ciprofloxacin susceptibility. All 71 isolates (100%) were susceptible to vancomycin (MIC ≥16 µg/ml). Resistance to the other antibiotics tested was observed in varying proportions. High frequency of resistance to rifampicin (MIC ≥16 µg/ml), erythromycin (MIC ≥8 µg/ml), ciprofloxacin (MIC ≥8 µg/ml), clindamycin (MIC ≥8 µg/ml) and ceftriaxone (MIC ≥64 µg/ml) was observed in 65 (91.5%), 63 (88.7%), 59 (83.1%), 57 (80.3%) and 36 (50.7%) of the isolates respectively. In contrast, metronidazole (MIC ≥16 µg/ml) and tetracycline (MIC ≥16 µg/ml) had lower resistance rates of 3 (4.2%) and 7 (9.9%) respectively.

Table 4.5: MIC breakpoints for the antibiotics tested against 71 *C. difficile* isolates

Antimicrobial agent	MIC range (mg/L)	MIC breakpoint criteria (µg/mL				Susceptibility frequency (%)		
		S	I	R	ECOFF	S	I	R
Vancomycin[a]	0.016-256	≤2		≥4		71 (100)	-	0
Metronidazole[a]	0.016-256	≤8	16	≥32		68 (95.8)	0	3 (4.2)
Clindamycin[a]	0.016-256	≤2	4	≥8		13 (18.3	1 (1.4)	57 (80.3)
Cefriaxone[a]	0.016-256	≤16	32	≥64		22 (31)	13 (18.3)	36 (50.7)
Erythromycin[a]	0.016-256	≤2	4	≥8		8(11.3)	-	63 (88.7)
Rifampicin[b]	0.002-32				0.004	6 (85)	-	65 (91.5)
Ciprofloxacin[a]	0.002-32	≤2	4	≥8		10 (14.1)	2 (	59 (83.1)
Tetracycline[a]	0.016-256	≤4	8	≥16		35 (49.3)	29 (40.8)	7 (9.9)

[a] Breakpoints as defined by CLSI MIC values for anaerobes. [b] Breakpoints as per EUCAST MIC guidelines for *C. difficile*. Abbreviations: MIC, minimum inhibitory concentration; S, susceptible; I, intermediate; R, resistant; ECOFF, epidemiological cut-off value.

4.7 Multiple antibiotic resistance

Among the 71 *C. difficile* isolates, 70 (98.6%) isolates were resistant to at least one antibiotic, while 68 isolates (95.7%) were resistance to more than two types of antibiotics. MDR was defined as 'non-susceptibility to at least one agent in three or more antimicrobial categories, where non-susceptibility refers to either resistant or intermediate results obtained from *in vitro* antimicrobial susceptibility testing'. To detect isolates with multiple antibiotic resistances, a cumulative resistance score (CRS) was generated in which susceptible, intermediate and resistant isolates were assigned scores of 0, 1 and 2 respectively. A fully susceptible isolate had a mean CRS of zero, whereas an MDR isolate had a mean score of five or higher. As a result, 61/71(85.9%) of the isolates were multidrug-resistant, which means they were resistant to more than three types of antibiotics. As illustrated in figure 4.5, two isolates (3.3%) were resistant to seven antibiotics, while the majority 26 (42.6%) were resistant to six antibiotics. The dominant resistance pattern included a combination of rifampicin, tetracycline, clindamycin, ceftriaxone, ciprofloxacin, and erythromycin. There was a tendency for slightly higher proportions of $A^+B^+CDT^-$variants to exhibit the highest resistance in all categories of antibiotics although the difference was not statistically significant (Table 4.6).

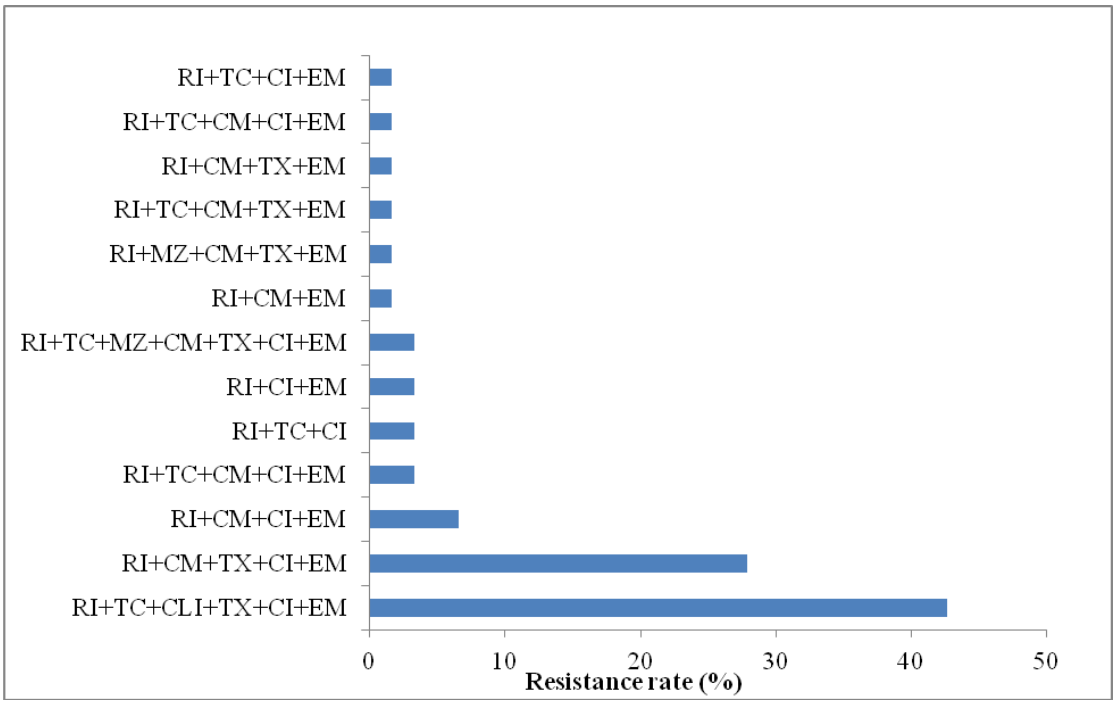


Figure 4.5: Resistance spectrum of MDR resistant *C. difficile* isolates to a combination of the antibiotics tested. The X-axis represents the resistance rate in percentage of the total MDR *C. difficile* phenotypes (61), while the Y-axis represents a series of antibiotic MDR phenotypes. Abbreviations: RI, rifampicin; TC, tetracycline; CI, ciprofloxacin; EM, erythromycin; CM, clindamycin; TX, ceftriaxone; MZ, metronidazole.

Table 4.6: Antimicrobial resistance of *C. difficile* strains based on toxin variant types

Resistance phenotype	Toxigenic strains									Nontoxigenic strains	*p*-value[#]
	$A^+B^+CDT^+$ (n=4)	$A^+B^+CDT^-$ (n=15)	$A^+B^+CDTA^+B^-$ (n=5)	$A^+B^-CDT^+$ (n=1)	$A^{-*}B^+CDT^+$ (n=16)	$A^{-*}B^+CDT^-$ (n=19)	$A^{-*}B^+CDTA^+B^-$ (n=8)	$A^{-*}BCDT^+$ (n=1)	$A^{-*}B^-CDT^-$ (n=1)	$A^-B^-CDT^-$ (n=1)	
Metronidazole (n=3)	-	1 (6.7)	-	-	2 (12.5)	-	-	-	-	-	1.000
Clindamycin (n=58)	4 (100)	12 (80.0)	3 (60.0)	1 (100)	16 (100)	14 (73.7)	7 (87.5)	-	-	1 (100)	1.000
Ceftriaxone (n=49)	2 (50.0)	9 (60.0)	2 (40.0)	1 (100)	13 (81.3)	13 (68.4)	7 (87.5)	1 (100)	-	1 (100)	0.724
Erythromycin (n=63)	3 (75.0)	14 (93.3)	4 (80.0)	1 (100)	16 (100)	16 (84.2)	7 (87.5)	-	1 (100)	1 (100)	0.613
Rifampicin (n=65)	3 (75.0)	15 (100)	3 (60.0)	1 (100)	16 (100)	17 (89.5)	8 (100)	-	1 (100)	1 (100)	0.492
Ciprofloxacin (n=61)	4 (100)	13 (86.7)	4 (80.0)	1 (100)	15 (93.8)	13 (68.4)	8 (100)	1 (100)	1 (100)	1 (100)	0.257
Tetracycline (n=36)	2 (50.0)	9 (60.0)	3 (60.0)	1 (100)	11 (73.3)	5 (26.3)	4 (50.0)	-	-	1 (100)	0.080
MDR (n=61)	3 (75.0)	14 (93.3)	3 (60.0)	1 (100)	16 (100)	14 (73.7)	8 (100)	-	1 (100.)	1 (100)	0.196
MDR patterns											
Three classes (n=6)	-	4 (26.7)	-	-	-	-	1 (12.5)	-	1 (100)	-	0.030
Four classes (n=6)	-	2 (13.3)	1 (20.0)	-	(6.3)	2 (10.5)	-	-	-	-	1.000
Five classes (n=26)	1 (25.0)	5 (33.3)	2 (40.0)	-	6 (37.5)	8 (42.1)	4 (50.0)	-	-	-	0.728
Six classes (n=23)	1 (25.0)	4 (26.7)	-	1 (100)	9 (56.3)	4 (21.1)	3 (37.5)	-	-	1 (100)	0.709

A^{-*}: isolates with a 110bp deletion in the tcdA gene compared to the wildtype tcdA gene. CDT^+: expressed both cdtA and cdtB genes of the binary toxin [#]P-value: obtained from a two-tailed Fishers exact test comparing $A^+B^+CDT^-$ isolates to $A^{-*}B^+CDT^-$ isolates. Abbreviations: MDR; multidrug resistance.

4.8 Risk factors of HO-CDI

4.8.1 Bivariate binary logistics regression

Initially, a binary logistics regression was applied to model the risk factors associated with HO-CDI. All variables with *p* values of <0.2 in the pairwise comparison in tables 4.2, 4.3, and 4.4 were individually fed into a bivariate (unadjusted) binary logistics regression model to assess the effect of each variable on the onset of HO-CDI. Variables with *p* values of <0.05 were considered significant when assessing the independent effect of input variables on the output of HO-CDI. The highest risk of HO-CDI was associated with antibiotic exposure (Odds ratio (OR): 10.973; 95% confidence interval (CI): 1.452-80.209), where the risk increased with additional antibiotic exposure as shown in table 4.7. However, significance was only detected in those exposed to three (OR: 14.583, 95% CI: 1.868-113.858) and more than four antibiotics (OR: 27.317, 95% CI: 3.249-210.258). Previous hospitalization was associated with a 3-fold increase in the risk of developing HO-CDI. Furthermore, invasive procedures such as colonoscopy and surgical procedures were related with a 3-fold and 1.5-fold increase in the odds of HO-CDI, respectively. Patients who received nasogastric tube feeding had a higher risk of HO-CDI (OR: 2.520, 95% CI: 1.090-5.829). Overall, patients with comorbidities had a greater unadjusted odd (OR: 3.480, 95% CI: 1.417-8.547) of developing HO-CDI than those without comorbidities.

Table 4.7: Bivariate binary logistic regression analysis of the clinical factors associated with HO-CDI

Variables	95% CI			
	OR	**LCI**	**UCI**	***p* value**
Age group in years				
≤2 (Ref)				
3-15	3.697	1.343	10.175	0.011
16-25	2.839	0.740	10.896	0.128
26-45	3.852	1.568	9.462	0.003
46-59	2.585	0.738	9.052	0.138
≥60	4.194	1.086	16.197	0.038
Admission duration (before diarrhoea onset)				
≤ 1 week (Ref)				
2 weeks	3.344	1.301	8.596	0.012
3 weeks	1.082	0.359	3.258	0.888
≥ 4 weeks	1.299	0.625	2.700	0.483
Number of Antibiotic administered				
None (Ref)				
One	2.549	0.256	25.389	0.425
Two	5.490	0.643	46.862	0.120
Three	13.023	1.514	112.029	0.019
≥ Four	25.188	2.946	215.385	0.003
NGT	2.931	1.011	8.496	0.048
Surgical procedure	2.464	1.131	5.371	0.023
Colonoscopy/sigmoidoscopy/endoscopy	3.069	0.802	11.743	0.102
Previous hospital admission	3.277	1.713	6.271	0.001
Comorbidity	3.480	1.417	8.547	0.007

Abbreviations: HO-CDI, Healthcare facility-onset *C. difficile* infection**;** OR, Odds ratio; LCI, Lower confidence interval; UCI, Upper confidence interval; Ref, Reference group.

Antibiotics identified as statistically significant risk factors for HO-CDI in the binary logistics regression analysis included amoxicillin/clavulanic acid (OR: 2.360, 95% CI: 1.361-4.093), benzylpenicillin/gentamicin (OR: 1.942, 95% CI: 1.030-3.660), meropenem (OR: 2.071, 95% CI: 1.044-4.109), ciprofloxacin (OR: 2.922, 95% CI: 1.425-5.990), clarithromycin (OR: 5.622, 95% CI: 1.728-18.293), trimethoprim-sulfamethoxazole (OR: 2.758, 95% CI: 1.380-5.513), clindamycin (OR: 5.205, 95% CI: 1.972-13.741), ceftriaxone (OR: 1.847, 95% CI: 1.062-3.211) and anti-tuberculosis agents (OR: 2.436, 95% CI: 1.186-5.002). For the non-antibiotic pharmacological agents, anti-retrovirals (OR: 2.111, 95% CI: 1.98-4.058) and chemotherapeutic agents (OR: 3.473, 95% CI: 1.214-9.931) significantly increased the risk of HO-CDI compared to not using these agents. Although the use of proton pump inhibitors and analgesics were relatively high, it resulted in a lower risk of developing

HO-CDI. Table 4.8 summarizes the association between different pharmacological agents and HO-CDI.

Table 4.8: Bivariate binary logistic regression analysis of pharmacological agents associated with HO-CDI

	95% CI			
Medication	**OR**	**LCI**	**UCI**	***p* value**
Metronidazole				
No	1			
Yes	1.613	0.914	2.845	0.099
Amoxicillin/Clavulanic acid				
No	1			
Yes	2.360	1.361	4.093	0.002
Benzylpenicillin/Gentamicin				
No	1			
Yes	1.942	1.030	3.660	0.040
Meropenem				
No	1			
Yes	2.071	1.044	4.109	0.037
Ciprofloxacin				
No	1			
Yes	2.922	1.425	5.990	0.003
Amikacin				
No	1			
Yes	1.703	0.770	3.768	0.188
Azithromycin				
No	1			
Yes	3.768	0.521	27.233	0.189
Clarithromycin				
No	1			
Yes	5.622	1.728	18.293	0.004
Trimethoprim-Sulfamethoxazole				
No	1			
Yes	2.758	1.380	5.513	0.004
Clindamycin				
No	1			
Yes	5.205	1.972	13.741	<0.001
Linezolid				
No	1			
Yes	7.565	0.676	84.659	0.101
Ceftriaxone				
No	1			
Yes	1.847	1.062	3.211	0.030
Anti-tuberculosis antibiotics				
No	1			
Yes	2.436	1.186	5.002	0.015
Proton pump inhibitors				
No	1			
Yes	1.686	0.975	2.914	0.061

Medication	95% CI			*p* value
	OR	LCI	UCI	
Anti-retrovirals				
No	1			
Yes	2.111	1.098	4.058	0.025
Chemotherapeutic agents				
No	1			
Yes	3.473	1.214	9.931	0.020
Analgesics				
No	1			
Yes	1.652	0.922	2.960	0.092

Abbreviations: HO-CDI, Healthcare facility-onset *C. difficile* infection; OR, Odds ratio; LCI, Lower confidence interval; UCI, Upper confidence interval; Ref, Reference group.

Table 4.9 summarizes the unadjusted odds of individual comorbidities, as well as the comorbidity scores and corresponding 95% confidence intervals. Patients with chronic pulmonary disease (OR: 7.055, 95% CI: 2.004-24.836, $p = 0.002$), chronic kidney disease (OR: 3.819, 95% CI: 1.608-9.074, $p < 0.001$), hypertension (OR: 2.984, 95% CI: 1.507-5.911, $p = 0.002$), diabetes (OR: 2.757, 95% CI: 1.259-6.038, $p < 0.015$), iron deficiency anemia (OR: 2.758, 95% CI: 1.380-5.513, $p = 0.006$) and tuberculosis (OR: 2.539, 95% CI: 1.257-5.130, $p = 0.012$) were more likely to have HO-CDI conditions compared to non-HO-CDI patients. Furthermore, patients with CCI scores of 2 (OR: 4.691, 95% CI: 1.962-11.213, $p = 0.001$) and ≥ 3 (OR: 3.193, 95% CI: 1.707-5.973, $p < 0.001$) were more likely to develop HO-CDI, as were those with ECI scores of 1 (OR:3.451, 95% CI: 1.755-6.789, $p < 0.001$), 2 (OR:3.804, 95% CI: 1.717-8.428 $p = 0.001$), and ≥ 3 (OR: 8.192, 95% CI: 3.202-20.963, $p < 0.001$). Peptic ulcer disease and HIV/AIDS were not significant, hence they were not included in the adjusted logistic regression model.

Table 4.9: Bivariate binary logistic regression analysis of comorbidities associated with HO-CDI

Comorbidity	95% CI			
	OR	**LCI**	**UCI**	***p* value**
Chronic obstructive pulmonary disease	7.055	2.004	24.836	0.002
Peptic ulcer disease	2.249	0.904	5.597	0.081
Diabetes	2.757	1.259	6.038	0.011
Chronic kidney Disease	3.819	1.608	9.074	0.002
HIV/AIDS	1.831	0.975	3.437	0.060
Hypertension	2.984	1.507	5.911	0.002
Solid tumor without metastasis	3.069	0.802	11.743	0.102
Iron deficiency anaemia	2.758	1.380	5.513	0.004
Tuberculosis	2.539	1.257	5.130	0.009
Rickets	0.183	0.024	1.389	0.100
Charlson comorbidity scores				
CCI=0 (Ref)				
CCI=1	2.985	.850	10.476	0.088
CCI=2	4.691	1.962	11.213	0.001
CCI≥3	3.193	1.706	5.973	0.000
Elixhauser comorbidity scores				
ECI = 0 (Ref)				
ECI = 1	3.451	1.755	6.789	0.000
ECI = 2	3.804	1.717	8.428	0.001
ECI ≥3	8.192	3.202	20.963	0.000

Abbreviations: HO-CDI, Healthcare facility-onset *C. difficile* infection; OR, Odds ratio; LCI, Lower confidence interval; UCI, Upper confidence interval; CCI, Charlson Comorbidity Index; ECI, Elixhauser Comorbidity Index Ref, Reference group.

4.8.2 Multiple binary logistics regression

To determine the predictors of HO-CDI, three separate multivariate regression models were constructed. All independent variables with *p*-values less than 0.05 from the unadjusted models were fed into multivariate regression models (Figures 4.7, 4.8, and 4.9). The models corresponded to the three models in section 4.8 that represented clinical factors (Table 4.7), pharmacological agents (Table 4.8), and comorbidities (Table 4.9).

After controlling for potential confounders, age was found to be a predictor of HO-CDI. The older patients were more likely to have HO-CDI than the younger patients, with those aged ≥ 60 years having a 4.19-fold (95% CI: 1.09-16.20, $p = 0.038$) higher risk of *C. difficile* infection than those aged two years and under. Patients aged 3-15 years had a higher risk (OR: 3.70, 95% CI: 1.34-10.18, $p = 0.011$), as did those aged 26-45 years (OR: 3.85, 95% CI: 1.57-9.46, $p = 0.003$). Furthermore, HO-CDI patients were more frequently exposed to an additional antibiotic, such that any additional antibiotic exposure resulted in an increased risk, with those receiving four or more types of antibiotics being highly likely to have HO-CDI

(OR: 25.188, 95% CI: 2.946-215.385, p = 0.003) compared to patients with no antibiotic exposure. Additionally, those who received three antibiotics had a 13-fold increased risk of infection.

Patients who underwent surgical procedures and nasogastric tube feeding were 2.46 times (95% CI: 1.131-5.371, p = 0.023) and 2.931-times (95% CI: 1.011-8.496, p = 0.048) more likely to get HO-CDI, respectively, than those who did not undergo any of these procedures. In this model, having comorbidity resulted in a 3.5 times increased risk of HO-CDI; however, a different model (Figure 4.8) was generated to assess the effect of each independent comorbidity.

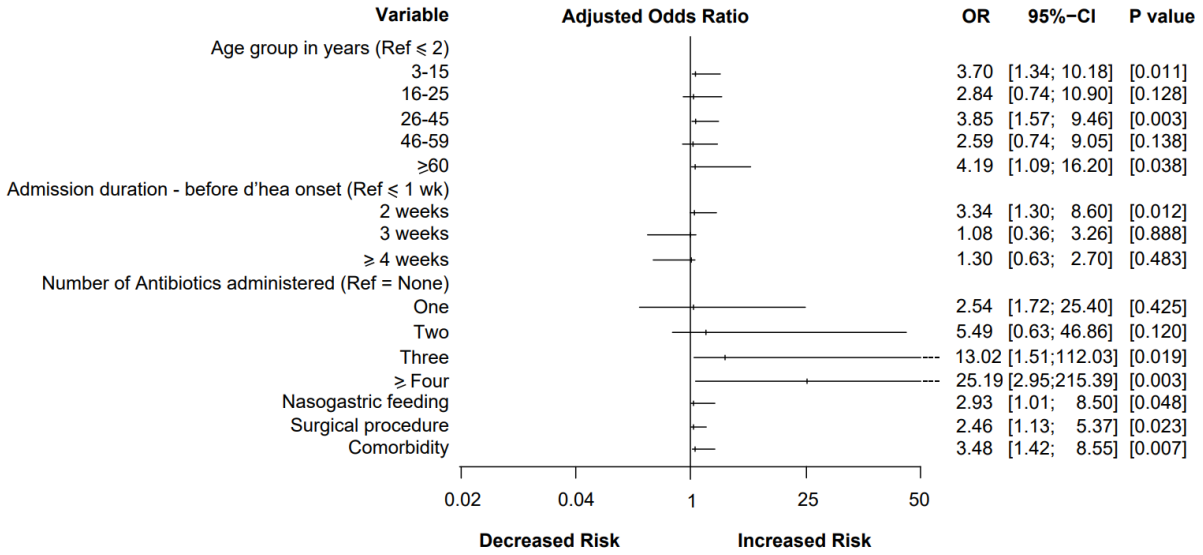


Figure 4.6: A Forest plot of clinical predictors for HO-CDI. The horizontal lines represent the width of the confidence interval, and the vertical marks on each horizontal line represent the odds ratios. An odds ratio greater than 1.0 indicates a higher risk. Abbreviations: OR- Odds ratio; CI- Confidence Interval; p value denoting the statistical significance ($p \leq 0.05$).

After controlling for age, clinical characteristics, and admission information, exposure to chemotherapeutic agents was associated with a higher risk of HO-CDI (OR: 11.011, 95% CI: 2.895-41.880), followed by clindamycin (OR: 8.547, 95% CI: 2.449-29.834), benzylpenicillin/gentamicin (OR: 7.992, 95% CI: 2.923-21.845), amoxicillin/clavulanic acid (OR: 4.441, 95% CI: 2.101-9.388), clarithromycin (OR: 4.345, 95% CI: 1.046-18.056), meropenem (OR: 2.839, 95% CI: 1.116-4.7.225), ciprofloxacin (OR: 2.771, 95% CI: 1.068-7.190) and ceftriaxone (OR: 2.267, 95% CI: 1.107-4.645). Exposure to trimethoprim-sulfamethoxazole, anti-tuberculosis agents, and anti-retrovirals resulted in a lower risk as shown in Figure 4.7.

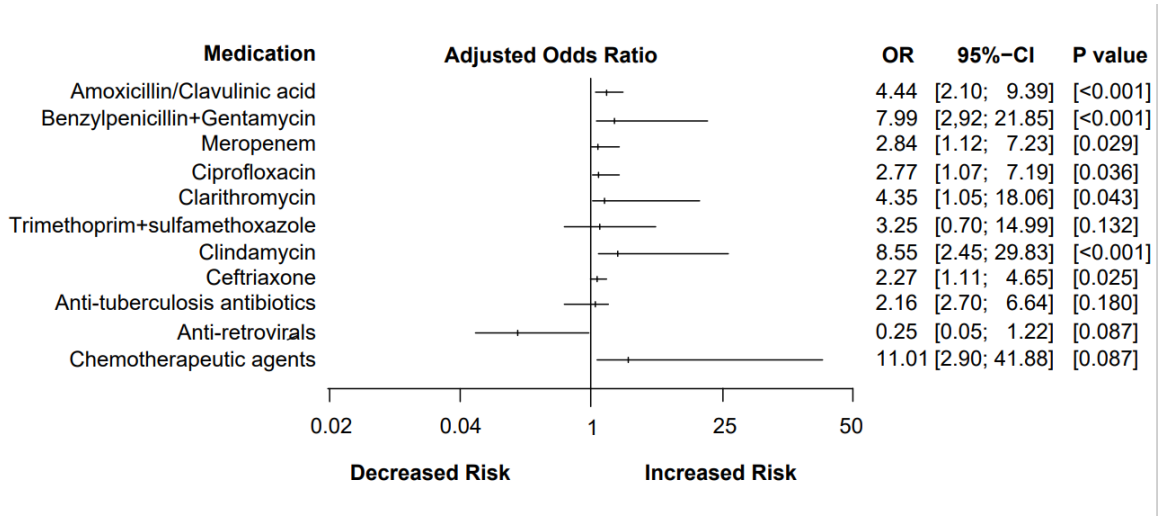


Figure 4.7: A Forest plot showing pharmacological agents predictors for HO-CDI. The horizontal lines represent the width of the confidence interval, and the vertical marks on each horizontal line represent the odds ratios. An odds ratio greater than 1.0 indicates a higher risk. Abbreviations: OR- Odds ratio; CI- Confidence Interval; p value denoting statistical significance ($p \leq 0.05$).

Taking into consideration the potential confounders, five independent comorbidities were identified as potential predictors of HO-CDI as shown in figure 4.8: chronic obstructive pulmonary disease (OR: 9.51, 95% CI: 1.80-50.10), diabetes (OR: 3.56, 95% CI: 1.11-11.384), chronic kidney disease (OR: 3.88, 95% CI: 1.57-9.62), iron deficiency anemia (OR: 3.67, 95% CI: 1.61-8.34) and hypertension (OR: 2.47, 95% CI: 1.00-6.07). Patients with tuberculosis were 48% less likely to develop HO-CDI than patients without tuberculosis, though this was not statistically significant. In terms of comorbidity scores, patients with CCI scores of 2 were 6.67-times (95% CI: 2.07-21.48, $p < 0.001$) more likely to have HO-CDI than patients without CCI comorbidities (i.e., CCI = 0), while patients with ECI scores of 1, 2, and ≥ 3 were associated with 4.07-times (95% CI: 1.72-9.65, $p < 0.001$), 2.86-times (95% CI: 1.03-7.89, $p < 0.05$), and 4.87-times (95% CI: 1.40-16.92, $p < 0.05$) increased odds of HO-CDI, respectively.

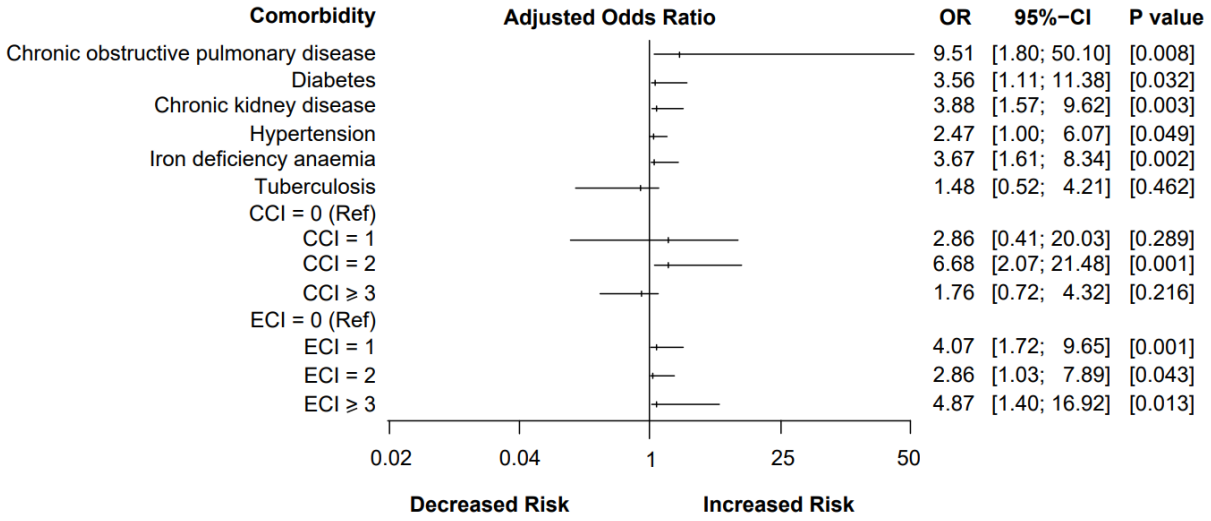


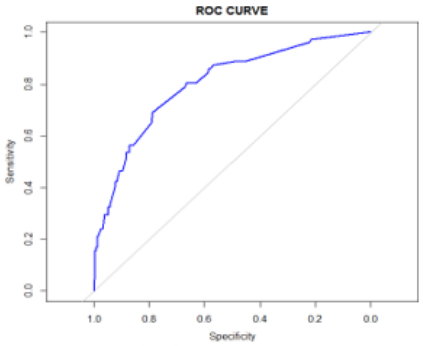
Figure 4.8: A forest plot showing comorbidity predictors for HO-CDI. The horizontal lines represent the width of the confidence interval, and the vertical marks on each horizontal line represent the odds ratios. An odds ratio greater than 1.0 indicates a higher risk. Abbreviations: OR- Odds ratio; CI- Confidence Interval; CCI, Charlson Comorbidity Index; ECI, Elixhauser Comorbidity Index; p-value denoting statistical significance ($p \leq 0.05$).

4.8.3 Model diagnosis

Efficiency calculated from the confusion matrix given by sum (diagonal)/sum showed that the three models performed better. The clinical information model was shown to explain the output of HO-CDI with 81.9% efficiency, with the pharmacological agents model having an efficiency of 80.7% while the comorbidity model had an efficiency of 80.7% with corresponding receiver operating characteristic (ROC) of 0.8075, 0.801 and 0.8258 respectively as shown in figure 4.9 (A, B, C) below.

A

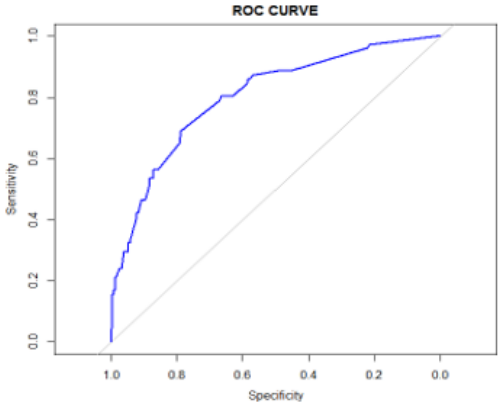
Confusion matrix for clinical factors model		
	Predicted negative	**Predicted positive**
Observed negative	239	16
Predicted positive	43	28
Efficiency of the model = 0.819		



Area under the curve = 0.8075

B

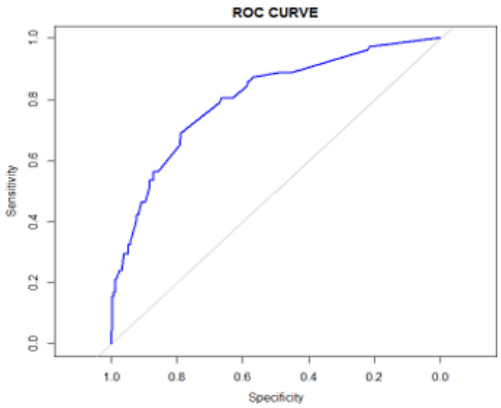
Confusion matrix for pharmacological agents model		
	Predicted negative	**Predicted positive**
Observed negative	240	15
Predicted positive	48	23
Efficiency of the model = 0.8067		



Area under the curve = 0.801

C

Confusion matrix for comorbidities model		
	Predicted negative	**Predicted positive**
Observed negative	255	7
Predicted positive	51	20
Efficiency of the model = 0.8067		



Area under the curve = 0.8258

Figure 4. 9: Confusion matrix and receiver operating characteristics (ROC) of the models.

4.9 Virulence, antibiotic resistant determinants and genetic relatedness of the isolates

Nine isolates were sequenced using Oxford Nanopore MinION Technology. The overview of the assembly statistics is shown in table 4.10. The selection criteria for the assemblies were based on the mismatches, misassembles, contigs, GC (%) content, N50 length, and the genome coverage using QUAST (http://bioinf.spbau.ru/quast). The largest N50 of the assemblies was 4255393, with the corresponding N50 being of the same length. The largest contig was 4269077 base pairs (bp) while the estimated size of the genomes ranged from 4141518 to 4342428. The genomes sequences were deposited in GenBank (GenBank Overview (nih.gov) under BioProject number PRJNA732612. Details of the genomes are provided in appendix X.

Table 4.10: Features of the nine genomes sequenced

Isolate no.	No. of contigs	Largest contig	Total length Genome Size (bp)	GC content (%)	N50
CD_045	2	4255393	4317689	29.11	4255393
CD_079	6	1723376	4305280	29.09	1501585
CD_147	5	3634268	4199657	29.05	3634268
CD_191	6	1259866	4141518	28.97	1117136
CD_199	1	4205639	4205639	28.49	4205639
CD_215	5	2633636	4326696	29.12	2633636
CD_248	3	4269077	4342428	29.1	4269077
CD_273	8	3198272	4246858	28.63	3198272
CD_283	3	4173937	4209625	28.71	4173937

4.9.1 Sequence types and antimicrobial-resistant determinants

The analysis of the nine genomes revealed two (2) novel MLST types. The other MLST subtypes include subtypes 37, 10, 58, and 743. MLST subtype 37 belongs to clade four (4) and often correlates to ribotype 017, while subtypes 10 and 58 belong to clade one (1) and are associated with ribotype 056. The analysis of the nine assembled genomes using ResFinder and CARD database revealed the acquired antibiotic resistance genes (*erm(G),* (*msr(D*), *erm(B*), *mef(A*), *tet(M*), *aac(6'*), *ant (6'*) that confer antimicrobial resistance to lincosamide, macrolide, tetracycline and aminoglycoside respectively. Certain strains also had point mutations on *gyrA* and *gyrB* and *rpoB* genes conferring resistance to fluroquinolone and rifampicin as shown in figure 4.10. The *CdeA* resistance gene was also found in all sequenced isolates. *In-silico* prokka analysis revealed the presence of *tcdA* and *tcdB* genes harboring toxin A and B respectively. Except for isolates 199, 273, and 283, the RepUS43 plasmid was found in all isolates. Detailed allelic polymorphisms is presented in appendix XI.

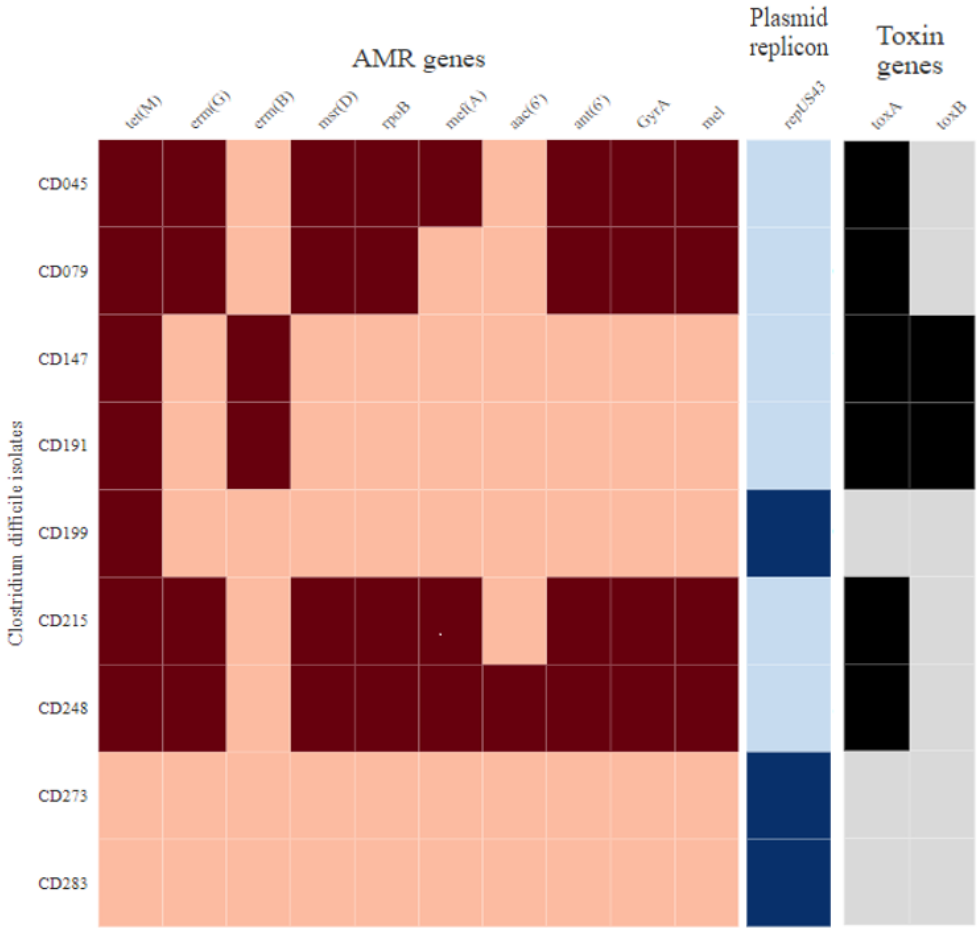


Figure 4.10: Heatmap of AMR genes and virulence determinants of nine *C. difficile* isolates. The antimicrobial resistance genes are shaded magenta, the plasmid replicon genes are shaded navy blue, and the toxin genes are shaded black. The isolates lacking these genes have a lighter shade of the respective colour.

4.9.2 Cluster analysis of the sequence types

Evolutionary analysis by the Maximum Likelihood method was conducted for nine isolates. The bootstrap consensus was set at 500 replicates to represent the evolutionary history of the taxa analyzed. The *C. difficile* strains belonging to similar sequence types clustered together. The analysis revealed two main cluster groups: Isolates 248, 079, 215, and 045 clustered in one group while isolates 199, 283, 191, 147, and 273 clustered alongside each other. Ribotyping based on the determined STs revealed that most isolates belonged to the RT017 lineage, 283, which belonged to the RT056 lineage except for isolates 147, 191, and 199 whose ribotypes haves not been associated with specific subtypes yet.

Phylogenetic analysis (figure 4.11) revealed that isolates 215, 248, 079, and 045 from this study were closely related to an Indonesian isolate (accession no.CP016104); isolates 283 and 273 were closely related to isolates from the United States (accession no. NZ_CP020424 and NZ_CP042267 respectively); isolate 199 was closely related to an Indonesian isolate (accession no. NZ_CP019860) and isolates 147 and 191 were closely related to a Ghanaian isolate (accession no. NZ_CP012321).

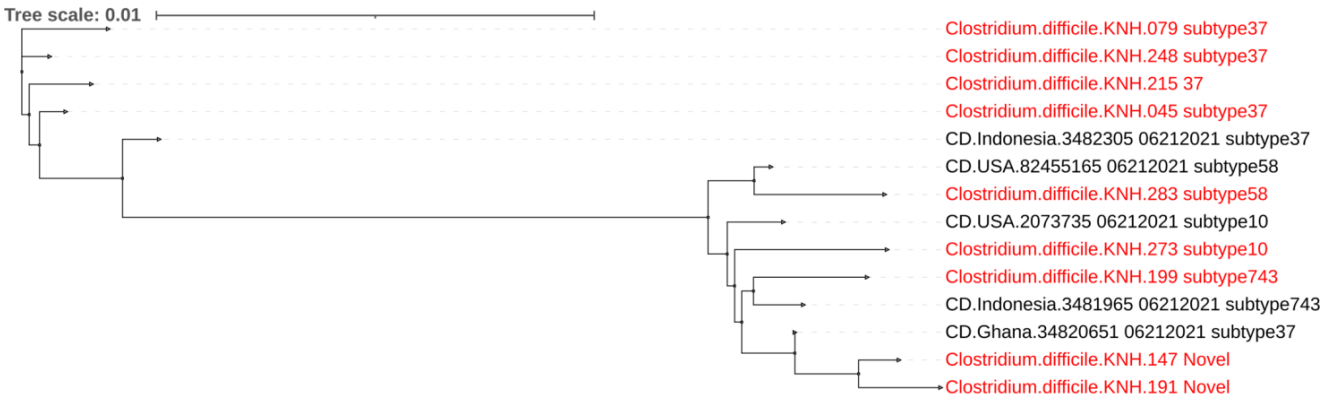


Figure 4.11: Phylogenetic analysis of multilocus sequence types (MLSTs). Phylogenetic tree of nine *C. difficile* genome sequences from this study (in red) and five genomes from different countries retrieved from GenBank (in black). The branch lengths are scaled in proportion to the extent of the change per position as indicated by the scale bar.

CHAPTER FIVE: DISCUSSION

5.1 Prevalence of HO-CDI in symptomatic patients

This study reports HO-CDI prevalence of 71 (21%) in hospitalized patients with healthcare facility-onset diarrhea, a figure that compares within the reported global range of 15-25% (Bartlett & Gerding, 2008). According to published data from African studies, *C. difficile* prevalence ranges from 0% to 93% (Beadsworth et al., 2014; Janssen et al., 2016; Brian Kullin, Meggersee, D'Alton, Galvao, Rajabally, Whitelaw, Bamford, Reid, & Abratt, 2015; Mwachari et al., 1998; Onwueme et al., 2011; Oyaro et al., 2018; Plants-Paris et al., 2019; M Seugendo et al., 2015; Simango & Uladi, 2014; Isaac Zulu et al., 2000). The observed variability may be influenced in part by the different diagnostic assays used and the population tested (age, outpatients and or hospitalized patients). While these differences make direct comparisons difficult, they underscore the pathogen's significance in an African population previously thought to be at low risk of developing CDI (Roldan et al., 2018).

Although infants are considered high carriers of both toxigenic and non-toxigenic *C. difficile*, there is still a knowledge gap regarding the impact of *C. difficile* in infants and children under the age of two (Emily Ann Lees et al., 2020; C. Rousseau et al., 2012; Schwartz et al., 2014; van Dorp et al., 2016; Wendt et al., 2014). Notably, we sampled a total of 101 (30.3%) children under the age of two; however, current guidelines on *C. difficile* diagnosis recommend that children under the age of two should not be tested for *C. difficile* due to the high carrier rate. As a result of this recommendation, a prevalence of 24.6% was recorded after excluding this population from the prevalence analysis. However, due to the rapidly changing epidemiology of CDI, little is known about *C. difficile* epidemiological dynamics in this population. Furthermore, children are not immune to CDI risk factors such as long hospitalization duration, immunodeficiency, pre-existing chronic conditions, and infections by multidrug organisms, which have exacerbated inappropriate and prolonged antibiotic use (Jason Kim et al., 2008; Migriauli et al., 2018; Samady et al., 2014). As a result, these conditions may gradually increase the risk of CDI in this population, just as they do in adults. Previous research has found that children under the age of one year are at a high risk of developing hospital-acquired diarrhea during their hospitalization due to malnutrition and pre-existing diseases, resulting in poor health outcomes. Hospitalized children with pediatric HO-CDI have had worse outcomes, including severe complications, death, prolonged hospitalization, relapse immunosuppression, bowel dysfunction, and inflammatory bowel disease (Julia Shaklee Sammons, Localio, et al., 2013; Schwartz et al., 2014). Notably,

Stoesser et al., in Oxfordshire demonstrated that colonizing *C. difficile* strains isolated from infants were genetically related to strains isolated from symptomatic CDI adult patients (Stoesser et al., 2017). This demonstrates unequivocally that healthy infants may serve as a reservoir for pathogenic strains in susceptible adults.

The proportion of patients who tested positive for HO-CDI was significantly higher than the proportion who tested negative for HO-CDI. The highest proportion was observed in patients exposed to antibiotics, where a linear increase in proportion to the additional antibiotic used was observed. Antibiotic exposure is critical for the establishment of toxigenic *C. difficile* in the gut (Webb et al., 2020). According to the findings of this study, antibiotic use and duration of use were significantly associated with the development of CDI. The most commonly prescribed antibiotics were ceftriaxone, amoxicillin/clavulanic acid, and metronidazole. This is consistent with findings from Kenya, Tanzania, Uganda, and other countries that took part in the Global Point Prevalence Survey (Global-PPS) (Kiguba et al., 2016; Momanyi et al., 2019; Sonda et al., 2019; Versporten et al., 2018). The growing body of evidence demonstrates that regulated use of fluoroquinolones not only significantly reduces the incidence of *C. difficile*, but also results in the replacement of major clones that express the *gyrA* Thre82Ile mutation with minor clones that exhibit a low level of resistance to fluoroquinolones (K. E. Dingle et al., 2017; Lawes et al., 2017; L. V. McFarland et al., 2016; Sarma et al., 2015; Stoesser et al., 2017). Following this successful intervention, additional multimodal antimicrobial stewardship interventions are required to promote antibiotic stewardship and thus reduce the burden of CDI. It is important to note that in the majority of the facilities in resource-limited settings, antibiotic prescription decisions are heavily influenced by the clinical situation of the patients, high cost of laboratory investigations, and a lack of facilities to perform culture and antibiotic sensitivity testing (Chem et al., 2018). Additionally, the high number of admissions to medical and pediatric wards for infectious clinical conditions may have influenced the antibiotic prescribing practices observed in this study. This, combined with exposure to multiple antibiotics, demonstrates the antibiotics' profound effect on the indigenous gut flora. As previously described, this practice imposes selective pressure and increases the risk of CDI. As a result, it is not surprising that the risk of HO-CDI increased significantly in direct proportion to the number of antibiotics prescribed.

In this study, admission within the last three months, NGT feeding, and comorbidities were all significantly associated with an increased risk of HO-CDI. HIV/AIDS was the most

prevalent comorbidity, followed by hypertension, iron deficiency anemia, tuberculosis, malnutrition, and diabetes. The majority of these comorbidities are related to polypharmacy and prolonged hospitalization, both of which have a detrimental influence on the shift from *C. difficile* colonization to subsequent HO-CDI, owing to a lower immune response to *C. difficile* (Vincent et al., 2016).

5.2 Toxin profiles and antimicrobial resistance phenotypes

Toxin profiles

As discussed in literature review section, CDI is primarily attributed to the production of toxins A and/or B, as well as binarity toxins on exceptional cases. Except for one isolate that did not express either of the toxin genes, the majority of isolated strains were PCR positive for at least one toxin. Notably, most isolates described in this study were $A^{-*}B^{+}$ variants harboring a previously described truncated *tcdA* gene (Ludovic Lemee, Dhalluin, Testelin, et al., 2004), in contrast to the previously described predominant $A^{+}B^{+}$ variants (Oyaro et al., 2018). Although $A^{-*}B^{+}$ variants are particularly common in Asia, they have been identified in many countries globally. It is believed that the early reliance on diagnostic tests that focused exclusively on toxin A allowed $A^{-*}B^{+}$ strains to circulate undetected for an extended period of time, thereby facilitating their spread (Imwattana, Wangroongsarb, et al., 2019). $A^{-*}B^{+}$ strains are capable of causing severe and recurrent disease and have been linked to a number of significant nosocomial outbreaks in Dublin (Denise Drudy et al., 2007), Canada (Al-Barrak et al., 1999), Australia (Elliott et al., 2011), Japan (Sato et al., 2004), Israel (Samra et al., 2002), and Netherlands (Kuijper et al., 2001). In Argentina, the increasing trend of this variant resulted in the complete replacement of the dominant $A^{+}B^{+}$ variant (Abraham Goorhuis et al., 2008). While both $A^{+}B^{+}$ and $A^{-*}B^{+}$ variants are modulators of nosocomial CDI, it is clear that $A^{-*}B^{+}$ variants are endemic in the patients sampled in this study. As a result, additional research is needed to determine whether the high prevalence of $A^{-*}B^{+}$ strains is due to a localized outbreak or simply reflects the pattern of strains found in the region.

The presence of strains expressing the binary toxin gene in Kenyan hospitalized patients is noteworthy because they have never been described in Kenya before, despite being linked to increased disease severity (Gerding et al., 2014). Similarly, a subset of isolated strains possessed *cdtA* and not *cdtB.* Although these strains are uncommon, they have been reported previously (Azimirad et al., 2018).

Interestingly, three of the isolates in the current study exhibited the rare A^+B^-variation. All three were PCR positive for the *tcdA* gene (both full length and truncated) but failed to yield a product for the *tcdB* gene, despite multiple attempts. A significant limitation of the *tcdA* primer set used in this study is that it does not detect additional deletions within the 5'- region of the gene, such as those found in toxinotype XI strains (Geric Stare & Rupnik, 2010). Although A^+B^- variants are uncommon, they have been reported previously (Monot et al., 2015), and it would be interesting to investigate the pathogenicity locus in these isolates further and its implications in disease onset.

Antimicrobial resistance

This study established high frequencies of resistance to the macrolide-lincosamide-streptogramin B (MLS_B) family of antimicrobials (clindamycin, erythromycin), fluoroquinolones (ciprofloxacin), cephalosporins (ceftriaxone), and rifamycins (rifampicin). A similar trend was reported by Spigaglia among clinical isolates of *C. difficile* (Spigaglia, 2016). Based on FDA adverse reporting system, antibiotic use specifically of penicillin combinations, carbapenems, cephalosporins, tetracyclines, macrolides, fluoroquinolones and trimethoprim-sulfamethoxazole was associated with an increased risk of developing CDI (Teng et al., 2019). The high-level of fluoroquinolone resistance has been linked to major outbreaks of "hypervirulent" *C. difficile* 027/BI/NAP1 strains (He et al., 2013; Spigaglia, 2016). Resistance to antibiotics in the fluoroquinolone class is caused by mutations in the quinolone resistance-determining regions (QRDR) of *gyrA* or *gyrB* genes (Dridi et al., 2002). However, because resistance is not associated with a cost to fitness, fluoroquinolone resistance can occur even in the absence of antibiotic pressure, allowing resistant strains to be stably maintained within *C. difficile* population (Vernon J J et al., 2019; Wasels et al., 2015). Additionally, *C. difficile* resistance to antimicrobials of the MLS_B class and tetracyclines is primarily mediated by *ermB* and *tet* genes, both of which are commonly found in mobile genetic elements that promote horizontal resistance transfer between strains.

Previously, prolonged rifampicin use, particularly in the treatment of tuberculosis (TB), was implicated in the emergence of rifampicin resistance in *C. difficile* (J. M. Choi et al., 2011). Kenya is one of the 30 countries with a high burden of TB, according to World Health Organization (WHO) (Enos et al., 2018). As a result, the high resistance (91.5%) of *C. difficile* isolates to rifampicin could be due to selective pressure caused by extensive use of rifampicin in first-line TB treatment regimens. Resistance mechanisms of rifampicin and

other rifamycin group of antibiotics in *C. difficile* are also attributed to the *rpoB* gene mutation, as is the case with other bacterial agents (Curry et al., 2009a; Dang et al., 2016). Thus, the findings of this study builds on those of a recent study conducted in Cape Town, South Africa, which reported an extremely high level of rifampicin resistance (~95% of strains resistant) in *C. difficile* isolated from tuberculosis patients undergoing treatment (B. R. Kullin et al., 2018).

Metronidazole and vancomycin continue to be the first line of defense against *C. difficile* infections, despite reports of decreased susceptibility to these antibiotics (Cohen et al., 2010). While almost all of the isolates in the current study were susceptible to metronidazole, three strains had MICs of ≥ 32 mg/L. Recent reports of metronidazole treatment failures due to substantial prolonged antimicrobial exposure are on the rise (Spigaglia, 2016). Interestingly, in this study, none of the three patients from whom these metronidazole resistant isolates were recovered had prior metronidazole exposure. Metronidazole resistance is primarily caused by alterations in metabolic pathways involved in DNA repair, iron metabolism, nitro-reductase carriage and the presence of transmissible plasmid (Boekhoud et al., 2020; Chong et al., 2014).

The results of this study indicate that $A^{-}B^{+}$ strains were resistant to more antimicrobials than other toxin profiles identified. RT017 strains are the most frequently described $A^{-}B^{+}$ isolates worldwide, and strains belonging to this ribotype have been shown in several studies to have a stronger association with MDR (A. Goorhuis et al., 2011; Jieun Kim et al., 2016; Putsathit et al., 2017; Spigaglia et al., 2011). Additionally, the majority of the isolates recovered exhibited MDR to a combination of rifampicin, tetracycline, clindamycin, ceftriaxone, ciprofloxacin, and erythromycin. Similar findings, in which more than half of *C. difficile* isolates recovered exhibited MDR to these antibiotics have been linked to major epidemics globally (Carman et al., 2018; Marcela Krutova et al., 2015; Piotr Obuch-Woszczatyński et al., 2014; Ramírez-Vargas et al., 2017; Spigaglia, 2016; Zhou et al., 2019). The mechanisms underlying these associations are unknown. However, antimicrobial resistance has most likely facilitated the spread of these isolates in the regions where they are found.

5.3 Predictors of HO-CDI

Several factors were linked to the development of HO-CDI, including age (3-15, 26-45 and ≥60 years), admission duration (2 weeks), antibiotic exposure to three or more than four antibiotics, previous hospitalization in the last three months, surgical procedures, nasogastric feeding, use of amoxicillin/clavulanic acid, benzylpenicillin/gentamicin, meropenem, ciprofloxacin, clarithromycin, trimethoprim-sulfamethoxazole, clindamycin, ceftriaxone,

anti-tuberculosis agents, antiretrovirals and chemotherapeutic agents, as well as the presence of comorbidities such as chronic pulmonary disease, chronic kidney disease, hypertension, diabetes, iron deficiency anemia, tuberculosis. After adjusting for potential confounders, almost all risk factors remained constant, with the exception of tuberculosis and the use of trimethoprim-sulfamethoxazole, antituberculosis agents, and antiretrovirals, which were associated with a decreased risk of developing HO-CDI.

Medication exposure

In this study antibiotic exposure was associated with fourfold increase in the risk of acquiring CDI. The strongest association was observed for benzylpenicillin/gentamicin and clindamycin with at least eightfold increase. Additionally, the number of antibiotics administered to a patient was associated with an increased linear odd of developing HO-CDI. As previously documented, exposure to broad spectrum antibiotics including amoxicillin/clavulanic acid, benzylpenicillin/gentamicin, meropenem, ciprofloxacin, clarithromycin, trimethoprim-sulfamethoxazole, clindamycin, ceftriaxone resulted in higher risk of infection (K. A. Brown et al., 2013; Furuya-Kanamori, Stone, et al., 2015). Prior antibiotic use increases the risk of developing *C. difficile* diarrhea by up to 60% by reducing the load of bacteria known to absorb the short chain fatty acids, thereby favoring the replication and vegetative growth of *C. difficile* in the gut of susceptible individuals, and facilitating toxin production and osmotic diarrhea from *C. difficile* (Donskey, 2004; Gregory et al., 2021; Pultz & Donskey, 2005; M Rupnik et al., 2009; Slimings & Riley, 2014). Furthermore, because *C. difficile* is resistant to most antibiotics, it continues to thrive and grow even during antibiotic therapy (M Rupnik et al., 2009). This is particularly true for the broad-spectrum antibiotics (Gupta et al., 2021; L. V. McFarland et al., 2016). Although this study did not find an association between HO-CDI and either metronidazole or vancomycin, which are both used to treat HO-CDI, these medications have been implicated as potential risk factors. (Pakyz et al., 2014; Stevens et al., 2011). Further, this study also noted that use of chemotherapeutic agents in oncology patients also increased the likelihood of developing CDI as previously documented (Abughanimeh et al., 2018).

Contrary to what was observed in the univariate analysis, trimethoprim-sulfamethoxazole, antituberculosis agents, and antiretrovirals were associated with a decreased risk of developing HO-CDI in the multivariate analysis. This was most likely due to the confounding effect of an underlying association. Given that the most commonly prescribed antibiotics during hospitalization were associated with an increased risk of HO-CDI, the findings of this

study emphasizes the importance of prudent antibiotic use in this population, particularly prescribing antibiotics with lower risk while managing patients at increased risk of HO-CDI.

Age

Advanced age is primarily an independent risk factor for CDI. Similar to previous studies, this study found individuals aged more than 60 years to have increased (4-times) risk of developing HO-CDI after adjusting for other potential confounding variables (Czepiel et al., 2015; Gupta et al., 2021; McDonald & Lee, 2015). Increased risk could be attributed to host physiological changes that impair immune responses towards pathogens of the gastrointestinal tract and make them more susceptible to infectious disease, as well as other complications such as surgery, extended hospitalization, and antibiotic therapy, all of which predispose to HO-CDI (Clabots et al., 1992; Hung et al., 2015; Johnson et al., 1991; L. V. McFarland et al., 2016a). Additionally, an increased risk was seen in pediatric patients younger than five years, consistent with findings from a study conducted to assess the epidemiology of CDI, which revealed an increased risk of HO-CDI in both extreme age groups (Lessa et al., 2012). Interestingly, this study did not observe the typical strong association between age and CDI observed in European and US studies. As noted in the multivariate logistic regression model, other than the elderly, individuals aged 26-45 years also had higher odds of developing CDI although the risk is lower than that seen in the elderly. Risk factors such as antibiotic use, hospitalization, comorbidities and care giving roles especially for infants and elderly who are thought to be high carriers of *C. difficle*. This disparity has been noted in African studies and may reflect the generally younger African population as well as differences in underlying risk factors (Rajabally et al., 2013) and in young adult patients without exposure to antibiotics (Jafari et al., 2013).

Invasive Procedures

The risk of HO-CDI was observed in patients who underwent invasive procedures such as surgery and nasogastric feeding. In comparison to other studies, the current study found that surgical procedures resulted in a 2.4-fold increase in the risk of HO-CDI (Abdelsattar et al., 2015; Nguyen et al., 2021). However, the risk varies depending on the surgical procedure, with the highest risk documented in those undergoing colectomy and resection of the small bowel or colon, resulting in poor disease outcomes (K. Kong et al., 2021). In practice, surgical patients receive preoperative prophylactic antibiotics. CDI is always preceded by

antibiotic use, and thus the frequent use of prophylactic antibiotics during surgical procedures may contribute to the increased risk of CDI.

NGT feeding was associated with a 2.9-fold increased risk of HO-CDI in this study, which is higher than the 1.8-fold observed in a meta-analysis (Wijarnpreecha et al., 2018), but lower than the 3.8-fold observed in China (OR: 3.8) (D. Wang et al., 2020). There are several hypotheses about how NGT feeding increases the risk of CDI. To begin, the tubes may become contaminated during insertion by HCW. Secondly, feeds, particularly those low in dietary fiber, decrease the colon's acidic pH, thereby capacitating *C. difficile*. Finally, NGT disrupts the normal gut flora, increasing susceptibility to CDI (O'Keefe, 2010; D. Wang et al., 2020; Wijarnpreecha et al., 2016).

Chronic kidney disease

The current study observed that 15.5% of HO-CDI patients had underlying chronic kidney disease (CKD), resulting in a significant odds ratio of 3.88 (95% CI, 1.57-9.62). Previous studies comparing patients with and without underlying CKD found that the former had a higher risk of developing initial and recurrent CDI episodes (Phatharacharukul et al., 2015; Ramesh & Yee, 2019). Similarly, a recent study found a fourfold increased risk of developing CDI in patients with underlying CKD (OR: 3.676, CI: 1.626-8.309, p=0.002) (Ziyu Yang et al., 2020). The risk is even higher in patients with both acute and chronic renal disease, as well as those on long-term dialysis (Keddis et al., 2012; S. C. Kim et al., 2016; Mullane et al., 2013). Given their impaired immune function to fight *C. difficile* toxins, CKD patients are more likely to contract infections, necessitating increased use of broad-spectrum antibiotics and hospitalization (Mihaescu et al., 2021; Ramesh & Yee, 2019). These factors are known CDI prerequisites and thus increase the likelihood of CDI occurrence. Predisposition to CDI is also increased in CKD patients with low gastric acid, *Clostridioides* species colonization, and coexisting conditions such as cancer and inflammatory bowel disease (IBD) (Aronsson et al., 1987; Dudzicz et al., 2021; Tariq, Singh, et al., 2017b). Reduced kidney function typically impairs the body's ability to eliminate toxins introduced by microbes, altering the functions of the intestinal microbiota and activating systemic inflammation, thereby increasing susceptibility to HO-CDI (Anders et al., 2013; Ramezani et al., 2016; Vaziri et al., 2013).

Hypertension

Hypertension was the second most prevalent comorbidity (12.6%) in this study, after HIV/AIDS, and was associated with an increased risk of developing HO-CDI (OR: 2.47, 95% CI: 1.00-6.07, p=0.049). Similar findings have been published from studies conducted in hospitals in the Netherlands and the United States (Hensgens et al., 2014; Malik et al., 2020). While hypertension has been widely documented to increase the risk of developing CDI, no causal relationship has been established. However, experimental evidence from human and animal models suggests that hypertension has an effect on dysbiosis of the gut microbiota (Silveira-Nunes et al., 2020; T. Yang et al., 2015). Furthermore, antihypertensive medications have been shown to either improve or compromise the intestinal microbiota (Jama et al., 2018; Robles-Vera et al., 2020). Verapamil, for instance, shields cells from *C. difficile* intoxication (Caspar et al., 1987). However, because no information on antihypertensive medication was gathered in this study, it was impossible to determine whether the increased odds were due to hypertension or the hypertensive medication.

Diabetes

Diabetes was another significant chronic disease predictor identified in this study. Diabetic patients were fourfold more likely than non-diabetic patients to develop HO-CDI (OR: 3.56, 95% CI 1.11-11.38, p=0.032). The relationship between CDI and diabetes has been extensively researched, where it has been identified as a possible independent risk factor for both primary and recurrent CDI (Hung et al., 2019; Shakov et al., 2011; Shoaei et al., 2020). Diabetes causes structural remodeling of the colon, which alters various gastrointestinal tract functions, including impaired motility and an altered composition of the intestinal microbiota, both of which contribute to *C. difficile*-driven diarrhea (Piper & Saad, 2017; Tottey et al., 2017). In contrast, Eliakim-Raz et al. found in their case-control study that diabetic patients treated with metformin, an anti-diabetic drug, had a lower risk of developing CDI (OR: 0.58; 95% CI, 0.37-0.93; p = 0.023) than their counterparts (Eliakim-Raz et al., 2015). Similarly, an interventional study discovered that diabetic patients receiving metformin had a lower number of *Clostridioides* spp. in their gut (Bryrup et al., 2019). Although the exact mechanism is unknown, one possibility is that metformin alters reabsorption of secondary bile acid, thereby inhibiting spore germination, vegetative growth, and toxin activity of *C. difficile* (Tam et al., 2020; Thanissery et al., 2017; Winston & Theriot, 2016). Thus, while no causal relationship has been established, it is clear that structural and functional changes in

the colon caused by diabetes or diabetes medication are likely to alter the composition of the gut microbiota, thereby increasing or decreasing the risk of CDI (Q. Zhang & Hu, 2020).

Tuberculosis

Tuberculosis was significantly more prevalent in HO-CDI positive cases than in HO-CDI negative cases (21.1% vs 9.5%, p=0.008). The univariate analysis revealed a correlation between tuberculosis and HO-CDI, which corresponded to similar studies conducted in Africa (B. R. Kullin et al., 2018; Legenza et al., 2018). However, after adjusting for potential confounders such as anti-tuberculosis treatment, no statistically significant difference between patients with and without HO-CDI was observed. Thus, the association between tuberculosis and HO-CDI may have arisen because of the confounding effect of anti-tuberculosis drug exposure. Rifampicin has previously been shown to induce CDI in patients receiving anti-tuberculosis therapy (Y. M. Lee et al., 2016) owing to its efficacy against a diverse array of gut bacteria other than *C. difficile* (Nakajima et al., 2000). Furthermore, long-term rifampicin use has resulted in high resistance rates, promoting the persistence of resistant *C. difficile* strains in tuberculosis patients (J. M. Choi et al., 2011; P. Obuch-Woszczatyński et al., 2013). This is essentially true as a large proportion of *C. difficile* isolates in this study exhibited high resistance to rifampicin.

HIV/AIDS

Although HIV/AIDS was a more prevalent comorbidity (17.7%) among study participants, and even more so in HO-CDI cases (25.4%), the association between HIV/AIDS and the development of HO-CDI was not statistically significant (OR: 1.8, p=0.06), resulting in a 42% lower risk of developing HO-CDI. Additionally, it was noted that the majority of patients (15.3%) received antiretroviral therapy, and as previously stated, antiretrovirals have been shown to reduce the risk of CDI (Collini et al., 2013). This hypothesis is supported by the results of the adjusted model of the current study which show that patients taking antiretrovirals were 75% less likely to develop HO-CDI (OR: 0.25, 95% CI: 0.05-1.22, p=0.087). This finding is consistent with previous research, though similar studies within the continent have suggested a possible association between pre-existing HIV/AIDS and CDI in both adults and children (Beadsworth et al., 2014; Collini et al., 2013; Imlay et al., 2016a; Mwachari et al., 1998; Onwueme et al., 2011; Isaac Zulu et al., 2000). The association could be attributed to low CD4 T cell counts; Haines et al. observed an increased risk of CDI in HIV patients with CD4 counts of ≤50 (Haines et al., 2013). T cell defects impair the anti-

toxin antibody response to *C. difficile* toxins, thereby increasing susceptibility to infection (P. F. Johnston et al., 2014). However, because no data on CD4 counts were collected in this study, this observation could not be evaluated.

Charlson Comorbidity Index (CCI) and Elixhauser Comorbidity Index (ECI)

In both CCI and ECI classification, there was sufficient evidence ($p < 0.001$) to reject the null hypothesis and conclude that there is an association between the CCI and ECI comorbidity scores and the primary outcome of HO-CDI. Despite their differences in weighting and number of comorbidities, both models performed well with minor differences in their validation values. It is interesting that patients with CCI ≥3 were no more likely to have HO-CDI than those with scores of 0. A possible reason for this might be that patients in this group are regarded as having moderate and severe comorbidity levels raising the likelihood that the diarrhoea, they experienced was due to causes other than CDI. In testing for goodness-of-fit, the adjusted/complex model was shown to fit the dataset significantly better (p value < 0.0001) for both the CCI and ECI groupings. However, most remarkable observation from the analysis was that the Elixhauser classification appeared as a better predictor than the Charlson classification in both the unadjusted (Pseudo R-squared 7.89 vs 6.09) and adjusted models (Pseudo R-squared 27.55 vs 27.04). These findings are consistent with earlier studies where the Elixhauser grouping was reported to be a better predictor of an outcome compared to the Charlson grouping, albeit by a small margin (Sharma et al., 2021).

In this study, there was no statistically significant association between HO-CDI and pre-existing peptic ulcer disease, liver disease, inflammatory bowel disease, low vitamin D levels (rickets), solid tumors without metastasis, or weight loss (malnutrition). This observation contradicted previous research findings (Abdalla et al., 2020; Furuya-Kanamori et al., 2017; H. Y. Lee et al., 2019; Nitzan et al., 2013; Perez-Cruz et al., 2018; W. J. Wang et al., 2014). The establishment of associations may have been hampered by the small number of cases presenting with the specific conditions. As a result, future clinical studies should investigate these associations considering the possibility of increased antibiotic use and hospitalization, in patients with these conditions predisposing to HO-CDI.

5.4 Sequence types, antibiotic resistant determinants and cluster analysis of nine *C. difficile* isolates

Despite the relatively low number of isolates that were subjected to comparative genomics, four sequence types were identified, with ST37 being the most prevalent. These strains clustered in a distinct population previously assigned to clade 4, which is linked to PCR

RT017 and $A^{-}B^{+}$ toxin profile (Griffiths et al., 2010). ST37 is a clinically significant emerging MLST that is dominant in Europe, Asia, and, China and has also been linked to epidemics in other countries (Cairns et al., 2015; Liu et al., 2018). Resistance to antibiotics, especially fluroquinolones (ciprofloxacin) and macrolides (clindamycin and erythromycin), has been linked to this variant. MLST ST743, ST10, and ST58 were also detected.

The phenotypic data from the nine isolates sequenced in this study demonstrated that nearly all *C. difficile* isolates were resistant to clindamycin and erythromycin. Macrolide resistance results from methylation of bacterial 23S rRNA by methylases enzymes encoded by the *erm* family of genes. Consistent with previous reports, WGS data showed that Macrolides, lincosamides and streptogramines (MLS) resistance was linked to *erm*B and *erm*G genes (Isidro et al., 2018; Waker et al., 2020). Among the sequenced isolates, four ST37 expressed the *ermG* gene, while two genomes (unknown STs) harboured the *erm*B gene. Interestingly, one of the three genomes that lacked these genes had MIC values for erythromycin and clindamycin of more than 256 μg/ml. *C. difficile* strains resistant to both erythromycin and clindamycin, or only erythromycin, but lack the *ermB* or *ermG* gene, have been described (Ackermann et al., 2003; Spigaglia et al., 2011). In absence of these genes, other determinants conferring either high-level or low-level resistance to MLS_B including chloramphenicol resistance gene (*cfr*) and efflux mechanisms, have been identified (Candela et al., 2017; K. E. Dingle et al., 2014; Isidro et al., 2018). As such resistance exhibited by the isolates in this study lacking the *erm* genes was likely conferred by a previously described *CdeA*, a multidrug efflux pump subunit that was present in all the sequenced *C. difficile* isolates (Dridi et al., 2004; Knetsch et al., 2018). Furthermore, the ST37 isolates that expressed the *ermG* and *cdeA* also expressed the *mel* gene, which is responsible for the efflux pump of macrolides. Indicating a dual efflux pump effect driven by the expression and regulation of both genes. A similar occurrence has been described in multidrug-resistant clone of ribotypes 017 and in the genomes of other non- *C. difficile* isolates (Isidro et al., 2018; Kartalidis et al., 2021). As a result, the presence of *cdeA* and *mel* genes emphasizes the importance of alternative resistance determinants in *C. difficile* that may trigger the spread of macrolide resistance. Therefore, additional research should investigate the interaction of these genetic determinants in mediating the efflux of macrolides in *C. difficile*.

In six sequenced isolates with phenotypic resistance and decreased susceptibility to tetracycline, *tetM* was found to be the encoding gene for tetracycline resistance. Although tetracycline use is linked to a decreased risk of *CDI*, the clonal spread of the epidemic strain

RT078 has been made possible by the emergence of tetracycline resistance in *C. difficile*, due to *tetM* gene.

Further the ST37 variants in this study encoded a aminoglycoside modifying enzymes *aac(6')-Ie-aph(2")* gene, which is common in Enterococcus with the possibility of horizontal genetic transfer to *C. difficile*. Although *C. difficile* is naturally resistant to aminoglycosides, the presence of *aac(6')-Ie-aph(2")* gene is imperative because it constitutes a natural reservoir of aminoglycoside resistant genes, contributing to the dissemination of these determinants to other bacterial agents that share the same environmental niche (Kartalidis et al., 2021).

While the four strains lacked *gyrB* gene, which confers fluoroquinolone resistance, the observed resistance in these strains was possibly due to alterations resulting in amino acid substitutions in the quinolone-resistance determining region (QRDR), as confirmed by the presence of the DNA gyrase subunit *gyrA*. This polymorphism in the *gyrA* gene, caused by an amino acid substitution from Thr82 to Ile, is consistent with findings from other regions of the world. This particular substitution is not only unique to ST37, but it has also been observed in epidemic strains, such as CD III/027/NAP1 (Denise Drudy et al., 2007; Spigaglia et al., 2008; B. Wang et al., 2018). Resistance to rifampicin was mediated by chromosomal mutations occurring in the *rpoB* gene of the four ST37 strains where double amino acid substitutions of H502N and R505K was observed. Similar multiple substitutions have been reported in previous studies and have been linked to the spread of epidemic strains including that of hypervirulent strains RT027 and RT017 (Chatedaki et al., 2019; Curry et al., 2009b; Miller et al., 2011; B. Wang et al., 2018).

Another intriguing finding from this study WGS analysis was the presence of the repUS43 plasmid in the four *C. difficile* isolates belonging to clade 4 and the two novel STs. The presence of this plasmid suggests the possibility of the isolates encoding antibiotic resistance genes on the plasmid, facilitating horizontal gene transfer of these genes between the isolates. Recently, a study conducted in South Africa determined that this plasmid was responsible for transporting genes encoding macrolides and tetracycline resistance (Asante et al., 2021).

The sequence types from this study clustered with isolates from Indonesia, USA and Ghana indicating that they share a common ancestor. Furthermore, the novel sequence types identified in this study are genetically related to a strain previously isolated in Ghana implying that these isolates are not unique to Kenya.

Overall, the existence of subtypes with at least one toxigenic profile, distinct antimicrobial resistance genes, and chromosomal changes imparting resistance in genetic traits all contribute to our understanding of circulating *C. difficile* variants among hospitalized patients at KNH. Despite the fact that only nine isolates were sequenced, the genomes provide baseline genetic data since no genomic epidemiology research on *C. difficile* has been conducted in East Africa.

5.6 Study limitations

This study has some limitations including: First, the participants were recruited from a one facility, therefore the findings may not be generalizable to other healthcare facilities in the country, thus, future studies should consider a multifacility approach. Secondly, data collection primarily relied on what was indicated in each patient's file, which may have led to underreporting of certain variables. Lastly, due to the small number of isolates sequenced for genotypic characterization, it was impossible to correlate the generated data with the different epidemiological profiles of the patients and phenotypic characteristics of the isolates.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study determined the prevalence and predictors of healthcare facility-onset *C. difficile* infection (HO-CDI) in symptomatic hospitalized patients admitted to Kenyatta National Hospital (KNH), in addition to assessing the toxin variants, antibiotic resistance determinants, sequence types, and evolutionary strains associated with this condition. The conclusion drawn from this study's findings is that:

1. *C. difficile* is a clinically significant cause of healthcare facility onset diarrhoea among patients receiving in-patient services at Kenyatta National Hospital. Although the prevalence was lower than that reported in a recent study in Kenya, it was generally consistent with the global picture of the documented *C. difficile* prevalence therefore contributing to the global burden of *C. difficile* in Africa.
2. Antibiotic exposure was associated with an increased risk of HO-CDI, and the proportion of cases increased as the quantity of antibiotics increased with an addition in the number of antibiotics.
3. *C. difficile* is potentially a relevant pathogen among those responsible for causing childhood diarrhoea.
4. The high rate of virulence and antibiotic resistance was associated with toxigenic *C. difficile* variants. This is the first study to report the predominance of $A^{-}B^{+}$ CDT^{-} variant expressing a truncated *tcdA* gene in a Kenyan population. In addition, the elevated levels of MIC found in the *C. difficile* isolates in this study represent potentially significant problem that may lead to untreatable infections or the use of expensive antimicrobials of last resort, such as vancomycin.
5. Age (3-15, 26-45, $\geq$ 60 years), surgical interventions, nasogastric feeding, antibiotic exposure (particularly to clindamycin, benzylpenicillin/gentamicin, amoxicillin/clavulanic acid, clarithromycin, meropenem, ciprofloxacin, ceftriaxone), multiple antibiotic use, chemotherapeutic drugs, and comorbidities (including, chronic obstructive pulmonary disease, chronic kidney disease, iron deficiency anemia, diabetes, and hypertension) can be used to predict the risk of HO-CDI in the in-patient population at KNH. As for classification of comorbidities, the comorbidity scores employed in Elixhauser Comorbidity Index (ECI) are appropriate for assessing the risk of comorbidities in the examined population.
6. *C. difficile* sequence type ST37, which encodes critical antibiotic-resistant determinants and pathogenicity loci, was the predominant ST linked with HO-CDI in

the KNH population studied. Additional distinct sequence types identified included ST10, ST58, and ST743.

7. The clustering of non-novel STs in the phylogenetic tree with STs of *C. difficile* isolated from Indonesia, Ghana, and the United States shows genetic relatedness with ST ancestors of these regions. Two novel sequence types with identical allelic patterns were identified, adding to the genome diversity of *C. difficile* on a global scale.

6.2 Recommendations

1. Health-care facilities should consider routine *C. difficile* screening in hospitalized patients, particularly those at high-risk (with determined predictors of HO-CDI). Moreover, investigation of *C. difficile* isolated from a more geographically diverse cohort of children is needed for a comprehensive understanding of the disease spectrum in children and for guiding clinical care and prevention initiatives of HO-CDI in this population. Health care facilities should also consider isolating and instituting contact precautions in suspected or confirmed cases to significantly reduce the risk of CDI transmission in hospitals.
2. It is of great concern that the $A^{-}B^{+}$ CDT^{-} variant linked to major outbreaks in several geographical regions throughout the world was the predominant variant identified in this study. As a result, this study recommends that more advanced molecular approaches be used to investigate the diversity of strain type to ascertain whether this strain is responsible for the HO-CDI outbreaks or reflects a clonal cluster specific to the population tested.
3. The substantial usage of antimicrobial agents coupled with the reported antimicrobial resistance highlights the need for regulated antibiotic prescription in hospitals and expanded antimicrobial stewardship initiatives aimed at reducing the occurrence and spread of HO-CDI.
4. The predictors of HO-CDI identified in this study should be noted at an early stage to limit potential exposures and monitor them for the development of HO-CDI and initiate targeted early treatment. Comorbidity stratification should also be considered as a suitable mechanism to simplify comorbidity assessment and support clinical management decisions.
5. Due to the limited resources available for comparative genomics at the time of this work, WGS and PCR ribotyping will be undertaken in the near future to expand on the molecular characterization of the remaining isolates. This will allow for a

comprehensive characterization and comparison of the genotypes of *C. difficile* isolated from this study. Evolutionary relationships with strains from other geographical regions will also be explored extensively.

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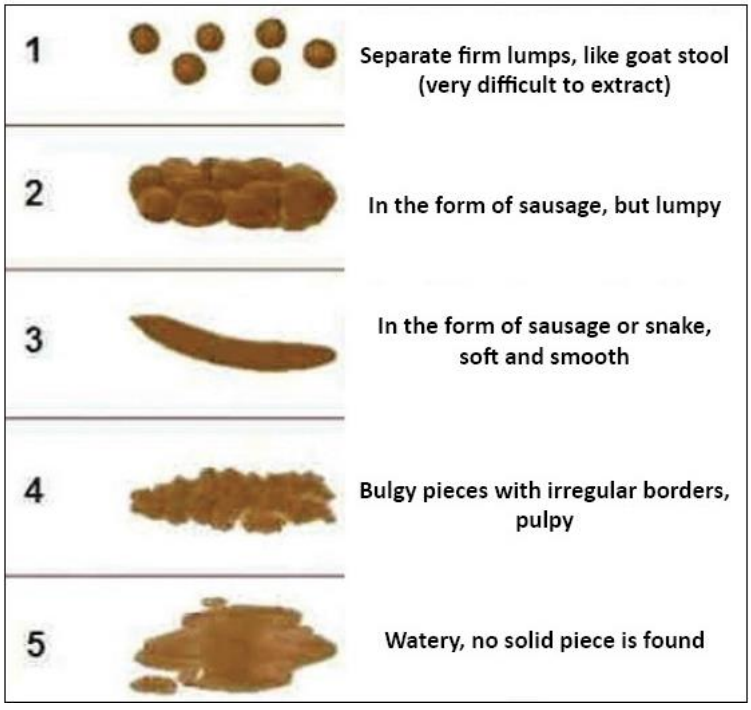
APPENDICES

Appendix I: Standard operating procedures for culture and identification of *C. difficile*

Sample preparation

1. Perform macroscopic examination of the stool sample and classify as per Bristol visual stool scale.
2. Pipette 100 µl of absolute alcohol into an Eppendorf tube.
3. Suspend a loopful of stool into the absolute alcohol to make an approximate 1:1 suspension.
4. Vortex and leave at room temperature for 60min .
5. Pipette 50 µl of the deposit into CCEY plate and streak it out.
6. inoculate the control organisms on CCEY from the stock culture.
7. Incubate anaerobically at 37C for 48 hr.

Modified Bristol stool scale



Culture interpretation and identification of *C. difficile*

1. Observe for the presence of colourless, flat, irregular-edged colonies with a characteristic phenolic odor.
2. Gram-stain the distinct colonies to reveal gram-positive rods with subterminal spores.
3. Perform qPCR for detection of triose phosphate isomerase (*tpi*) housekeeping gene.

Stock cultures

1. Prepare BHI broth and dispense in bijou bottles.
2. Pick discrete colonies from the primary culture and suspend in BHI broth.
3. Incubate overnight anaerobically at 37 ℃ anaerobically.
4. Add 500 µl of the overnight culture to 500 µl of 50% glycerol in a 1.5 mL cryovial.
5. Tightly cap the cryovial, mix well and freeze the glycerol stock tube at -80℃ for long-term storage.

Gel electrophoresis

1. Prepare 2% agarose by measuring 2g of agarose.
2. Dissolve in 100ml TAE.
3. Microwave for 3 minutes mixing within intervals of 30 sec to dissolve the agarose.
4. Let agarose solution cool down to about 50 ℃ the add 2 µl of ethidium bromide or prosafe.
5. Pour the agarose into a gel tray with the well comb in place and allow it to solidify.
6. Load the molecular weight ladder into the first lane of the gel.
7. Add loading buffer to each of the DNA samples and load into the wells.

Quality control

Each new batch of culture were counter checked for growth and gram stain with the reference strain n *Clostridioides difficile* DSMZ-27147. The DNA template for this strain was also used as an *internal* positive control to validate the PCR assays.

Laboratory work sheet

Lab ID:……

Date sample received: ___ / ___ / _____(dd/mm/yyyy)

Culture results on CCEY: O Growth O No growth

Odor: O yes O no

Gram stain results: O **Gram** positive rods O Other

AST:

Antimicrobial agent	**MIC (mg/L)**	**Interpretation**
Vancomycin		
Metronidazole		
Clindamycin		
Ceftriaxone		
Erythromycin		
Rifampicin		
Ciprofloxacin		
Tetracycline		

Molecular assay:

tpi gene	O positive	O negative
Production of toxins A full-length	O positive	O negative
Production of toxins A truncated	O positive	O negative
Production of toxins B	O positive	O negative
Presence of binary toxin genes	O positive	O negative

Toxin Profile: _____……..

Appendix II: Ethical approval

UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
P O BOX 19676 Code 00202
Telegrams: varsity
(254-020) 2726300 Ext 44355

KNH/UON-ERC
Email: uonknh_erc@uonbi.ac.ke
Website: www.uonbi.ac.ke

KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

Ref: KNH-ERC/A/152 Link:www.uonbi.ac.ke/activities/KNHUoN 15th May 2014

Winnie Chepkurui Mutai
Dept.of Medical Microbiology
School of Medicine
University of Nairobi

Dear Winnie

RESEARCH PROPOSAL: MOLECULAR EPIDEMIOLOGY OF *CLOSTRIDIUM DIFFICILE* IN A SELECTED POPULATION AT KENYATTA NATIONAL HOSPITAL (P8/01/2014)

This is to inform you that the KNH/UoN-Ethics & Research Committee (KNH/UoN-ERC) has reviewed and **approved** your above proposal. The approval periods are 15th May 2014 to 14th May 2015.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH/UoN ERC before implementation.
c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH/UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH/UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH/UoN-Ethics & Research Committee for each batch of shipment.
g) Submission of an *executive summary* report within 90 days upon completion of the study
This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

For more details consult the KNH/UoN ERC website **www.uonbi.ac.ke/activities/KNHUoN.**

Protect to Discover

Yours sincerely

PROF. M. L. CHINDIA
SECRETARY, KNH/UON-ERC

c.c. The Principal, College of Health Sciences, UoN
The Deputy Director CS, KNH
The Chairperson, KNH/UoN-ERC
The Assistant Director, Health Information, KNH
The Dean, School of Medicine, UoN
The Chairman, Dept.of Medical Microbiology, UoN
Supervisors: Prof. Omu Anzala, Dr.Marianne Mureithi, Prof. Gunturu Revathi

Appendix III: informed consent and assent

Informed Consent Documents

Project Title: MOLECULAR EPIDEMIOLOGY OF *CLOSTRIDIOIDES DIFFICILE* IN A SELECTED POPULATION AT KENYATTA NATIONAL HOSPITAL

Principal investigator: WINNIE CHEPKURUI MUTAI

Description: *Clostridioides difficile* is bacteria that produces spore which can survive harsh environments and common sterilization techniques. The bacteria are transmitted through fecal-oral, from person to person, from fingers and from hospital furniture. The bacteria causes can range from uncomplicated diarrhea to sepsis and even death. Currently there are no reports showing the extent to which CDI is common in Kenya. We would wish to conduct a study to find out what is the proportion of patients and health workers are infected with different strains of CD. We would also like to find out the factors that makes people susceptible. Finally, we will find out if the strains from patients, health workers and the hospital environment are susceptible to which antibiotics and whether or not they are genetically related. This information would help us to design accurate methods to prevent future infection as well as provide accurate management of your infection. We will ask you to provide us with stool or rectal swabs during your normal hospital visitation which will be stored for specialized testing. At a later date, part of your samples will be transported to a laboratory abroad for additional confirmation and specialized testing. **Risks and discomfort:** One potential risk of being in the study is the loss of privacy. However, we will do our best to make sure that the personal information gathered during this study is kept private. You will not feel any discomfort when collected stool or rectal swabs. There is no monetary benefit for your participation in this study. **Benefits:** Finding of CD infection will help us design accurate preventive measures as well provide best management for those infected. **Confidentiality: Y**our participation in the study will be confidential. Your samples will be identified only by coded number. In any reports generated from this study none will use your names. **Voluntary participation:** The decision to participate in this study is purely your choice. Your decision whether or not to participate in this study will not affect your treatment or working at KNH. **Time involvement**: This study will be part of your routine out and in patient visit to KNH. You will be engaged for at least 30 minutes **Questions:** You are free to ask any questions at any time about the study as well as regarding your rights as a research volunteer. You will not be giving up any of your rights by signing this consent form. **Further information**: Please contact the following: **Winnie Mutai of** Department of Medical Microbiology; College of Health Science-University of Nairobi. PO Box: 19676 Nairobi. Tel: 0724886584

If you have questions about your rights as a study participant, or are dissatisfied at any time with any aspect of this study, you may contact - anonymously, if you wish the Chairperson of the University of Nairobi/Kenyatta National Hospital Research and Ethics Committee (UoN/KNH-ERC), PO Box 19676, Nairobi, Kenya; Tel: +254 020 2726300 ext 44102.

Statement of consent: I have read this form or had it read to me in a language that I understand. I have discussed the information with study staff. My questions have been answered. My decision whether or not to take part in the study is voluntary. If I decide to join the study I may withdraw at any time. By signing this form I do not give up any rights that I have as a research participant.

I have read or have had the document read to me: YES___ NO____

I agree to participate in this research study: YES___ NO____

I agree to have my stool/swabs collected/stored and analyzed for study assays: YES___NO___

_____ _____

Participant Signature Date

I, the undersigned have fully explained the relevant details of this research study to the participant named above and believed that the participant has understood and has knowingly given his consent.

_____ _____ _____

Study Staff Conducting Study Staff Signature Date

CHILD ASSENT

Project Title: MOLECULAR EPIDEMIOLOGY OF *CLOSTRIDIOIDES DIFFICILE* IN A SELECTED POPULATION AT KENYATTA NATIONAL HOSPITAL

Principal investigator: WINNIE CHEPKURUI MUTAI

My name is Winnie Chepkurui Mutai a PhD student at the University of Nairobi. I am currently pursuing my research work that will involve collecting feaces from patients who develop diarrhoea after two to three days of admission following use of antibiotics. I will then take your sample to the laboratory to check the bug making you have the diarrhoea. Therefore if you agree, you will be asked afew questions including your age, why you were hospitalized, when you developed the diarrhoea and if you are currently using any medication. Once you have answered the questions you will be given a container labeled with you name to provide stool sample at your own convenience. The research assistant will then come back and check if you have provided the sample and take it to the laboratory for analysis.

The analysis takes about one week if we find the bug in your stool sample we will inform the doctor attending to you so that he/she can do a follow up.

You may be helping us understand if the medication you are using is causing you to develop the diarrhoea. This will help the doctors get the right treatment that you will not react to.

If you agree to help us, you should know that your friends will not know that you were involved in this study and that we collected sample from you and also they will not know your results. You have a right to say no that is you do not wish to participate in this study and by you declining does not mean that you will not receive treatment or you will be treated differently. Remember the procedure does not involve any pricking so you will not feel pain; you will just give us you stool sample that's all.

There are no right or wrong answers to the questions that you will be asked. As for the complicated questions we will look into your file to check the answers for example you may not know the antibiotics you are using and hence we will check in your file to know. Kindly talk to your parent or you guardian before you make a decision to participate in this study or not. Permission will be sought from your parent/ guardian as well for you to be involved in this study, but even if your parents say "yes," you can still say "no" and decide not to be in the study. If you wish not to continue with the study once we begin that's okay too.

You are free to ask any questions concerning this study. If you have any concerns, you can always call me or ask you guardian/parent to call me on this no. 0724886584

I agree to take part in the study on Molecular Epidemiology of *Clostridioides difficile* in a selected population in Nairobi, Kenya. I have read and understood the accompanying letter and information leaflet. I have been given a preview of what the study is about and the part I will be involved in. I know that I do not have to answer all of the questions and that I can decide not to continue at any time. Name _____

Signature _____ Age_____

I have read and understood the child assent and I give permission as a parent/Guardian for the child (named above) to be included.

Name _____

Relationship to child _____

Signature _____

Parental/Guardian Consent Form for child's participation in a research project

Project Title: MOLECULAR EPIDEMIOLOGY OF *CLOSTRIDIOIDES DIFFICILE* IN A SELECTED POPULATION AT KENYATTA NATIONAL HOSPITAL

Principal investigator: WINNIE CHEPKURUI MUTAI

Introduction

You are invited to consider allowing your child to participate in this research study. Please take as much time as you need to make your decision. Feel free to discuss your decision with whomever you want, but remember that the decision to allow your child to participate, or not to participate, is yours. If you decide that you allow your child to participate, please sign and date where indicated at the end of this form.

Purpose

The purpose of this research is to determine whether *Clostridioides difficile* causes nosocomial diarrhoea among patients who have been admitted in hospitals following treatment or medical procedures. The results of this study will guide on treatment options for such cases and inform on how prevalent the infections due to this bug is

Procedures

Your child is being asked to take part in this study because she/he has been admitted for two days and has diarrhoea. About 370 patients will be recruited from different hospitals both adults and children as long as they meet the criteria.

If you agree for your child to participate in this study he/she will be asked to provide a stool sample to the laboratory to check the bug making him/her to diarrhoea. You will also be asked afew questions prior to sample collection including the age, reason for hospitalization, when did the diarrhea start and if your child is currently using any antibiotics. The interview will take roughly 10-15minutes. The research assistant will fill in the details you have provided and some will be extracted from the child's file as well. The sample will be taken to the laboratory for analysis.

The analysis takes about one week if we find the bug in your child's stool sample we will inform the doctor treating your child so that she/he can do a follow up.

You or your child can stop participation at any time. However, if you decide to stop participating in the study, we encourage you to talk to the researcher first.

Risks: There are no direct risks associated with participating in this research but one likely risk of being in the study is the loss of privacy. However, we will do our best to make sure that the personal information gathered during this study is kept private. Your child will not feel any discomfort when collected stool or rectal swabs. There is no monetary benefit for allowing your child to participate in the study. **Benefits:** Finding of *Clostridioides* associated diarrhea will help us design accurate preventive measures as well provide best management for those infected. **Confidentiality: Y**our child participation in the study will be confidential. His/her sample will be identified only by coded number. In any reports generated from this study none will use his/her name. **Voluntary participation:** The decision to participate in this study is purely your choice. Your decision as to allow or not to allow your child to be part of this study will not affect your treatment at KNH. **Time involvement**: You will be engaged for at least 10 to 15 minutes **Questions:** You are free to ask any questions at any

time about the study as well as regarding your rights as a research volunteer. You will not be giving up any of your child's rights by signing this consent form. **Further information**: Please contact the following: **Winnie Mutai of** Department of Medical Microbiology; College of Health Science-University of Nairobi. PO Box: 19676 Nairobi. Tel: 0724886584

If you have questions about your rights as a study participant, or are dissatisfied at any time with any aspect of this study, you may contact - anonymously, if you wish the Chairperson of the University of Nairobi/Kenyatta National Hospital Research and Ethics Committee (UoN/KNH-ERC), PO Box 19676, Nairobi, Kenya; Tel: +254 020 2726300 ext 44102.

Statement of consent: I have read this form or had it read to me in a language that I understand. I have discussed the purpose and procedures, the possible risks and benefits, and that my child participation in this research is completely voluntary. My questions have been answered. I freely and voluntarily agree to allow my child to participate in this study

_____ _____

Name of Minor Minor's Date of Birth

_____ _____

Signature of Parent/Guardian Date

I, the undersigned have fully explained the relevant details of this research study to the participant named above. I have invited the participant to ask questions and I have given complete answers to all of the participant's questions and the participant has knowingly given his consent.

_____ _____ _____

Study Staff Conducting Study Staff Signature Date

Appendix IV: Comorbidity Index assigned weight

	Charlson Comorbidity Index (CCI)	Elixhauser Comorbidity Index (ECI)
Condition[a]	**Assigned weight**	**Assigned weight[b]**
Comorbidity Variables		
Congestive heart failure	1	7
Cardiac arrhythmias	-	5
Pulmonary circulation disorders	-	4
Hypertension	-	0
Peptic ulcer disease	1	-
Hemiplegia	2	7
Hypothyroidism	-	0
Renal disease	2	5
Liver disease	-	11
Solid tumour without metastasis	2	4
Metastatic solid tumour	6	12
HIV/AIDS	6	0
Lymphoma	-	9
Weight loss (malnutrition)	-	6
Deficiency anaemia	-	-2
Depression	-	-3

Appendix V: Questionnaire
Date: _____

Date of Admission: _____

Patient identification number _____

Ward (Unit) ID: _____

Ward specialty (see code list): _____

Gender: _____

Age: _____

1. Have you experienced diarrhea?

   O yes O no O unknown

2. For how long have you had the diarrhea?

   O <1 week O 1 to 3 weeks O >3 weeks O unknown

3. Did you have stomach cramps?

   O yes O no O unknown

4. Was the stool bloody?

   O yes O no O unknown

5. What other symptoms are you experiencing?
   - Fever
   - Vomiting
   - Gas
   - Bloating
   - others

6. Onset date of symptoms _____
7. Are you taking any antibiotics currently?

   O yes O no O unknown

   If answer is "yes":

   Name: _____ Dated started _____

8. Are you receiving any chemotherapeutic drugs?

   O yes O no O unknown

9. Duration of hospitalization in weeks
   - 0-1
   - 1-2
   - 2-3

- 3-4
- 5>

10. Reason for hospitalization

- Delayed procedure
- Delayed healing
- Lack of financial resources
- Others (specify)_____

11. Has the patient been admitted to a hospital in the past 3 months (apart from a possible current admission)?

12. Has the patient used proton pump inhibitors?

O yes O no O unknown

If yes which PPI?_____

13. Has the child received any enemas?

O yes O no O unknown

If yes which?

_____

14. Has the patient been using NGT for feeding?

O yes O no O unknown

15. Has the patient had a bowel disease?

O yes O no O unknown

If yes which?

_____

16. Has the patient had GI surgery?

O yes O no O unknown

17. Has the patient had any hematological malignancies?

O yes O no O unknown

18. Has the patient had bone marrow transplant?

O yes O no O unknown

19. What are the co-morbidities in the patient? (**Tick the attached table**)

Comorbidity Name	**Yes**	**No**	**Comorbidity Name**	**Yes**	**No**
Congestive heart failure			Iron deficiency anaemia		
Cardiac arrhythmias			Depression		
Pulmonary circulation			Chronic pulmonary disease		

Hypertension			Diabetes		
Peptic ulcer disease			Chronic kidney disease		
Hemiplegia			Peripheral vascular disease		
Hypothyroidism			Renal failure		
Renal disease			Obesity		
Liver disease			Paralysis		
Solid tumour without			Psychoses		
Metastatic solid tumour			Coagulopathy		
HIV/AIDS			Alcohol abuse		
Lymphoma			Rheumatoid arthritis		
Weight loss (malnutrition)			Valvular Disease		

20. Has a sigmoidoscopy or colonoscopy been performed during the last three months?

O yes O no O unknown

If answer is “yes”:

Date of first sigmoidoscopy or colonoscopy: _____

21. Were pseudomembranes seen during the (first) sigmoidoscopy or colonoscopy?

O yes O no O unknown

22. Was ulceration seen during the (first) sigmoidoscopy or colonoscopy?

O yes O no O unknown

23. Has an abdominal CT scan been performed during the current episode of *Clostridioides difficile* infections?

O yes O no O unknown

If answer is “yes”:

Date of (first) abdominal CT scan: _____

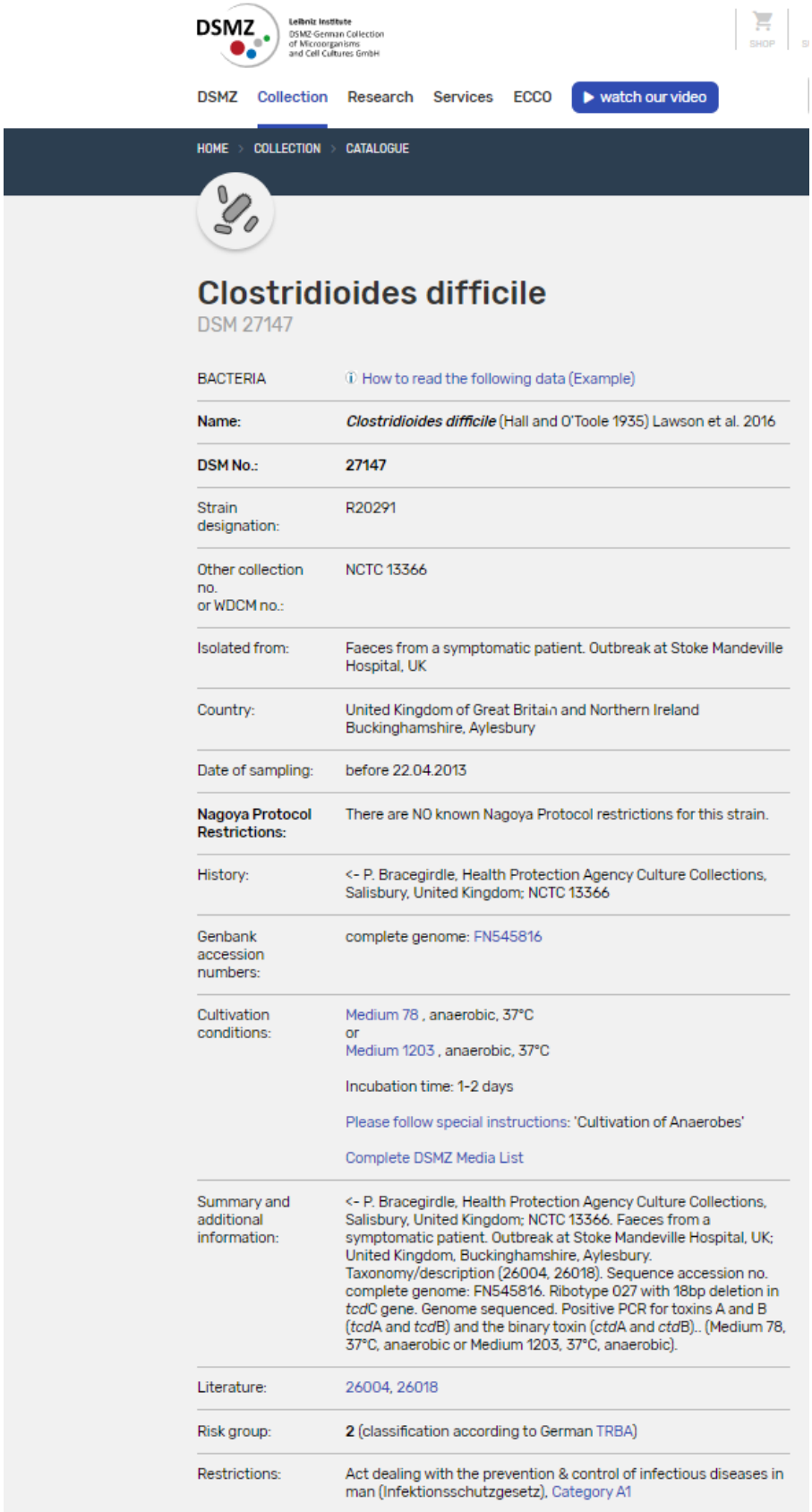
24. Was colonic wall thickening seen on the (first) abdominal CT scan?

O yes O no O unknown

25. Was pericolonic fat stranding seen on the (first) abdominal CT scan?

O yes O no O unknown

Appendix VI: Quality control strain



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Clostridioides difficile

DSM 27147

BACTERIA	ⓘ How to read the following data (Example)
Name:	***Clostridioides difficile*** (Hall and O'Toole 1935) Lawson et al. 2016
DSM No.:	**27147**
Strain designation:	R20291
Other collection no. or WDCM no.:	NCTC 13366
Isolated from:	Faeces from a symptomatic patient. Outbreak at Stoke Mandeville Hospital, UK
Country:	United Kingdom of Great Britain and Northern Ireland Buckinghamshire, Aylesbury
Date of sampling:	before 22.04.2013
Nagoya Protocol Restrictions:	There are NO known Nagoya Protocol restrictions for this strain.
History:	<- P. Bracegirdle, Health Protection Agency Culture Collections, Salisbury, United Kingdom; NCTC 13366
Genbank accession numbers:	complete genome: FN545816
Cultivation conditions:	Medium 78 , anaerobic, 37°C or Medium 1203 , anaerobic, 37°C Incubation time: 1-2 days Please follow special instructions: 'Cultivation of Anaerobes' Complete DSMZ Media List
Summary and additional information:	<- P. Bracegirdle, Health Protection Agency Culture Collections, Salisbury, United Kingdom; NCTC 13366. Faeces from a symptomatic patient. Outbreak at Stoke Mandeville Hospital, UK; United Kingdom, Buckinghamshire, Aylesbury. Taxonomy/description (26004, 26018). Sequence accession no. complete genome: FN545816. Ribotype 027 with 18bp deletion in *tcdC* gene. Genome sequenced. Positive PCR for toxins A and B (*tcdA* and *tcdB*) and the binary toxin (*ctdA* and *ctdB*).. (Medium 78, 37°C, anaerobic or Medium 1203, 37°C, anaerobic).
Literature:	26004, 26018
Risk group:	**2** (classification according to German TRBA)
Restrictions:	Act dealing with the prevention & control of infectious diseases in man (Infektionsschutzgesetz), Category A1

Appendix VII: Oxford MinION Sequencing Protocol
Preparing input DNA for Oxford MinION Sequencing

The quantity of DNA was assessed by DeNovix DS-11 fluorometer, a benchtop fluorometer designed to measure DNA accurately. The required input mass of DNA to yield a good sequencing run should be one ug. Checking for computer requirements needed for a sequencing run

The Oxford nanopore MinION sequencing device uses the MinKNOW software to collect sequencing data and processes it into basecalls. This can be done in real-time or on a local host computer. A minimum of 1 TB storage space is recommended to avoid the risk of losing data.

Checking the flow cell

Flow cells, when shipped, always contain a QC DNA molecule present in a buffer. This molecule produces a distinctive nanopore signal. The MinKNOW software uses the signal to check the nanopore array's integrity before the flow cell is used by giving the number of simultaneously available channels for the experiment. A minimum of 800 nanopores is required to conduct a successive sequencing run.

DNA repair and end-prep

DNA repair is achieved using NEBNext FFPE DNA repair mix (M6630). NEBNext Ultra II End Repair/dA-Tailing Module was also utilized in the end-prep step to prepare 1000ng of sheared gDNA (E7546). In a 1.5 ml Eppendorf DNA LoBind tube, the following will combined;

i) gDNA (48µl)
ii) NEBNext FFPE DNA Repair Buffer (3.5µl)
iii) NEBNext FFPE DNA Repair Mix (2µl)
iv) Ultra II End-prep reaction buffer (3.5µl)
v) Ultra II End-prep enzyme mix (3µl)

Then the Eppendorf DNA LoBind tube was incubated using a thermal cycler at 20°C for 5 minutes and 65° C for 5 minutes. Agencourt AMpure XP beads was added to the end-prep reaction and mixed by pipetting. The DNA will have freed of contaminants using a 1:1 volume of AMPure XP beads (A63880, Beckman Coulter) and eluted in 25 µl of nuclease free water for each isolate.

Native barcode ligation

Blunt/TA Ligase Master Mix (M0367S, NEB) was used to ligate native barcode adapters to end prepared gDNA. A unique barcode was selected for each isolate to be run together on the same flow cell, from the provided 24 barcodes. Only up to 24 samples can be barcoded and combined in one experiment. The end-prep gDNA (500ng) was diluted in 22.5 ul in nuclease-free water for each isolate. Blunt/TA ligase Master Mix (25ul), native barcode (2.5ul) and 500ng end-prepped DNA (22.5ul) was mixed and incubated for 10 minutes at room temperature for each isolate. Agencourt AMpure XP beads (50ul) was added to the reaction to remove any contaminants and eluted in 26ul nuclease free water.

1ul of the eluted sample was quantified using DeNovix DS-11 flourometer. Equimolar amounts of twenty-four different barcoded gDNA samples of VRE was pooled into one DNA LoBind Eppendorf tube. This was done ensuring that sufficient sample is combined to produce a pooled sample of 700ng. The pooled sample is diluted in nuclease free water (65ul)

Adapter ligation and clean-up

The adapter Mix II was ligated to the pooled DNA using quick T4 DNA ligase (E6056). The DNA fragments was enriched by mixing, vortexing and spinning down with the long fragment buffer supplied in the ligation sequencing kit.

In an Eppendorf DNA LoBind tube, the following will combined;

i) 700ng pooled barcoded sample- 65ul
ii) Adapter Mix II (AMII)- 5ul
iii) NEBNext Quick Ligation Buffer (5X)- 20ul
iv) Quick T4 DNA ligase- 10ul

The reaction was incubated for 10 minutes at room temperature. The reaction was purified using AMPure Xp beads (50ul). The beads are in the next step washed by adding 250ul of long fragment buffer then placed on a magnetic rack to allow the beads to pellet. The resuspended pellet is eluted in 15ul Elution buffer and incubated for 10 minutes in room temperature.

Priming and loading the SpotON flow cell

The lid of the nanopore sequencing device is opened and slide the flow cell's priming port cover clockwise so that the priming port is visible. After opening the priming port, check for small bubble under the cover. Draw back a small volume to remove any bubble (a few µls):

1. Set a P1000 pipette to 200 µl
2. Insert the tip into the priming port

3. Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip

Visually check that there is continuous buffer from the priming port across the sensor array. Prepare the flow cell priming mix: add 30 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FLB), and mix by pipetting up and down. Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes. Thoroughly mix the contents of the Loading Beads (LB) by pipetting

In a new tube, prepare the library for loading as follows:

Sequencing Buffer (SQB)- 37.5 µl

Loading Beads (LB), mixed immediately before use- 25.5 µl

DNA library- 12 µl

Mix the prepared library gently by pipetting up and down just before loading. Add 75 µl of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next. Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION lid.

ONT NGS Library Prep worksheet

Date:----------8/04/2021---------- Name of Staff:_____Winnie_____ No. of Samples:_____16_____

Sample no	Sample ID	Before end repair[a] (ng/ul)	1ug DNA[b] (ul)	Adjust to 47ul	After end prep conc[c]	Volume for 500ng[d]	Adjust to 22.5ul	Barcode no.[e]	After barcoding conc[f]	Equimolar pooling 700ng total [g]
1										
2										
3										
4										

[a]Reading before end prep repair/after extraction. ≥25ng/µl for LSK109 naïve barcoding kit id recommended.
[b]DNA volume needed with concentration at (a) to make 1µg.
[c]Adjust to final volume of 49µl with nuclease free water.
[d]Qubit concentration after end prepped DNA. Recovery aim >700ng (~28ng/µl).
[e]Volume needed to make up to 500ng of end prepped sample to be barcoded.
[f]Unique barcode for sample.
[g]Barcode concentration.
Equimolar pooling volume for 700ng (based on sample concentration and total number of samples).
Example: for 10 samples, each contributes 70ng. The volume to pick is based on sample concentration.

ONT NGS Pooled Library Prep worksheet

Pooled barcoded library concentration (ng/µl[h)]	Volume for 700ng[i]	Adjust to 65µl[j]	Library concentration[n]

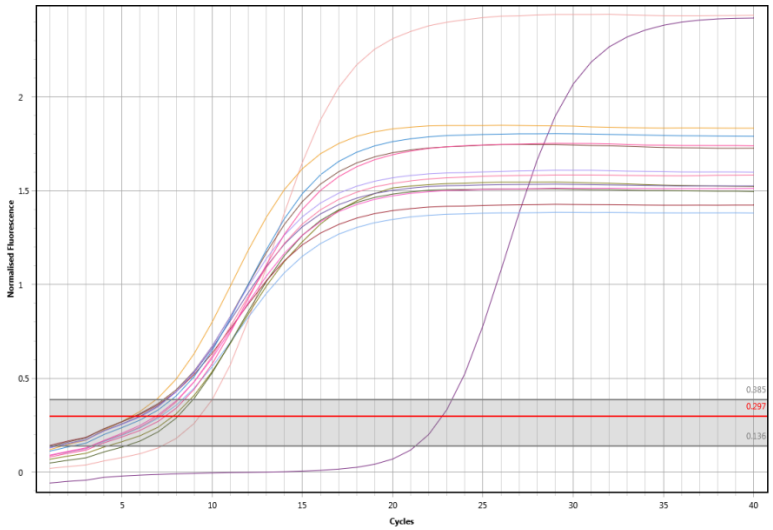
[h]Library contraction after pooling.
[i]Volume of pooled library to make 700ng.
[j]Adjust to 65µl with nuclease free water (depends on the library contraction; if too concentrated, dilution may be necessary).
[n]Adaptor ligated library concentration, aim for 430ng total volume.

Appendix VIII: Genomic information of sequence types from different geographical regions

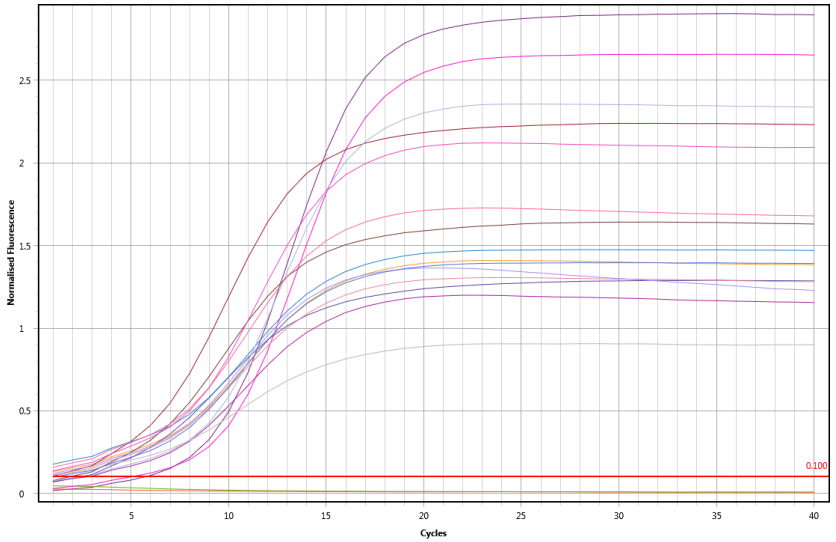
Geographical location	BioProject	BioSample	Accession	Taxonomy	Sample ID
Indonesia	PRJNA323780	SAMN05188758	CP016104	*Clostridium difficile*	DSM 29629
USA:VA	PRJNA231221	SAMN04875593	NZ_CP020424	*Clostridioides difficile*	FDAARGOS_267
Ghana	PRJNA281317	SAMN03487129	NZ_CP012321	*Clostridioides difficile*	DSM 28666
Indonesia	PRJNA224116	SAMN05188775	NZ_CP019860	*Clostridioides difficile*	DSM 29632
USA: Houston, TX	PRJNA556848	SAMN12370807	NZ_CP042267	*Clostridioides difficile*	Mta-79

Appendix IX: Ampificatin curves for *tcdA*, *tcdB*, *cdtA* and *cdtB*

tcdA



tcdB



Appendix X: Accession numbers of the sequenced isolates

SUBID	BioProject	BioSample	Accession	Taxonomy	Sample ID
SUB9730321	PRJNA732612	SAMN19328071	JAHFVV000000000	*Clostridioides difficile*	CD045
SUB9730375	PRJNA732612	SAMN19328148	JAHFVW000000000	*Clostridioides difficile*	CD079
SUB9731398	PRJNA732612	SAMN19331478	JAHFVX000000000	*Clostridioides difficile*	CD147
SUB9731806	PRJNA732612	SAMN19331538	JAHFVY000000000	*Clostridioides difficile*	CD191
SUB9731934	PRJNA732612	SAMN19332189	JAHFVZ000000000	*Clostridioides difficile*	CD215
SUB9731988	PRJNA732612	SAMN19332272	JAHFWA000000000	*Clostridioides difficile*	CD248
SUB9732052	PRJNA732612	SAMN19332400	JAHFWB000000000	*Clostridioides difficile*	CD273
SUB9732113	PRJNA732612	SAMN19332463	JAHFWC000000000	*Clostridioides difficile*	CD283

Appendix XI: Allelic polymorphism of the sequenced isolates

Strain	ST	Clade	RT	MLST_loci							Rif^R	Flu^R		Tet^R	$MLSB^R$	
				adk	atpA	dxr	glyA	recA	sodA	tpi	RpoB	GyrA	GyrB	tetM	ermG	ermB
CD045	37	4	RT017	adk(3)	atpA(7)	dxr(3)	glyA(8)	recA(6)	sodA(9)	tpi(11)	H502N, R505K	T82I	-	+	+	-
CD079	37	4	RT017	adk(3)	atpA(7)	dxr(3)	glyA(8)	recA(6)	sodA(9)	tpi(11)	H502N?, R505K	T82I?	-	+	+	-
CD147	-	-	-	adk(1)	atpA(1)	dxr(2)	glyA(~1)	recA(~1)	sodA(5)	tpi(1)	-	-	-	+	-	+
CD191	-	-	-	adk(1)	atpA(~1)	dxr(2)	glyA(~1)	recA(~1)	sodA(5)	tpi(1)	-	-	-	+	-	+
CD199	743			adk(1)	atpA(1)	dxr(2)	glyA(1)	recA(5)	sodA(3)	tpi(91)	--	-	-	-	-	-
CD215	37	4	RT017	adk(3)	atpA(7)	dxr(3)	glyA(8)	recA(6)	sodA(9)	tpi(11)	H502N, R505K	T82I	-	+	+	-
CD248	37	4	RT017	adk(3)	atpA(7)	dxr(3)	glyA(8)	recA(6)	sodA(9)	tpi(11)	H502N, R505K	T82I	-	+	+	-
CD273	10	1	RT015	adk(2)	atpA(1)	dxr(2)	glyA(1)	recA(1)	sodA(3)	tpi(1)	-	-	-	-	-	-
CD283	58	1	RT056	adk(1)	atpA(5)	dxr(7)	glyA(1)	recA(52?)/recA(1)	sodA(13)	tpi(1)	-	-	-	-	-	-

Appendix XII: Dissertation submission notice

UNIVERSITY OF NAIROBI
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Our Ref: H80/99967/2015

26th August, 2021

Winnie Mutai
Department of Medical Microbiology
Faculty of Health Sciences

Dear Ms. Mutai,

NOTICE OF INTENT TO SUBMIT YOUR Ph.D THESIS

We wish to acknowledge receipt of your notice of intent to submit your Ph.D thesis dated 28th July, 2021 entitled; **"Molecular epidemiology of Clostridioides defficile isolated from diarrhoeal inpatients in Kenya."** We also wish to acknowledge receipt of the abstract of the thesis. Please submit a soft copy of the thesis to the **Chairman, Department of Medical Microbiology.**

In addition you should run and submit and anti – plagiarism test on your thesis whose tolerance levels should be 15% and below.

Please note that you will be expected to show proof of acceptance for publication of two (2) papers in referred journals as a requirement for full Ph.D students before graduation.

We look forward to receiving soft copy of your thesis within three (3) months from the date of this letter subject to having received and approved by the Committee of Examiners from the Chairman.

Yours sincerely,

OCIATE DEAN POSTGRADUATE
UDENTS AND RESEARCH,
ACULTY OF HEALTH SCIENCES
UNIVERSITY OF NAIROBI

PROF. EVELYN WAGAIYU
ASSOCIATE DEAN
POSTGRADUATE STUDENTS & RESEARCH

CC. Chairman, Department of Medical Microbiology
Prof. Omu Anzala

JN/ekc

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Appendix XIII: Publication abstracts

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 08 February 2021
doi: 10.3389/fcimb.2020.604986

High Prevalence of Multidrug-Resistant *Clostridioides difficile* Following Extensive Use of Antimicrobials in Hospitalized Patients in Kenya

OPEN ACCESS

Edited by:
Xingmin Sun, University of South Florida, United States

Reviewed by:
Masoumeh Azimirad, Shahid Beheshti University of Medical Sciences, Iran
William Thomas Self, University of Central Florida, United States

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***Winnie C. Mutai*[1*], *Marianne W. Mureithi*[1], *Omu Anzala*[1], *Gunturu Revathi*[2], *Brian Kullin*[3], *Magdaline Burugu*[1], *Cecilia Kyany'a*[4], *Erick Odoyo*[4], *Peter Otieno*[4] *and Lillian Musila*[4]**

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Introduction: *Clostridioides difficile* is a neglected pathogen in many African countries as it is generally not regarded as one of the major contributors toward the diarrheal disease burden in the continent. However, several studies have suggested that *C. difficile* infection (CDI) may be underreported in many African settings. The aim of this study was to determine the prevalence of CDI in hospitalized patients, evaluate antimicrobial exposure, and detect toxin and antimicrobial resistance profiles of the isolated *C. difficile* strains.

Methods: In this cross-sectional study, 333 hospitalized patients with hospital-onset diarrhoea were selected. The stool samples were collected and cultured on cycloserine-cefoxitin egg yolk agar (CCEY). Isolates were presumptively identified by phenotypic characteristics and Gram stain and confirmed by singleplex real-time PCR (qPCR) assays detecting the species-specific *tpi* gene, toxin A (*tcdA*) gene, toxin B (*tcdB*) gene, and the binary toxin (*cdtA/cdtB*) genes. Confirmed *C. difficile* isolates were tested against a panel of eight antimicrobials (vancomycin, metronidazole, rifampicin, ciprofloxacin, tetracycline, clindamycin, erythromycin, and ceftriaxone) using E-test strips.

Results: *C. difficile* was detected in 57 (25%) of diarrheal patients over the age of two, 56 (98.2%) of whom received antimicrobials before the diarrheal episode. Amongst the 71 confirmed isolates, 69 (97.1%) harbored at least one toxin gene. More than half of the toxigenic isolates harbored a truncated *tcdA* gene. All isolates were sensitive to vancomycin, while three isolates (2.1%) were resistant to metronidazole (MIC >32 mg/L). High levels of resistance were observed to rifampicin (65/71, 91.5%), erythromycin (63/71, 88.7%), ciprofloxacin (59/71, 83.1%), clindamycin (57/71, 80.3%), and ceftriaxone (36/71, 50.7.8%). Among the resistant isolates, 61 (85.9%) were multidrug-resistant.

PLOS GLOBAL PUBLIC HEALTH

RESEARCH ARTICLE

Assessment of independent comorbidities and comorbidity measures in predicting healthcare facility-onset *Clostridioides difficile* infection in Kenya

Winnie C. Mutai[1]*, Marianne Mureithi[1], Omu Anzala[1], Brian Kullin[2], Robert Ofwete[1], Cecilia Kyany' a[3], Erick Odoyo[3], Lillian Musila[3], Gunturu Revathi[4]

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OPEN ACCESS

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Abstract

Introduction

Clostridioides difficile is primarily associated with hospital-acquired diarrhoea. The disease burden is aggravated in patients with comorbidities due to increased likelihood of polypharmacy, extended hospital stays and compromised immunity. The study aimed to investigate comorbidity predictors of healthcare facility-onset *C. difficile* infection (HO-CDI) in hospitalized patients.

Methodology

We performed a cross sectional study of 333 patients who developed diarrhoea during hospitalization. The patients were tested for CDI. Data on demographics, admission information, medication exposure and comorbidities were collected. The comorbidities were also categorised according to Charlson Comorbidity Index (CCI) and Elixhauser Comorbidity Index (ECI). Comorbidity predictors of HO-CDI were identified using multiple logistic regression analysis.

Results

Overall, 230/333 (69%) patients had comorbidities, with the highest proportion being in patients aged over 60 years. Among the patients diagnosed with HO-CDI, 63/71(88.7%) reported comorbidities. Pairwise comparison between HO-CDI patients and comparison group revealed significant differences in hypertension, anemia, tuberculosis, diabetes, chronic kidney disease and chronic obstructive pulmonary disease. In the multiple logistic regression model significant predictors were chronic obstructive pulmonary disease (odds ratio [OR], 9.51; 95% confidence interval [CI], 1.8–50.1), diabetes (OR, 3.56; 95% CI, 1.11–11.38), chronic kidney disease (OR, 3.88; 95% CI, 1.57–9.62), anemia (OR, 3.67; 95% CI,