CHARACTERIZATION OF METRONIDAZOLE- AND VANCOMYCIN-RESISTANT CLINICAL ISOLATES OF CLOSTRIDIUM DIFFICILE

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CHARACTERIZATION OF METRONIDAZOLE- AND VANCOMYCIN-
RESISTANT CLINICAL ISOLATES OF CLOSTRIDIUM DIFFICILE

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CHARACTERIZATION OF METRONIDAZOLE- AND VANCOMYCIN-RESISTANT CLINICAL ISOLATES OF CLOSTRIDIUM DIFFICILE

A

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for the Degree of

MASTER OF SCIENCE

by

Chioma Odo, M.S.

Houston, Texas

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Dedication

I dedicate this to my late parents Joseph and Lucy Odo. Thank you for teaching me how to be strong no matter what life throws at me and thank you for believing in me more than I even believe in myself. I know you will be very proud of the woman I have become.

To my late brother Tonna Odo, I still miss you so much and I did it just like you would have motivated me to do, to keep pushing especially when it hurts so badly. I did it again and I know you will be very proud.
Acknowledgment

I thank God the Father, Son and the Holy Spirit for giving me the grace, wisdom, knowledge and opportunity to be in this program and meet the right people who have been of immense help. To my mentor Dr. Darkoh, thank you so much for believing in me, for giving me the opportunity to realize my dream, for your patience in nurturing me and other things too numerous to mention. I am indebted to you. I am very grateful to Dr. Heidi Kaplan for her constant support and encouragement. To my other advisors Dr. Norris, I appreciate your kindness and support, Dr. Volk and Dr. Xia, thank you for all the advice and support throughout this project. To Dr. Chappell, I am very grateful for your support and guidance.

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To my most loving family both here in the US and in Nigeria, you guys are the best. I tell anyone that cares to listen that I have the best family and I would not be where I am today without you. I am so grateful and love you to the moon and back.
Characterization of Metronidazole- and Vancomycin-Resistant Clinical Isolates of

*Clostridium difficile*

Chioma Odo, MS.

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**ABSTRACT**

The incidence of *C. difficile* infections (CDI) has been increasing at an alarming rate. This was precipitated by the emergence of strains with increased virulence, disease severity, and high recurrence rates. These strains also exhibit high propensity for resistance to antibiotics such as fluoroquinolones and beta lactams, which has made the treatment of CDI very challenging. Currently, metronidazole and vancomycin are the most commonly used drugs for the treatment of primary CDI. Metronidazole is used for the treatment of mild to non-severe cases of CDI while vancomycin is reserved for severe CDI cases. In 25-30% of the patients treated with these antibiotics, the infection may recur, and this further complicates CDI treatment. Because *C. difficile* strains have an intrinsic ability to resist multiple antibiotics, it was hypothesized that there may be strains with high resistance to metronidazole and vancomycin circulating in the patient population. To investigate this hypothesis, 536 clinical CDI stool samples obtained from patients who presented with diarrhea at St. Luke’s Episcopal Hospital at the Texas Medical Center Houston, Texas, Kenyatta National Hospital, Nairobi, and Kisii Teaching and Referral Hospital, Kisii, Kenya were screened for resistant *C. difficile* strains. The stool samples were analyzed on *C. difficile*-specific differential medium containing either metronidazole (8 µg/ml) or vancomycin (4 µg/ml). These are concentrations designated by the Clinical
and Laboratory Standards Institute to be the resistant breakpoint for each of the antibiotics. Stools that grew resistant colonies were identified and colonies were selected for further analysis. The minimum inhibitory concentration (MIC) of the isolates was determined by E-test and broth microdilution. The results showed that 33.1% (145/438) of the CDI patients from Texas had *C. difficile* strains in their stools that were resistant to both metronidazole and vancomycin. Remarkably, 93.9% of the CDI patient stools from Kenya had both metronidazole- and vancomycin-resistant *C. difficile* strains. The resistant strains from both patient populations also exhibit high level of tolerance for these antibiotics that far exceed the previously reported MICs (>1024 µg/ml compared to 256 µg/ml for metronidazole and >1024 µg/ml compared to 16 µg/ml for vancomycin). All of the vancomycin-resistant strains isolated from the patients in both populations had the homologue of *vanA* gene, which has been shown to confer a high degree of vancomycin resistance in Gram-positive bacteria. Together, the results demonstrate high prevalence of metronidazole- and vancomycin-resistant *C. difficile* strains circulating in the patient populations from Texas and Kenya. The spread of *C. difficile* strains that are resistant to these two antibiotics of last resort may have serious public health implications and underscores the urgent need for a more in-depth analysis of the circulating resistant strains to help inform clinical decisions.
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INTRODUCTION

Epidemiology of *Clostridium difficile* Infections

*Clostridium difficile* is a multidrug-resistant, spore forming, Gram-positive anaerobic pathogen. It is the most common cause of hospital-acquired and healthcare-associated infectious diarrhea (1). Broad spectrum antibiotics use is the most common risk factor for *Clostridium difficile* infection (CDI), as it alters colonization resistance, thereby rendering the microbiota susceptible to *C. difficile* colonization and infection (2). Clinical symptoms of CDI range from mild to severe diarrhea, pseudomembranous colitis, toxin megacolon, septic shock or even death (3). The Centers for Disease Control and Prevention (CDC) has classified *C. difficile* as an urgent threat with approximately 453,000 reported cases, 29,000 deaths, and $1.1 to 7 billion in treatment costs annually in the US (4). Recently, the incidence of CDI has exceeded that of methicillin-resistant *Staphylococcus aureus* (MRSA) in the community hospitals (5). There is an increased report of CDI among the young under 18 and also in communities outside the hospital, such as assisted care facilities for the elderly.

The emergence of *C. difficile* strains with resistance to multiple antibiotics, especially the most prominent hyper-virulent strain ribotype (RT) 027, has added another complication to the treatment of CDI (6). (RT) 027, which is commonly found in the US, Europe and Canada is completely intractable to traditional treatment with increased recurrence and mortality rate (6). The emergence is associated with fluoroquinolone exposure (6). *C. difficile* RT027, also known as fluoroquinolone-resistant (FQR) *C. difficile*, is the most common cause of outbreaks in North America
and the UK (with more than 40% of the reported cases in the UK) and it was recently reported to be common in other parts of Europe and Australia (7).

**Clostridium difficile Pathogenesis**

*C. difficile* is spread through ingestion of its spores, which tolerates both stomach acidic and harsh environmental conditions. Under favorable conditions, such as the presence of bile acids in the small intestine, the ingested spores germinate into vegetative cells that colonize the large intestine following disruption of normal microbiota by antibiotic therapy (15).

Spore germination is initiated when a receptor within the inner membrane of the spore interacts with a germinant such as ions, sugar, nucleotides, bile acids or surfactants (48). When the spore receptor senses or recognizes a germinant, it triggers an irreversible spore germination process which leads to the release of Ca\(^{2+}\)-dipicolinic acid, water uptake, spore cortex degradation, and outgrowth of the vegetative cells and under appropriate conditions, produce toxins that cause disease (41).

The vegetative form of *C. difficile* thrives in the lumen of the large intestine and produces two major toxins: toxins A (an enterotoxin) and B (a cytotoxin) (15). These two major virulence factors are encoded by the *tcdA* and *tcdB* genes, respectively, and are located within 19.6 kb of the pathogenicity locus (PaLoc) in the genome (Fig.1). They are members of large clostridial toxins, a family of toxins that modifies GTPases (16, 95, 96). Also found in the pathogenicity locus is *tcdC*, which negatively regulates expression of *tcdA* and *tcdB*. The *tcdE* gene, encodes a holin (essential for the extracellular release of
toxins A and B) and tcdR, encodes a sigma factor that is required for toxin A and B expression. The PaLoc, located at the same site in all toxin-producing strains, can be horizontally transferred to non-toxin producing strains, however, it is not intrinsically mobile (38).

The amino acid sequences of TcdA and TcdB are 44% identical and 66% similar and share a common structure (95). The C-terminus has a high degree of sequence diversity. Both toxins have a similar three-dimensional structure and a similar mode of entry into host cells (38). They both have a “pincher-like” head delivery domain, a receptor-binding domain, also known as the long tail domain at the C-terminus, and a short inner tailed (52) glucosyltransferase domain at the N-terminus. Generally, both toxins share similar enzymatic activities, as well as multi-modular domain structure, described as the ABCD model (A: biological activity, B: binding, C: cutting and D: delivery) (Fig. 1B) (38, 52). Region A, which is the short tail region, is located at the N-terminus and it is the site of biological activity of the toxins. They contain the 63 kDa glucosyltransferase domain (GTD) that modifies the small GTPases, which regulates the host cytoskeleton (38, 42). Region B, the long tail region, is located at the C-terminus and consists of combined repeated oligopeptides (CROPs) that form the receptor binding domain (RBD). The RBD binds to receptors on the host cell and becomes internalized through receptor-mediated endocytosis. The region C contains the cysteine protease domain (CPD), which is responsible for autoprocessing of the glucosyltransferase domain, endocytosis, and translocation of the toxin into the cytosol of target cells. Also contained in region C of both TcdA and TcdB is a three-helix bundle region, conserved in both toxins and located at the junction of GTD-CPD region (39, 42,
The D region contains the delivery hydrophobic domain, which is responsible for translocation into the cytosol and binding to the GTPase of target cells in the host (38). Also, part of the D domain is the small globular sub-domain (SGD) and an elongated hydrophobic helical stretch containing four α-helices (38, 52).
Figure 9: (A) The schematic representation of the components of the *C. difficile* toxin pathogenicity locus (PaLoc) and (B) the multi-modular domain structure of TcdA and TcdB. The PaLoc comprises of *tcdR*, *tcdB*, *tcdE*, *tcdA*, and *tcdC* genes. The *tcdR* is a 555 bp, positive regulator that encodes an alternative RNA polymerase sigma factor that regulates *tcdA* and *tcdB* expression. *tcdB* is 7101 bp and encodes toxin B. The 501 bp *tcdE* encodes a putative holing domain/protein, which has been suggested to be responsible for extracellular release of both toxin A and B (38). The *tcdA* (8133 bp) encodes toxin A, whereas the 699 bp *tcdC* encodes a negative regulator of toxin A and B (38). The ABCD model (A, biological activity; B, binding; C, cutting; D, delivery) domain structure of the two toxins contains the A and C domains, which corresponds to the N-terminal glucosyltransferase domain (GTD) in red and cysteine protease domain (CPD) in cyan, followed by the three-helix domain 3HB in blue (38). The D domain corresponds to the delivery hydrophobic domain (DD) (in yellow), which contains the small globular domain (SGD). The B domain (in pink), corresponds to the receptor binding domain (RBD) (38).
The mechanism of action of toxin A and B on the mammalian target cells involves receptor-mediated endocytosis, which leads to protein translocation out of the endosome. Translocation and release of the toxins in the host cell triggers autoproteolytic processing and subsequently monoglucoyslation of host Rho and Ras proteins (39, 97, 98). The highly repetitive C-terminal domain of the toxin binds to the target cell and is internalized through endocytosis (52). The N-terminal translocation across the membrane is facilitated by the low pH of the endosome. The delivery domain, which is the central region, has hydrophobic residues that changes its conformation at low pH leading to membrane insertion and pore formation (39, 41, 52). The N-terminal glucosyltransferase is translocated through the pore and is released into the cytosol of the target cell to disrupt the small GTPases, such as Rho and Ras proteins. The Rho and Ras proteins are important in maintaining the integrity of the cytoskeleton. Rho monoglucoyslation results in its inactivation and loss of ability to polymerize actin filament leading to deregulation of the actin cytoskeleton and loss of cell-to-cell contact at the tight junctions due to signal disruption (32, 97). This leads to the release of cytokines from mast cells resulting in fluid secretion, intestinal inflammation, and apoptosis (16).

Another putative virulence factor, CDT binary toxin, also contributes to C. difficile pathogenesis (32). This toxin disrupts the cytoskeleton and forms microtubule-based projections that facilitate C. difficile adherence to the surface of epithelial cells. About 35% of C. difficile strains secret the binary toxin, which is encoded by cdtA and cdtB (35). There are two domains: CDTa, the biologically active ADP-ribosyltransferase that modifies actin and CDTb, the binding component, which is involved in binding and
transportation of the enzymatic component into the cytosol. ADP-ribosyltransferase modifies monomeric G-actin and inhibits its polymerization as ADPribosylated actin attaches to the barbed end of the actin filament. This inhibits elongation and formation of F-actin leading to cell rounding and death. (34, 36).

**Treatment of C. difficile Infections**

Antimicrobial agents play an important role in CDI as they are involved in both induction and resolution (4). Third generation antibiotics cephalosporins and clindamycin were previously used for treatment of CDI, but they are currently associated with promoting risk of CDI (4). CDI treatment depends on the disease classification. CDI can be classified as non-severe to mild, recurrent, severe or complicated, and the type of treatment depends on the disease severity. Metronidazole is commonly prescribed for non-severe to mild cases, whereas vancomycin is usually reserved for severe cases (10). Other antibiotics, such as rifaximin and teicoplanin, are also used (54). Rifaximin is a non-absorbable oral antibiotic usually prescribed for first and recurrent CDI. Teicoplanin, a glycopeptide, is similar to vancomycin but is not approved in the United States (44). Metronidazole is neurotoxic and so is not used for long-term or recurrent treatment. Vancomycin is usually reserved for severe cases of CDI. Fidaxomicin, a novel macrocyclic antibiotic, is now being considered as a vancomycin substitute (44, 45).

In addition to treating severe CDI, vancomycin is active against both metronidazole-resistant *C. difficile* and epidemic strains with high metronidazole tolerance. Second
and subsequent recurrences of CDI are usually treated with tapered and/or pulsed doses of vancomycin (10). In severe cases of CDI, vancomycin and metronidazole can be administered through different routes. Vancomycin is usually given directly into the colon, though oral and rectal delivery are also recommended. Failure of treatment is believed to stem from a dynamic ileus (paralysis of the small bowel), which may prevent the oral vancomycin from reaching the colon and thus be ineffective. Therefore, vancomycin is injected directly into the colon (75, 76). Combination of intravenous metronidazole with intracolonic vancomycin administered by nasogastric tube is recommended by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guideline to be used in severe cases where oral therapy is not effective (3, 46). Vancomycin is recommended by Infectious Disease Society of America for suspected severe cases. A study that evaluated the appropriateness of CDI empiric treatment showed that many CDI patients receive inappropriate empirical treatment without meeting the criteria for severe CDI. Half of this empirical treatment is reportedly dispensed without confirmation. Therefore, patients are often treated inappropriately (15).

Metronidazole- and vancomycin-induced collateral damage of the microbiota has been found to be associated with a high incidence of CDI recurrence following conventional treatment (14). Although most patients respond well to treatment and have their standard metronidazole and/or vancomycin antibiotic therapy discontinued after 10 to 14 days, some patients have symptoms that persist or recur after treatment. The rate of CDI recurrence is between 20 to 35%, and the rate of recurrence increases with each
subsequent episode (68). One of the identified significant risk factors for relapse is an initial infection with the BI/NAP1/027 epidemic clone (56).

The recommended treatment following recurrence of CDI is not straightforward, as metronidazole is not appropriate for prolonged administration (53) and there has also been a gradual increase of the *C. difficile* strain with resistance to metronidazole (28, 30). Using the Clinical laboratory standard institute (CLSI) breakpoints, 8% of *C. difficile* clinical isolates were found to be resistant to vancomycin in Iran (53) based on a report of increased resistance and reduced sensitivity to both metronidazole and vancomycin (54, 55). Treatment failures with these last resort antibiotics is a result of *C. difficile* developing resistance to these antimicrobial agents (54).

**Antimicrobial Resistance in *C. difficile***

Antimicrobial resistance is the ability of microorganisms (bacteria, fungi, viruses, and parasites) to resist the effects of antimicrobial drugs (antibiotics, antifungals, antivirals, antimalarials, and antihelmintics) (58, 59). Pathogens that are able to resist multiple antimicrobials are often referred to as “superbugs”. Antibiotic resistance has become a global threat. In the United States alone, about 2 million people become infected with bacteria that are resistant to antimicrobials designed to kill them and about 23,000 patients die as a result of antimicrobial-resistant bacteria (27). New forms of resistance can easily spread across continents with significant speed. The mortality rate due to antimicrobial resistance is expected to reach about 10 million patients by the year 2050, with an estimated global cost of $100 trillion (40). The most important cause
of antimicrobial resistance is the overuse of antibiotics, which is also the leading risk factor for CDI (4).

*C. difficile* is resistant to multiple antibiotics (14). Antibiotic resistance is a significant factor in CDI dissemination among some hospitalized, elderly, and immunocompromised patients (14). *C. difficile* is currently resistant to the following antibiotics previously used for treatment: penicillin, clindamycin, erythromycin, cephalosporins, fluoroquinolones and tetracycline (27, 28). Clindamycin was identified as the highest risk factor in 1970, and was replaced by cephalosporin in the 80s, and most recently fluoroquinolones (6). Studies of antimicrobial resistance of different *C. difficile* isolates in North America, Europe, and Asia show that clindamycin resistance ranges from 15 to 97% (28). The *C. difficile* strains most reported to be resistant to different antibiotics in different countries and continents are as follows: (i) 30% of ribotype 027 strains are resistant to clindamycin, moxifloxacin, and rifampin in North America; (ii) in the United States, about 98% of ribotype 027 strains are resistant to moxifloxacin; and (iii) in the Netherlands, ribotype 078, (another hypervirulent strain) is resistant to ciprofloxacin, erythromycin, imipenem, and moxifloxacin (29, 31).

The hypervirulent BI/NAP1/027, also known as FQ-resistant *C. difficile* strain, is characterized by efficient sporulation, high toxin production, and enhanced cytotoxicity (57). Spore production in *C. difficile* is an important mechanism of persistence that facilitates resistance to antibiotics and to the host immune system (28).

Although metronidazole and vancomycin remain the most effective mode of treatment for most CDI cases, approximately 25% of patients treated with either antibiotics can recur typically within 4 weeks of completing the primary therapy (15).
Currently, *C. difficile* isolates with increased resistant to metronidazole and vancomycin are increasing (30).

**Metronidazole Mode of Action**

Metronidazole (Fig. 2) causes its antimicrobial action by damaging bacterial DNA through reduction of the nitro group into cytotoxic nitrosoimidazole and nitroradical anion by thioredoxin reductase (17, 20). The most important system involved in metronidazole activation is the pyruvate-ferredoxin/flavodoxin oxidoreductase (PFOR) system, which works in concert with ferredoxin and glycerol-3-phosphate hydrogenase to reductively activate metronidazole directly (19). The nitroradical anion reduces O$_2$ to generate reactive oxygen species, which is highly deleterious to the cell. Nitrosoimidazole on the other hand, forms an adduct when reacted with non-protein thiols or proteins. Adduct formation causes both depletion of non-protein thiols and modification of metronidazole’s target proteins: purine nucleoside phosphorylase (PNP), thioredoxin, thioredoxin reductase, and superoxide dismutase (SOD) leaving the cell vulnerable to oxidative stress and eventually death (19, 20).
**Figure 10: Mechanism of action of metronidazole.** Under anaerobic conditions, metronidazole is reduced to nitroradical anion or nitrosoimidazole by thioredoxin reductase. The nitroradical anion formed reduces O$_2$ and thereby generates reactive oxygen species causing oxidative damage to the cell. Nitrosoimidazole in the presence of non-protein thiols forms protein adducts, which modify thioredoxin reductase (TrxR), thioredoxin (Trx), superoxide dismutase (SOD), metronidazole target protein 1 (Mtp1), and purine nucleoside phosphorylase (PNP), and eventually causes cell death (17).
The Proposed Mechanisms of Metronidazole Resistance in *C. difficile*.

The exact mechanism of metronidazole resistance has not yet been established in *C. difficile*. The *nimA-J* gene cluster is associated with metronidazole-resistance in different genera of both Gram-positive and Gram-negative anaerobic bacteria, including *Bacteroides* species (80). The *nimA-J* genes encode an alternative reductase, which convert nitroimidazoles to a stable and non-cytotoxic amine derivative to circumvent the toxic species that causes DNA breakage (4, 17, 80). However, homologs of the *nim* genes are not present in the *C. difficile* genome.

Comparative whole genome sequencing analysis of a stable metronidazole-resistant (RT) 027 strain, a clone resistant to metronidazole and became susceptible upon freeze-thaw (63) revealed the following mutations: (i) mutations within the sporulation gene (*spo0A*) and germination (*cspC*) loci; (ii) mutations in the ferric uptake regulator (*fur*), a putative nitroreductase gene; and (iii) a mutation in the coproporphyrinogen III oxidase gene (*hemN*) (60, 61, 62, 4). In another study, a proteomic analysis of a similar (RT) 027 strain revealed no evidence of association of deficiencies in the PFOR system. However, an increase in production of the ferric uptake regulator protein (a central regulator of iron homeostasis in bacteria) was observed in the metronidazole-resistant (RT) 027 strain (4, 19).

The proposed mechanism of metronidazole resistance is complex (17) (Fig. 3) and manifests as either a reduced rate of internal concentration of metronidazole due to increased efflux or by a reduced rate of metronidazole reductive activation by altering pyruvate fermentation. Other mechanisms that have been suggested to be involved are reduced iron transport and increased DNA repair efficiency (19), reduced rate of
glucose uptake, which alters pyruvate fermentation leading to downregulation of PFOR and upregulation of lactic acid dehydrogenase (17, 51). During fermentation, PFOR reduces pyruvate to acetyl-CoA and CO₂ releasing electrons. The electrons flow through PFOR to reduce ferredoxin then proceed to hydrogenase, which is a low redox carrier (17). It was originally proposed that PFOR in collaboration with ferredoxin were the only proteins with adequately low redox potential to actively reduce metronidazole in anaerobic bacteria, but it was later discovered that effectors with high negative midpoint redox capacities, such as flavodoxin and glycerol-3-phosphate hydrogenase, were equally able to reductively activate metronidazole directly in C. pasteurianum (17). This process is inhibited in metronidazole-resistant isolates, as deficiencies in pyruvate dehydrogenase activity is consistent with changes in the end products of glucose metabolism (64).

Other proposed mechanisms of resistance include overexpression of efflux pump genes, which have been shown to be associated with metronidazole resistance in other bacteria (65). In Bacteroides species, decrease in metronidazole uptake because of increased efflux are facilitated by an over active multidrug efflux pump system and this results in a reduced subsequent reductive activation (19, 65). Also, inactivation of the recA gene, which is required for generalized DNA repair and recombination, improved susceptibility of H. pylori to metronidazole and expression of a cloned recA gene from a metronidazole-resistant strain reportedly increased the level of metronidazole resistance in E. coli (66). Another mechanism involved in resistance is the mutation of the ferrous iron transporter FeoAB, which inhibits iron uptake (17, 18).
Figure 11: Mechanisms of metronidazole resistance. The mechanisms of metronidazole resistance are complex. Loss of function in various enzymes results in reduced susceptibility to metronidazole. The enzymes include pyruvate: ferredoxin oxidoreductase (PFOR), lactate dehydrogenase (LDH), hydrogenase (HYD), DNA helicase (RecQ), and DNA repair effectors recombinase A (RecA) (18, 19). The *Bacteroides* multidrug efflux pump system (BME) also facilitates metronidazole efflux. Oxygen-insensitive nitroreductase (NfsA) and the *nim* genes (NIM) reductively inactivate the nitro group attached to the amino derivative of metronidazole and reduces it into a stable amino derivative. Crosses (X) indicate reduced activity or uptake whereas the red arrows indicate the change expression that confers metronidazole resistance (18).
Peptidoglycan Biosynthesis and Mechanism of Action of Vancomycin

There are three stages involved in the biosynthesis of cell wall polymers (26) (Fig. 4). The first stage is the precursor synthesis in the cytoplasm, which involves the conversion of L-alanine to D-alanine (D-Ala) by the enzyme racemase and then joining of two molecules of D-Ala by D-alanine-D-alanine ligase (Ddl) to form dipeptide D-Ala-D-Ala (26). The second stage involves the formation of the subunit bound to mobile lipid undecaprenylphosphate. Uracil diphosphate–N-acetylmuramyl-tripeptide is added to the dipeptide D-Ala-D-Ala to form uracil diphosphate–N-acetylmuramyl-pentapeptide. Uracil diphosphate–N-acetylmuramyl-pentapeptide is bound to the mobile lipid undecaprenylphosphate for translocation to the outer surface of the membrane. The third and the final stage is transglycosylation and transpeptidation, in which N-acetylmuramyl-pentapeptide is attached to the nascent glycan chain by transglycosylation and finally cross bridge formation by transpeptidation (24, 26).

Vancomycin binds to the C-terminus of D-alanine-D-alanine during cell wall peptidoglycan biosynthesis to block transglycosylation and transpeptidation reactions (18, 25). It interferes with the late stage of peptidoglycan assembly. This is evident in the assembly of cell wall precursor UDP-MurNAc-pentapeptide in the cytoplasm (26). Vancomycin interaction with the precursor takes place only after translocation to the outer membrane, since vancomycin cannot penetrate the cell into the cytoplasm.
Figure 12: Peptidoglycan biosynthesis and mechanism of action of vancomycin.
Vancomycin binds to the C-terminal D-Ala–D-al of peptidoglycan precursors preventing the function of transglycosylases, transpeptidases, and the carboxypeptidases. D-Ala-D-Ala ligase (Ddl) joins the two D-alanines and MurF, the pentapeptide synthetase flips the precursor (UDP N-acetylmuramyl-pentapeptide and N-acetylglucosamine) to get attachment to the growing chain of peptidoglycan precursor (26).
Vancomycin Resistance Mechanisms in Gram-Positive Bacteria

The mechanism of acquired vancomycin resistance is well established in *Staphylococcus aureus* and *Enterococcus* spp. However, the mechanism of vancomycin-resistance in *C. difficile* is not known. Vancomycin is not target enzyme-specific and does not interact with enzymes responsible for cell wall biosynthesis, but rather is substrate-specific (23, 24). Seven types of vancomycin-resistance associated genes have been described in *Enterococci* spp. These are *vanA*, *B*, *C*, *D*, *E*, *G* and *L*. Strains that harbor the *vanA*, *B* and *D* genes synthesize D-Ala-D-lactate, instead of D-Ala-D-Ala, whereas *vanC*, *E*, *G* and *L* synthesize D-Ala-D-serine. They are all responsible for creating reduced cell wall affinity to vancomycin and complete elimination or reduction in the cell wall terminating precursor D-Ala-D-Ala. VanA-type resistance is the most common and was first to be reported (23). It is characterized by a high level of resistance to vancomycin and teicoplanin that can also be induced (26).

Vancomycin resistance has also been reported to be regulated by a two-component system VanSR (67). Changes in the peptidoglycan biosynthetic pathway results in high level of resistance to vancomycin and other glycopeptides in strains with the *vanA* phenotype in both *Enterococcus faecium* and *E. faecalis* (69). In a medium containing glycopeptide, VanS, (Fig. 5) a membrane-associated sensor kinase which contains a histidine residue in its cytoplasmic domain, is phosphorylated. VanR, a transcriptional activator containing an aspartate catalyzes the transfer of the phosphoryl group from activated VanS. Thus, the level of phosphorylation of VanR is controlled by VanS. Co-transcription of the *vanH*, *vanA*, *vanX*, and *vanY* genes is activated by the phosphorylated form of VanR by binding to the PRES promoter (74). Phosphorylated
VanR also activates transcription of the \textit{vanR} and \textit{vanS} genes by binding to the \textit{P}_{\text{REG}} promoter (73).

VanA and VanH synthesize D-Ala-D-lactate (69) and incorporate it into the peptidoglycan precursors to replace D-Ala-D-Ala (70, 71, 72). A dipeptidase VanX, hydrolyses D-Ala-D-Ala synthesized by the ligase thereby decreasing the production of the normal peptidoglycan precursor UDP-MurN Ac-L-Ala-D-isoGlu-L-Lys-D-Ala-D-Ala (UDP-MurN Ac-pentapeptide(Ala)) and VanY, a membrane-bound D, D-carboxypeptidase enzyme removes the C-terminal D-Ala residues of late peptidoglycan precursors (67, 73). All of these mechanisms increase resistance to vancomycin.
**Figure 13: Mechanism of vancomycin resistance.** Vancomycin prevents transglycosylation and cross-linking of the peptidoglycan during cell wall synthesis. Gene clusters that produces peptidoglycan D-Ala-D-Lactate instead of D-Ala-D-Ala have been found to be associated with VanA resistance. They include the vanH, vanA, vanX, vanS, vanR, and vanZ gene clusters (21). VanS is a sensor histidine kinase that senses the presence of vancomycin, VanR is the regulator that activates transcription of the operon. VanH is a dehydrogenase that produces lactate by converting pyruvate to D-lactic acid. VanX is a D, D-dipeptidase, which cleaves D-Ala-D-Ala, and VanA is a ligase that forms D-Ala-D-Lactate. VanY is a carboxypeptidase that cleaves the terminal D-alal (from pentapeptide to tetrapeptide). The role of VanZ is unknown (99).
Both *in-vivo* and *in-vitro* transfer of the *vanA* gene from *Enterococci* species to *S. aureus* results in a strain that is resistant to vancomycin. The first MRSA with a very high resistance to vancomycin (minimum inhibitory concentration >256 μg/ml) was isolated in 2002 (22).

The past decade has recorded a remarkable increase in CDI treatment failure with both antibiotics (28). Therefore, a metronidazole-resistant strain was used to explore potential impact decreased susceptibility may have on the pathophysiology of recurrent CDI. High level of resistance was once not considered to be responsible (80), however, recent reports have demonstrated a causal relationship (30).

A preliminary data (not shown) generated from our laboratory showed the present of *C. difficile* strains with significantly high-level resistant to metronidazole and vancomycin in the community. Presently, it is unclear how prevalent metronidazole and vancomycin resistant *C. difficile* strains are in healthcare communities. It is important to establish the prevalence of metronidazole and vancomycin resistance in *C. difficile*, determine the minimum inhibitory concentrations (MIC) of the different isolates and identify the mechanism of resistance in order to be able to design novel targeted therapies. Therefore, the purpose of this study was to determine the prevalence of metronidazole-and-vancomycin resistance in *Clostridium difficile* strains isolated from patients and uncover the resistance mechanism.
MATERIALS AND METHODS

Stool Samples

Clinical stool samples were obtained from inpatients, who presented with diarrhea at St. Luke’s Episcopal Hospital in the Texas Medical Center (TMC), Houston, Texas, and two hospitals in Kenya: Kenyatta National Hospital, Nairobi, and Kisii Teaching & Referral Hospital, Kisii. All the obtained clinical stool samples for this study was approved by the Institutional Review Boards of The University of Texas Health Science Center at Houston and St. Luke’s Episcopal Hospital (Houston, Texas), and the ethical review boards of Kenyatta National Hospital and Kisii Teaching and Referral Hospital. All the stool samples from patients were also de-identified. The samples from St. Luke’s were tested by their medical laboratory and reported as C. difficile positive, whereas the Kenyan samples were not tested for C. difficile by their respective microbiology laboratories. All samples were stored at -80°C until analyzed.

C. difficile Detection

The presence of C. difficile in the stools was determined using the C. difficile Plate Assay (CDPA). This method utilized an agar-based selective culture medium developed in our laboratory for differential analysis of C. difficile isolates (11). The assay differentiates toxin producing C. difficile from non–toxin producing colonies and concurrently inhibits growth of non- C. difficile colonies (11). CDPA is composed of brain heart infusion (BHI) medium (Becton Dickinson, Cockeysville, MD) (37 g), agar (14 g), defibrinated horse blood (7%) (Fisher Scientific, Pittsburgh, PA), 150 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Zymo Research Irvine, CA), p-cresol (0.025%), D-cycloserine (300 mg) and cefoxitin (8.5 mg) (Fisher Scientific), per liter.
A total of 572 stool samples were tested, of which 466 were obtained from St Luke’s Episcopal Hospital, 106 were from Kenyatta National Hospital, and Kisii Teaching and Referral Hospital. A loopful of each stool sample was spread on the CDPA plate and incubated anaerobically in an atmosphere of 10% H₂, 5% CO₂, and 85% N₂ in a Controlled Atmosphere Anaerobic Chamber (Plas Labs, Lansing, MI) for 48 hours to facilitate both colony formation and toxin production. To enable the chromogenic reaction, which differentiates toxin from non-toxin producing *C. difficile*, the plates were exposed to oxygen at room temperature after the anaerobic incubation. Toxin producing colonies appeared blue while undetectable or non-toxin producing colonies appear pale white.

**Screening for Resistant Isolates**

CDPA plates containing 8 µg/ml of metronidazole (CDPA-Metro) and CDPA plates containing 4 µg/ml of vancomycin (CDPA-Van) were used in screening for metronidazole and vancomycin resistant isolates, respectively. The initial concentrations of vancomycin and metronidazole used were based on the CLSI break point (30). Using a sterilized loop, each *C. difficile*-positive stool sample was spread directly onto the CDPA-Metro and CDPA-Van plates and were incubated anaerobically at 37°C for 48 hours. All stool samples were grouped into metronidazole-resistant only (Met-R only), these are stool samples that formed viable colonies on CDPA-Metro plates only, vancomycin-resistant only (Van -R only), these are stool samples that formed viable colonies on CDPA-Van plates only and both metronidazole and vancomycin resistant
(Met-Van-R) are stool samples that grew viable colonies on both CDPA-Metro and CDPA-Van plates (Fig. 6).

**Figure 14: Screening steps for isolating metronidazole- and vancomycin-resistant C. difficile colonies from stool samples.** This chart details steps employed to screen the 466 stool samples from Texas and 102 stool samples from Kenya. CDPA plates were used to determine *C. difficile* positive stool samples, whereas CDPA + Metronidazole (8 μg/ml) and CDPA + Vancomycin (4 μg/ml) were used to screen for metronidazole- and vancomycin-resistant positive stool samples.
**DNA Isolation**

Frozen isolates (stock of resistant isolates) were cultured in 5 ml BHI media supplemented with D-cycloserine (250 µg/ml) and cefoxitin (8.5 µg/ml), and were incubated anaerobically at 37°C overnight. DNA was isolated using Gen Elute Bacteria Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO), following the manufacturer’s recommended procedure. The concentration of the isolated DNA was measured using a NanoDrop 2000 Spectrophotometer (Thermos Scientific, Waltham, MA). All DNA was stored at -20°C until analyzed.

**Toxin Analysis**

The *C. difficile* toxin activity (CdiffTox) assay was used to measure toxin activity in the resistant isolates. The CdiffTox assay employs the ability of the *C. difficile* toxins to cleave ρ-nitrophenyl-β-D-glucopyranoside into chromogenic nitrophenol, which can be measured spectrophotometrically at 410 nm (11). For the toxin test, 20 different resistant isolates with different MICs were cultured in BHI broth containing D-cycloserine (250 µg/ml) and cefoxitin (8.5 µg/ml) and incubated at 37°C in anaerobic chamber (PLAS LABS, Lansing, MI) for 48 hours and the cells were harvested by centrifuging at 4000 rpm for 15 minutes. The culture supernatants (250 µl) were added into a sterile Costar polystyrene 96-well plate and 30 µl of substrate (sterilized 30 mM of ρ-nitrophenyl-β-D-glucopyranoside in water) was added and incubated aerobically at 37°C for 4-24 hours. The cleaved substrate was measured at 410 nm wavelength using SPECTRAmax Plus 384 spectrophotometers (Molecular Devices, Sunnyvale, CA).
**Determination of Minimum Inhibitory Concentrations**

The minimum inhibitory concentrations (MIC) of metronidazole and vancomycin were determined using two methods. These are the E-test Strip method (also called the (Epsilometer test) method (60), and the broth microdilution methods (60).

**(i) E-test Strip Method**

E-test strip (Biomerieux inc, Durham, NC) is a gradient diffusion assay recommended by CLSI for determining antimicrobial susceptibility profile in both clinical and epidemiological surveillance (60). To assess the level of resistance, 53 resistant isolates from Texas were randomly selected (Table 1) and MICs for metronidazole and vancomycin were determined using the E-Strip method. The MIC range on the stripe was from 0.16 μg/ml to 256 μg/ml for both metronidazole and vancomycin. For the test, single colonies were picked from a CDPA plate and suspended in 1000 μl of sterile 1X phosphate buffered saline (PBS). A sterilized cotton swab was used to spread the culture onto a BHI agar plate containing 7% horse blood. Two different strips impregnated with gradient concentrations (0.16-256 μg/ml) of metronidazole or vancomycin were placed onto each plate using sterilized forceps and incubated anaerobically at 37° for 24 hours. The MICs were determined based on the zone of inhibition.
Table 1: Different number of Isolates and their corresponding resistance group.

The minimum inhibitory concentration of metronidazole and vancomycin was determined on colonies selected from the metronidazole-resistant only (Met-R only), vancomycin-resistant only (Vanc-R only) and both metronidazole and vancomycin resistant (Met-Van-R) isolates. Overnight cultures of single colonies of each isolate were spread on BHI plates containing blood using a sterilized cotton swab. E-test strips containing graduated concentration of metronidazole and vancomycin (0.5 - 256µg/ml) were separately placed onto the plate and incubated anaerobically overnight at 37°C to determine their various MICs.

<table>
<thead>
<tr>
<th>Category</th>
<th>Number of Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met-R Only</td>
<td>15</td>
</tr>
<tr>
<td>Van-R Only</td>
<td>4</td>
</tr>
<tr>
<td>Met-Van-R</td>
<td>34</td>
</tr>
</tbody>
</table>
(ii) MIC Determination by the Broth Microdilution Method

Overnight cultures prepared from stored resistant colonies selected from CDPA plates were used. For the test, 4 µl of overnight cultures of each isolate was added in duplicate to a sterile Costar polystyrene 96-well plate (Corning Inc., NY) containing 200 µl of BHI supplemented with D-cycloserine (250 µg/ml), cefoxitin (8.5 µg/ml), gradient concentrations of metronidazole or vancomycin (2-1024 µg/ml) separately and incubated anaerobically at 37°C for 24 hours. The optical densities of the culture were measured at a wavelength of 600 nm.

PCR Amplification of the vanA gene in the isolates

PCR was used to examine the presence of the vanA gene in the vancomycin-resistant C. difficile stored isolates. The amplification was performed using the primers: Forward 5’GGGAAAACGACAATTGC3’ and Reverse 5’GTACAATGCGGCCGTAA3’. The conditions used were: denaturation at 94°C for 2 min, 40 cycles of 94°C for 30 secs, 55°C for 60 seconds, and 68°C for 90 seconds and a final elongation step at 68°C for 5 min. The PCR products were analyzed using 1% agarose gel electrophoresis and ethidium bromide staining.

Ligation and Cloning

Following amplification, the PCR products were purified with the Nucleic Acid Purification Kit (Epoch Life Science) following manufacturer directions. The DNA concentration was quantified using a Nanodrop 2000 Spectrophotometer. The purified PCR products were cloned into PCR II vector (Thermo Scientific) and sequenced at Lone Star Labs, Houston, Texas. For cloning and ligation, the manufacturer’s protocol was followed. Briefly, 10 µl of ligation reaction was set up using 50-200 ng DNA, 2 µl of
5X ExpressLink T4 DNA Ligase Buffer, 2 μl PCR II vector (25 ng/μl), 3μl of water, and 1μl of ExpressLink T4 DNA Ligase. The ligation reaction was incubated at room temperature for 15 minutes and used for transformation as described below.

**Transformation**

Top10 competent cells were thawed on ice and 100 μl of the thawed competent cells was added into a microfuge tube on ice, 2 μl of each ligation reaction was added separately in each tube containing the competent cells. The content of each tube was gently mixed with a pipette, incubated on ice for 30 minutes, and 100 μl of the transformation reaction was spread on Luria Broth (LB) agar plates containing 100 μg/ml of ampicillin and 150 μg/ml of X-gal. The plates were incubated at 37 °C overnight. The transformants (3 colonies each) were picked from each plate and cultured in LB broth containing 100 μg/ml ampicillin and incubated at 37 °C in a MaxQ 4000 shaker (Thermo Scientific) at 275 rpm overnight. Stocks were saved, and plasmids were isolated using Plasmid Isolation Kit (BioBasic Inc), as directed by the manufacturer. The concentration of each isolated plasmid was quantified using a Nanodrop 2000 Spectrophotometer and the plasmids were sent for sequencing at LoneStar Labs (Houston, Texas).

**Sequence Analysis**

The transformants containing the inserts with the plasmids were isolated and sequenced. The plasmid was identified and removed from the sequence. Both the forward and reverse sequences were merged using MAFFT and multiple sequence alignment was performed using CLUSTALW.
Biofilm Formation Analysis

To evaluate the rate of adhesiveness or the amount of biofilm produced by the vancomycin-resistant isolates when exposed to vancomycin, three resistant isolates 62-4, 255-1, and 67-2 were tested. The vancomycin MICs of these isolates determined by the broth microdilution method are (i) low resistance, MIC 4 µg/ml (isolate 62-4); (ii) intermediate resistance, MIC 64 µg/ml (isolate 255-1); and (iii) high resistance, MIC 1024 µg/ml (67-2).

Overnight cultures of the isolates were prepared in BHI supplemented with D-cycloserine (250 µg/ml) and cefoxitin (8.5 µg/ml) and incubated anaerobically at 37°C. BHI medium supplemented with 1% glucose was prepared for the control, while BHI supplemented with 1% glucose and vancomycin at concentrations corresponding to ½ and ¼ of the MICs of the isolates was prepared for the experimental as follows:

(1) Low resistance, MIC 4 µg/ml (62-4): BHI supplemented with 1% glucose containing 2 µg/ml or 1 µg/ml vancomycin.

(2) Intermediate resistance, MIC 64 µg/ml (isolate 255-1): BHI supplemented with 1% glucose containing 32 µg/ml or 16 µg/ml of vancomycin.

(3) High resistance, MIC 1024 µg/ml (67-2): BHI supplemented with 1% glucose containing 512 µg/ml or 256 µg/ml vancomycin.

For the control without vancomycin, 200 µl of overnight culture of each of the isolates were added in triplicate into 24-well polystyrene plates containing 1800 µl of BHI supplemented with 1% glucose. For the experimental group, 200 µl of the culture were added in triplicate into 24-well polystyrene plates and 1800 µl of BHI supplemented with
1% glucose containing different concentrations of vancomycin, as described above. All the samples were incubated at 37\(^0\)C anaerobically for 72 hours.

Following the incubation period, the amount of biofilm produced was measured (59). Briefly, the supernatant of all the wells was gently removed and each well was washed twice with 2000 µl of sterilized 1X PBS. The plates were dried at 60\(^0\)C for 1 hour. The dried wells were stained with 150 µl of 2% crystal violet and incubated at 37\(^0\)C for 15 minutes. The excess stain was washed out with deionized water and the plates were dried at 60\(^0\)C for 10 minutes. The crystal violet bound to the adherent cells was extracted with 1800 µl of 100% ethanol. The optical density was measured at 570 nm wavelength using SPECTRAmax Plus 384 spectrophotometers. The experiment and the assay were performed three times.
RESULTS

To determine the prevalence of metronidazole-and-vancomycin resistance in *C. difficile*, all the *C. difficile* positive stool samples and stool samples containing metronidazole-resistant and vancomycin-resistant *C. difficile* were enumerated and classified as follows. Stool samples that contains *C. difficile* that grew on CDPA plates were classified as *C. difficile* positive stool samples whereas, those that did not grow were classified as *C. difficile* negative stool samples. All stool samples that contain *C. difficile* strains that grew on plates containing metronidazole only were grouped as metronidazole only resistant stool sample (Met-R), all stool samples that contain *C. difficile* strains that grew on plates containing vancomycin only were grouped as vancomycin only resistant stool sample (Van-R) and all stool samples that contain *C. difficile* strains that grew on both metronidazole and vancomycin plates were grouped as metronidazole-vancomycin resistant stool sample (Met-Van-R).
Table 2: Distribution of the number of patients having metronidazole- and vancomycin-resistant *C. difficile* strains in their stool. The samples were grouped as described, Met-R, Van-R and Met-Van-R. The total number and percent relative to the *C. difficile* positive samples, metronidazole- and vancomycin- resistant from both Texas and Kenya patients is shown.

<table>
<thead>
<tr>
<th></th>
<th>Texas</th>
<th>Kenya</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (%)</td>
<td>N (%)</td>
</tr>
<tr>
<td><em>C. difficile</em> positive stools</td>
<td>438 (93.9)</td>
<td>98 (96)</td>
</tr>
<tr>
<td>Met-R Only</td>
<td>31 (7)</td>
<td>26 (26.5)</td>
</tr>
<tr>
<td>Vanc-R Only</td>
<td>17 (3.9)</td>
<td>9 (9.1)</td>
</tr>
<tr>
<td>Met-Van-R</td>
<td>97 (22.1)</td>
<td>57 (58.2)</td>
</tr>
<tr>
<td>Total Met-R</td>
<td>128 (29.2)</td>
<td>83 (84.7)</td>
</tr>
<tr>
<td>Total Van-R</td>
<td>114 (26)</td>
<td>66 (67.3)</td>
</tr>
<tr>
<td>Total (Met-R + Van-R)</td>
<td><strong>145 (33.1)</strong></td>
<td><strong>92 (93.9)</strong></td>
</tr>
</tbody>
</table>
Distribution of Metronidazole- and Vancomycin-Resistant *C. difficile* in CDI Patients

To determine the percent of *C. difficile* positive stool samples, metronidazole and vancomycin resistant stool in samples from Texas and Kenya, the number of *C. difficile* positive and negative stool samples were enumerated and grouped (Table 2) as metronidazole resistant stool samples (Met-R only), vancomycin-resistant only (Van-R only) samples, and both metronidazole and vancomycin resistant stool samples (Met-Van-R).

To establish the prevalence of metronidazole- and vancomycin-resistant *C. difficile*, CDI-positive stool samples were plated on CDPA plates containing either metronidazole or vancomycin at their reported CLSI cutoff values (8 µg/ml and 4 µg/ml respectively). All stool samples that grew viable colonies only on the metronidazole-containing plates were grouped as (Met-R only), stools that grew viable colonies only on the vancomycin-containing plates (Van-R only) and stools samples that grew colonies on both metronidazole and vancomycin plates (Met-Van-R). From Texas stool samples 438 (93.9%) contained colonies that were *C. difficile* positive, 31 (7%) were Met-S only, 17 (3.9%) were Vanc-R only and 97 (22.1%) were Met-Van-R. From the Kenya stool samples, 98 (96%) were *C. difficile* positive, 26 (26.5%) were Met-R only, 9 (9.1%) were Vanc-R only and 57 (58.2%) were Met-Van-R. (Table 2). Overall, the total numbers of resistant stool samples were Met-R, 128 (29.2%), Van-R, 114 (26%) and Met-Van-R 145 (33.1%).

For further analysis, five different colonies were selected from each plate containing resistant stool samples from Texas and at least two from the Kenyan stools. All the
colonies were picked and cultured anaerobically overnight in BHI broth containing D-cycloserine (250 µg/ml), cefoxitin (8.5 µg/ml) and their respective antibiotics (8 µg/ml of metronidazole or 4 µg/ml of vancomycin). The number of viable colonies that represent the number of stored independent isolates from all the metronidazole and vancomycin resistant stool samples was recorded (Fig. 7).

Figure 15: Isolation of metronidazole- and vancomycin-resistant *C. difficile* colonies from the stools. CDI stools were plated on CDPA plates containing either metronidazole (8µg/ml) or vancomycin (4 µg/ml). Following a 24-hour incubation, colonies (5 from Texas and at least 2 from Kenya stools) were selected for further analysis.
Characteristics of the Colonies Based on Morphology and Toxin Production

Of the *C. difficile* positive stool samples from Texas and Kenya, 157 (35.9%) and 5 (5.1%), respectively, of the samples produced only blue colonies on the CDPA plates, indicative of active toxin production. On the other hand, the plates for 122 (27.9%) of the Texas samples and 9 (9.2%) of the Kenya samples produced had only white colonies (Table 3). This suggests that the colonies were not producing active toxins or cannot produce active toxins. The colonies from 159 (36.3%) of Texas and 84 (85.7%) of the Kenyan patients had mixed blue and white (both toxin and non-toxin) colonies on the plates.

The sizes of the colonies in each plate were also analyzed and classified as (a) small, (b) medium, (c) large, and (d) a mixture. The classification is the different sizes observed and therefore, subjective. Of the stool samples analyzed, 245 of the patients from Texas and 45 patients from Kenya had *C. difficile* isolates that formed only small colonies. Further, 49 of the Texas and 3 of the Kenyan samples formed medium sized colonies only, whereas 18 of the Texas and 6 of the Kenyan samples formed large colonies only. Also, 58 of the Texas and 20 of the Kenyan samples formed a mixture of small and medium colonies, while 10 (Texas) and 1 (Kenya) samples formed a mixture of medium and large colonies. Finally, 42 of the Texas and 19 of the Kenyan samples formed a mixture of small and large colonies whereas 16 (Texas) and 4 (Kenyan) samples formed a mixture of small, medium and large colonies. These results indicated that many Texas and most Kenyan CDI patients are infected with *C. difficile* strains that are morphologically different.
Table 3: The characteristics of the *C. difficile* colonies isolated from patients from Texas and Kenya.

<table>
<thead>
<tr>
<th>Morphology</th>
<th>Texas</th>
<th>Kenya</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of Samples</td>
<td>Number of Samples</td>
</tr>
<tr>
<td>Small colonies only</td>
<td>245</td>
<td>45</td>
</tr>
<tr>
<td>Medium colonies only</td>
<td>49</td>
<td>3</td>
</tr>
<tr>
<td>Large colonies only</td>
<td>18</td>
<td>6</td>
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<tr>
<td>Mixture of small and medium colonies</td>
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<td>20</td>
</tr>
<tr>
<td>Mixture of medium and large colonies</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Mixture of large and small colonies</td>
<td>42</td>
<td>19</td>
</tr>
<tr>
<td>Mixture of small, medium and large</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>438</strong></td>
<td>98</td>
</tr>
<tr>
<td>Blue colonies only</td>
<td>157</td>
<td>5</td>
</tr>
<tr>
<td>White colonies only</td>
<td>122</td>
<td>9</td>
</tr>
<tr>
<td>Mixture of white and blue colonies</td>
<td>159</td>
<td>84</td>
</tr>
</tbody>
</table>
The Minimum Inhibitory concentrations (MIC) of the *C. difficile* strains

To determine the MICs of the isolates from metronidazole- and vancomycin-resistant stools, the E-test strip assay was used to test 53 randomly selected isolates from Texas. The result revealed that the metronidazole MIC of 52 (98.1%) of the isolates was greater than 256 µg/ml, and only 1 (1.9%) was sensitive with an MIC 1.0 µg/ml (Table 4). Also, the vancomycin MIC of 35 of the isolates (77.4%) was greater than 256 µg/ml, 5 had MICs between 8-16 µg/ml, whereas 12 (22.6%) had MICs less than 4 µg/ml.

Since the majority of the 53 samples initially tested by the E-test strip method have MICs greater than 256 µg/ml, the broth microdilution method was used to determine the exact MICs of all the resistant isolates. This is because this method does not have a limitation on the antibiotic concentration to be tested, contrary to the E-test strip method whose maximum concentration is 256 µg/ml for both metronidazole and vancomycin.

Out of 145 resistant isolates from Texas, three isolates did not grow in the liquid, so 142 were tested for their MIC using broth microdilution method. From this analysis, 80% (114 out of 142 resistant isolates) from Texas colonies were vancomycin resistant with MIC >16 µg/ml whereas 41% (38 out of 92 resistant isolates) from Kenya were vancomycin resistant with MIC >16 µg/ml. 97.8% (139 out of 142 resistant isolates) from Texas population were metronidazole resistant with MICs > 256 µg/ml whereas 97% (90 out of 92 resistant isolates) from Kenya were metronidazole resistant with MICs > 256 µg/ml.
Table 4: The MIC of 53 *C. difficile* isolates using E-test strip method. The MIC breakpoint was based on guideline recommended for anaerobes by Clinical and Laboratory Standard Institute (CLSI), which is 8 µg/ml for metronidazole and 4 µg/ml for vancomycin.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Number of Isolates</th>
<th>Metronidazole</th>
<th>Vancomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
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<td>8</td>
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<tr>
<td>8</td>
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</tr>
<tr>
<td>12</td>
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</tr>
<tr>
<td>16</td>
<td>-</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>&gt;256</td>
<td>52</td>
<td>35</td>
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</tbody>
</table>
Pearson’s test of association between resistance and location showed a high tendency of *C. difficile* isolates to be metronidazole resistant in Kenya than Texas (p-value ≤ 0.001). This might be as a result of frequent self-medication with metronidazole.

Unexpectedly, the tendency to develop vancomycin resistant is equally higher in Kenya than Texas (p-value ≤ 0.001), even with no history of vancomycin treatment. Both metronidazole- and- vancomycin resistance were prevalent in Kenya than Texas.
Table 5: The MICs of the metronidazole and vancomycin-resistance *C. difficile* isolates determined using the broth microdilution method.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Texas (n= 142)</th>
<th>Kenya (n= 92)</th>
<th>Texas (n= 142)</th>
<th>Kenya (n= 92)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3</td>
<td>2</td>
<td>11</td>
<td>42</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>1</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>32</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>64</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>128</td>
<td>3</td>
<td>-</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>256</td>
<td>2</td>
<td>4</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>512</td>
<td>7</td>
<td>8</td>
<td>23</td>
<td>4</td>
</tr>
<tr>
<td>&gt;1024</td>
<td>123</td>
<td>76</td>
<td>76</td>
<td>15</td>
</tr>
</tbody>
</table>
Table 6: Distribution of the isolates based on both CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. CLSI and EUCAST cut off values are as follows: EUCAST: Susceptible is ≤2mg/L, Resistant is >2mg/L and the Cut off is ≤2mg/L, for both metronidazole and vancomycin. CLSI: ≤8/16/ ≥32 (Sensitive/Intermediate/Resistant) for metronidazole and ≥4µg/ml for vancomycin.

<table>
<thead>
<tr>
<th></th>
<th>Texas (n = 142)</th>
<th></th>
<th>Kenya (n = 92)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CLSI</td>
<td>EUCAST</td>
<td>CLSI</td>
<td>EUCAST</td>
</tr>
<tr>
<td>Metronidazole</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>resistant only</td>
<td>11</td>
<td>11</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>Vancomycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>resistant only</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Both</td>
<td>128</td>
<td>128</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>Metronidazole</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>and</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vancomycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>resistant</td>
<td></td>
<td></td>
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</tbody>
</table>
Cluster Analysis of the MICs

Cluster analysis reveals that a significant number of the isolates (123/145 for the Texas isolates and 76/91 for the Kenya isolates) had MIC >1024 µg/ml for metronidazole. There was a wide gap in MIC range among the metronidazole isolates in both patient populations with 139 (97.9%) having MIC >64 µg/ml (for the Texas samples) and 89 (96.7%) for Kenyan samples (Fig. 8). The MICs for the vancomycin-resistant isolates were spread across different concentrations in both patient populations. However, a higher number of the isolates 99 (68.3%) had vancomycin MIC of ≥512 µg/ml for the Texas samples. Remarkably, isolates with resistance to high levels of metronidazole was predominant in both Texas and Kenyan patients. Based on the distribution of the MICs, the resistant isolates can be grouped into three categories: (i) Low resistance (4-16 µg/ml); (ii) intermediate resistance (32-64 µg/ml); and (iii) high resistance (64 - >1024 µg/ml).
Figure 16: Distribution of metronidazole- and vancomycin-resistant Texas and Kenyan *C. difficile* isolates based on MICs. The red line is the median and the solid line represents number of highly resistant isolate with MIC of 1024 µg/ml. Each dot represents a colony.
Analysis of Toxin Production in Both Texas and Kenya Resistant Isolates

Twenty isolates with different levels of resistance (Fig. 9) from Texas and Kenya were selected to explore the association between toxin production and antibiotic resistance. The isolates were grown in BHI containing D-cycloserine (250 µg/ml) and cefoxitin (8.5 µg/ml) anaerobically at 37°C for 48 hours. The culture supernatants were tested for toxin activity by mixing 250 µl of the supernatant with 30 µl of the substrate. Sixteen out of twenty isolates from Texas were toxin positive (Tox⁺) whereas 15 out of the twenty 20 isolates from Kenya were Tox⁺.
Figure 17. Analysis of toxin production by the metronidazole and vancomycin resistant isolates. Both toxin-producing (Tox+) and non-toxin-producing (Tox-) isolates are represented from different groups of resistant isolates: low resistance (4-16 μg/ml), intermediate resistance (32-64 μg/ml) and high resistance (32-64 μg/ml). Stored isolates (20) were cultured for 48 hours and 250 μl of each culture supernatant was incubated with toxin substrate (30 mM p-nitrophenyl-β-D-glucopyranoside) for 4 hours. The toxin present in culture supernatant cleaves the chromogenic toxin substrate and generates yellow colored nitrophenol whose absorbance is read at 410nm in a spectrophotometer.
Analysis of Biofilm Formation of the Resistant Isolates

Biofilm has been suggested to be associated with vancomycin resistance (30). To investigate the effect of vancomycin on the ability of the resistant isolates to form biofilms, isolates with different MICs were evaluated. This experiment was based on the hypothesis that if the low- and intermediate-resistant isolates, but not highly-resistant isolates, use biofilm as a defensive mechanism when exposed to vancomycin, then the degree of biofilm formation in those isolates should increase. To test this hypothesis, the isolates were exposed to sub-inhibitory concentrations of vancomycin for 72 hours and biofilm production was measured. There was no significant difference ($p=0.3554$) in the amount of biofilm produced in the presence or absence of vancomycin in all the isolates tested (Fig. 10).
Figure 10: Effect of vancomycin on biofilm formation on vancomycin-resistant *C. difficile* isolates. Cultures with different vancomycin MICs were exposed to different amounts of vancomycin for 72 hours and biofilm produced was measured. The blue, purple, and green bars represent the low, intermediate and high resistant isolates respectively. The different isolates were grown without vancomycin, or in the presence of vancomycin at concentrations equivalent to $\frac{1}{4}$ MIC or $\frac{1}{2}$ MIC values of the isolates. Error bars represent the standard deviation from three independent replicates.
PCR- Based Detection of the *vanA* Gene in the Vancomycin Resistant Isolates.

To evaluate the presence of *vanA* gene in the resistant isolates, PCR was performed using a primer pair specific for *vanA* in *Enterococcus faecium*. A total of 40 vancomycin resistant isolates were tested and all were positive for the *vanA* gene (Fig. 11).

![Figure 11](image)

**Figure 11:** A representative agarose gel electrophoresis images showing the expected *vanA* bands in the *C. difficile* isolates tested. Genomic DNA was isolated from the resistant isolates and used as templates for PCR with primers specific for the *vanA* gene in *E. faecium*. The PCR product was resolved on 1% agarose and stained with ethidium bromide. M is 1kb DNA marker, lanes 1 and 14 are the *vanA* amplicons and –VE is the negative control with no DNA.
Sequence Comparison of Putative *C. difficile vanA* gene with the *Enterococcus Faecium* Homolog

To confirm the presence of the *C. difficile vanA* gene and also enable comparison to the *E. faecium* homolog, the vanA PCR products were purified and sequenced. The sequence alignment is shown (Fig. 12). When *C. difficile vanA* sequence is compared to *vanA* of *E. faecium* there is a significant measure of divergence in the 5’ end compared to 3’ end which is more conserved.

![Sequence alignment from the PCR product of the amplified segment of the *C. difficile vanA* gene.](image)

The alignment appears to be divergent at the 5’ region and more conserved in the 3’.
VanA Amino Acid Sequence Alignment

The DNA sequences obtained from the isolates were translated into amino acid and aligned to the vanA amino acid sequence from *Enterococcus faecalis* (Fig. 13). Cluster analysis was performed to determine the percentage of similarity and most of the sequences were 97.27-100% similar. It appears the differences in the sequence did not impact the antibiotic resistance activity as the isolate with the lowest similarity in the sequence has a high degree of vancomycin resistance of 512 μg/ml compared to as the others. However, due to the low quality of the sequences obtained at the 5’ and 3’ ends, we were not able to obtain the full-length version of the *C. difficile* vanA from the isolates. This will be addressed in a planned future study.
Figure 13: VanA amino acid sequence alignment of the resistant isolates.
DISCUSSION

*Clostridium difficile* infections are the most common hospital acquired infection and currently treated with metronidazole and vancomycin (4). The unexpectedly high percent of *C. difficile* metronidazole and vancomycin resistant isolates from a preliminary study in our laboratory was of great concern and led to the hypothesis that metronidazole and vancomycin resistant *C. difficile* is more widespread in the patient population that previously envisaged. The objective of this study was to determine the prevalence of metronidazole- and- vancomycin resistance in *C. difficile* strains and uncover the resistance mechanism.

The result of the initial analysis of the metronidazole and vancomycin resistant *C. difficile* strains in stools of diarrhea patients from Texas and Kenya demonstrated that metronidazole and vancomycin resistance is prevalent in CDI patients. Our analysis to determine the level of metronidazole and vancomycin resistance of each isolate from the stool sample revealed a high degree of resistance to either or both antibiotics. The two methods of MIC determination (E-test strip and broth microdilution assays) showed *C. difficile* isolates with high MICs to both antibiotics and for the first time *C. difficile* isolate with vancomycin MIC >1024 µg/ml was isolated. Cluster and statistical analysis revealed that the percentage of metronidazole resistant isolates was greater than vancomycin resistant isolates in both patient populations examined, and there is a greater tendency for a *C. difficile* isolate to be metronidazole – or – vancomycin resistant in Kenya than Texas. PCR analysis of the vancomycin-resistant isolates revealed the presence of a *vanA*, gene responsible for vancomycin resistance in other
Gram-positive bacteria. Further studies will be necessary to uncover the mechanism of resistance.

To determine the prevalence of metronidazole- and- vancomycin resistance in clinical isolates of *C. difficile* from Texas and Kenya, the stool samples were analyzed using *C. difficile* specific agar selective medium. The stool samples that contained *C. difficile* strains resistant to either or both antibiotics were grouped into three categories, Met-R, Van-R and Met-Van-R, and comprised of 33% of the Texas stool samples and unexpectedly 93.7% of the Kenya stool samples. These prevalence rates significantly exceed prior reports from other studies such as, the 2011 to 2014 longitudinal surveillance report from the US and Europe, which reported a low percent of both metronidazole and vancomycin resistance (28). The sudden increase in prevalence could be a result of an unexplored potential link between CDI treatment failure and antimicrobial resistance. CDI treatment failure has not been shown to have a relationship with antimicrobial resistance (4). A reduced 8 µg/ml fecal concentration of metronidazole may result in a simultaneous decrease of concentration that accompanied reduction of colonic inflammation (4). This concentration is high enough to kill susceptible *C. difficile* strains and low enough to be a sub-inhibitory concentration for resistant strains so that it could play a role in selecting and sustaining colonies with a gradually increase in their MICs (81). In addition, as the concentration decreases following cessation of metronidazole treatment, persistent spores could germinate into vegetative cells (4). What appears to be a gradual increase in the prevalence of metronidazole and vancomycin resistance from the previous (28) to the present
indicates gradual dissemination of both vancomycin and metronidazole resistant isolates in the community.

One interesting finding from this study was that a greater number of the isolates were metronidazole resistant than vancomycin resistant. Although considerable variation in C. difficile resistance patterns has generally been observed, the percent of vancomycin resistance has been shown to exceed metronidazole resistance in most previous studies (30). However, in this study, the rate of metronidazole resistance exceeded that of vancomycin: 29.2% metronidazole and 26% vancomycin resistant isolates in Texas and 84.7% metronidazole and 67.3% vancomycin in the examined Kenya population. Conversely, previous published reports showed that 3.6% of stools from CDI patients in the US, contained C. difficile strains that are resistant to metronidazole and 17.9% to vancomycin (77). Similarly, prevalence rates of 0.11% and 2.29% were reported for metronidazole and vancomycin, respectively, in Europe (77). Another study also reported that 94% of the C. difficile isolates collected from Iran were susceptible to metronidazole at 2 μg/ml and only 5.3% were vancomycin resistant (84). However, a study conducted in Israel reported 47% vancomycin and 18% metronidazole resistance (88). Interestingly, one study here in Texas reported 13.3% metronidazole resistance and 0% vancomycin resistance (89), which is consistent with our report, although no vancomycin resistance was isolated. The variation in resistance is probably as a result of geographical location, and local or national antibiotics treatment policies. It is noteworthy that, the hypervirulent strain (RT) 027 was consistently isolated in most metronidazole and vancomycin resistance studies (4, 30). Its emergence underscored the potential importance of antimicrobial resistance in spreading of epidemic C. difficile.
clones. Eleven (11) out of twenty (20) resistant strains from a European antimicrobial surveillance study were ribotype 027 (77).

To determine the predisposition of an isolate becoming resistant to either or both antibiotics in the two populations studied, Pearson’s statistical analysis was employed. The analysis indicated that there is a higher tendency for a *C. difficile* isolate to be metronidazole resistant in Kenya than Texas. This may be as a result of frequent self-medication with metronidazole that occurs in Kenya. Unexpectedly, the tendency for vancomycin resistance is also higher in Kenya than Texas even though, there is no significant vancomycin treatment of CDI in Kenya. Both metronidazole and vancomycin resistance are more prevalent in Kenya than Texas. However, there was a greater number of highly vancomycin resistant isolates in the Texas samples than those from Kenya, suggesting that the reported inappropriate empirical treatment of CDI patients who do not meet the criteria for severe CDI may be responsible (15).

To determine the level of antibiotic resistance in the isolated *C. difficile* strains, the E-test strip assay was used. Isolates from the three groups of resistance (Met-R, Van-R and Met-Van-R) were represented. Most of the metronidazole sensitive isolates (3 out of 4) became metronidazole resistant and even more (12 out of 15) vancomycin sensitive became resistant. This unanticipated change from sensitive to resistant suggests heterogenous resistance resulting from some *C. difficile* sensitive strains expressing resistance after freeze-thawing of stored samples. Previous reports have also shown a similar phenotypic change after frozen samples were thawed for analysis (4, 90). Also, a slow-growing metronidazole resistant sub-population of *C. difficile* have been observed after an extended incubation period of the E-test assay (4). This was
also observed in our study. Sub-population of the isolates grew colonies inside the zone of inhibition after a period.

To further confirm the E-test determined MICs of the resistant isolates and to determine the MICs beyond the 256 µg/ml limit of the E-test strip used in this study, broth microdilution method was used. Eighty (80%) and 41% of vancomycin resistant isolates from Texas and Kenya, respectively, had vancomycin MIC >16 µg/ml, which is significantly higher than the currently reported maximum MIC 16 µg/ml (30). Whereas the proportion of the metronidazole resistant isolates with metronidazole MIC > 256 µg/ml (78), was 97.8% and 86.6% for Texas and Kenya respectively. It was not unexpected to isolate metronidazole-resistant isolates with significantly high MIC as sub-inhibitory concentration of metronidazole measured in the colon (0.8 to 24.2 µg/g) might play a role in selecting and sustaining colonies with a gradual increase in MICs (81). However, similar MIC levels in vancomycin-resistant isolate was unanticipated as the fecal concentration of vancomycin is high, range from 520 to 2200 µg/ml (82, 89). The observed high MIC could also be because of horizontal gene transfer from other Gram-positive bacteria possessing vancomycin resistance genes. The *C. difficile* genome (11%) contains mobile genetic element that includes antibiotics resistant genes. These could be transferred between different *C. difficile* strains or between *C. difficile* and other bacteria (30,90). High level of resistance in vancomycin-resistant *Staphylococcus aureus* was found to be a result of horizontal gene transfer from coinfecting vancomycin-resistant *Enterococcus* (91).

The report of this study calls for attention to the significant percent of Texas and Kenyan patients (95%) who may not respond to metronidazole treatment and 80% and 41% of
Texas and Kenyan respectively, who may equally not respond to vancomycin treatment (Fig. 8). These are the percent of *C. difficile* isolates from patient stool samples with MICs higher than four times the currently prescribed dosage of metronidazole and vancomycin which is the maximum concentration of antibiotics that will effectively clear the bacteria from the system (93). The highest dosage of metronidazole currently prescribed is 500 mg (91, 92) and 125 mg for vancomycin (92). Use of metronidazole for long-term chronic therapy or beyond the first recurrence of CDI is not recommended because of its potential cumulative neurotoxicity (54). Nephrotoxicity is also a potential serious side effect associated with high and prolong vancomycin therapy (92). As a result, an increase in dosage or prolonged therapy with either of the antibiotics is greatly discouraged.

No correlation between toxin production and the levels of resistance was observed for the 20 isolates with different MICs selected from Texas and Kenya suggesting that different pathways control toxin production and antibiotics resistance. However, no measurable toxin production was recorded in the isolates that had grown poorly as it has been shown that toxin production in *C. difficile* is quorum sensing regulated (12) and so these experiments will be repeated with high level of growth for all the isolates.

In this study, the effect of sub-inhibitory concentrations of vancomycin on biofilm formation showed no significant effect on the isolates with high resistance, intermediate resistance, and low resistance. On the contrary, a similar study that tested the effect of sub-inhibitory concentrations of metronidazole (not vancomycin) on biofilm formation in *C. difficile* strains (79) revealed a significant increase in biofilm formation when treated with metronidazole in the strains with reduced antibiotic susceptibility and
susceptible strain but their stable-resistant strain had no change in biofilm formation when the antibiotic was in the medium. Our experiment was performed using crystal violet assay which has limitations and this result could be improved with a more comprehensive assay.

The recognized genes for vancomycin resistance include \textit{vanA}, \textit{B}, and \textit{D} that are responsible for the biosynthesis D-alanine-D-lactate instead of the normal terminating precursor D-alanine-D-alanine. Vancomycin-resistant \textit{E. faecalis} and \textit{E. faecium} that possess \textit{vanA} have been associated with inducible high level of resistance to vancomycin (MICs, \geq 64 \mu g/ml). Initially \textit{vanB} was believed to induce modest levels of vancomycin resistance (MICs, 32 to 64 \mu g/ml), however, recent reports revealed that \textit{vanB} gene may afford a maximum resistant up to \geq 1000 \mu g/ml (122). \textit{vanC}, \textit{E}, \textit{G}, and \textit{L} are responsible for D-alanine-D-serine biosynthesis instead of normal D-ala-D-ala-terminating precursors and are associated with low-level vancomycin resistance. This characterized low-level resistance to vancomycin have been found and well-studied in \textit{Staphylococcus aureus} and \textit{Enterococcus} spp (30, 94). To date, no functional vancomycin resistance genes have been identified in \textit{C. difficile} (30). Since one of these listed nine genes is always expressed (30) in vancomycin resistant in other Gram-positive bacteria, we decided to perform a PCR analysis using PCR primers for the nine genes to explore potential amplification of any of the genes in our isolates. Interesting, the \textit{vanA} primer successfully amplified the expected 800bp fragment in all the resistant isolates tested. The \textit{vanA} gene has been previously shown to mediate high level of resistance to vancomycin and it is possible that the observed high level of resistance in our isolates is related to the \textit{vanA} gene.
The *vanA* gene encodes the enzyme that synthesizes D-alanine-D-lactate and functions in conjunction with three other enzymes *Van H*, *X* and *Y*. The mechanism is well studied and defined in both *Staphylococcus aureus* and *Enterococcus* spp (30). To begin our studies of the vancomycin resistance mechanism in *C. difficile*, we will first attempt to identify the *vanH* *X* and *Y* genes in the genome of our vancomycin resistant isolates either by PCR or whole genome sequencing. The mechanism of vancomycin resistance may be different in the different *C. difficile* strains as a previous study has shown the diversity in the genome of different strains of *C. difficile*. For instance, the hypervirulent strain (R20291) that encode both accessory gene regulator (*agr1* and *agr2*) and the non-hypervirulent strain (630) encodes only *agr1* (107).

Multiple studies have investigated the diversity in the genome of *C. difficile*, however, this is the first time the *vanA* gene is identified in vancomycin-resistant *C. difficile* isolates. The PCR products amplified using the primers were directly sequenced and compared with *vanA* gene of *E. faecieum*, the observed differences in the 5’ end of the sequence alignment strongly suggest poor amplification of some of the PCR products resulting in poor sequence that impeded the data evaluation. As a result, the PCR products were cloned into a vector, transformed and the clones were sequenced. They gave better result (13).

In the future, the potential relationship between the degree of resistance and the colony appearance (sizes or shape) will be explored. Each plate containing stool samples with viable *C. difficile* colonies was analyzed and the different sizes and shapes of colonies recorded. Some of the colonies possessed irregular, Webb-like shapes or smooth edges. The study of colony morphology is important, as other studies have shown that
the surface layer (S-layer), is responsible for bacteria adhesion to host cells and that a mutation in one of the cysteine proteases involved in assembly of S-layer, resulted in the inability to retain a specific cell wall protein leading to altered cell morphology (86). Another study that compared an assay for selective and differential C. difficile isolation from feces showed that colony size enables possible identification within 24 hours (87).

In summary, the data reported here, established the prevalence of metronidazole- and-vancomycin resistance C. difficile isolates from Texas and Kenyan CDI patients, determined the level of resistance of each of the isolates, reported a high vancomycin MIC greater than the currently reported and suggest for the first time the mechanism responsible for vancomycin resistance.

**Summary and Perspective**

The goal of the thesis was to establish the prevalence of metronidazole- and vancomycin-resistance in C. difficile in two different populations and also to uncover the potential resistance mechanisms involved. We isolated and characterized metronidazole- and- vancomycin resistant isolates from stool samples from both Texas and Kenya and analyzed their prevalence according to the location and the tendency of acquiring each resistance with respect to location. C. difficile resistance to both antibiotics is prevalent in both Texas and Kenya. Interestingly higher resistance to both antibiotics is prevalent in Kenya.

Analysis of the degree of resistance of the isolates revealed relatively high MICs in the two the populations. The two methods used for MIC determination (the E-test strip and broth microdilution methods), were compared. The E-test method had an agreement of
>90% in comparison to broth microdilution method. There is a significantly high level of metronidazole-resistance in both populations when compared to vancomycin. The degree of vancomycin resistance is more spread across all levels of resistance although a high proportion had a high degree of resistance with >256 µg/ml. On the other hand, a relatively significant percent of metronidazole resistance isolates is highly resistant.

Three distinct groups were identified after MIC determination, the low resistance, intermediate resistance, and high resistance. These three groups will be used for further analysis. In the future, whole genome sequence analysis will allow a comparison between the three groups and analysis of gene expression can also be used. This analysis will help to uncover the resistance mechanism and possibly be useful in the identification of a small molecule inhibitor as an alternative therapy.

Colonies were classified in order to explore potential relationship between the degrees of resistance or etiology to the sizes of the different colonies. Viable colonies had different sizes with some possessing irregular edges, Webb-like shapes and smooth round edges. Further studies will explore these phenotypes. The study of colony morphology is important, as other studies have shown that the surface layer (S-layer), is responsible for bacteria adhesion to host cells and that a mutation in one of the cysteine proteases involved in assembly of S-layer, resulted in the inability to retain a specific cell wall protein leading to altered cell morphology (113). Another study that compared an assay for selective and differential *C. difficile* isolation from feces showed that size of colony enables possible identification within 24 hours (114).

Further analysis is required to examine the spore formation ability of the different colonies, toxin production and explore their relationship with antibiotic resistance.
Our hypothesis that sub-inhibitory concentration of vancomycin will induce more biofilm formation in the reduced susceptible and intermediate isolates, but not high resistant was proven false, although as expected, the high resistant isolates that may not need biofilm as a defense mechanism in the presence of vancomycin had minimal biofilm formation. Further analysis is recommended in the biofilm analysis with a more accurate assay or technique.

**Future Experiments**

1. A study to isolate *Enterococci* species from the same stool samples containing *C. difficile* isolates with high vancomycin-resistance in order to analyze and compare the *vanA* gene is recommended.

2. VanA-type resistance is mediated by transposon Tn1546 (20). A whole genome sequence analysis is recommended to determine if the same transposon also mediates the *vanA* gene in *C. difficile* and to determine whether the resistance is due to a genomic mutation or plasmid-encoded.

3. A detailed study of the pathway that mediates *vanA* resistance is suggested to develop a novel target, such as small nonantibiotics molecules that will address the high level of resistance.
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