8-2020

Mechanistic Insights into Vancomycin Resistance in Clostridioides difficile

Ayesha Mahmood

MECHANISTIC INSIGHTS INTO VANCOMYCIN RESISTANCE

IN *CLOSTRIDIOIDES DIFFICILE*

by

AYESHA MAHMOOD

APPROVED:

_____

Charles Darkoh, Ph.D.

_____

Cesar Arias, M.D, Ph.D.

_____

Anne-Marie Krachler, Ph.D.

_____

Steven Norris, Ph.D.

_____

Shane Cunha, Ph.D.

APPROVED:

_____

Dean, The University of Texas MD Anderson Cancer Center UT Health Graduate School of Biomedical Sciences

MECHANISTIC INSIGHTS INTO VANCOMYCIN RESISTANCE IN *CLOSTRIDIOIDES DIFFICILE*

A

THESIS

Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center

UTHealth Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

by

Ayesha Mahmood, B.S.

Houston, Texas

August 2020

DEDICATION

I dedicate this work to the only person I love more than microbiology. You have been with me on this journey from the moment I knew I wanted to be a scientist. You reminded me of my love for science even when I had doubts and was ready to give up. You never let me lose sight of my dream and have been supporting and encouraging me through every rejection, missed opportunity, and adversity. Not to mention, you have been my number one fan for even the most inconsequential victories. You've sacrificed so much to help me achieve my dream, and I will forever be grateful to you for that.

ACKNOWLEDGEMENTS

This would not have been possible without the support and encouragement of my family and friends, so thank you from the bottom of my heart.

Thank you to all the members of the Darkoh lab for their support in all matters. Thank you, Dayna for your friendship. Thank you, Kim for always taking the time to answer all of my statistical analysis questions and for sharing a curiosity about science that I admire. Thank you, Kadi for help with all things sequencing and your patience with me as I pestered you with a million questions. A special thank you for your kindness and support as I maneuvered through this program. Your advice and positive attitude did so much to help me get through this, and I want you to know how much I appreciate that. To Dr. Darkoh, thank you for welcoming me into your lab. I've learned a lot from this experience and hope that you were able to gain something valuable as well.

To my committee members, thank you for sharing your expertise with me and taking the time to be a part of my research journey. Your attentiveness and participation in my project have elevated it.

I am grateful for all that I have learned from my time at GSBS and from my professors and peers in the MID program. I am especially thankful for Drs. Moore and Swindell. I always felt like your number one priority even though I'm sure you had a million other things to worry about. Without your guidance and advice, I don't know if I would have made it through this process with my sanity. GSBS students are lucky to have individuals so devoted to their students' success.

MECHANISTIC INSIGHTS INTO VANCOMYCIN NON-SUSCEPTIBILITY IN *CLOSTRIDIOIDES DIFFICILE*

Ayesha Mahmood, B. S.

Advisor: Charles Darkoh, Ph.D.

C. difficile is a nosocomial, opportunistic pathogen that has become more hypervirulent over the last decade. Vancomycin is currently a frontline antibiotic for the treatment of *C. difficile* infections. Yet, strains of *C. difficile* are becoming non-susceptible to vancomycin. Emergence of the vancomycin non-susceptible phenotype has led to various questions including: What resistance elements are present that might contribute to the phenotype? Are these elements located on the chromosome or a plasmid? Where did these elements originate from? To address these questions, whole genome sequence analysis was performed to survey genes that may be involved in the vancomycin non-susceptible phenotype. Bioinformatic analysis was used to answer whether putative vancomycin-resistance genes were chromosomal or plasmid based. Culture-dependent methods were used to detect enterococci from stools of patients from Texas and Kenya infected with *C. difficile* and this was supplemented with patient data to describe co-colonization in the gut and its effects on patients.

The sequencing analysis revealed the presence of a putative *van* gene cluster in all isolates tested. The presence of vancomycin-resistance elements varied among

the isolates and included: *vanRS, vanW, vanH,* and *vanZ*. *In silico* analysis of sequences suggested that the putative vancomycin-resistance genes were chromosomal. A high prevalence of enterococci and vancomycin-resistant enterococci (VRE) was observed in the stools of *C. difficile* patients from both Kenya and Texas. In addition, the presence of enterococci increased the duration of CDI symptoms and this might also promote selection of non-spore forming *C. difficile* isolates. Finally, high proportions of VRE and non-susceptible *C. difficile* isolates were found in the same stools, suggesting a potential source of resistance elements.

Although rigorous functional analysis is needed to confirm the specific genes responsible for vancomycin non-susceptibility in *C. difficile*, the presence of vancomycin-resistance genes underlines a promising avenue for further experimentation. Knowing that the genes are likely chromosomal will help in downstream characterization of these genes. Given the correlation between vancomycin-resistant enterococci and the vancomycin non-susceptibility phenotype in *C. difficile,* and the shared epidemiology between the two bacteria, it is plausible that these resistance elements may have originated from *Enterococci* spp, however, further genetic analysis is needed to confirm this observation. Overall, this study has shed some light on the mechanism of vancomycin non-susceptibility in *C. difficile* and has highlighted avenues for further exploration. Moreover, the relationship between *Enterococci* spp. and *C. difficile* during infection has become clearer and might enhance future treatment strategies.

Table of Contents

List of Tables

List of Figures

1. Introduction

1.1. *C. difficile* characteristics and epidemiology

C. difficile was first isolated and described from the stools of infants in 1935 (1). These novel bacteria were characterized as obligate anaerobic, “heavy-bodied rods” containing spores (1). Additionally, it was determined that bacterial cultures and toxigenic supernatant could cause severe disease in guinea pigs, indicating pathogenicity of the newfound bacterium (1). Based on the physical properties and difficult cultivation processes, this unknown pathogen was named *Bacillus difficilis.* Now, this Gram-positive bacterium has been reclassified as *Clostridioides (Clostridium) difficile* (2). Over time, *C. difficile* infection (CDI) became associated with gastrointestinal disease initiated from antibiotic use (3, 4), and today, it is the number one cause of antibiotic associated diarrhea and an important nosocomial pathogen (5-7). *C. difficile* can be an asymptomatic colonizer in about 0-15% of healthy adults (8). In neonates, the prevalence is more varied but can be substantial (18-90%) (7-15).

One of the biggest risk-factors for the development of CDI is broad-spectrum antibiotic use, as this diminishes colonization resistance by gut commensals and provides an ideal environment for *C. difficile* overgrowth (16-18). Moreover, the use of proton-pump inhibitors can contribute to CDI development (19). Immunocompromised patients, individuals with underlying conditions, or individuals of advanced age may also be more susceptible to CDI (16, 20).

Over the past 20 years, the emergence of hypervirulent strains have made the treatment and control of CDI even more difficult (3). Hypervirulent strains sporulate earlier, thus aiding in recurrence of disease since the spores are not affected by

antibiotics (3, 21). This is complicated further with antibiotic resistance seen in hypervirulent strains. Clinical *C. difficile* ribotype (RT) 027 has shown resistance to erythromycin, fluoroquinolones, and ciprofloxacin, in addition to second-generation cephalosporins, and to a lesser extent, third-generation cephalosporins (3). Also, multiple β-lactamases can be encoded in *C. difficile*, which contributes to β-lactamase resistance (3). Genes associated with conferring macrolide resistance have also been found in *C. difficile* (3). RT 027 was first described in Canada, but has quickly become the dominant strain worldwide and is endemic to the U.S. (3). Another hypervirulent strain, RT 078, has exhibited similar levels of infection severity throughout Europe (3), while RT 001, RT 017, and RT 018 have shown clindamycin resistance (19).

In addition to emergence of hypervirulent strains, the changing epidemiology of CDI has expanded to include groups not classically regarded as at-risk for *C. difficile* infection including children, pregnant woman, and individuals with no previous healthcare contact (5, 7, 22). The treatment costs of CDI in the United states is estimated to be $1.3-7 billion annually (7, 23-25). The growing severity and burden of this pathogen necessitates further examination of its pathogenesis and epidemiology.

1.2. *C. difficile* pathogenesis

Disruption of the commensal gut microbiota is a key determinant in *C. difficile* colonization. This commonly occurs through the use of broad-spectrum antibiotics or proton pump inhibitors (16-19). Commensal gut microorganisms metabolize short chain fatty acids and free sialic acid in the gut, however, when these bacteria are depleted, *C. difficile* is able to utilize the abundant metabolites and grow unchecked

(26). Furthermore, bacteriocin production by some commensals can limit *C. difficile* overgrowth (26-28). The lack of interactions with competing bacteria and abundance of growth resources, space, and nutrients, lay the groundwork for CDI development (26).

The pathogenesis of *C. difficile,* including genetic variation among isolates, and host immune responses lead to a spectrum of disease states (18). Presentation includes asymptomatic carriage, diarrhea, pseudomembranous colitis, and toxin megacolon. In some cases, these symptoms culminate in death (18). As an obligate anaerobe, *C. difficile* produces spores to survive in normally toxic aerobic environment. These spores are transferred between surfaces via other individuals and can eventually be ingested. The spore coat allows for *C. difficile* survival in the acidic gastric environment, and upon entry into the small intestines, bile acids induce transformation of spores into vegetative cells. When combined with reduced colonization resistance in the gut from broad-spectrum antibiotic use, these vegetative cells can proliferate and begin their virulence process. Once *C. difficile* bacteria have reached a critical density, they start producing their major virulence factors, toxins A and B. These toxins, encoded by TcdA and TcdB, respectively (18, 26) contain Rho and Rac glucosyl transferase domains (GTDs) that can glycosylate and inactivate Rho and Rac GTPases of colonic epithelial cells (26, 29). This leads to cytoskeleton deformation (actin polymerization) and cell rounding in target cells (20, 29). Moreover, the cytoskeletal restructure results in the degeneration of tight junctions between cells. Loss of epithelial barrier function leads to increased leakiness in the gut which results in diarrheal symptoms in the affected individual. Local *C. difficile* toxin production

promotes recruitment of immune factors such as tumor necrosis factor-α (TNF-α), interferon- γ (IFN-γ), and proinflammatory interleukins (ILs) (26, 30). This, in turn, exacerbates vascular permeability and recruitment of monocytes and neutrophils (30, 31). Local production of hydrolytic enzymes by immune cells can induce connective tissue degradation and subsequent colitis in the patient (26). Accumulation of immune cells can also lead to pseudomembrane formation, another hallmark symptom of CDI (30). In certain cases, extracolonic manifestations can appear in the form of bacteremia, small bowel infection, and reactive arthritis (17, 32, 33).

In the endogenous form of CDI, the individual is already colonized with *C. difficile,* which can overgrow with loss of colonization resistance. Although treatment with oral antibiotics has been the standard of care, it does not promote recolonization of normal gut flora, potentiating recurrent cases of CDI in about 15-30% of adult patients (34).

1.3. *C. difficile* treatment

CDI treatment is focused around bacterial clearance and promotion of normal flora to prevent recurrence. Classification of CDI severity (i.e. first infection, recurrent infection, severe, etc.) also aids in intervention determination (3, 35). Previously, the first line of drug used for treatment was metronidazole (36-40), a nitroimidazole antibiotic that deforms and destabilizes the helical DNA structure, preventing protein synthesis, and eventually leads to bacterial death (41). It was previously thought that metronidazole treatment would limit the use of vancomycin and prevent downstream overgrowth of vancomycin-resistant *enterococci* (VRE) (36). However, recent studies have shown that metronidazole use can be associated with VRE and that VRE colonization seem to be at similar levels with either oral metronidazole or oral

vancomycin treatment (36, 42). Due to neurotoxicity from prolonged use, metronidazole is not ideal for long-term or recurrent cases of *C. difficile* (41). In addition, there have been increasing reports of its ineffectiveness in CDI cases (43-45). Consequently, the Infectious Diseases Society of America (IDSA) and the Society for Healthcare Epidemiology of America (SEHA) recently recommended vancomycin, followed by fidaxomicin, as the drug of choice (36, 37, 46, 47).

Other first-line antibiotics are available for use but remain underutilized. Rifaximin can be applied for a first-time infection, as well as teicoplanin, however *C. difficile* isolates have shown resistance to rifaximin in about 30% of samples (48) while the latter is not approved for use in the U.S. (3, 7). More recently, one study by Alberto, et al. did not show any statistically significant differences in infection rates between a cohort of patients with advanced liver disease that were either treated or not treated with rifaximin (49).

The glycopeptide vancomycin has been used for the treatment of severe and recurrent cases of CDI effectively (37), but now it has become the major antibiotic of choice for treatment (50, 51).

Fidaxomicin is a relatively newer antibiotic and has been shown to be effective for CDI treatment by inhibiting RNA synthesis, spore formation, and toxin production (33, 52-55). Unfortunately, it is quite costly for use as a first-line antibiotic, and combined with limited data on outcomes, prevents its widespread use (18, 56). Given its ability to reduce recurrence rates, it is a promising drug for CDI treatment, but requires a reduction of cost and more patient studies to be widely accepted.

Fecal microbial transplants (FMTs) have also been a widely debated treatment option for CDI. Many studies have looked at the efficacy of FMTs, but their use has only been approved for recurrent cases of CDI (26). Although FMTs have shown success in up to 89% of patients (57), FMT usage has been met with hesitance due to perceived downstream side effects, such as reports of obesity and long-term health concerns (58).

Spore-formulation is a method similar to FMT, but is more selective of which microbial species are introduced into the infected individual. Spores of competitive bacterial strains can be introduced into the gut microenvironment, which has been successful in a few pilot studies (59, 60). In one case, spores of nontoxigenic *C. difficile* strains were introduced into patients with recurrent CDI and showed a decrease in recurrence when compared to the control group (59). However, the treatment group still exhibited a large percentage of adverse effects linked to treatment (59). In another example, firmicute spores from healthy donors were used as competitive remodeling of the gut microbiota, and the results showed a reduction in recurrence, however, the study itself had some limitations that questioned the validity of the study (60). This relatively new avenue of therapeutic intervention is promising but requires more studies to determine the efficacy of such a treatment.

An over the counter (OTC) treatment option to alleviate symptoms is the use of probiotics. Probiotics can be defined as microbes consumed for the purpose of conferring some sort of health benefit (61). Probiotics have three major modes of conferring advantage to the host including (1) immunomodulation, (2) providing colonization resistance by preventing other, potentially pathogenic bacteria from

adhering to intestinal epithelia and invading, and (3) interacting with harmful bacterial effectors, such as *Saccharomyces boulardii* providing protection against *C. difficile* toxin A (3, 61). Probiotics can be consumed through foods containing the beneficial microbes or through manufactured, lyophilized pills of the microbes (61). Probiotics have been shown to prevent recurrence in CDI patients (62, 63). Generally regarded as safe, there is an underlying concern of transfer of antibiotic resistance from the probiotic bacteria to resident gut bacteria. Concurrent probiotic and antibiotic use can promote a reservoir of antibiotic-resistance determinants (64, 65).

Bacteriocins are anti-microbial proteinaceous molecules produced by bacteria to target other bacteria. Three bacteriocins show the most activity against *C. difficile.* Lacticin 3147 is a two-component antibiotic produced by *Lactobacillus lactis* (66). Not only does Lacticin 3147 provides *C. difficile* growth reduction in various *in vitro* models (67). Thuricin CD, produced by *Bacillus thuringiensis,* has been shown to be potent against *C. difficile* (27, 68). Finally, Nisin is a polypeptide bacteriocin produced by *Lactobacillus lactis.* It has been shown to be as effective as vancomycin and inhibits growth of *C. difficile* post spore germination (69). These bacteriocins, and many others, may be effective against treatment of CDI. While Nisin is generally recognized as safe, and can be used as food additive, lacticin and Thuricin CD do not survive gastric transit and must rely on administration via enema, which is not always the easiest treatment route for CDI patients (3).

Other non-antibiotic treatments are also being actively explored, including small molecule inhibitors for TcdB receptors (70). Also, bis-cyclic guanidine compounds that mimic host defense proteins seem promising for CDI treatment (71).

Monoclonal antibodies against toxins A and B have been attempted in patients at high-risk for recurrence and did show a lower rate of recurrence. However, when compared to vancomycin, antibody treatment showed a different microbial succession profile which could have unknown consequences (32, 72).

There are a variety of treatments available for CDI, but pitfalls exist with each, especially with the relative novelty of some therapeutics and unknown consequences. Vancomycin is currently the most widely used drug for the treatment of CDI.

1.4. Gram-positive cell wall biogenesis

In Gram-positive bacteria, the process of cell wall biogenesis begins with D-alanine, formed by conversion of L-alanine via a racemization. Two molecules of D-alanine are joined together by a Ddl ligase in the cytosol (73). A uracil dipeptide N-acetylmuramyl-tripeptide (NAM), consisting of L-Ala, D-Glu, and L-Lys, is ligated to the D-Ala-D-ala forming a pentapeptide that is linked to an undecaprenol lipid carrier. Once this molecule is attached to N-acetyl glucosamine (NAG), the lipid carrier flips across the cell membrane (73). Transglycosylation activity by penicillin binding proteins (PBPs) aids in elongating nascent peptidoglycan while transpeptidation activity catalyzes the formation of a glycosidic cross-links between peptide chains (74) (Figure 1).

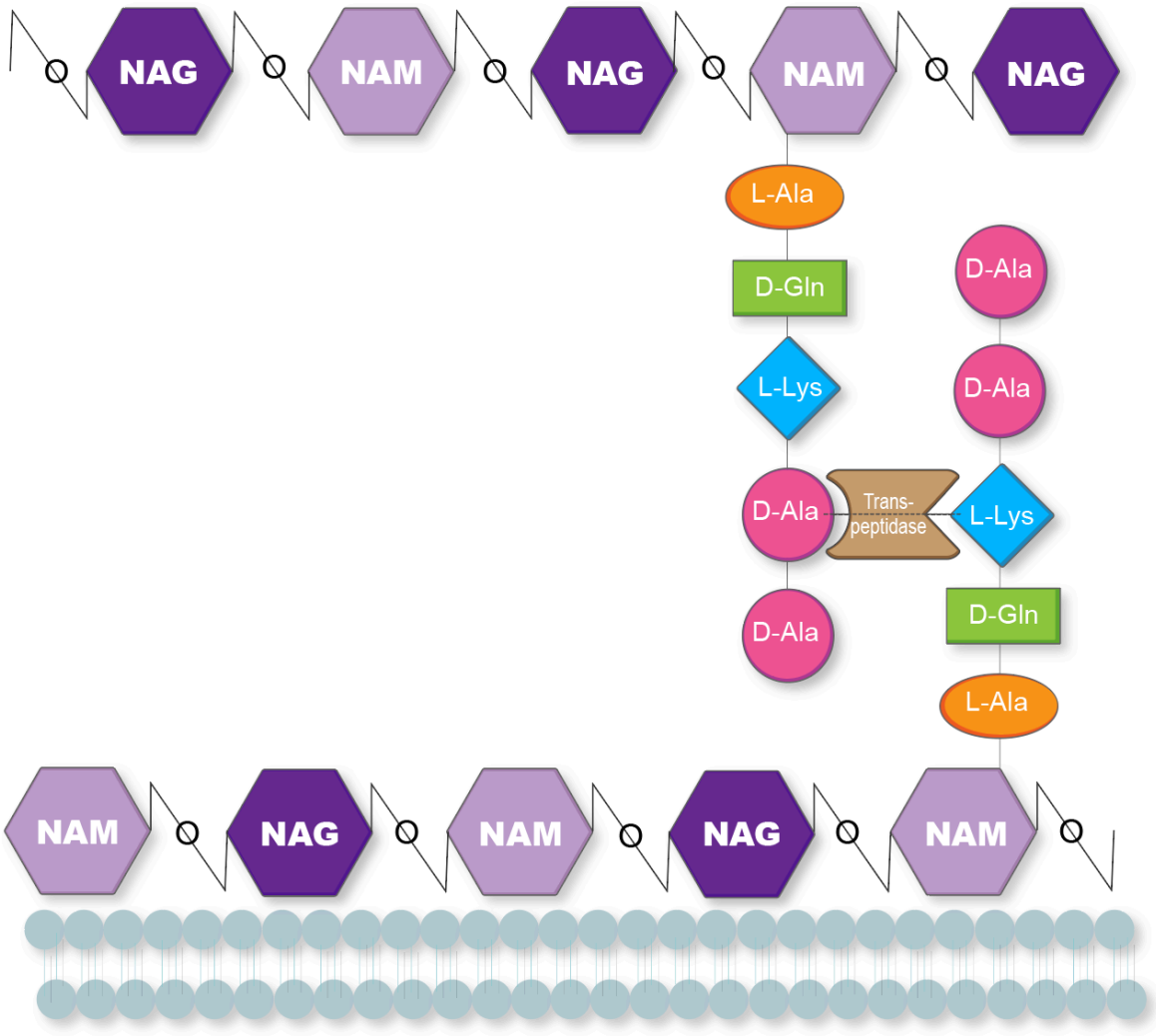

Figure 1: Gram-positive peptidoglycan structure is characterized by a N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) backbone and crosslinked pentapeptide chains (73, 74). The alternating NAG and NAM subunits are shown in purple hexagons. Each NAM subunit is attached to a pentapeptide chain composed of L-Ala, D-Gln, L-Lys, D-ala, and D-ala. A transpeptidase (brown) creates a covalent bond between pentapeptide chains.

1.5. Vancomycin mode of action

Vancomycin works by preventing polymerization of the peptidoglycan cell wall. During bacterial cell wall biogenesis, vancomycin binds to the terminal D-alanyl-D-alanine moiety, sterically hindering penicillin binding proteins (PBPs) from enzymatic cross-linking of peptidoglycan precursors. This creates disruption of the cell wall and eventual bacterial cell lysis (75, 76) (Figure 2).

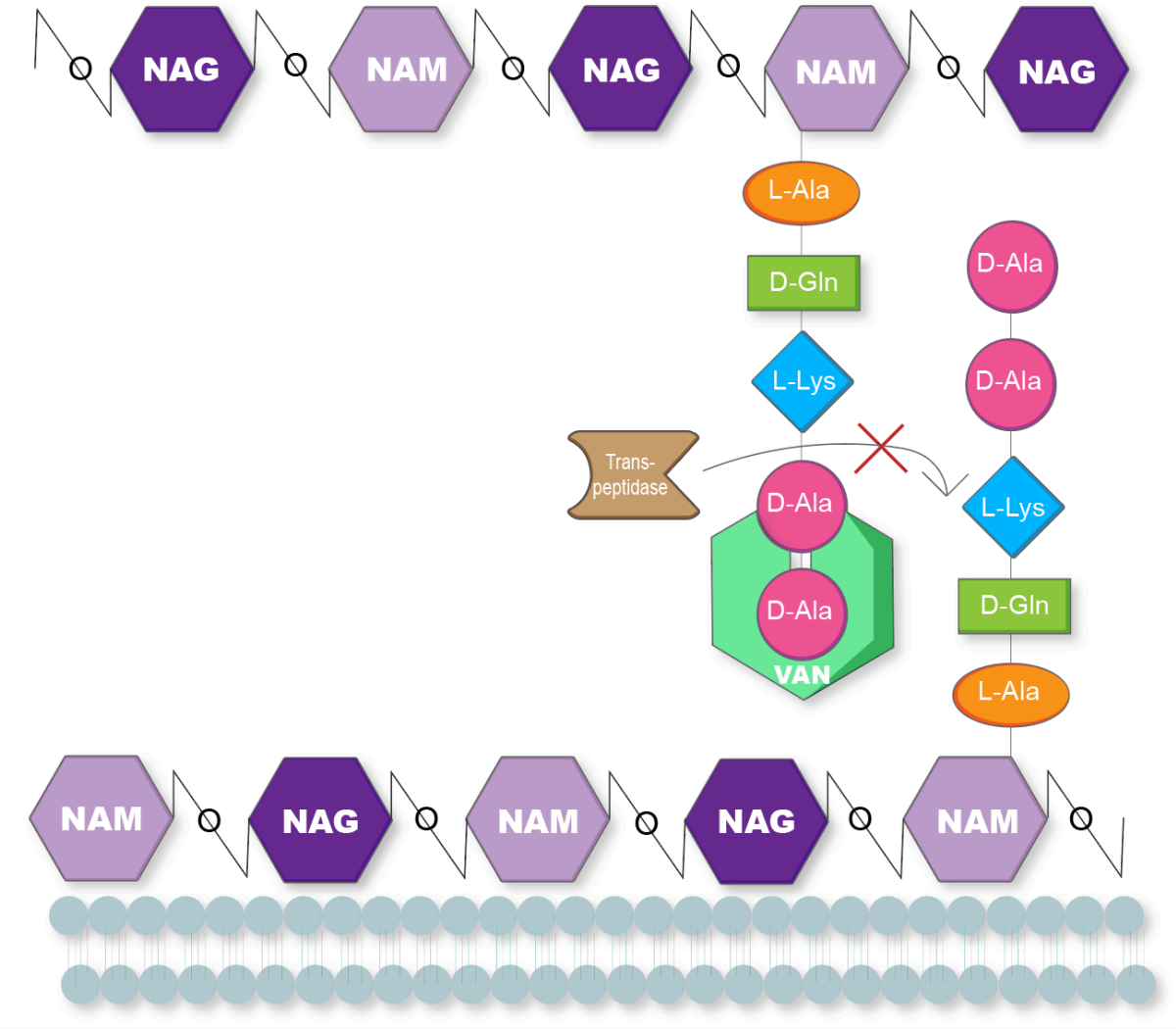

Figure 2: Vancomycin prevents peptidoglycan elongation (76, 77). Vancomycin binds to the terminal D-Ala-D-ala peptides, sterically hindering penicillin binding proteins (PBPs) from performing transpeptidation activities. The peptidoglycan structure is destabilized, and the bacterium is killed.

1.6. Vancomycin resistance

As described earlier, vancomycin works by binding to the terminal D-alanyl-D-alanine amino acids of the pentapeptide chain. To combat this, some bacteria can convert their terminal residues to alternative precursors, including D-alanine-D-lactate and D-alanine-D-serine (Figure 3). Most commonly, high level vancomycin-resistance phenotypes have terminal endings of D-ala-D-lactate, which lowers the binding affinity of vancomycin up to 1000-fold (78).

Vancomycin resistance in certain species of bacteria (e.g. enterococcus) is encoded on a *van* operon (78), although alternatives exist. For example, Gram-negative bacteria are intrinsically resistant to glycopeptides given the impenetrable outer membrane, non-conductive to the passage of glycopeptides (79). There are various phenotypes associated with glycopeptide resistance and are differentiated based on resistance characteristics. The *van* operon usually contains a regulatory region carrying the *vanR* and *vanS* genes. *VanRS* is a two-component sensor-response regulator system that functions by detecting disruptions in the cell wall and/or by sensing vancomycin (78, 80). The essential components of some operons include *vanH, vanT*, a van ligase, and *vanX*. *VanH* encodes a D-hydroxyacid dehydrogenase responsible for reducing free pyruvate in the cell into D-lactate; this gene is found with bacteria producing D-ala-D-lactate precursors (73). *VanT* is usually found in organisms producing D-ala-D-serine precursors and encodes a serine racemase (81).

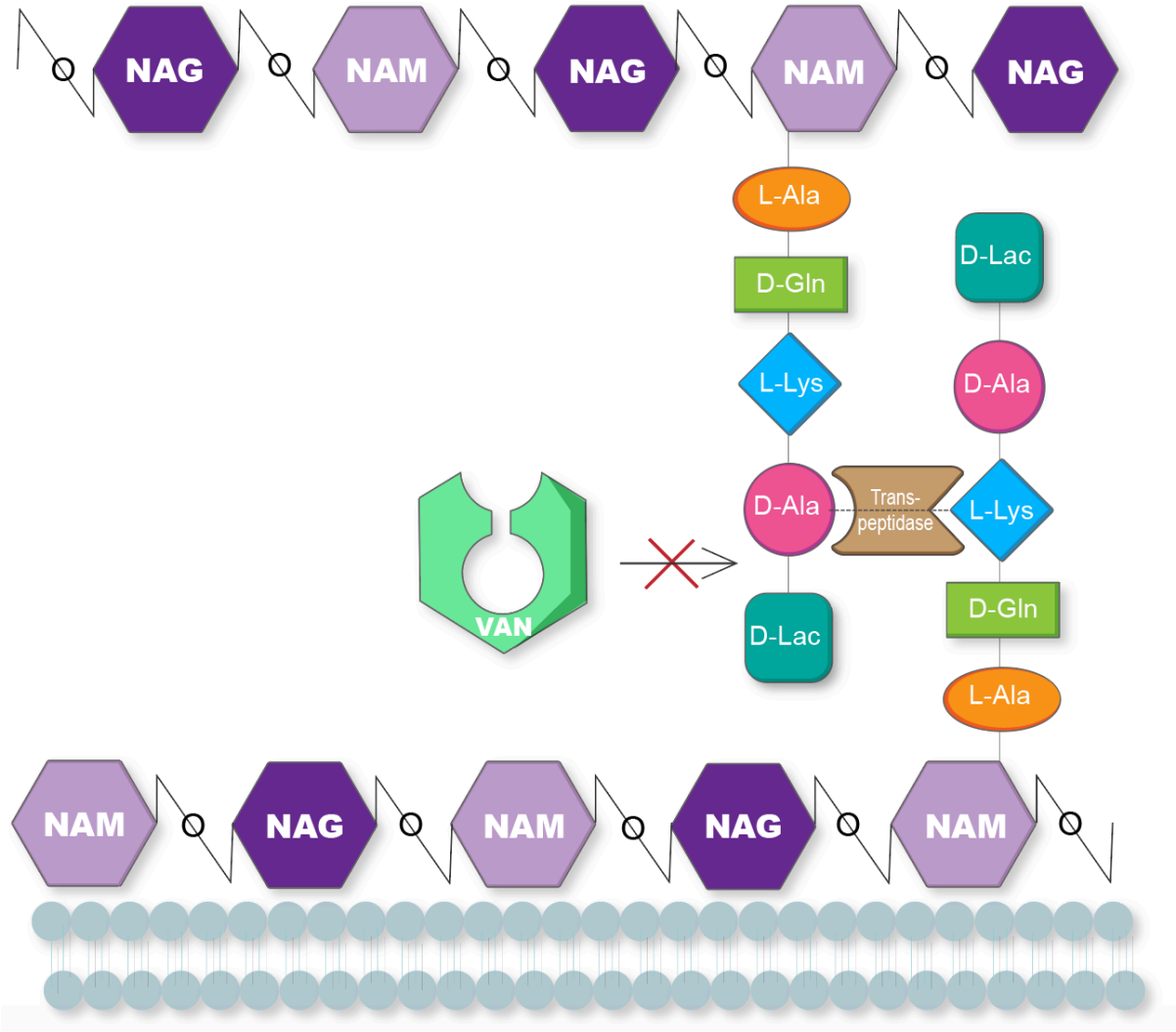

Figure 3: Bacteria may respond to vancomycin by altering terminal peptides (77). Some bacteria may replace the terminal residues of the pentapeptide chain to either a D-alanine-D-lactate or D-alanine-D-serine (not shown) to reduce vancomycin binding affinity, resulting in a resistance phenotype that allows normal transglycosylation and transpeptidation activities.

In *vanA*-type resistance, the *van* ligases form an ester bond between D-D-alanine and D-lactate (75). *vanX* is a D,D-dipeptidase responsible for increasing free D-alanine in the cell by cleaving the terminal peptide repeats (82). The accessory component of the operon includes *vanY*, a D,D carboxypeptidase that increases D-alanine amounts by cleaving them directly from the cell membrane, and *vanZ*, which is only found in *vanA* carrying bacteria and confers resistance to teicoplanin (83).

Besides *Enterococcus* spp.*,* there has been an isolate of *Bacillus circulans* (VR0709) that contains a *vanA*-like, chromosomally encoded gene cluster (84). Although all genes in the *vanA* operon were present, genes for a transposase and resolvase similar to some *Enterococcus* spp. were absent, and instead were replaced by an open reading frame with a low percentage (28%) homology to the *vanA* operon carried on *Enterococcal* Tn*1546*, yielding in a 9.2 kb fragment (84). Overall this study showed that vancomycin resistance in this case did not arise from an *Enterococcus* species member (84).

Some isolates of *Staphylococcus aureus* have also demonstrated vancomycin resistance. Due to an increase of methicillin-resistant *S. aureus* (MRSA), the antibiotic of choice for treatment has been vancomycin (85). Initially, decreased susceptibility to vancomycin was described in a MRSA isolate from Japan (86). Given the low levels of vancomycin resistance (MIC = 3-8 μg/ml), this, and other isolates with similar MIC phenotypes were called vancomycin intermediate *Staphylococcus aureus* (VISA) (85). It was shown that these isolates did not gain resistance from an extrachromosomal element, but rather from mutations in two-component systems regulating transcription of cell wall components that arose during prolonged vancomycin treatments (85).

C. difficile has largely been regarded as susceptible to vancomycin, but some studies have indicated the presence of non-susceptible isolates (87). An initial study with a cohort of samples between 1983 and 2004 showed that about 6 out of 110 samples showed non-susceptibilities between 2-4 µg/ml vancomycin (88). One study showed that 3 out of 38 samples were non-susceptible to vancomycin in Poland (89). Samples taken between 1993-2001 in Spain showed that 10% of samples were non-susceptible up to 16 µg/ml vancomycin (90). In a 2013 study, Goudarzi et al. showed that 8% of their isolates (6/75) were not susceptible to vancomycin up to 4 µg/ml (91). An Israeli study from 2014 indicated that about 8% of isolates were non-susceptible (92). Of these isolates, about 2% were of strain 027, which had the most instances of non-susceptibilities, whereas strain cr-02 had the highest degree of non-susceptibilities (up to 6 µg/ml vancomycin) (92). A more recent European study showed that about 3% (n=918) of samples were non-susceptible up to 8 µg/ml with notable ribotypes including 014, 027, 078, 126, 001/072 (93). In another US Study from 2011, about 18% of isolates (n=925) were considered resistant according to EUCAST guidelines up to 4 µg/ml (94).

1.6.1. Vancomycin-resistant phenotypes

Even though VanA is the most common vancomycin-resistance phenotype, there are many other variations that have been described in *Enterococcus* spp. (Figure 4, Table 1).

Table 1: Features of glycopeptide resistances described in *Enterococcus* spp. (95-99). Resistance types are classified based on MIC, mode of transfer, genetic determinant (encoded on the chromosome [ch] or plasmid [p]), and mode of expression, either constitutive (C) or inducible (I). ND indicates not determined.

Operon	MIC (µg/ml)	Mode of Transfer	Genetic Determinant	Mode of Expression	Refs
VanA	64-1024	Acquired	Ch or P	I	(75, 100-102)
VanB	4-1024	Acquired	Ch or P	I	(100, 103, 104)
VanC	2-32	Intrinsic	Ch	C or I	(100, 105, 106)
VanD	64-128	Acquired	Ch	C or I	(107)
VanE	8-32	Acquired	ND	C or I	(108, 109)
VanG	16-32	Acquired	ND	C or I	(110, 111)
VanL	8	Acquired	Ch	I	(112)
VanM	ND	Acquired	ND	I	(113)
VanN	16	Acquired	P	C	(114)

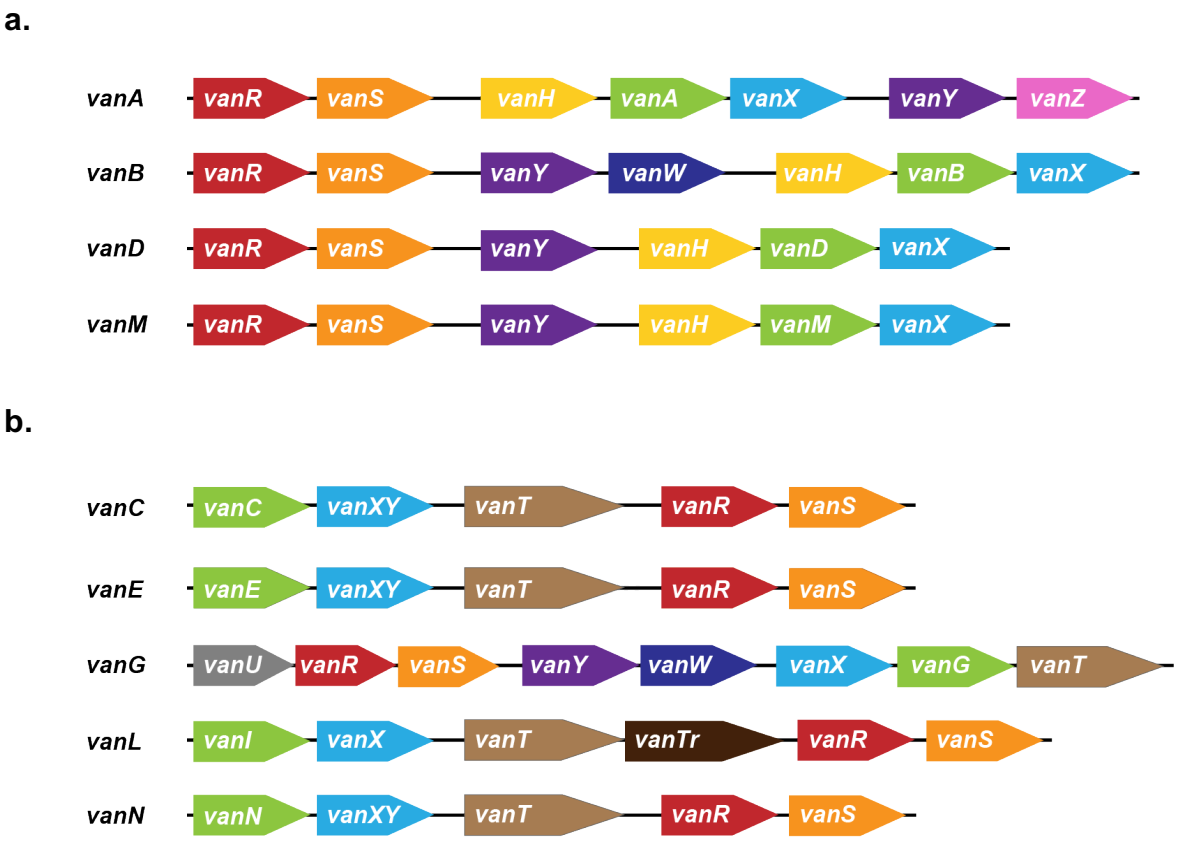

Figure 4: Arrangements of vancomycin-resistance types described in *Enterococcus* spp. (78, 97, 115). Vancomycin-resistance operons described in *Enterococcus* spp. are classified based on the presence of either a (a) D-alanine-D-lactate ligase or (b) D-alanine-D-serine ligase.

1.7. Enterococci

Enterococci species are Gram-positive, opportunistic pathogens and are classified as lactic acid bacteria that produce bacteriocins (100). They are asymptomatic colonizers of the human gut, but can be found in other locations including the oropharynx, soft tissue wounds, and the perineal region (116). Major diseases caused by these pathogens include urinary tract infections (UTIs), endocarditis, and bacteremia, many in a nosocomial fashion (116). *Enterococcus faecium* and *E. faecalis* are responsible for most of these infections. However, *E. gallinarum, E. casseliflavus, E. durans, E. avium,* and *E. raffinosis* have been reported to cause up to 5% of Enterococcal infections (83, 117, 118). Similar to CDI, enterococcal infections can be initiated by prior broad-spectrum antibiotic use, such as cephalosporins, fluoroquinolones, and macrolides (78, 119).

The usual treatment for enterococcal infections includes a combination of penicillin and ampicillin, or vancomycin in conjunction with an aminoglycoside (117). Alarmingly, multi-drug resistant strains of enterococci have been emerging since the 1980's, which has made them more difficult to treat (120-123). In human feces, enterococci may represent up to 1% of the indigenous microflora (120, 124-126). In children, one study of infants in the intensive care unit indicates a percentage of 23% of enterococcal colonization, with 57% of those being multidrug resistant (127). In a cohort of healthy adults from Laos, 73% were colonized with *Enterococci* spp (125). Of those, about 45% were found to be *E. faecalis,* and 55% were *E. faecium,* as determined by biochemical and physical characteristics (125). Further examination of the two *Enterococci* species showed a vancomycin resistance rate of 13.8% among

volunteers (125). Other estimates of VRE in the United States indicate prevalences of about 30%, with 77% caused by *E. faecium,* and 9% by *E. faecalis* (78). There are other species that can be implicated in VRE infections, including *E. gallinarum, E. caseliflavus, E. avium* and *E. raffinosus* (78). VRE prevalence can increase to 99% in hospital patients (120, 128) .

1.7.1. Vancomycin-resistant enterococci

Vancomycin resistance has been documented in *Enterococcus* since the 1980's (42). Two species are of primary importance, *E. faecalis* and *E. faecium*, as they cause severe infections, sometimes associated with multi-drug resistance. *Enterococcus* can be intrinsically resistant to β-lactam antibiotics and can acquire resistance to other antibiotic types such as glycopeptides (78). Glycopeptide resistance in enterococci is acquired via transposons or other genetic elements carrying the appropriate genes. These elements can be found as plasmids or can become integrated chromosomally (78). In one case, it was posited that two distinct *vanA* type elements could be carried by certain isolates that localize both to the chromosome and a conjugative plasmid (129).

Vancomycin resistance can be encoded in multiple ways. *E. faecalis* (V583) encodes resistance on mobile element EF1955-EF1963, which shares sequence homology with *vanB* type genes found on Tn*1549*, but differs significantly in the remaining portion of the element (130).

1.8. *C. difficile* and enterococci co-colonization of the colon

C. difficile and VRE are commonly found co-colonized in stools. Proliferation and subsequent disease development of both bacteria is dependent on similar risk factors, such as treatment with fluoroquinolones (78, 131). Patients who are immunocompromised, have underlying health conditions, or are of advanced age may also be more susceptible to both pathogens (132, 133). Treatment of CDI by vancomycin is also a risk factor for VRE development (119, 131). In pediatric settings (1 month to 18 years old), one study shows a VRE colonization rate of 18.6% among pediatric intensive care unit patients (134). Another study reports 63.3% VRE colonization in hospitalized children (135). Surveillance studies from 1994 to 2018 indicated a VRE-*C. difficile* co-colonization rate between 3.2- 55% in adult patients (119, 136-145). Some common characteristics associated with co-colonization include a higher percentage of VRE colonization in stools containing toxin-producing *C. difficile* isolates (119, 140). Leber et al. (2001) showed a 3.2% VRE colonization in patients with toxigenic *C. difficile*, with an odds ratio of 2.3 times that of VRE co-colonization with non-toxigenic *C. difficile* (141). Other reports are as high as 17% (140) and 21% VRE colonization in toxigenic *C. difficile* (142). One study even describes CDI patients having 55.7% colonization with VRE (145).

C. difficile and *Enterococcus* spp. have become two of the most prevalent agents of hospital-acquired infections. Given the emergence and increase of *C. difficile* hypervirulent strains and the rise in multidrug-resistant enterococci, understanding the relationship between these two opportunistic pathogens is vital.

1.9. Genetic transfer between gut microbiota

C. difficile has a highly plastic, mosaic genome, with up to 11% of its whole genome (~473 kbp) consisting of mobile genetic elements [MGE] (146-148). The *Clostridia* genus displays examples of insertion sequences, conjugative and non-conjugative transposons, phages, and plasmids that may aid in genetic transfer of resistance elements, but at the forefront remains integrative conjugative elements (146, 148). Genetic transfer of antibiotic resistance encoded by these genetic elements have been well-documented between *Clostridia* and other firmicutes (148). Transposon *5397* carries tetracycline resistance genes and was shown to be transferred from *C. difficile* to *E. faecalis* JH2-2 *in vitro* (149). Additionally, the same study described the transfer from *E. faecalis* transconjugant EF20 to a *C. difficile* isolate belonging to the 027 ribotype, which is considered endemic (149).

Enterococcus faecalis Tn*916* encodes tetracycline resistance via the *tet (M)* determinant (146), and belongs to a family of conjugative transposons that also contains the *Streptococcus pneumoniae* Tn*1545*, which encodes erythromycin resistance via *erm(AM)* (146). This family of Tn*916*-like/Tn1*545*-like transposons have been described in multiple bacteria including *C. difficile.* Tn*5397*, one of these Tn*916*-like elements, has been shown to share homology with tetracycline encoding regions of Tn*916* in *C. difficile* (146). Another study has observed the transfer of Tn*916* from *C. difficile* 630 to *B. subtilis* and vice versa.

In *C. difficile,* resistance to the macrolide-lincosamide-streptogramin B group of antibiotics conferred by the *erm*(B) genes is contained on the conjugative transposon

Tn*6194* (150). Transfer of this element was shown to occur with *C. difficile* conspecifics and with *E. faecalis* (150). Another study showed transfer from *C. difficile* to *Staphylococcus aureus* (151). These studies indicate the propensity of conjugative transfer between enterococcus and *C. difficile.*

C. difficile itself does not have many plasmids characterized and most are cryptic or do not contribute to a virulence phenotype (146, 152). To date, two plasmids have been characterized in *C. difficile:* pCD630 (7.8 kb) and pCD6 (6.8 kb) (147, 153). Also, in a study identifying and characterizing plasmids from *C. difficile,* only 15 of the 82 samples showed presence of plasmids (ranging in size from 4.5 to 75 kb) and did not correlate to any resistance genes (154). Some work has been done to show plasmid transfer from one bacterium to *C. difficile,* but this was done artificially to increase genetic tractability of *C. difficile* (153, 155). Overall, these studies require supplementation with newer ones given the genetic plasticity of *C. difficile* and the rise of antibiotic resistance in general.

Whether contained on a plasmid or other MGE, *C. difficile* has shown an ability for horizontal gene transfer (HGT) not only between conspecifics but also with other bacterial species. Given the rise of antibiotic resistance in other bacteria residing in the gut, *C. difficile* is poised to acquire new types of antibiotic resistance given the proper selection pressure.

1.10. Other vancomycin-resistant bacteria in the gut

In order to fully appreciate antibiotic resistance present in the gut microbiome, it is necessary to discuss other bacterial species that exhibit vancomycin resistance as

these may play a role in the emergence of vancomycin-resistant *C. difficile.* So far, major consideration has been given to enterococcus as they have a well-documented history of vancomycin resistance and they occupy the same niche as *C. difficile.* Another potential source of vancomycin resistance could be *Staphylococcus aureus.* Although not necessarily a part of the gut microbiome during CDI, probiotics have been used increasingly as therapeutics for diarrheal symptoms. These microbes could be another reservoir given their rise in popularity for recolonizing the normal gut microbiota. *Lactobacillus casei* group (LCG), composed of *L. casei, L. paracasei,* and *L. rhamnosus.* are common probiotic bacteria (156). They are Gram-positive, rod-shaped, non-spore forming, and non-motile that colonize the gastrointestinal and urogenital tracts (156). These bacteria are also commonly found in various foods (156). It has been known for a while that vancomycin-resistance has been circulating in the *Lactobacillus* population. In one study, it was shown that 100% of lactobacillus strains tested were resistant to vancomycin (157). Other studies have shown that exposure to probiotic bacteria is a significant risk-factor for vancomycin-resistant enterococci colonization in neonates (158)

Weisella spp. were first recognized in 1993 (159). They share many properties with *C. difficile* bacteria (i.e. short rods/coccobacilli, Gram-positive, etc.) but are unique in that they are non-spore forming and are intrinsically resistant to vancomycin (159). They are commonly confused for members of the genus *Lactobacillus* and are disregarded as contaminants in clinical samples (159). *Weisella* inhabits many foods and can be a part of the normal human flora; it has also been recovered from the stool of many healthy individuals (159, 160). A review by Kamboj, et al. describes how *W.*

confusa can cause cases of bacteremia, so it is a relevant human pathogen. Vancomycin resistance has also been described in these bacteria (161, 162). Taken together, these bacteria pose as alternative reservoirs of vancomycin-resistant elements.

1.11. Evaluation of vancomycin non-susceptibility *in C. difficile*

Analysis of *C. difficile*-positive stool samples from Texas for vancomycin non-susceptibility showed that 29% (n = 274) of the Texas samples were not susceptible to vancomycin (Figure 5).

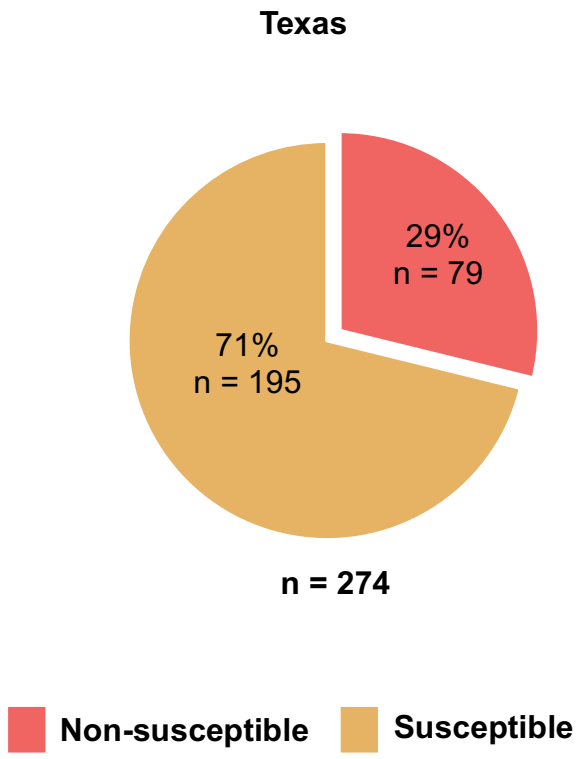

Figure 5: CDI-infected stools from Texas contain vancomycin non-susceptible *C. difficile*. CDI patient stools from Texas were screened for non-susceptibility to vancomycin on *C. difficile* selective plates containing 4 µg/ml vancomycin.

So far, no clear mechanism exists for vancomycin resistance in *C. difficile*. The literature has described the presence of a *vanG* cluster originating from *E. faecalis* in many clinical isolates of *C. difficile.* The inducibility and functionality of these genes have not been confirmed and they are not recognized as promoting a vancomycin non-susceptibility phenotype, as there are minimal changes in MICs and an absence of modified peptidoglycan precursors (110, 163, 164).

Knowledge of the mechanism may provide therapeutic targets with long-term significance for clinical treatment and public health. Many intestinal gut bacteria such as *Enterococcus* and *Staphylococcus* species encode vancomycin resistance on the plasmid Tn*1546*. However, it is not known whether the vancomycin non-susceptibility observed in *C. difficile* isolates is conferred by genes located in the chromosome or on a plasmid. The overall goal of this project is to demonstrate the source of vancomycin resistance in clinical *C. difficile* isolates. Analysis of whole-genome *C. difficile* sequences available on NCBI demonstrated a lack of vancomycin-resistance associated genes in the genomes of sequenced strains, except the *vanG* homologs described above. This led to the hypothesis that the vancomycin resistance observed in the *C. difficile* isolates may be transferred from other co-inhabiting gut bacteria, such as *Enterococcus* species and may be plasmid mediated. Two aims were designed to address the hypothesis, and these are:

Specific Aim 1: To survey vancomycin-resistance genes in the *C. difficile* genome and isolate and characterize plasmids from vancomycin-resistant clinical *C. difficile* isolates. The goal of this aim was to determine what vancomycin-resistance genes were present that may contribute to the resistance phenotype and

to describe if putative vancomycin resistance genes in *C. difficile* are plasmid mediated or chromosomally encoded. Whole genome sequencing, along with comprehensive *in silico* analysis were employed to address this aim.

Specific Aim 2: To estimate the prevalence of *Enterococcus spp*. in stools containing *C. difficile* and to test if vancomycin-resistance may have originated from *Enterococcus* species. Patients with *C. difficile* stools were investigated for co-colonization with enterococcus, and vancomycin-resistance transfer between the two bacteria was investigated. CDI stool samples were screened for the presence of (1) enterococcus and (2) vancomycin-resistant enterococcus. An epidemiological approach was used to characterize CDI infection in terms of enterococcus co-colonization. Finally, vancomycin-susceptible *C. difficile* isolates were co-cultured with enterococci to determine if vancomycin-resistance can be transferred to the *C. difficile* isolates.

2. Materials and Methods

2.1. Stool sample collection

Clinical stool samples were obtained from inpatients presenting with diarrhea at St. Luke's Episcopal Hospital in Houston, TX from 2012-2018. Additional samples were collected from Kenyatta National Hospital in Nairobi, Kenya, and Kisii Teaching & Referral Hospital in Kisii, Kenya from 2016-2018. This study was approved by the Institutional Review Board (IRB) of The University of Texas Health Science Center at Houston and the Ethics Review Board of Kenyatta National Hospital and University of Nairobi (KNH/UoN-ERC). In Houston, patients identified to be positive for *C. difficile* toxin by the hospital's laboratory were consecutively enrolled. All the stool samples

were initially tested by real-time PCR for the toxin genes and classified as *C. difficile* positive by the Medical Microbiology Laboratory at the hospital. In Nairobi, adult patients that reported to the two hospitals with diarrhea were sequentially enrolled and stool samples were collected for analysis. Stool samples were also obtained from children 5 years or younger presenting with diarrhea and gastroenteritis at Kenyatta National Hospital (Nairobi County, Kenya) from 2015 to 2018. All children presenting with diarrhea during the study period were sequentially enrolled in the study. The IRB approval stipulated that all the stool samples be de-identified so no patient information could be included in the current study.

2.2. Isolating vancomycin non-susceptible *C. difficile* from stool

Each patient stool sample was spread on CDPA medium using a sterilized loop and incubated anaerobically at 37 ^{o}C for 48 h (165, 166). The *C. difficile* culture medium (CDPA) contains brain heart infusion (BHI) broth (Becton Dickinson, Cockeysville, MD) (37 g/l), agar (14 g/l), defibrinated horse blood (7%) (Quad Five, Ryegate, Montana), 150 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Zymo Research, Irvine, CA), *p*-cresol (0.025%), 0.1% sodium taurocholate (Sigma-Aldrich, St. Louis, MO), D-cycloserine (500 µg/ml), and cefoxitin (16 µg/ml) (Fisher Scientific, Hampton, NH). Anaerobic conditions were maintained with an atmosphere of 10% H_2, 5% CO_2, and 85% N_2 in a Bactron 600 anaerobic chamber (Sheldon Manufacturing, Inc, Cornelius, OR). To identify stool samples containing non-susceptible *C. difficile* isolates, the stools were plated on CDPA only, and CDPA containing 4 µg/ml vancomycin based on CLSI and EUCAST guidelines (167, 168).

The presence of *C. difficile* in the stools was confirmed using toxigenic *C. difficile* culture and PCR (169-172). Isolates confirmed as *C. difficile* positive and that demonstrated the vancomycin non-susceptible phenotype were saved as freezer stocks at -80 ^{o}C.

2.3. Determining minimum inhibitory concentration (MIC)

To test the minimum inhibitory concentrations for the *C. difficile* vancomycin non-susceptible isolates, the broth microdilution method was used (173, 174). Overnight *C. difficile* cultures inoculated from freezer stocks were used to inoculate 96-well plates. MICs for the wild type control and clinical isolates were done using both Müeller-Hinton broth and brain-heart infusion (BHI) broth, a complex growth media commonly used for culturing *C. difficile* (175).

2.4. DNA Isolation and purification

Freezer stocks were streaked onto CDPA containing 4 µg/ml vancomycin and incubated for 48 h anaerobically at 37 ^{o}C. Isolates were then cultured anaerobically at 37 ^{o}C in BHI broth containing 4 µg/ml vancomycin overnight. Cultures were pelleted at 4000 rpm for 15 minutes at 4 ^{o}C. DNA was isolated and purified using the Qiagen DNeasy Kit, according to the manufacturer's instructions. DNA concentrations and purity were assessed using a NanoDrop 2000 (Thermo Scientific, Waltham, MA).

2.5. DNA sequencing and *in silico* genome analysis

To assess whether the vancomycin non-susceptible C. difficile isolates harbored vancomycin-resistance elements, whole-genome sequencing was performed at the Genomics & Bioinformatics Service (Texas A&M University) on Illumina MiSeq Platform with paired-end reads. This analysis was performed on six of the non-susceptible isolates with MICs ranging from 4 μg/ml to 64 μg/ml. These isolates were CD6 (4 μg/ml), TMC109 V (16 μg/ml), TMC544V_2 (32 μg/ml), TMC579 (32 μg/ml), TMC 68V_1B (64 μg/ml), and TMC024S (64 μg/ml).

For the analysis, the adapters were first removed by Trimmomatic (Version 0.39) (176) and reads were examined for quality with FastQC (Version 0.11.9) (177). SPAdes (version 3.14.0) was used to create *de novo* assemblies for the clinical isolates (178).

Assembly quality was evaluated in several ways. Initially, QUAST was used to determine quality as well as GC content (179). This report also provided information including the total length, N(50), and N(75) values. QUAST was utilized through the Patric web service (version 3.6.5) (180). The number of reads generated by Illumina sequencing was calculated by counting the number of lines present in the respective fastq files through a command line prompt and dividing that number by 4, to account for the three lines of descriptive data for each read. Coverage was determined in terms of depth and breadth. Empirical coverage depth was calculated using the following equation and an average read length of 290 bp for CD 6 isolate and 150 bp for all other samples as indicated by the assembly report:

$$Coverage\ depth = \frac{number\ of\ reads\ x\ average\ read\ length}{assembly\ size}$$

Coverage breadth was calculated using the following equation:

$$Coverage\ breadth = \frac{assembly\ size}{reference\ genome\ size}\ x\ 100$$

Coverage breadth is denoted as average percent total coverage; contigs were ordered using various *C. difficile* assemblies available from NCBI (Table 2) (181). Progressive Mauve alignment was used to align contigs (182-184) and a score assembly was used to assess the percent of bases missed with each alignment. An average of percentages was determined to give the final value.

Table 2: Reference genomes from NCBI used to determine coverage for *C. difficile* clinical isolates.

Strain	Size	GC content	Refseq	Reference
020711	4.17	28.70	NZ_CP028530.1	(185)
AK	4.32	28.72	NZ_CP027014.1	(186)
ATCC43255	4.21	28.55	NZ_CM000604.1	(187)
BR81	4.12	28.70	NZ_CP019870.1	(188)
CBA7204	4.04	28.50	NZ_CP029566.1	(189)
CD-17-01474	4.30	28.90	NZ_CP026591.1	(190)
DH/NAP11/106/ST-42	4.09	28.60	NZ_CP022524.1	(191)
FDAARGOS723	4.18	28.70	NZ_CP046327.1	(192)
Mta-79	4.12	28.70	NZ_CP042267.1	(193)
W0003A	4.08	28.60	NZ_CP025047.1	(194)

Assembled sequences were subjected to two different annotation processes. Primary annotations were done on the Rapid Annotation using Subsystem Technology (RAST) Server (Version 0.1.1) (195). RAST annotations were compared against secondary annotations made in Prokka (Version 2.1.1) (196).

Annotation quality was determined via EvalIG which utilized the CheckM algorithm to determine completeness of sequence in comparison to a reference (197). Additionally, EvalCon was used to describe the coarse and fine consistency of the annotations (198); EvalIG and EvalCon were utilized through the Patric web service (version 3.6.5) (180).

Regions of interests determined through annotation programs were searched through NCBI Open Reading Frame (ORF) Finder. Consensus annotations were manually assigned to genes using SnapGene Viewer (Version 5.0.7). To describe the evolutionary relationships between sequences, MEGA X (199) was used to construct a phylogenetic tree via the Neighbor-Joining method (200). Evolutionary distances were calculated based on the maximum composite likelihood method and are described in units of base substitutions per site (201). All ambiguous positions were removed for each sequence pair (pairwise deletions).

BioEdit (Version 7.2.5) (202, 203) was as used to align *C. difficile* sequences based on the ClustalW algorithm and to construct sequence identity matrices (204).

To determine if genes were located on the chromosome or on a plasmid, putative plasmid sequences were extracted from whole genome sequences using plasmidSPAdes (version 3.14.0) (205, 206). The resulting contigs were compared against sequences in the NCBI database via NCBI BLAST to determine if they

matched a plasmid or chromosomal DNA. Contig sequences less than 500 bp were excluded from the analysis. To validate the results, existing *C. difficile* plasmid sequences on NCBI (Table 3) were compared with extracted sequences from the plasmidSPAdes program (Table 3). Finally, both plasmid and chromosomal sequences were annotated with RAST to determine if vancomycin-resistance genes were present.

Table 3: Plasmid sequences from *C. difficile* deposited in the NCBI database. None of the plasmids contain vancomycin-resistance elements. NCBI has 18 published plasmid sequences associated from *C. difficile*. No vancomycin-resistance elements were found in these plasmid sequences.

Strain	Plasmid	RefSeq	Size (kb)	GC (%)	CDS	*Van* elements (Y/N)	Ref.
C. difficile strain	pCD6	NC_005326.1	6.83	24.50	5	N	(155)
630 delta erm	pCD630	NZ_CP016319.1	7.881	27.90	9	N	(207)
FDAARGOS_267	unnamed1	NZ_CP020425.2	45.187	28.13	64	N	(208)
FDAARGOS_267	unnamed2	NZ_CP020426.2	131.326	26.43	151	N	(208)
AK	pAK1	NZ_CP027015.1	142.753	26.62	159	N	(186)
AK	pAK2	NZ_CP027016.1	56.372	28.30	74	N	(209)
CD161	unnamed1	NZ_CP029155.1	130.529	26.23	151	N	(210)
CD161	unnamed2	NZ_CP029156.1	48.594	28.53	68	N	(210)
CDT4	unnamed1	NZ_CP029153.1	48.594	28.53	68	N	(211)
12038	unnamed1	NZ_CP033215.1	10.52	37.21	3	N	(212)
CD21062	unnamed1	NZ_CP033217.1	109.346	33.30	80	N	(212)
TW11	p_TW11	NZ_CP045225.1	42.254	25.08	43	N	(213)
TW11-RT078	p1	CP035500.1	42.254	25.08	43	N	(214)
630	pCD630	NC_008226.2	7.881	27.90	10	N	(215)
ATCC9689 = DSM1296	unnamed	NZ_CP011969.1	45.187	28.13	64	N	(216)
BI1	pCDBI1	FN668942.1	45.258	28.03	0	N	(217)
BI1	unnamed	FN668943.1	300.869	25.00	0	N	(217)
ERR022513	pCD-WTSI2	NZ_MG019960.1	12.526	26.55	15	N	(218)

2.6. Estimating prevalence of enterococci in CDI patients

To detect presence of vancomycin-sensitive and vancomycin-resistant enterococci, patient stool samples were streaked onto Slanetz-Bartley Media (SBM)(Oxoid) (219). A loop-full of stool was streaked onto the medium containing either no vancomycin or 4 µg/ml vancomycin based on EUCAST breakpoints (168). The plates were incubated aerobically for 48 h at 37 ºC. Any consistent, non-contaminating growth on SBM was considered positive for enterococci. The entire bacteria growth on the plate was pooled and inoculated into 4 ml of BHI broth. The culture was allowed to grow overnight at 37 °C. Stocks were saved by combining 900 µl of culture with 100 µl of dimethyl sulfoxide (DMSO) (Fisher Scientific) in a microcentrifuge tube. The tubes were vortexed for a few seconds and stored at -80 °C. Samples that did not grow were re-plated with the same procedure to confirm results.

2.7. Evaluating genetic transfer between enterococci and *C. difficile*

Freezer stocks of select vancomycin-resistant enterococci were streaked onto (1) SBM plates to isolate single colonies, (2) SBM containing vancomycin to confirm the resistance phenotype, and (3) CDPA plates to ensure the samples would not grow on the *C. difficile* selective plates. *C. difficile* isolates demonstrating a vancomycin susceptible phenotype were streaked onto (1) CDPA plates to isolate single colonies, (2) CDPA with vancomycin to confirm the susceptibility phenotype, and (3) SBM plates to ensure *C. difficile* samples did not grow on the enterococcus selective media. Plates were incubated anaerobically at 37 °C for 48 h. Then, single colonies of both bacteria

were inoculated into 4 ml of BHI broth and incubated anaerobically overnight at 37 °C. The next day 500 µl of the enterococci culture and 500 µl of the *C. difficile* culture were combined into a 15 ml conical tube containing 3 ml of fresh BHI. Control tubes were also made by transferring either 1 ml of *C. difficile* culture into 3 ml of BHI or 1 ml of enterococcal culture into 3 ml BHI. All tubes were incubated anaerobically overnight at 37 °C. The following day, 200 µl each of overnight culture was deposited onto SBM, CDPA, and CDPA + 4 µg/ml vancomycin for the mixed culture, CDPA, CDPA + vancomycin, and SBM for the *C. difficile* control, and CDPA and SBM for the enterococcus control. These plates were incubated for 24 h anaerobically at 37 °C and observed for growth/no growth. Additionally, 1 ml overnight culture of each sample was transferred into 3 ml of fresh BHI. Propagation of culture and plating continued for 10 days.

2.8. Determining toxin production of *C. difficile* isolates

Toxin activity was done using the Cdifftox activity assay as described previously (165, 169-171, 220, 221). Briefly, supernatant recovered from 48 hour *C. difficile* culture was combined with 30 µl of 30 mM p-nitrophenyl-β-D-glucopyranoside (Sigma-Aldrich) in a sterile 96-well plate. Absorbance was measured at 410 nm after incubation at 37 °C for 24 h. The *C. difficile* TOX A/B II ELISA test (TechLab, Blacksburg, VA) was used for toxin production by testing 200 µl of the culture supernatant according to the manufacturer's instructions.

2.9. Determining spore production of *C. difficile* isolates

Freezer stocks were streaked on CDPA with 250 µg/ml D-cycloserine and 8 µg/ml cefoxitin and incubated for 48 hours. Single colonies were selected from the plates, inoculated in 4 ml BHI broth, and incubated for 5 days anaerobically. Following the incubation period, 1 ml of culture was transferred into a microcentrifuge tube and incubated at 65 ºC in a hot water bath for 30 minutes. Then, 200 µl of heat-shocked culture was streaked onto CDPA containing 0.1% sodium taurocholate and incubated for 48 hours anaerobically. Colony growth on the plate indicated presence of spores.

2.10. Statistical analysis

Statistical analysis was performed using RStudio (version 1.2.1335) with the Epitools Package (222, 223). Pearson's Chi-Squared Test for Independence with Yate's Continuity Correction was used to determine if proportions between vancomycin-resistant Enterococcus and vancomycin-resistant *C. difficile* were significant, if co-colonization proportions affected chronic CDI symptoms, and if type of antibiotic used affected chronic CDI symptoms. A *p*-value of less than 0.05 was considered significant. Odds ratios and confidence intervals were calculated for prevalence of enterococci in *C. difficile* stools, prevalence of vancomycin-resistant enterococci in *C. difficile* stools, and presence of *C. difficile* spores in *C. difficile*-only stools versus co-colonized stools.

3. Results

3.1. *C. difficile* genomes reveal presence of vancomycin-resistance elements

Multiple *de novo* assemblies of the vancomycin non-susceptible *C. difficile* isolates yielded assembled genomes ranging from 4.07 to 4.26 Mbp, with relatively high N(50) and N(75) values (Table 4). N(50) and N(75) values describe the minimum length (bp) of either 50% or 75% of contigs, respectively.

The assemblies for each isolate were broken down into contig size and coverage (Table 14-Table 19, Appendix A). For CD6, 2,178,790 paired reads produced 41 contigs above 300 bp with a mean length of 99,156 and 24 contigs of ≥1,000 bp with a mean length of 169,032 bp (Table 14). For TMC109V, 4,456,654 paired reads produced 62 contigs above 300 bp with a mean length of 66,101 and 40 contigs of ≥1,000 bp with a mean length of 102,183 bp (Table 15). For TMC544V_2, 4,175,631 paired reads produced 60 contigs above 300 bp with a mean length of 68,256 and 51 contigs of ≥1,000 bp with a mean length of 80,211 bp (Table 16). For TMC579, 4,449,583 paired reads produced 70 contigs above 300 bp with a mean length of 58,147 and 57 contigs of ≥1,000 bp with a mean length of 71,301 bp (Table 17). For TMC68V_1B, 4,184,738 paired reads produced 58 contigs above 300 bp with a mean length of 70,773 and 49 contigs of ≥1,000 bp with a mean length of 83,680 bp (Table 18). Finally, for TMC024S, 4,313,776 paired reads produced 133 contigs above 300 bp with a mean length of 38,152 and 71 contigs of ≥ 1,000 bp with a mean length of 71,002 bp (Table 19). No DNA from exogenous bacterial sources were found in these isolates.

Table 4: Whole genome sequencing characteristics reflect quality assemblies of the sequences obtained from the *C. difficile* clinical isolates. Table values are based on assemblies produced by the SPAdes *de novo* assembly and QUAST pipelines.

Sample	Reads	# of Contigs	Total Length (bp)	N(50) (bp)	N(75) (bp)	Coverage Depth	% Avg. Total Coverage	GC Content
CD6	2,178,790	41	4,065,404	429,529	205,873	155X	92.5	28.44
TMC109V	4,456,654	62	4,098,287	162,289	133,781	163X	93.7	28.42
TMC544V_2	4,175,631	60	4,095,333	140,966	81,329	153X	93.3	28.51
TMC579	4,449,583	70	4,070,284	146,718	81,329	164X	92.9	28.50
TMC68V_1B	4,184,738	58	4,104,836	128,719	81,329	153X	93.5	28.49
TMC024S	4,313,776	133	4,260,622	233,385	85,764	152X	93.5	28.58

The annotated contigs revealed several vancomycin-resistance elements including a vancomycin resistance two-component system (VanRS) and a putative D-alanine-(R)-lactate ligase (Table 5). Further analysis of the annotation data revealed a variety of resistance elements.

Table 5: Resistance elements found in *C. difficile* clinical isolates. Annotation by RAST via the PATRIC web server revealed the presence of vancomycin-resistance genes in *C. difficile* genomes. These results were consistent with gene annotations from Prokka.

Element
Copper Homeostasis
Mercury Resistance Operon
Multidrug Resistance Efflux Pumps
Cobalt-zinc-cadmium Resistance
Multidrug Resistance, Gram-positive Bacteria
Resistance to Vancomycin
Resistance to Fluoroquinolones
Copper Homeostasis
Zinc Resistance
Beta-lactamase
Mercuric reductase
Methicillin Resistance in Staphylococci
Arsenic Resistance

Evaluation of the annotation data showed that the mapped genes were reliable with relatively high coarse consistency values (>94%), while the fine consistency values span a range of values from 68.8% to 98.7% (Table 6). The EvalCon program uses a reference genome to determine which genes are expected and in what quantity (198). The coarse consistency indicates the percentage of expected genes that are present. Fine consistency indicates the number of genes that are present. A lower fine consistency would indicate a different number of genes than what was predicted based on the reference genome.

Table 6: Annotation quality of the assemblies. Assemblies were submitted for annotation using RAST via the PATRIC web server, and EvalIG and EvalCon was used to determine the reliability of the annotations made by RAST.

Sample	Completeness (%)	Coarse Consistency (%)	Fine Consistency (%)
CD6	100	98.8	98.7
TMC109V	100	95.6	80.9
TMC579	100	97.8	93.6
TMC544V_2	100	98.3	95.3
TMC68V_1B	100	98.7	98.2
TMC024S	100	94.8	68.8

The annotations were confirmed using NCBI BLAST and maps were created of regions showing any vancomycin-resistant elements. In the CD6 isolate, vancomycin resistant elements were found in 4 separate loci. (Note: the node/loci designation is arbitrary and used only for organizational purposes). In node 1, locus 2 there was a *vanW* ligase upstream of a D-alanyl-D-alanine carboxypeptidase; a second distinct *vanW* gene is present in node 4, locus 3. A *vanZ* gene associated with teicoplanin resistance and commonly found as part of the VanA operon is also found in the genome (node 4, locus 1). Finally, a vancomycin-resistance operon containing a putative van ligase (green) is present (node 1, locus 5). This operon contains other elements including *vanRS* and *vanXY*, but *vanH,* needed to form precursors terminating in lactate, is absent. Additionally, *vanT*, an alanine/serine racemase, is found in the operon (Figure 6).

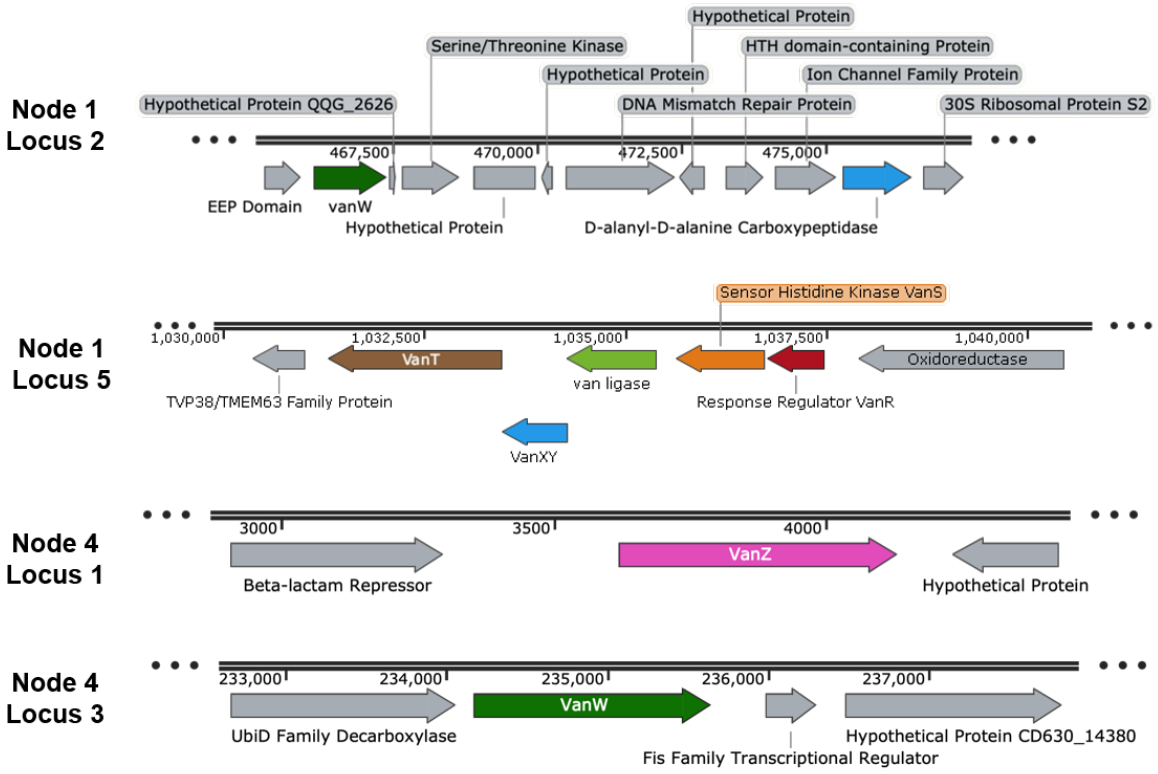

Figure 6: The *C. difficile* vancomycin non-susceptible isolate CD6 contains a putative vancomycin resistance operon and additional putative vancomycin resistance genes. Gene annotation software and sequence examination through NCBI BLAST revealed the location and identity of vancomycin-resistance elements including: D-alanyl-D-alanine carboxypeptidase (blue), putative van ligase (green), vanS (orange), vanR (red), vanW (dark green), and vanZ (pink).

The TMC109V isolate has five copies of the *vanZ* gene (node 1, locus 1; node 2; node 3; node 10, locus 2; and node 13) and two copies of *vanW* genes. The other vancomycin-resistance operon is located on node 12 (Figure 7).

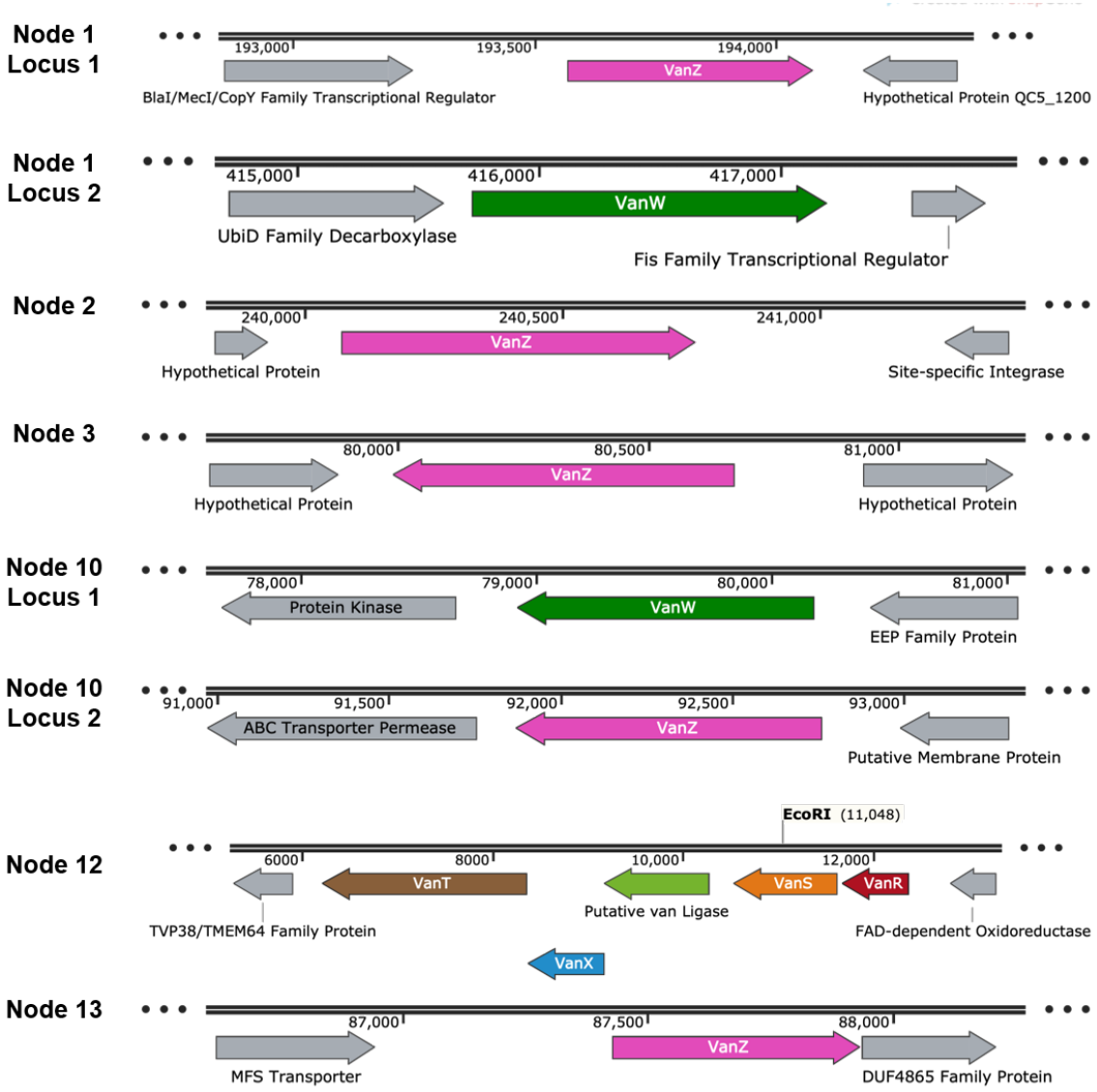

Figure 7: The *C. difficile* vancomycin non-susceptible isolate TMC109V contains a putative vancomycin resistance operon and additional putative vancomycin resistance genes. Gene annotation software and sequence examination through NCBI BLAST revealed the location and identity of vancomycin-resistance elements including: D-alanyl-D-alanine carboxypeptidase (blue), putative van ligase (green), *vanS* (orange), *vanR* (red), *vanW* (dark green), and *vanZ* (pink).

One feature in the TMC544V_2 isolate is that the one instance of *vanRS* is flanked by a HAMP-domain containing protein and a hypothetical protein, while the other is found in the complete resistance operon in node 2. The sequences for both *vanRS* are not identical. Several vanZ genes (node 3, locus 1; node 3, locus 3; node 4, locus 2; node 6) and vanW genes (node 3, locus 2; node 4, locus 1) are also present in the genome (Figure 8).

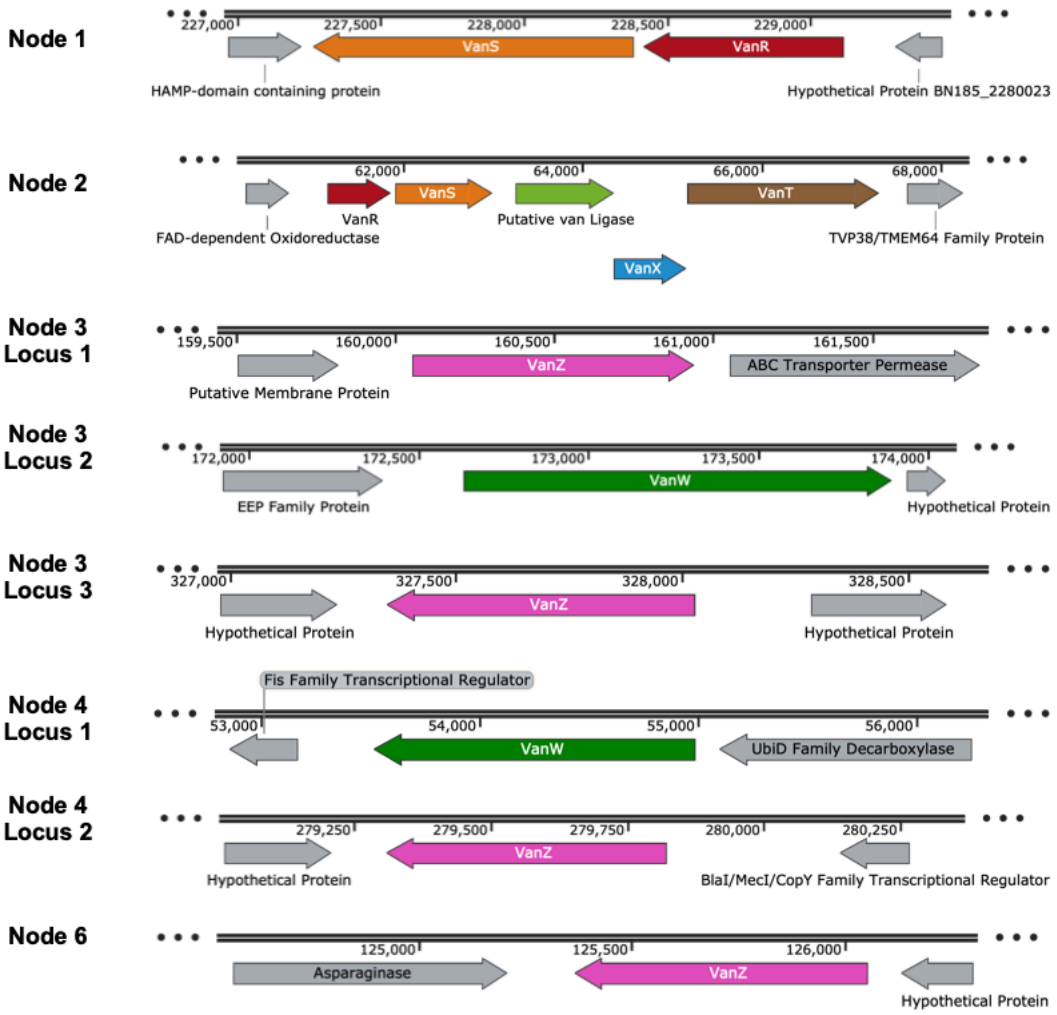

Figure 8: The *C. difficile* vancomycin non-susceptible isolate TMC544 V_2 contains a putative vancomycin resistance operon and additional putative vancomycin resistance genes. Gene annotation software and sequence examination through NCBI BLAST revealed the location and identity of vancomycin-resistance elements including: D-alanyl-D-alanine carboxypeptidase (blue), putative van ligase (green), vanS (orange), vanR (red), vanW (dark green), and vanZ (pink).

The TMC579 isolate had elements almost identical to TMC544V_2. The vancomycin resistance operon (node 1, locus 1) and the *vanRS* two-component system (node 2) are present, in addition to the same number of *vanZ* gene copies (node 1, locus 2; node 1, locus 4; node 3, locus 1; node 4) and *vanW* genes (node 1, locus 3; node 3, locus 2) (Figure 9).

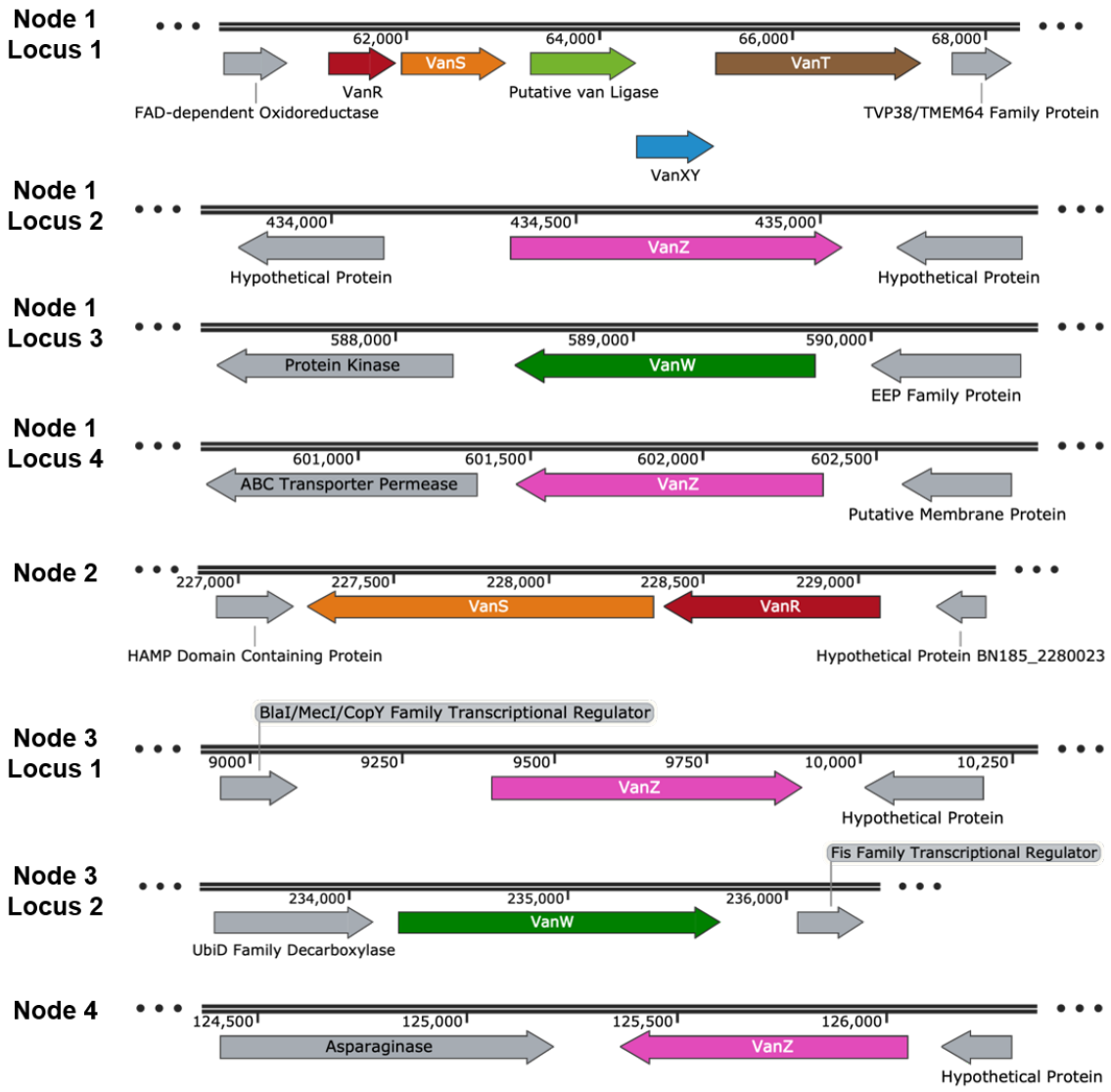

Figure 9: The *C. difficile* vancomycin non-susceptible isolate TMC579 contains a putative vancomycin resistance operon and additional putative vancomycin resistance genes. Gene annotation software and sequence examination through NCBI BLAST revealed the location and identity of vancomycin-resistance elements including: D-alanyl-D-alanine carboxypeptidase (blue), putative van ligase (green), vanS (orange), vanR (red), vanW (dark green), and vanZ (pink).

The same trend is observed in the TMC68V$_1$ B isolate with the resistance operon (node 2) and the *vanRS* two-component system (node 1) present. The *vanZ* genes copies (node 3, locus 1; node 3, locus 3; node 4, locus 2; node 6) and *vanW* genes (node 3, locus 2; node 4, locus 1) are also present (Figure 10).

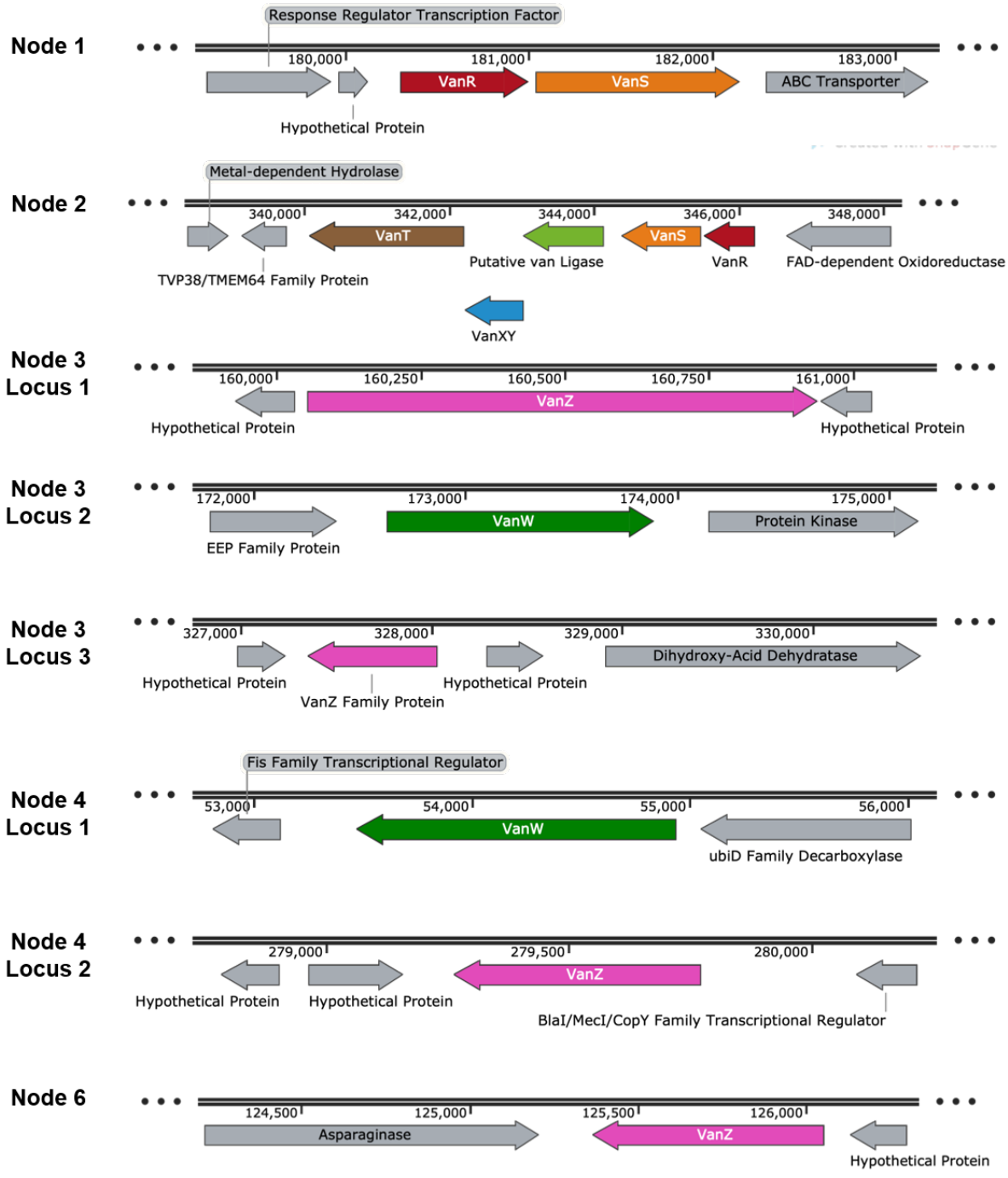
Node 1
Response Regulator Transcription Factor
180,000
181,000
182,000
183,000
VanR
VanS
ABC Transporter
Hypothetical Protein
Node 2
Metal-dependent Hydrolase
340,000
342,000
344,000
346,000
348,000
VanT
VanS
Putative van Ligase
VanR
FAD-dependent Oxidoreductase
TVP38/TMEM64 Family Protein
VanXY
Node 3
Locus 1
160,000
160,250
160,500
160,750
161,000
VanZ
Hypothetical Protein
Hypothetical Protein
Node 3
Locus 2
172,000
173,000
174,000
175,000
VanW
Protein Kinase
EEP Family Protein
Node 3
Locus 3
327,000
328,000
329,000
330,000
Dihydroxy-Acid Dehydratase
Hypothetical Protein
Hypothetical Protein
VanZ Family Protein
Node 4
Locus 1
Fis Family Transcriptional Regulator
53,000
54,000
55,000
56,000
VanW
ubiD Family Decarboxylase
Node 4
Locus 2
279,000
279,500
280,000
VanZ
Hypothetical Protein
Hypothetical Protein
BlaI/MecI/CopY Family Transcriptional Regulator
Node 6
124,500
125,000
125,500
126,000
Asparaginase
VanZ
Hypothetical Protein

Figure 10: The *C. difficile* vancomycin non-susceptible isolate TMC68V$_1$B contains a putative vancomycin resistance operon and additional putative vancomycin resistance genes. Gene annotation software and sequence examination through NCBI BLAST revealed the location and identity of vancomycin-resistance elements including: D-alanyl-D-alanine carboxypeptidase (blue), putative van ligase (green), vanS (orange), vanR (red), vanW (dark green), and vanZ (pink).

The TMC024S isolate shows features unique to all the other isolates. In addition to the vancomycin-resistance operon that is present in the other isolates (node 13), there are two *vanW* genes (node 646 and node 8870). Unique features of this isolate include an isolated VanB-type *vanS* gene (node 425), an isolated *vanX* (node 843), and two copies of *vanB* (node 6425, and node 8788). Finally, a *vanH* gene was present (node 1002) (Figure 11).

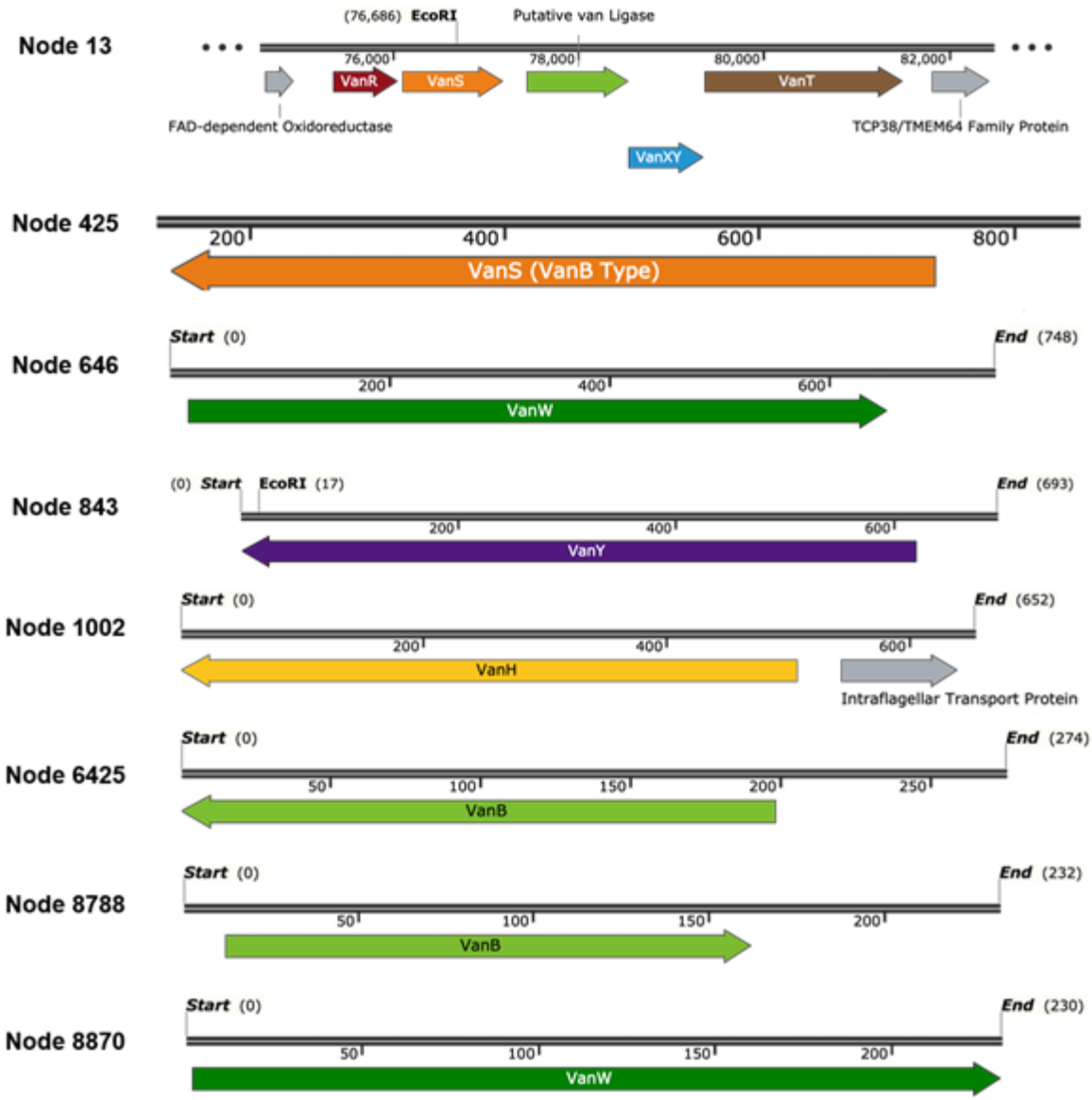

Figure 11: The *C. difficile* vancomycin non-susceptible isolate TMC024S contains a putative vancomycin resistance operon and additional putative vancomycin resistance genes. Gene annotation software and sequence examination through NCBI BLAST revealed the location and identity of vancomycin-resistance elements including: D-alanyl-D-alanine carboxypeptidase (blue), putative van ligase (green), *vanS* (orange), *vanR* (red), *vanW* (dark green), and *vanZ* (pink).

To determine how closely related the *C. difficile* clinical isolates were, a sequence alignment was performed between the putative *van* ligase of the conserved operon, which showed a high sequence identity. Percent identity among the samples ranged between 99.6% and 100%. CD6, TMC109V and TMC68V_1B were most closely related followed by TMC024S, and finally, TMC579 and TMC544V_2 (Figure 12). The phylogenetic tree had a sum branch length of 0.005 with a total of 6 sequences and 12995 positions in the final data set (Figure 12).

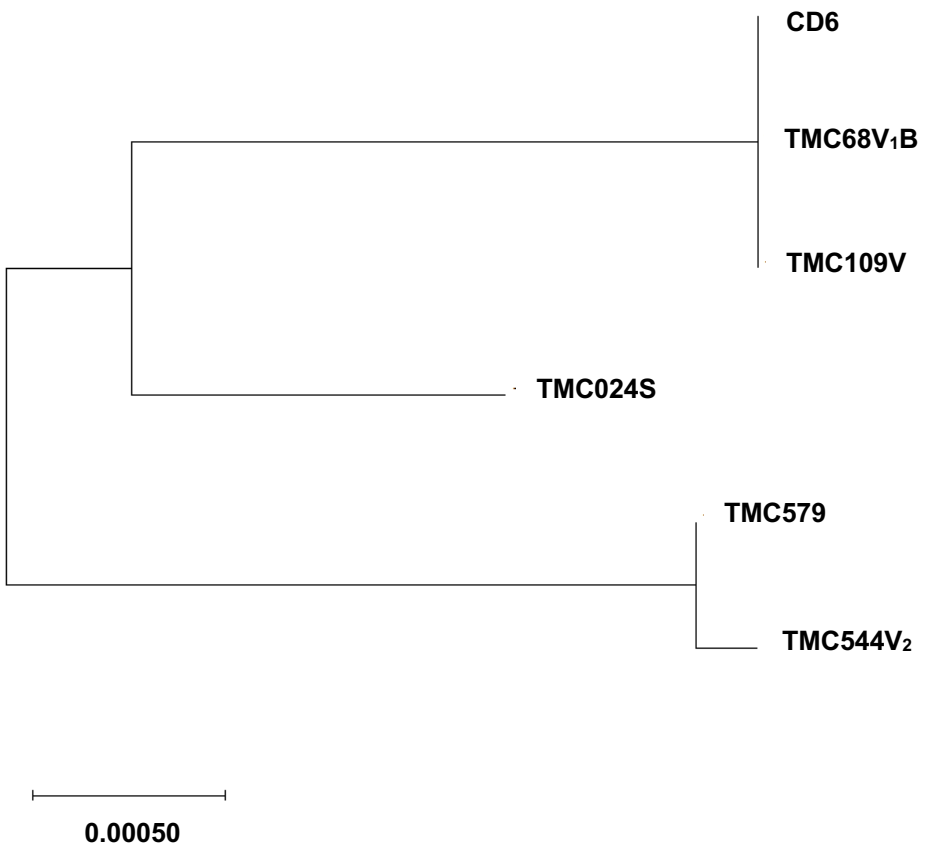

Figure 12: High level of relatedness among putative *van* ligases found in vancomycin non-susceptible *C. difficile* isolates. The phylogenetic tree was created using the neighbor-joining method with a sum branch length of 0.005. Tree is drawn to scale.

Several vancomycin resistance elements from the isolates were searched through the NCBI BlastP database. The putative *van* ligases from the clinical isolates matched most closely with the *vanG* from other *C. difficile* strains, followed by *vanG* from other members of the *Clostridium* genus (Figure 13). These similarities were also observed with the putative *vanT* elements present in each of the six isolates (Figure 14). Both elements from the VanG operon were not similar to enterococcal *vanG* and *vanT* elements.

In TMC024S, the putative *vanH* element unique to this isolate matched most closely with $vanH_B$ from *Enterococcus* spp. with a sequence identity of 97% (Figure 15).

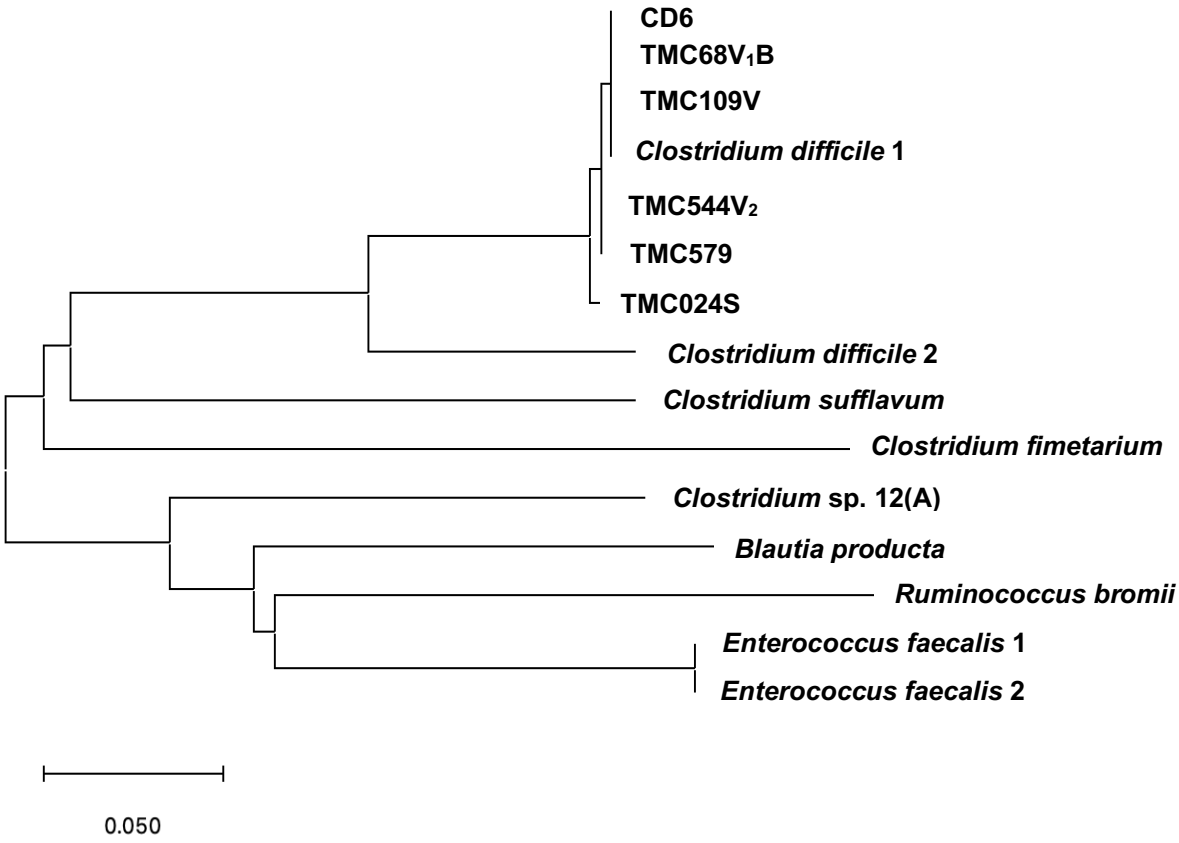

Figure 13: Putative *van* ligases matched most closely with the *vanG* ligases from other *Clostridium difficile* strains. The phylogenetic tree had a branch length of 1.2536 with a total of 368 positions. *VanG* ligases from the NCBI database were used from the following organisms*: Clostridium difficile* 1 (WP_021360551.1), *Clostridium difficile* 2 (WP_021382963.1), *Clostridium sufflavum* (WP_110462065.1), *Clostridium fimetarium* (WP_092455360.1), *Clostrdium* sp. 12(A) (WP_024835184.1), *Blautia producta* (WP_171285627.1), *Ruminococcus bromii* (WP_015523973.1), *Enterococcus faecalis* 1 (WP_063856695.1), and *Enterococcus faecalis* 2 (ABA71731.1).

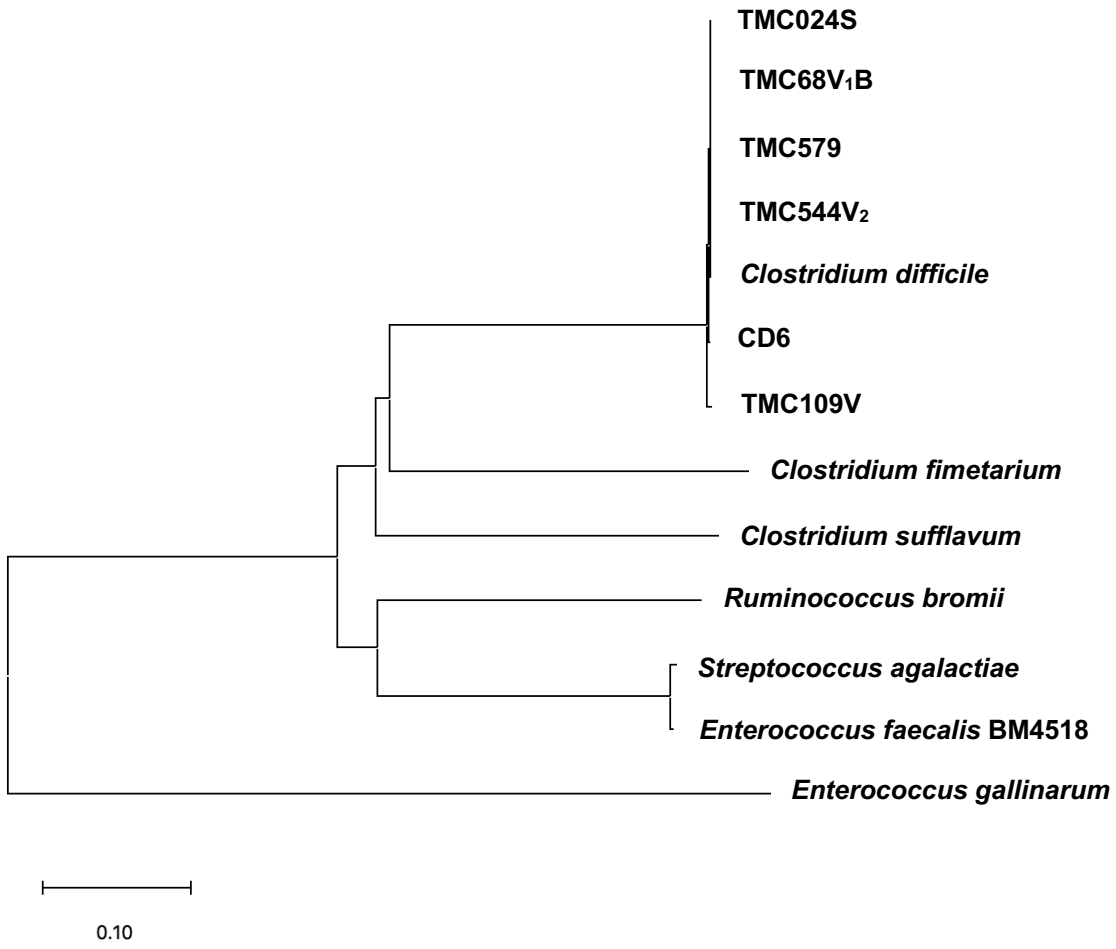

Figure 14: **putative *vanT* genes matched most closely with *Clostridium* spp. members.** The phylogenetic tree had a sum branch length of 1.92778 with a total of 720 positions in the final dataset. The following *vanT* sequences from the NCBI database were used: *Clostridium difficile* (WP_009893196.1), *Clostridium fimetarium* (WP_092455364.1), *Clostridium sufflavum* (WP_110462027.1), *Ruminococcus bromii* (WP_015523975.1), *Streptococcus agalactiae* (WP_041330235.1), *Enterococcus faecalis* BM4518 (AAQ16274.1), and *Enterococcus gallinarum* (Q9X3P3).

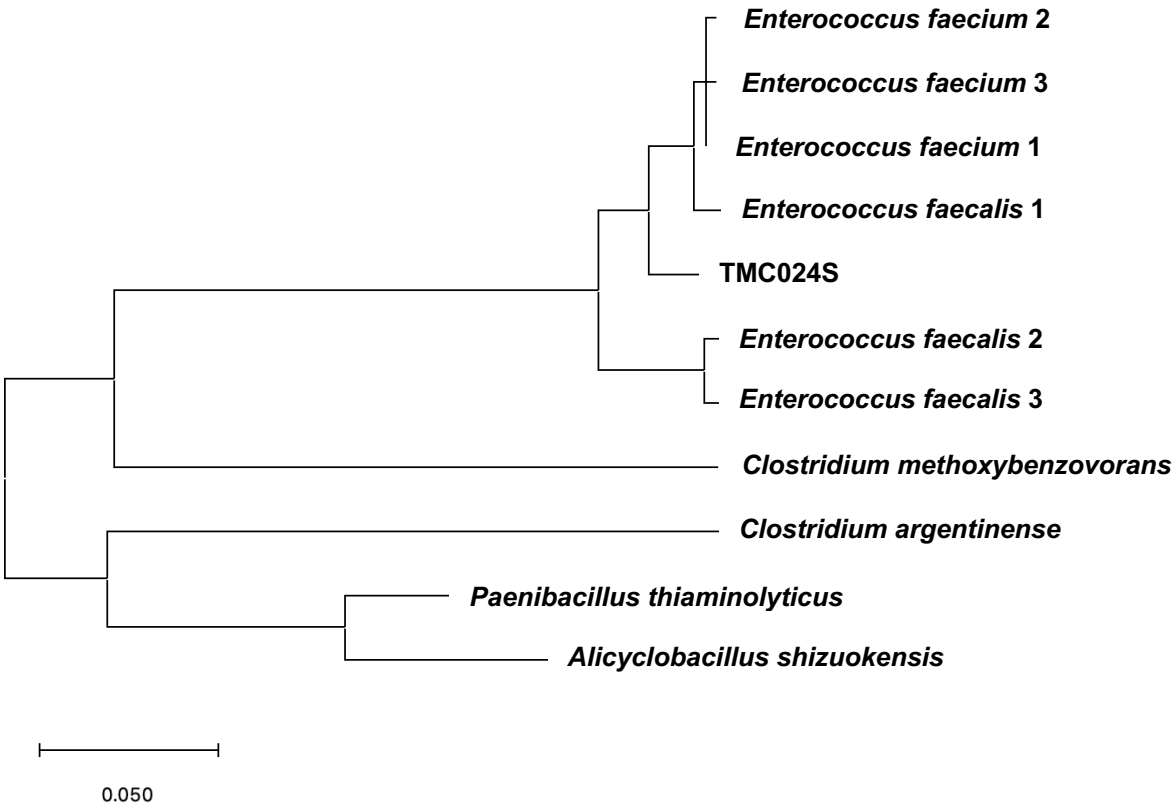

Figure 15: TMC024S putative *vanH* matched most closely with *E. faecium* and *E. faecalis*. The phylogenetic tree had a sum branch length of 0.7991 with a total of 323 positions in the final dataset. The following *vanH* sequences from the NCBI database were used: *Enterococcus faecium* 1 (WP_063856701.1), *Enterococcus faecalis* 1 (WP_032489745.1), *Enterococcus faecalis* 2 (WP_172863810.1), *Enterococcus faecalis* 3 (WP_099156040.1), *Enterococcus faecium* 2 (WP_127841138.1), *Enterococcus faecium* 3 (WP_115250287.1), *Clostridium methoxybenzovorans* (WP_024345223.1), *Clostridum argentinense* (WP_039635344.1), *Paenibacillus thiaminolyticus* (WP_063856708.1), *Alicyclobacillus shizuokensis* (WP_083517214.1).

3.2. Sequence analysis suggests vancomycin-resistance elements are located on the chromosome

Several plasmids have been isolated and annotated for *C. difficile* (Table 3). Sequences derived from our plasmid extraction pipeline were searched through the NCBI database to determine if they were plasmid or chromosomal DNA for each isolate (Table 7-Table 12). TMC024S showed presence of plasmid DNA, but these were plasmids that have already been annotated for *C. difficile* and do not contain vancomycin-resistance genes. Conversely, annotation of chromosomal DNA via RAST indicated the presence of one *vanZ* gene on node 7 of TMC68V_1B; no other vancomycin-associated genes were found with this approach. Attempts to isolate plasmid DNA from the isolates were also unsuccessful. Taken together, these data suggest that vancomycin-resistance elements are not found on a plasmid.

Table 7: CD6 putative plasmid DNA was found to be chromosomal without any resistance elements

Node	Length (bp)	Chromosome (C) or Plasmid (P)
1	2,334	C
2	2,140	C
3	1,579	C
4	1,219	C
5	864	C
6	758	C
7	586	C
8	544	C

Table 8: TMC109V putative plasmid DNA was found to be chromosomal without any resistance elements

Node	Length (bp)	Chromosome (C) or Plasmid (P)
1	39,391	C
2	2,925	C
3	2,192	C
4	1,615	C
5	856	C
6	749	C
7	724	C
8	619	C
9	575	C
10	524	C

Table 9: TMC544V_2 putative plasmid DNA was found to be chromosomal without any resistance elements.

Node	Length (bp)	Chromosome (C) or Plasmid (P)
1	99,342	C
2	94,916	C
3	72,596	C
4	2,724	C
5	2,392	C
6	1,575	C
7	1,311	C
8	1,301	C
9	885	C
10	809	C
11	708	C
12	669	C
13	620	C
14	593	C
15	541	C

Table 10: Putative plasmid DNA was found to be chromosomal without any resistance elements in TMC579.

Node	Length (bp)	Chromosome (C) or Plasmid (P)
1	99,499	C
2	97,665	C
3	94,916	C
4	72,881	C
5	2,724	C
6	2,392	C
7	1,575	C
8	1,386	C
9	809	C
10	620	C
11	594	C
12	541	C
13	535	C
14	512	C
15	508	C

Table 11: Putative plasmid DNA was found to be chromosomal without any resistance elements in TMC68V$_1$B.

Node	Length (bp)	Chromosome (C) or Plasmid (P)
1	100,904	C
2	99,462	C
3	97,425	C
4	94,916	C
5	72,596	C
6	2,724	C
7	2,392	C
8	1,575	C
9	1,028	C
10	809	C
11	734	C
12	620	C
13	617	C
14	600	C
15	597	C
16	569	C
17	541	C

Table 12: Putative plasmid DNA mapped to existing *C. difficile* plasmids, but it was largely chromosomal in TMC024S.

Node	Length (bp)	Chromosome (C) or Plasmid (P)
1	44,851	ATCC9689 = DSM 1296 unnamed plasmid
2	41,219	C
3	12,653	plasmid pCD-WTSI2
4	2,925	C
5	2,287	C
6	1,520	C
7	854	C
8	821	C
9	622	C
10	620	C
11	575	C
12	525	C

3.3. Enterococci co-colonization of *C. difficile* patients

The prevalence of *Enterococcus* species in stools of CDI patients from Texas and Kenya was determined by plating stools on enterococci-specific media. In adults, 70% of the CDI patient stools contained enterococcus, while 50% of stools from children had enterococcus. The odds of enterococcus colonization in CDI stools was 3 times (95% CI [1.39-5.85]) the odds of *C. difficile*-only colonization in Kenya children, 5 times (95% CI [1.11-22.66]) in Kenyan adults, and 4 times (95% CI [1.39-2.53]) in Texas adults with CDI (Figure 16).

Because of the importance of vancomycin in CDI treatment, the proportion of CDI stools containing VRE was determined by culturing stool samples on SBM and incubating aerobically for growth (219). VRE was present in 6% of the stools of Kenyan children, 27% of Kenyan adults, and 31% of Texas adults with CDI (Figure 17). The stools were further examined to determine whether there were vancomycin-resistant *C. difficile* strains colonizing the same patients. Co-colonization by vancomycin-resistant isolates of both bacteria occurred in 42% of the Texas patients and 45% of the Kenyan adult patients.

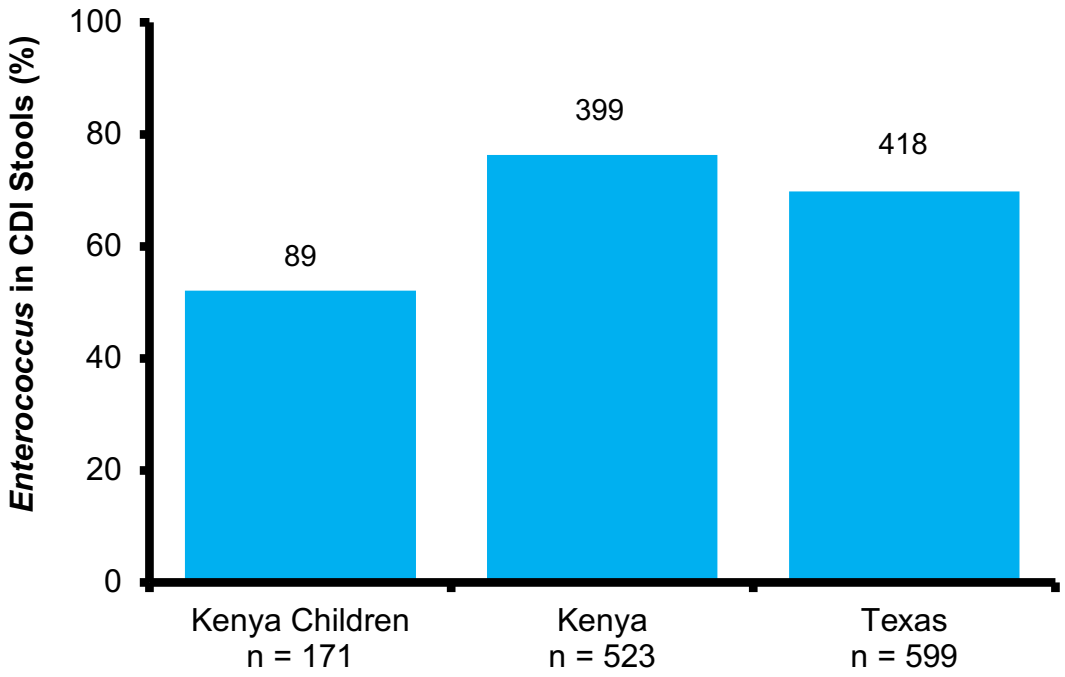

Figure 16: Enterococci colonization in CDI stools. Stools were streaked onto Slanetz-Bartley Media (SBM) and incubated aerobically at 37 ºC for up to 48 hours. Samples were considered presumptively positive for enterococcus if colony growth was apparent on the plates. Colonies varied from an opaque, white color to a deep maroon color. Colony size also varied. In some cases, multiple colony types were present on the plate. Negative results indicated that no colony growth was observed on the plate.

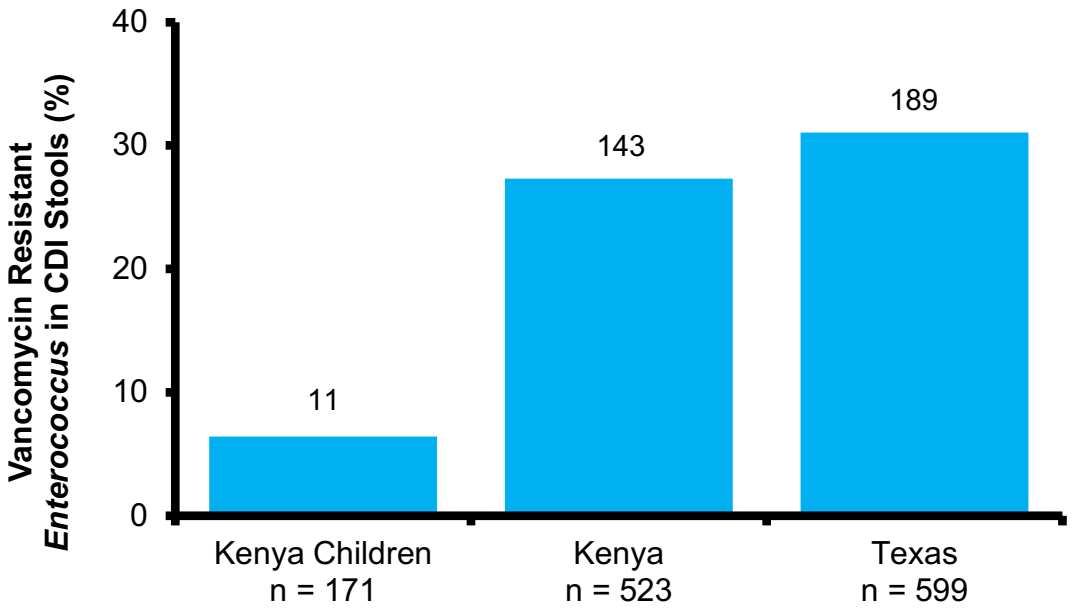

Figure 17*:* Proportion of vancomycin-resistant enterococci in CDI stools*.* Stools were streaked onto SBM containing 4 μg/ml vancomycin and incubated aerobically at 37 ºC for up to 48 hours. Samples were considered positive for vancomycin-resistance if colony growth was apparent on the plates. Colonies varied from an opaque, white color to a deep maroon color. Colony size also varied. In some cases, multiple colony types were present on the plate. Negative results indicated that no colony growth was observed on the plate.

The stools were further examined to determine whether there were vancomycin-resistant *C. difficile* strains colonizing the same patients. Co-colonization by vancomycin-resistant isolates of both bacteria occurred in 42% of the Texas patients and 45% of Kenyan patients. By comparing frequencies, both Texas (χ^2 = 30.6, p < 0.0001, df = 1) and Kenyan adults (χ^2 = 29.8, p < 0.0001, df = 1) with CDI showed a statistically significant correlation between vancomycin-resistant *C. difficile* and VRE in the same stool (Figure 18). These results suggested that both types of vancomycin-resistant bacteria are not present together in the same stool by chance.

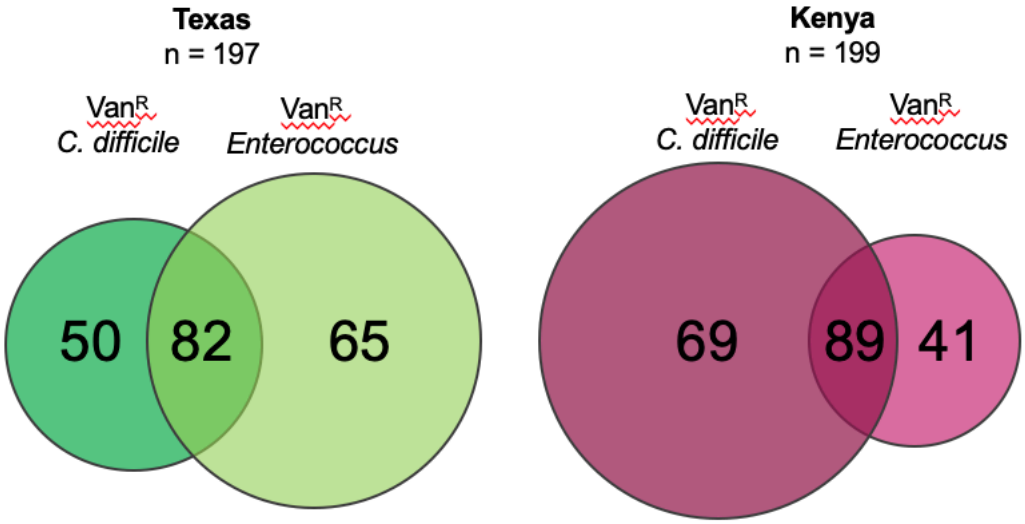

Figure 18: **Association between vancomycin-resistant *C. difficile* and vancomycin-resistant enterococci.** Data analysis was performed in RStudio (version 1.2.1335) using Pearson's Chi-Squared Test for Independence with Yate's Continuity Correction and revealed a p-value of <0.001 for both Texas and Kenya cohorts.

3.4. Effect of Enterococci on CDI virulence factors

Toxin production and sporulation were examined for isolates grown from stool samples to determine if enterococci co-colonization in CDI stools affect *C. difficile* pathogenesis. The results showed that enterococci co-colonization did not impact *C. difficile* toxin production (χ^2 = 0.18, p = 0.67, df = 1). However, there was a significantly higher percentage (45.2%) of spore-producing isolates in *C. difficile*-only stools versus co-colonized stools (χ^2 = 12.03, p = 0.0005, df = 1) (Figure 19). The odds of having spores in *C. difficile*-only stools was 3 times higher than the odds of having spores in co-colonized stools (95% CI = [1.48-4.37]).

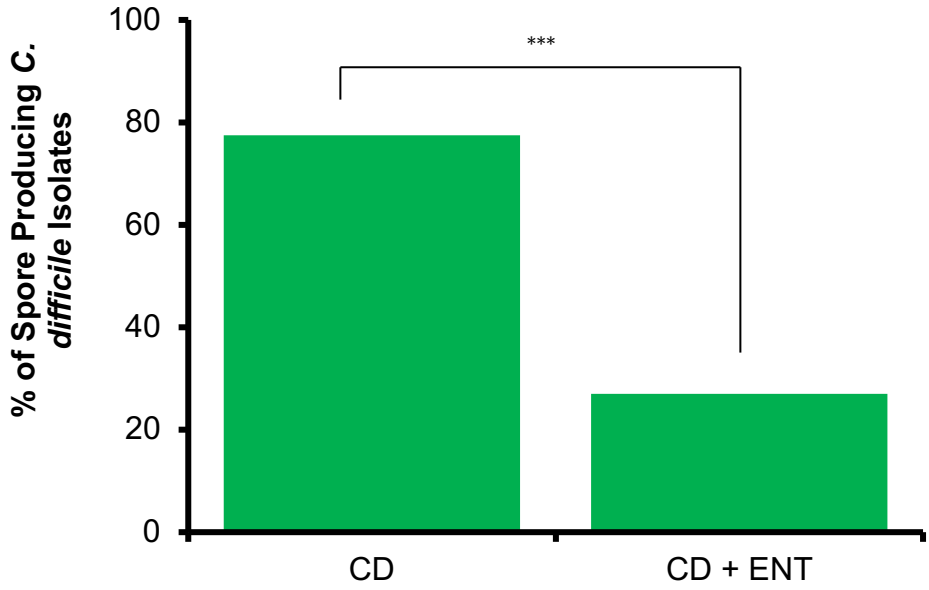

Figure 19: Proportion of spore-producing *C. difficile* isolates in CDI and co-colonized stools. There was a significantly higher number of spore-producing isolates in the *C. difficile*-only colonized stool versus the co-colonized stool (χ^2 = 37.72, $p < 0.0001$, df = 1).

3.5. Effect of Enterococci co-colonization on CDI patients

A subset of the Kenyan CDI patients whose medical data were available was examined to further understand the clinical implication of enterococci co-colonization in CDI patients. In adults, enterococci-*C. difficile* co-colonization was observed most often in the 30-39 age group (45%), followed by 40-49 age group (22.5%), and 50-59 age group (18.5%). *C. difficile*-only colonization was most common among the 40-49 age group. More than 60% of all groups were married, which may have increased risk of infection in these patients. A higher percentage of the females (57%) were co-colonized compared to the males (Table 13, A). For the children, 0-12 month age group had the highest percentage of co-colonization (52%). However, 68% of this age group were neither infected with *C. difficile* nor enterococci, suggesting that diarrhea in these children was caused by other agents. The 13-24 month age group had the next highest percentage (43%) of co-colonization (Table 13, B).

Table 13: Characteristics of Kenyan patients with *Enterococci* and *C. difficile* co-colonization in (A) adult stool and (B) children stool.

A. Adults

Metric	ENT$^+$/CDI$^+$		ENT$^+$/CDI$^-$		ENT$^-$/CDI$^+$		ENT$^-$/CDI$^-$	
	N = 49	%	N = 5	%	N = 6	%	N = 5	%
Gender								
Female	28	57	2	40	3	50	2	40
Male	21	43	3	60	3	50	3	60
Age (Years)								
19-29	7	14	0	0	1	17	1	20
30-39	22	45	1	20	2	33	1	20
40-49	11	22.5	1	20	3	50	2	40
50-59	9	18.5	2	40	0	0	1	20
60-69	0	0	1	20	0	0	0	0
Married	39	80	5	100	5	83	3	60
Employed	24	49	2	40	3	50	2	40

B. Children

Metric	ENT$^+$/CDI$^+$		ENT$^+$/CDI$^-$		ENT$^-$/CDI$^+$		ENT$^-$/CDI$^-$	
	N = 42	%	N = 46	%	N = 19	%	N = 63	%
Gender								
Female	20	48	16	35	6	32	21	33
Male	22	52	28	61	13	68	40	64
Unknown	0	0	2	4	0	0	2	3
Age (Months)								
0-12	22	52	22	48	10	53	43	68
13-24	18	43	17	37	8	42	12	19
25-36	0	0	1	2	0	0	3	5
Unknown	2	5	6	13	1	5	5	8

Duration of CDI diarrhea was also examined in relation to co-colonization. There was a significantly higher number of patients (n = 32) with chronic diarrhea in the co-colonized group versus patients that were only colonized with *C. difficile* (n = 4) (χ^2 = 9.62, p = 0.001925, df = 1). The odds of having chronic diarrhea in co-colonized patients was 5 times higher than that of chronic diarrhea from *C. difficile*-only colonization (95% CI = 1.70-15.90). These data suggested that co-colonization may play a role in the duration of diarrhea (Figure 20).

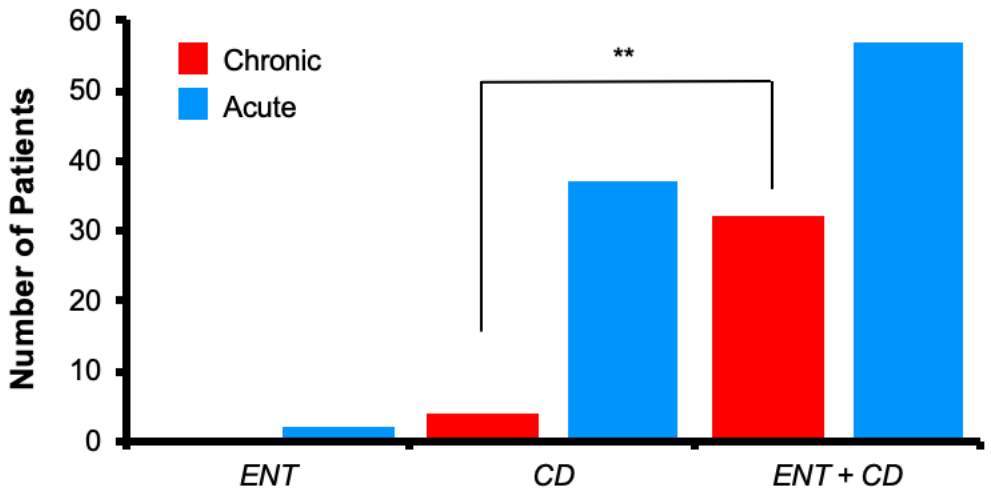

Figure 20: Effect of enterococci and *C. difficile* co-colonization on disease duration in the Kenyan adult patients. Each group indicates whether patient stool contained enterococci only, *C. difficile* only or co-colonization by both bacteria. These groups are broken down further into the number of patients that presented with either chronic (> 2 weeks) or acute (≤ 2 weeks) diarrhea. *p*-values less than 0.05 were considered significant.

Antibiotic usage by the patients prior to developing diarrhea was examined, since it is a major risk factor for intestinal overgrowth for both *C. difficile* and enterococci. Penicillin was the antibiotic class taken most often by the patients before their hospital visit (83.6%), followed my nitroimidazoles (40.8%), macrolide (24.5%), fluoroquinolone (24.5%), proton-pump inhibitor (PPI) (24.5%), sulfonamide (22.4%), and cephalosporins (20.4%). Fluoroquinolones showed a significant increase in percent (16.3%) of patients with chronic CDI symptoms versus acute CDI symptoms (χ^2 = 5.85, p = 0.0155, df = 1). There was no statistically significant difference between chronic and acute diarrhea in patients taking the other antibiotics. Together, these data suggest that previous broad-spectrum antibiotics use may promote enterococci-*C. difficile* co-colonization, thereby prolonging the duration of diarrhea (Figure 21).

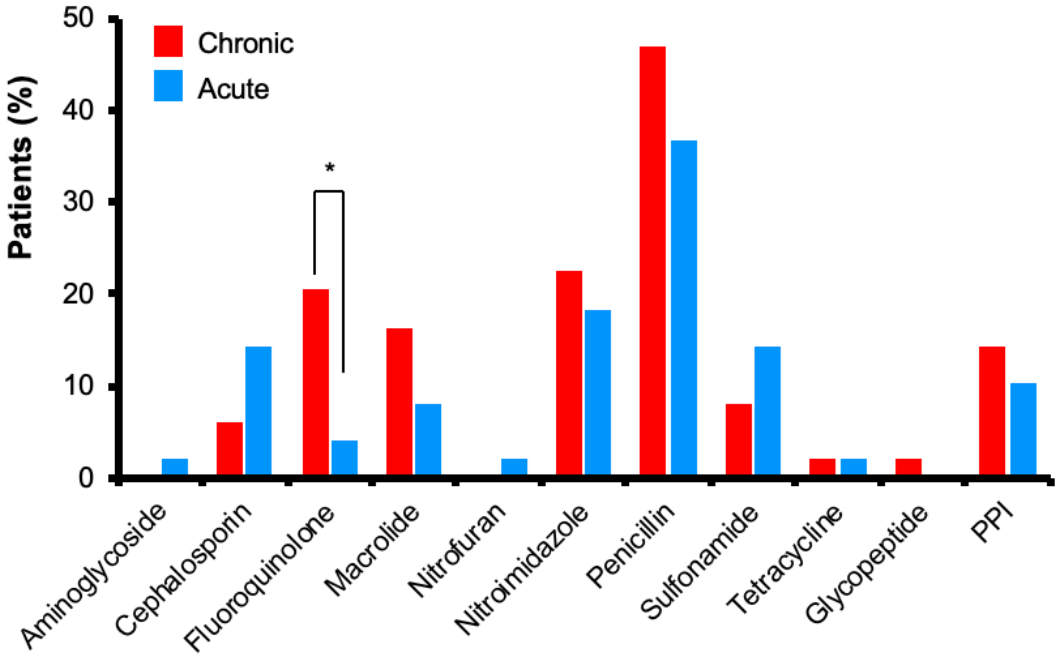

Figure 21: Prior antibiotic use in Kenyan patients co-colonized with enterococci and *C. difficile*. Patient data was analyzed with RStudio (version 1.2.1335) using Pearson's Chi-Squared Test for Independence with Yate's Continuity Correction ($\chi 2 = 5.85$, $p = 0.0155$, $df = 1$).

3.6. Examining potential transfer of vancomycin-resistance elements from enterococci to *C. difficile*

As vancomycin resistance has not been frequently observed in *C. difficile* and it is not intrinsic to the bacterium, we sought to determine the origin of these resistance genes. To that end, we attempted to assess whether there VRE could transfer resistance elements to susceptible *C. difficile* isolates. Initially this was done using VRE isolates and susceptible *C. difficile* isolates from the same stool (Figure 22). No transfer was observed over the course of 10 days of co-culture cycles.

Another approach was taken by utilizing one susceptible *C. difficile* isolate (CD 507) and culturing it with various vancomycin-resistant enterococci isolates and propagating for a total of 5 days. Again, there was no change in susceptibility of the CD507 isolate (Figure 23). These results suggested that transfer may not be occurring between *Enterococcus* spp. and *C. difficile.*

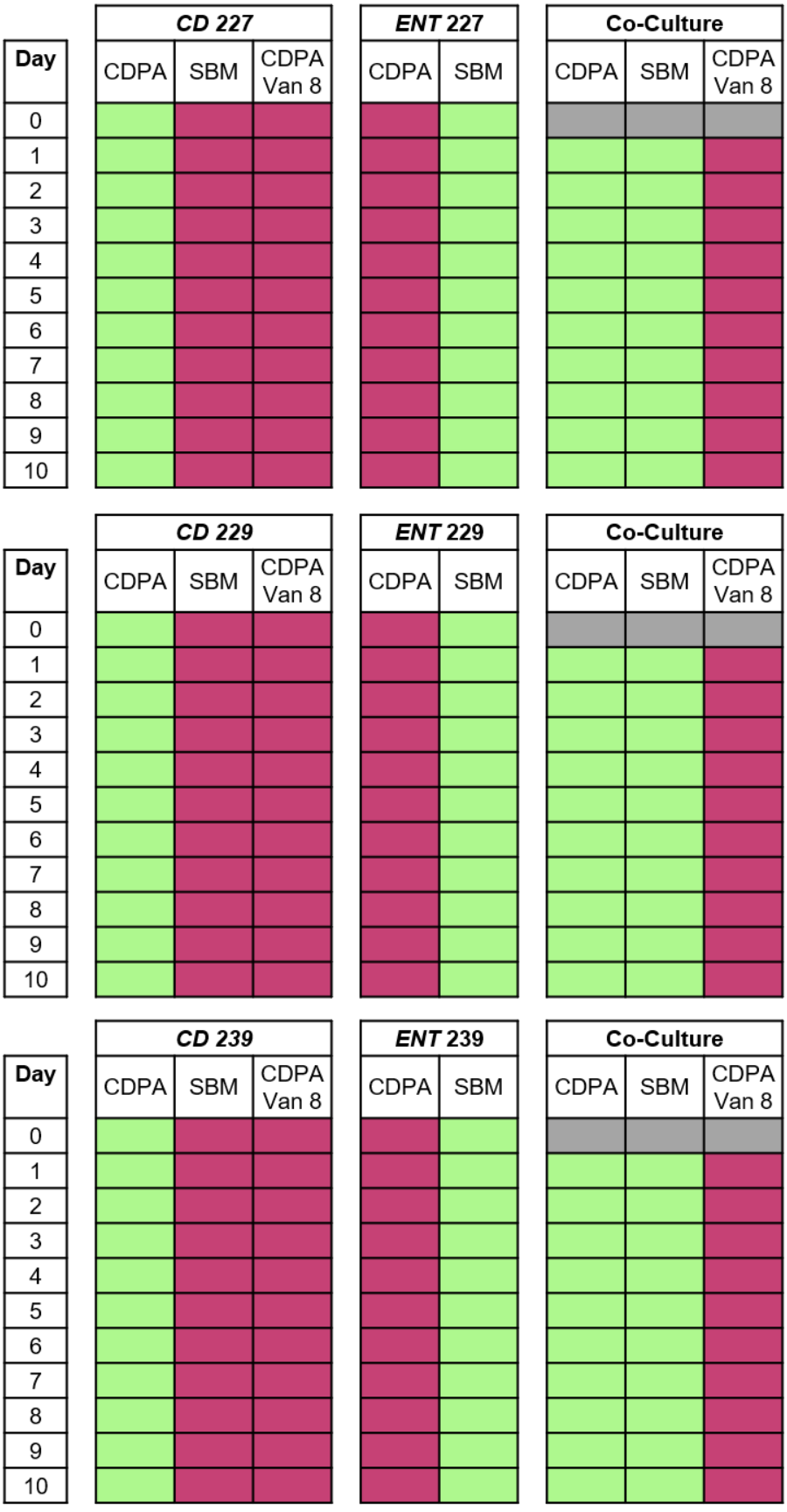
CD 227
ENT 227
Co-Culture
Day
CDPA
SBM
CDPA Van 8
0
1
2
3
4
5
6
7
8
9
10
CD 229
ENT 229
Co-Culture
Day
CDPA
SBM
CDPA Van 8
0
1
2
3
4
5
6
7
8
9
10
CD 239
ENT 239
Co-Culture
Day
CDPA
SBM
CDPA Van 8
0
1
2
3
4
5
6
7
8
9
10

Figure 22: Continued susceptibility of *C. difficile* isolates after co-culture with vancomycin-resistant enterococcus from the same stool. Susceptible *C. difficile* isolates were co-cultured with VRE taken from the same stool. Various media types were used to check for growth and contamination. Samples were propagated for 10 days. Green indicates growth, pink represents no growth.

Day	*CD 507*			*ENT* CAA		Co-Culture		
	CDPA	SBM	CDPA Van 8	CDPA	SBM	CDPA	SBM	CDPA Van 8
0								
1								
2								
3								
4								
5								

Day	*CD 507*			*ENT* 308		Co-Culture		
	CDPA	SBM	CDPA Van 8	CDPA	SBM	CDPA	SBM	CDPA Van 8
0								
1								
2								
3								
4								
5								

Day	*CD 507*			*ENT 466*		Co-Culture		
	CDPA	SBM	CDPA Van 8	CDPA	SBM	CDPA	SBM	CDPA Van 8
0								
1								
2								
3								
4								
5								

Day	*CD 507*			*ENT* 008B		Co-Culture		
	CDPA	SBM	CDPA Van 8	CDPA	SBM	CDPA	SBM	CDPA Van 8
0								
1								
2								
3								
4								
5								

Figure 23: Continued susceptibility of *C. difficile* isolates after co-culture with vancomycin-resistant enterococcus isolate from a different stool sample. One susceptible *C. difficile* isolate was co-cultured with a VRE from different patient stool. Different media types were used to check for growth and contamination. Samples were propagated for 5 days. Green indicates growth, pink describes no growth.

4. Discussion

4.1. *C. difficile* genomes reveal vancomycin-resistance elements

Although *de novo* sequence assemblies from multiple *C. difficile* isolates yielded a variety of genome and GC contents (Table 4), assembled genomes sizes in this study are consistent with published *C. difficile* genome sizes that range from 4.07 Mbp for common *C. difficile* reference genome 630 (224) to between 4.1 and 4.3 Mbp for other strains (217, 225-228). This indicates that our *de novo* assembly method was valid and produced sequences within the normal range of *C. difficile* genomes.

Despite the MiSeq analysis having high depth coverage, long read resequencing of the isolates may be needed to properly assemble the genomes and to enable us to accurately identify components of the vancomycin resistance operons. This can be achieved by utilizing PacBio or Oxford Nanopore sequencing platforms to produce long reads and using a program such Unicycler to produce a hybrid *de novo* assembly with the current MiSeq data. One advantage to using a hybrid assembly is sequence refinement of putative genes and potential variants (229). Duplicated genes and tandem repeats are easily recovered from long-read technologies and can help determine linage of certain gene regions, especially those involved in antimicrobial resistance (230). Additionally, plasmid DNA reconstruction is more reliable when supplemented with long-read sequencing (231). Overall, using hybrid assemblies would greatly refine vancomycin resistance gene sequences identified and would provide a more accurate depiction of the genes present in the vancomycin non-susceptible *C. difficile* isolates.

The suggested coverage depth for bacterial genomes to ensure adequate single nucleotide polymorphisms (SNPs) discovery is 60X (232). Our coverage depths were well over double what the literature considers to be adequate for sequencing SNPs.

Even though the N(50) values give some measure of the average contig length, it is difficult to compare this value among the isolates sequenced because they vary in length. As expected, the N(75) values are less than the N(50) values but still give a good indication of the sequence assemblies. Overall, the high coverage depth and N(50) values indicate thorough sequencing experiments for the isolates. In terms of the average percent total coverage, the isolates were all close to 93% coverage. Depending on the isolate, the values ranged from 90-97% when compared to a single isolate.

Both RAST and Prokka use different algorithms for annotation and thus, the consistency between these two annotation methods confirmed presence of vancomycin-resistance elements. In evaluating the quality of annotation with RAST, various “functional roles” or genes associated with *C. difficile* were correctly identified (Table 6). The coarse consistencies are all relatively high, indicating that annotations are self-consistent. The fine consistency, although expected to be lower than the coarse consistency, was very low in the case of TMC024S and, to a lesser extent, TMC109V. This means that the number of gene instances predicted was lower for these samples than the actual number. Given that annotations for regions of interest were also checked manually through NCBI BLAST, the chance of incorrect annotations was low. Overall, utilizing RAST as a system for gene annotation was successful in pinpointing vancomycin-resistance elements in *C. difficile* isolates.

Mapping of the vancomycin resistance genes showed a variety of elements both contained as a set of genes and isolated elements. Additionally, all operons in the *C. difficile* isolates were contained between the same two genes: a FAD-dependent oxidoreductase upstream of the operon and a TCP38/MEM64 family protein downstream (Figure 6-Figure 11). This putative vancomycin resistance operon is conserved among our isolates. Examination of the gene order shows a *vanT* gene, indicative of a D-alanine-D-serine type resistance. This arrangement matches the VanG operon found in *Enterococcus* species, however, the *C. difficile* putative vanG operon lacks *vanU, vanW*, and *vanY* elements. This suggests that a direct transfer was unlikely, and if a transfer did occur, it occurred much earlier in time. The gene arrangement seems widespread in the *C. difficile* isolates, with about 85% of isolates containing *vanG*-like clusters, but various studies have shown that these genes are not functional and do not contribute to significantly to vancomycin resistance (110, 163, 164). Several VanG arrangements have been described in enterococci and do not seem to be well conserved among *E. faecium* (233, 234). In other *Clostridium* species, the arrangement of *vanG*-like operons is not well conserved. While the arrangement of *C. difficile* VanG has been described as VanRSGXYT, *Clostridium acidurici* contains VanRWGXYT, and *Clostridium argentinense* has VanYGT$_m$T$_r$ (235). Analysis of *vanG*-like operon arrangements could not be compared for *Clostridia* species most closely related to our isolates, as that information was unavailable. Even though there are differences in gene arrangements among *Clostridia,* the most probable evolutionary scenario is an ancient event involving *Clostridium* species members, causing divergence from other Gram-positive anaerobes.

Other interesting features include the presence of isolated *vanZ* and *vanW* genes in the isolates. The *vanW* gene is reported as a putative hydrolase and has only been reported in *C. difficile* associated within a silent *vanB2* operon (236). In all the isolates, there were two *vanW* genes present. One gene is flanked by an exonuclease/endonuclease/phosphatase (EEP) family protein and a protein kinase, while the other resides between a fis-family transcriptional regulator and a ubiD family decarboxylase. One exception is the TMC024S isolate. Because the *vanW* genes were present on shorter contigs, they were not large enough to capture sequences flanking *vanW* and we were unable to confirm if the same trend was seen in this isolate. Again, the presence of these genes seems to have been derived from an earlier ancestor. The presence of the *vanW* gene needs to be explored further in terms of expression, contribution to vancomycin non-susceptibility, and gene regulation.

Four orthologs of *E. faecium vanZ* have been identified in *C. difficile* and have been shown to confer modest teicoplanin resistance despite the lack of an associated VanA operon (237). Additionally, it seems expression of *vanZ* in *C. difficile* is induced by the antimicrobial peptide LL-37 (237). Because *vanZ* is closely associated with the VanA operon (238) many questions arise about how *vanZ* became isolated in *C. difficile.* Even though it has been shown that this gene does not contribute to vancomycin resistance, it is important to understand the origin of this gene to find avenues to test other isolated resistance genes.

Three isolates (TMC68V_1B, TMC544V_2, and TMC579) contained a *vanRS* two-component system without any other vancomycin-resistance genes associated with

it. The purpose of these genes remains unclear. A future study could address functionality and regulation of these genes.

TMC024S was unique compared to the other isolates. It contained the *van* operon, but also a VanB-type sensor kinase, and a *vanH* gene, absent from all the other isolates. Since *vanH* is a feature of the VanA operon, and other resistance types producing precursors terminating in D-ala-D-lactate, a thorough investigation needs to be conducted to determine its role, if any, in non-susceptibility.

The presence of a vancomycin-resistance operon in non-susceptible isolates was expected, however, the presence of isolated genes from various resistance types was not. It is possible that *C. difficile*-specific recombination systems could have altered conjugation or transposition products yielding one or two gene components throughout the genome (239). Given that *vanZ* has been shown to be functional independent of an operon, it opens the door to many questions about how the other individual genes interact in the *C. difficile* genome. Perhaps a more likely explanation is that the short-read sequencing was not adequate for accurate assembly of the genome and may need to be supplemented with long read sequencing data to resolve areas where only one or two genes may be present.

Although it was hypothesized that vancomycin resistance elements may originate from *Enterococcus* spp., phylogenetic analysis of *vanG* ligases and the *vanT* racemase among *Clostridium* species, other Gram-positive anaerobes, and enterococci showed the putative ligases in the isolates had the highest similarity among the *Clostridium* genus. However, it is important to note that this gene may not contribute to vancomycin non-susceptibility in *C. difficile* (163), so this phylogeny may

not be relevant in determining the origin of genes conferring vancomycin resistance. In terms of the putative *vanH* in TMC024S, phylogenetic and multiple sequence analyses suggested that this gene originated from enterococci, however, functional analysis needs to be performed in order to determine if this gene is contributing to the resistance phenotype.

The two groups of vancomycin resistance elements in the isolates (putative *vanG* operon, and VanB-like elements in TMC024) may follow different evolutionary scenarios. It is unlikely that putative *vanG* genes were transferred from enterococci because of a low sequence identity to enterococcal *vanG*-like elements when compared to *vanG* from other organisms. It is more likely based on the phylogenetic analysis that an ancient event occurred diverting *Clostridia* from other Gram-positive anaerobes (Figure 13).

The putative vanH from TMC024S presents a potential horizontal gene transfer event from enterococci given the high sequence similarity and relatedness (Figure 15). Although this needs to be evaluated more thoroughly, the presence of VanB-like elements in *C. difficile* in one non-susceptible isolate may indicate more than one mechanism of vancomycin non-susceptibility. Again, more robust sequencing needs to be employed to help resolve areas containing these vancomycin resistance genes, as it is unlikely that they are found isolated throughout the genome. This will also address the issue of our putative *vanH* having a partial sequence, which may change the hypothesized horizontal gene transfer event.

4.2. *Sequence analysis suggests vancomycin-resistance elements are located on the chromosome*

By subjecting the clinical isolates to the plasmidSPAdes pipeline, putative plasmid sequences were extracted from the sequencing data. This program operates under the assumption that plasmid DNA is present at a higher copy number than the chromosome and should have a higher coverage (205). These sequences all mapped to either existing chromosomal or plasmid DNA. The recognition of two known plasmid sequences from our sequencing samples validates the use of this program. Analysis of the known plasmid sequences indicated an absence of vancomycin-resistance elements. With failed attempts at experimentally isolating plasmid DNA from these isolates, it is likely that the vancomycin-resistance genes reside on the chromosome.

4.3. Enterococci co-colonization of *C. difficile* patients

C. difficile and enterococci occupy the same niche in the human gut. Establishment of disease and proliferation of these bacteria is often mediated by congruent risk factors. Because of this, we sought to explore the prevalence of enterococci and vancomycin-resistant enterococci in CDI stools and describe how their presence might affect CDI patients. We analyzed stool samples of patients from Kenya and Texas and used patient information for a subset of the Kenyan patients to help inform the discussion.

Our data showed that enterococci colonization varies between the adult and children populations. In terms of enterococci colonization in children, our results showed a higher colonization rate than in the literature. Although enterococci are normal gut commensals, antibiotic usage can increase colonization of *Enterococci* spp. in infant stools (127, 240), one explanation for the increase. There may be other

factors that caused diarrheal symptoms favorable for enterococcal colonization. Not many studies have been done looking at enterococci colonization in children as VRE colonization studies are more relevant in the health landscape at present. Because of this, it is difficult to gauge what is considered higher than normal. Given that gut microbiota changes occur rapidly in the children (240), colonization information may be based on a variety of factors not explored in this study.

Our results indicated a high level of *C. difficile*-vancomycin-resistant enterococci co-colonization among adult patients, which is a little lower than the rates reported by others (145). One reason for the discrepancy between the Fujitani, et. Al study and others would be that only toxin-positive *C. difficile* stools were screened for VRE presence and other studies taking a more unbiased approach have shown that *C. difficile* toxin-positive stools were highly correlated with VRE colonization (119, 140). Kenyan patients may have been treating their diarrhea with antibiotics without the guidance of a medical professional. The use of one or more antibiotics could have exacerbated overgrowth conditions, allowing for an increase in transfer of resistance elements between *Enterococci* spp. Despite the Texan patients having regulated antibiotic use, the proportions of co-colonization were still very high, indicating that over time, VRE may be more prevalent among CDI patient populations around the world. The increase in VRE colonization in CDI patients could increase the risk of a secondary VRE infection as seen with other patient types. For example, in patients with cancer, although only a small percentage may be colonized with VRE (~6%), 60% of those patients go on to develop a VRE-associated infections (241).

4.4. Effects of enterococci on CDI virulence factors

Our data showed a significantly high proportion of *C. difficile* non-susceptibility to vancomycin in stools containing VRE in both Texas and Kenya, describing a possible reservoir of vancomycin-resistance elements. Since the shift to vancomycin as the recommended treatment for CDI (36, 37, 46, 47), increased use of vancomycin may select for *C. difficile* isolates containing vancomycin resistance elements. Coupled with the fact that enterococci and *C. difficile* can occupy the same niche during CDI, a perfect storm may be created for emergence of vancomycin non-susceptible *C. difficile.* There has been evidence of genetic transfer of other antibiotic resistance elements to *C. difficile* (149, 242), so it may be possible in this case as well. Genetic analysis through next generation sequencing (NGS) would allow further insight into the origin of these elements.

Several studies indicated that individuals with toxin-producing *C. difficile* strains also showed a higher level of enterococcal colonization, however, this was not the case in the present study. Possibly, compensatory virulence factors might prevail in this cohort of isolates or some other epidemiological factors could be at play. Interestingly, spore-producing *C. difficile* isolates were significantly more present in co-colonized stools versus singly colonized stools; to our knowledge, this is a result not yet reported in the literature. The selection of spore-producing isolates in the presence of enterococcal colonization might indicate competition between the two bacteria and lead to persistence of CDI symptoms as spore-producing isolates are responsible for CDI recurrence (243).

4.5. Effects on enterococci co-colonization on CDI patients

In terms of CDI disease presentation with enterococcal co-colonization, CDI symptoms appear to be prolonged, with patients experiencing diarrhea for over 2 weeks. As previously stated, this could be due the inability for normal gut microbiota from reestablishing itself. Additionally, fluoroquinolone use prior to a CDI diagnosis also correlates with a longer duration of the diarrheal symptoms. As a risk-factor antibiotic for both enterococci and *C. difficile* overgrowth in the gut, prolonged or improper use of this broad-spectrum antibiotic prevents recolonization of normal gut flora and potentially drives acquisition of antibiotic resistance elements.

Taken together, the data leads us to hypothesize the following model for the role of vancomycin-resistant enterococcal colonization during a *C. difficile* infection. During CDI, co-colonization may lead to chronic diarrhea. Prior antibiotic use by patients can promote *C. difficile* overgrowth in the gut followed by CDI disease presentation. Loss of colonization resistance can also allow enterococcus overgrowth. Presentation of CDI symptoms is combated with vancomycin treatment, allowing VRE to persist, preventing normal flora from re-establishing in the gut. Continued vancomycin treatment will also impede recolonization by normal flora. Spore-producing *C. difficile* isolates may germinate, and reestablish a CDI without normal flora, necessitating more treatment. This perpetuates a cycle in which vancomycin is readily used and selects for VRE, allowing for a gut landscape that may promote recurrence and horizontal gene transfer of vancomycin-resistance elements from enterococcus to *C. difficile.*

This model would be enhanced by knowledge of recurrence rates of CDI in patients co-colonized with enterococci, however, given our present dataset, this was not possible to examine, nor has this been explored in the literature to our knowledge. Sequencing data of the vancomycin non-susceptible *C. difficile* isolates may reveal if resistance elements have been transferred from enterococci. The present study demonstrates that co-colonization of CDI patients by enterococci may impact duration of CDI symptoms, and presence of VRE may drive vancomycin non-susceptibility in *C. difficile.* Our data highlight the importance of further study examining VRE co-colonization in CDI patients.

4.6. Transfer of vancomycin-resistance elements

The co-culture propagation experiments to explore potential transfer of resistance elements via conjugation were unsuccessful, however, there are other factors to explore before concluding that transfer of vancomycin-resistance elements does not happen between enterococci and *C. difficile.* Additionally, lack of transfer with this experiment does not preclude transfer by transformation, a low frequency event.

One thing to be considered is the strain of *C. difficile* isolate used. In our experiment, the strain of clinical isolate was unknown. Although laboratory *C. difficile* strains (i.e. CD630) show a high conjugation efficiency, ribotype 027 strains R20291 of *C. difficile*, which are becoming more endemic in the clinical setting, have low conjugation frequencies (244). Since our isolates are from a clinical setting, this may be a factor in the failure of the experiment. Designing experiments that increase conjugation frequency between the bacteria might help answer the question of

transference. For example, a filter mating technique or direct plating method might have a better chance of yielding transconjugants.

4.7. Future Directions

Due to laboratory closures as a result of the COVID-19 pandemic, the experiments suggested below could not be fully explored but still present avenues for furthering this work. As mentioned previously, supplementing current MiSeq data with data from long-read sequencing technologies would result in more refined assemblies and will allow us to conclusively determine the arrangement of the vancomycin-resistance genes in the vancomycin non-susceptible *C. difficile* isolates. Additionally, functional studies of the genes found would be a logical next step, including qRT-PCR to measure expression, gene knockouts, and HPLC analysis of the peptidoglycan precursors. Assays determining racemase activity for *vanT* and dipeptidase activity for *vanXY* can be used to describe the functionality of these genes.

One avenue to pursue moving forward would be to classify this phenotype based on the ribotypes of the isolates. If the non-susceptibility phenotype is isolated to one or a few ribotypes, then this may help inform intervention strategies to limit spread of the resistance genes.

In terms of epidemiology, the impact of vancomycin non-susceptibility needs to be described in terms of disease severity, patient outcomes, and recurrence rates. A comprehensive examination of the relationship between enterococci and *C. difficile* is necessary and may be important in understanding how *C. difficile* pathogenesis may be impacted by other co-inhabiting gut bacteria. Assessing patient outcomes and

recurrence rates in CDI patients co-colonized by enterococci would help define this relationship further.

Finally, presence of the putative *vanH* and other putative VanB-like elements in TMC024S share a high sequence identity with enterococcal orthologs, suggesting that these may have transferred from *Enterococcus* species members. Although functionality of these genes needs to be assessed, it could indicate potential transfer of vancomycin-resistance elements. Although our transfer experiments assessing conjugative transfer were initially unsuccessful, experiments testing for transformation could be pursued before searching for other sources of vancomycin resistance elements in the gut.

5. Bibliography

1. Hall, I. C., and E. O'Toole. 1935. Intestinal Flora in New-Born Infants With a Description of a New Pathogenic Anaerobe, Bacillus difficilis *American Journal of Diseases of Children* 49: 390-402.
2. Lawson, P. A., D. M. Citron, K. L. Tyrrell, and S. M. Finegold. 2016. Reclassification of Clostridium difficile as Clostridioides difficile (Hall and O'Toole 1935) Prevot 1938. *Anaerobe* 40: 95-99.
3. Fernández-García, L., L. Blasco, M. López, and M. Tomás. 2017. Clostridium difficile Infection: Pathogenesis, Diagnosis and Treatment. In *Clostridium Difficile - A Comprehensive Overview*.
4. Bartlett, J. G., N. Moon, T. W. Chang, N. Taylor, and A. B. Onderdonk. 1978. Role of Clostridium difficile in antibiotic-associated pseudomembranous colitis. *Gastroenterology* 75: 778-782.
5. Freeman, J., M. P. Bauer, S. D. Baines, J. Corver, W. N. Fawley, B. Goorhuis, E. J. Kuijper, and M. H. Wilcox. 2010. The changing epidemiology of Clostridium difficile infections. *Clin Microbiol Rev* 23: 529-549.
6. Lessa, F. C., C. V. Gould, and L. C. McDonald. 2012. Current status of Clostridium difficile infection epidemiology. *Clin Infect Dis* 55 Suppl 2: S65-70.
7. Ananthakrishnan, A. N. 2011. Clostridium difficile infection: epidemiology, risk factors and management. *Nat Rev Gastroenterol Hepatol* 8: 17-26.
8. Halstead, F. D., A. Ravi, N. Thomson, M. Nuur, K. Hughes, M. Brailey, and B. A. Oppenheim. 2019. Whole genome sequencing of toxigenic Clostridium

difficile in asymptomatic carriers: insights into possible role in transmission. *J Hosp Infect* 102: 125-134.

9. Jangi, S., and J. T. Lamont. 2010. Asymptomatic colonization by Clostridium difficile in infants: implications for disease in later life. *J Pediatr Gastroenterol Nutr* 51: 2-7.

10. Penders, J., C. Thijs, C. Vink, F. F. Stelma, B. Snijders, I. Kummeling, P. A. van den Brandt, and E. E. Stobberingh. 2006. Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics* 118: 511-521.

11. Rousseau, C., F. Levenez, C. Fouqueray, J. Dore, A. Collignon, and P. Lepage. 2011. Clostridium difficile colonization in early infancy is accompanied by changes in intestinal microbiota composition. *J Clin Microbiol* 49: 858-865.

12. Miyajima, F., P. Roberts, A. Swale, V. Price, M. Jones, M. Horan, N. Beeching, J. Brazier, C. Parry, N. Pendleton, and M. Pirmohamed. 2011. Characterisation and carriage ratio of Clostridium difficile strains isolated from a community-dwelling elderly population in the United Kingdom. *PLoS One* 6: e22804.

13. Arvand, M., V. Moser, C. Schwehn, G. Bettge-Weller, M. P. Hensgens, and E. J. Kuijper. 2012. High prevalence of Clostridium difficile colonization among nursing home residents in Hesse, Germany. *PLoS One* 7: e30183.

14. Galdys, A. L., J. S. Nelson, K. A. Shutt, J. L. Schlackman, D. L. Pakstis, A. W. Pasculle, J. W. Marsh, L. H. Harrison, and S. R. Curry. 2014. Prevalence and

duration of asymptomatic Clostridium difficile carriage among healthy subjects in Pittsburgh, Pennsylvania. *J Clin Microbiol* 52: 2406-2409.

15. Manzoor, S. E., C. A. M. McNulty, D. Nakiboneka-Ssenabulya, D. M. Lecky, K. J. Hardy, and P. M. Hawkey. 2017. Investigation of community carriage rates of Clostridium difficile and Hungatella hathewayi in healthy volunteers from four regions of England. *J Hosp Infect* 97: 153-155.
16. Bartlett, J. G. 2006. Narrative Review: The New Epidemic of *Clostridium difficile*– Associated Enteric Disease. *Annals of Internal Medicine* 145: 758-764.
17. Brazier, J. S., and S. P. Borriello. 2000. Microbiology, Epidemiology and Diagnosis of Clostridium difficile Infection. *Current Topics in Microbiology and Immunology* 250: 1-33.
18. Burke, K. E., and J. T. Lamont. 2014. Clostridium difficile infection: a worldwide disease. *Gut Liver* 8: 1-6.
19. Baines, S. D., and M. H. Wilcox. 2015. Antimicrobial Resistance and Reduced Susceptibility in Clostridium difficile: Potential Consequences for Induction, Treatment, and Recurrence of C. difficile Infection. *Antibiotics (Basel)* 4: 267-298.
20. Di Bella, S., P. Ascenzi, S. Siarakas, N. Petrosillo, and A. di Masi. 2016. Clostridium difficile Toxins A and B: Insights into Pathogenic Properties and Extraintestinal Effects. *Toxins (Basel)* 8.
21. Merrigan, M., A. Venugopal, M. Mallozzi, B. Roxas, V. K. Viswanathan, S. Johnson, D. N. Gerding, and G. Vedantam. 2010. Human hypervirulent

Clostridium difficile strains exhibit increased sporulation as well as robust toxin production. *J Bacteriol* 192: 4904-4911.

22. Kim, J., S. A. Smathers, P. Prasad, K. H. Leckerman, S. Coffin, and T. Zaoutis. 2008. Epidemiological features of Clostridium difficile-associated disease among inpatients at children's hospitals in the United States, 2001-2006. *Pediatrics* 122: 1266-1270.

23. Dubberke, E. R., and A. I. Wertheimer. 2009. Review of current literature on the economic burden of Clostridium difficile infection. *Infect Control Hosp Epidemiol* 30: 57-66.

24. Leffler, D. A., and J. T. Lamont. 2015. Clostridium difficile infection. *N Engl J Med* 372: 1539-1548.

25. Zimlichman, E., D. Henderson, O. Tamir, C. Franz, P. Song, C. K. Yamin, C. Keohane, C. R. Denham, and D. W. Bates. 2013. Health care-associated infections: a meta-analysis of costs and financial impact on the US health care system. *JAMA Intern Med* 173: 2039-2046.

26. Abt, M. C., P. T. McKenney, and E. G. Pamer. 2016. Clostridium difficile colitis: pathogenesis and host defence. *Nat Rev Microbiol* 14: 609-620.

27. Rea, M. C., A. Dobson, O. O'Sullivan, F. Crispie, F. Fouhy, P. D. Cotter, F. Shanahan, B. Kiely, C. Hill, and R. P. Ross. 2011. Effect of broad- and narrow-spectrum antimicrobials on Clostridium difficile and microbial diversity in a model of the distal colon. *Proc Natl Acad Sci U S A* 108 Suppl 1: 4639-4644.

28. Trzasko, A., J. A. Leeds, J. Praestgaard, M. J. Lamarche, and D. McKenney. 2012. Efficacy of LFF571 in a hamster model of Clostridium difficile infection. *Antimicrob Agents Chemother* 56: 4459-4462.

29. Orrell, K. E., Z. Zhang, S. N. Sugiman-Marangos, and R. A. Melnyk. 2017. Clostridium difficile toxins A and B: Receptors, pores, and translocation into cells. *Crit Rev Biochem Mol Biol* 52: 461-473.

30. Madan, R., and W. A. Petri, Jr. 2012. Immune responses to Clostridium difficile infection. *Trends Mol Med* 18: 658-666.

31. Rupnik, M., M. H. Wilcox, and D. N. Gerding. 2009. Clostridium difficile infection: new developments in epidemiology and pathogenesis. *Nat Rev Microbiol* 7: 526-536.

32. Goudarzi, M., S. S. Seyedjavadi, H. Goudarzi, E. Mehdizadeh Aghdam, and S. Nazeri. 2014. Clostridium difficile Infection: Epidemiology, Pathogenesis, Risk Factors, and Therapeutic Options. *Scientifica (Cairo)* 2014: 916826.

33. Vaishnavi, C. 2010. Clinical spectrum & pathogenesis of *Clostridium difficile* associated diseases. *Indian J Med Res* 131: 487-499.

34. Kyne, L. 2010. *Clostridium difficile*-Beyond Antibiotics. *The New England Journal of Medicine* 362: 264-265.

35. Marra, F., and K. Ng. 2015. Controversies Around Epidemiology, Diagnosis and Treatment of Clostridium difficile Infection. *Drugs* 75: 1095-1118.

36. Al-Nassir, W. N., A. K. Sethi, Y. Li, M. J. Pultz, M. M. Riggs, and C. J. Donskey. 2008. Both oral metronidazole and oral vancomycin promote persistent overgrowth of vancomycin-resistant enterococci during treatment of

Clostridium difficile-associated disease. *Antimicrob Agents Chemother* 52: 2403-2406.

37. Bartlett, J. G. 2008. The case for vancomycin as the preferred drug for treatment of Clostridium difficile infection. *Clin Infect Dis* 46: 1489-1492.

38. Gerding, D. N., S. Johnson, L. R. Peterson, M. E. Mulligan, and J. Silva, Jr. 1995. Clostridium difficile-associated diarrhea and colitis. *Infect Control Hosp Epidemiol* 16: 459-477.

39. Gerding, D. N. 2005. Metronidazole for Clostridium difficile-associated disease: is it okay for Mom? *Clin Infect Dis* 40: 1598-1600.

40. Prevention, C. f. D. C. a. 1995. Recommendations for preventing the spread of vancomycin resistance: recommendations of the Hospital Infection Control Practices Advisory Committee (HIC-PAC). *American Journal of Infectious Control* 23: 87-94.

41. Weir, C. B., and J. K. Le. 2019. *Metronidazole*. StatPearls.

42. Edmond, M. B., J. F. Ober, D. L. Weinbaun, M. A. Pfaller, T. Hwang, M. D. Sanford, and R. P. Wenzel. 1995. Vancomycin-Resistant *Enterococcus faecium* Bacteremia Risk Factors for Infection. *Clinical Infectious Diseases* 20: 1126-1133.

43. Dingsdag, S. A., and N. Hunter. 2018. Metronidazole: an update on metabolism, structure-cytotoxicity and resistance mechanisms. *J Antimicrob Chemother* 73: 265-279.

44. Fernandez, A., G. Anand, and F. Friedenberg. 2004. Factors associated with failure of metronidazole in Clostridium difficile-associated disease. *J Clin Gastroenterol* 38: 414-418.

45. Zar, F. A., S. R. Bakkanagari, K. M. Moorthi, and M. B. Davis. 2007. A comparison of vancomycin and metronidazole for the treatment of Clostridium difficile-associated diarrhea, stratified by disease severity. *Clin Infect Dis* 45: 302-307.

46. 2019. New Clostridium difficile Guidelines. The Society for Healthcare Epidemiology of America.

47. Campbell, C. T., M. O. Poisson, and E. O. Hand. 2019. An Updated Review of Clostridium difficile Treatment in Pediatrics. *J Pediatr Pharmacol Ther* 24: 90-98.

48. Reigadas, E., P. Munoz-Pacheco, S. Vazquez-Cuesta, L. Alcala, M. Marin, A. Martin, and E. Bouza. 2017. Rifaximin-resistant Clostridium difficile strains isolated from symptomatic patients. *Anaerobe* 48: 269-272.

49. Alberto, F., K. Kuper, A. Haider, J. Sackey, and D. Finkel. 2019. The Relationship Between Rifaxamin Use and the Prevalence of *Clostridiodes difficile* and Vancomycin-Resistant Enterococcus in Patients with Advanced Liver Disease. . *Open Forum Infectious Disease* 6: S823.

50. Cohen, S. H., D. N. Gerding, S. Johnson, C. P. Kelly, V. G. Loo, L. C. McDonald, J. Pepin, M. H. Wilcox, A. Society for Healthcare Epidemiology of, and A. Infectious Diseases Society of. 2010. Clinical practice guidelines for Clostridium difficile infection in adults: 2010 update by the society for

healthcare epidemiology of America (SHEA) and the infectious diseases society of America (IDSA). *Infect Control Hosp Epidemiol* 31: 431-455.

51. McDonald, L. C., D. N. Gerding, S. Johnson, J. S. Bakken, K. C. Carroll, S. E. Coffin, E. R. Dubberke, K. W. Garey, C. V. Gould, C. Kelly, V. Loo, J. Shaklee Sammons, T. J. Sandora, and M. H. Wilcox. 2018. Clinical Practice Guidelines for Clostridium difficile Infection in Adults and Children: 2017 Update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). *Clin Infect Dis* 66: e1-e48.

52. Al Momani, L. A., O. Abughanimeh, B. Boonpheng, J. G. Gabriel, and M. Young. 2018. Fidaxomicin vs Vancomycin for the Treatment of a First Episode of Clostridium Difficile Infection: A Meta-analysis and Systematic Review. *Cureus* 10: e2778.

53. Artsimovitch, I., J. Seddon, and P. Sears. 2012. Fidaxomicin is an inhibitor of the initiation of bacterial RNA synthesis. *Clin Infect Dis* 55 Suppl 2: S127-131.

54. Babakhani, F., L. Bouillaut, A. Gomez, P. Sears, L. Nguyen, and A. L. Sonenshein. 2012. Fidaxomicin inhibits spore production in Clostridium difficile. *Clin Infect Dis* 55 Suppl 2: S162-169.

55. Babakhani, F., L. Bouillaut, P. Sears, C. Sims, A. Gomez, and A. L. Sonenshein. 2013. Fidaxomicin inhibits toxin production in Clostridium difficile. *J Antimicrob Chemother* 68: 515-522.

56. Bartsch, S. M., C. A. Umscheid, N. Fishman, and B. Y. Lee. 2013. Is fidaxomicin worth the cost? An economic analysis. *Clin Infect Dis* 57: 555-561.

57. Burke, K. E., and J. T. Lamont. 2013. Fecal transplantation for recurrent Clostridium difficile infection in older adults: a review. *J Am Geriatr Soc* 61: 1394-1398.

58. Russell, L., T. Monaghan, and D. Kao. 2018. The need to move away from fecal transplant towards targeted, refined microbiome therapy. *J Thorac Dis* 10: E755-E757.

59. Gerding, D. N., T. Meyer, C. Lee, S. H. Cohen, U. K. Murthy, A. Poirier, T. C. Van Schooneveld, D. S. Pardi, A. Ramos, M. A. Barron, H. Chen, and S. Villano. 2015. Administration of spores of nontoxigenic Clostridium difficile strain M3 for prevention of recurrent C. difficile infection: a randomized clinical trial. *JAMA* 313: 1719-1727.

60. Khanna, S., D. S. Pardi, C. R. Kelly, C. S. Kraft, T. Dhere, M. R. Henn, M. J. Lombardo, M. Vulic, T. Ohsumi, J. Winkler, C. Pindar, B. H. McGovern, R. J. Pomerantz, J. G. Aunins, D. N. Cook, and E. L. Hohmann. 2016. A Novel Microbiome Therapeutic Increases Gut Microbial Diversity and Prevents Recurrent Clostridium difficile Infection. *J Infect Dis* 214: 173-181.

61. Suez, J., N. Zmora, E. Segal, and E. Elinav. 2019. The pros, cons, and many unknowns of probiotics. *Nat Med* 25: 716-729.

62. Surawicz, C. M. 2003. Probiotics, antibiotic-associated diarrhoea and Clostridium difficile diarrhoea in humans. *Best Practice & Research Clinical Gastroenterology* 17: 775-783.

63. Na, X., and C. Kelly. 2011. Probiotics in clostridium difficile Infection. *J Clin Gastroenterol* 45 Suppl: S154-158.

64. Wong, A., D. Y. Ngu, L. A. Dan, A. Ooi, and R. L. Lim. 2015. Detection of antibiotic resistance in probiotics of dietary supplements. *Nutr J* 14: 95.

65. Mathur, S., and R. Singh. 2005. Antibiotic resistance in food lactic acid bacteria--a review. *Int J Food Microbiol* 105: 281-295.

66. McAuliffe, O., M. P. Ryan, R. P. Ross, C. Hill, P. Breeuwer, and T. Abee. 1998. Lacticin 3147, a Broad-Spectrum Bacteriocin Which Selectively Dissipates the Membrane Potential. *Appl Environ Microbiol* 64: 439-445.

67. Rea, M. C., E. Clayton, P. M. O'Connor, F. Shanahan, B. Kiely, R. P. Ross, and C. Hill. 2007. Antimicrobial activity of lacticin 3147 against clinical *Clostridium difficile* strains. *J Med Microbiol* 56: 940-946.

68. Rea, M. C., D. Alemayehu, P. G. Casey, P. M. O'Connor, P. G. Lawlor, M. Walsh, F. Shanahan, B. Kiely, R. P. Ross, and C. Hill. 2014. Bioavailability of the anti-clostridial bacteriocin thuricin CD in gastrointestinal tract. *Microbiology* 160: 439-445.

69. Lay, C. L., L. Dridi, M. G. Bergeron, M. Ouellette, and I. L. Fliss. 2016. Nisin is an effective inhibitor of Clostridium difficile vegetative cells and spore germination. *J Med Microbiol* 65: 169-175.

70. Tam, J., G. L. Beilhartz, A. Auger, P. Gupta, A. G. Therien, and R. A. Melnyk. 2015. Small molecule inhibitors of Clostridium difficile toxin B-induced cellular damage. *Chem Biol* 22: 175-185.

71. Li, C., P. Teng, Z. Peng, P. Sang, X. Sun, and J. Cai. 2018. Bis-Cyclic Guanidines as a Novel Class of Compounds Potent against Clostridium difficile. *ChemMedChem* 13: 1414-1420.

72. Peterfreund, G. L., L. E. Vandivier, R. Sinha, A. J. Marozsan, W. C. Olson, J. Zhu, and F. D. Bushman. 2012. Succession in the gut microbiome following antibiotic and antibody therapies for Clostridium difficile. *PLoS One* 7: e46966.

73. Courvalin, P. 2006. Vancomycin Resistance in Gram-Positive Cocci. *Clinical Infectious Diseases* 42.

74. Siegel, S. D., J. Liu, and H. Ton-That. 2016. Biogenesis of the Gram-positive bacterial cell envelope. *Curr Opin Microbiol* 34: 31-37.

75. Dutka-Malen, S., C. Molinas, M. Arthur, and P. Courvalin. 1990. The VANA glycopeptide resistance protein is related to D-alanyl-D-alanine ligase cell wall biosynthesis enzymes. *Mol Gen Genet* 224: 364-372.

76. Reynolds, P. E. 1961. Studies on the mode of action of vancomycin. *Biochimica et Biophysica Acta* 52: 403-405.

77. Dutka-Malen, S., S. Evers, and P. Courvalin. 1995. Detection of Glycopeptide Resistance Genotypes and Identification to the Species Level of Clinically Relevant Enterococci by PCR. *Journal of Clinical Microbiology* 33: 24-27.

78. Faron, M. L., N. A. Ledeboer, and B. W. Buchan. 2016. Resistance Mechanisms, Epidemiology, and Approaches to Screening for Vancomycin-Resistant Enterococcus in the Health Care Setting. *J Clin Microbiol* 54: 2436-2447.

79. Fernandes, M. M., K. Ivanova, J. Hoyo, S. Perez-Rafael, A. Francesko, and T. Tzanov. 2017. Nanotransformation of Vancomycin Overcomes the Intrinsic

Resistance of Gram-Negative Bacteria. *ACS Appl Mater Interfaces* 9: 15022-15030.

80. Arthur, M., C. Molinas, and P. Courvalin. 1992. The VanS-VanR Two-Component Regulatory System Controls Synthesis of Depsipeptide Peptidoglycan Precursors in *Enterococcus faecium* BM4147. *Journal of Bacteriology* 174: 2582-2591.

81. Arias, C. A., M. Martin-Martinez, T. L. Blundell, M. Arthur, P. Courvalin, and P. Reynolds. 1999. Characterization and modelling of VanT a novel, membrane-bound, serine racemase from vancomycin-resistant *Enterococcus gallinarum* BM4174. *Molecular Microbiology* 31: 1653-1664.

82. Roper, D. I., T. Huyton, A. Vagin, and G. Dodson. 2000. The molecular basis of vancomycin resistance in clinically relevant *Enterococci:* Crystal structure of D-alanyl-D-lactate ligase (VanA). *PNAS* 97: 8921-8925.

83. Cetinkaya, Y., P. Falk, and C. G. Mayhall. 2000. Vancomycin-Resistant Enterococci. *Clinical Microbiology Reviews* 13: 686-707.

84. Ligozzi, M., G. Lo Cascio, and R. Fontana. 1998. *vanA* gene cluster in a vancomycin-resistant clinical isolate of *Bacillus circulans*. *Antimicrobial Agents and Chemotherapy* 42: 2055-2059.

85. Gardete, S., and A. Tomasz. 2014. Mechanisms of vancomycin resistance in Staphylococcus aureus. *J Clin Invest* 124: 2836-2840.

86. Hiramatsu, K., H. Hanaki, T. Ino, K. Yabuta, T. Oguri, and F. C. Tenover. 1997. Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced

vancomycin susceptibility. *Journal of Antimicrobial Chemotherapy* 40: 135-136.

87. Peng, Z., D. Jin, H. B. Kim, C. W. Stratton, B. Wu, Y. W. Tang, and X. Sun. 2017. Update on Antimicrobial Resistance in Clostridium difficile: Resistance Mechanisms and Antimicrobial Susceptibility Testing. *J Clin Microbiol* 55: 1998-2008.

88. Hecht, D. W., M. A. Galang, S. P. Sambol, J. R. Osmolski, S. Johnson, and D. N. Gerding. 2007. In vitro activities of 15 antimicrobial agents against 110 toxigenic clostridium difficile clinical isolates collected from 1983 to 2004. *Antimicrob Agents Chemother* 51: 2716-2719.

89. Dworczyński, A., B. Sokół, and F. Meisel-Mikołajczyk. 1991. Antibiotic resistance of Clostridium difficile isolates. *Cytobios* 65: 149-153.

90. Pelaez, T., R. Alonso, C. Perez, L. Alcala, O. Cuevas, and E. Bouza. 2002. In vitro activity of linezolid against Clostridium difficile. *Antimicrob Agents Chemother* 46: 1617-1618.

91. Goudarzi, M., H. Goudarzi, M. Alebouyeh, M. Azimi Rad, F. S. Shayegan Mehr, M. R. Zali, and M. M. Aslani. 2013. Antimicrobial susceptibility of clostridium difficile clinical isolates in iran. *Iran Red Crescent Med J* 15: 704-711.

92. Adler, A., T. Miller-Roll, R. Bradenstein, C. Block, B. Mendelson, M. Parizade, Y. Paitan, D. Schwartz, N. Peled, Y. Carmeli, and M. J. Schwaber. 2015. A national survey of the molecular epidemiology of Clostridium difficile in Israel:

the dissemination of the ribotype 027 strain with reduced susceptibility to vancomycin and metronidazole. *Diagn Microbiol Infect Dis* 83: 21-24.

93. Freeman, J., J. Vernon, K. Morris, S. Nicholson, S. Todhunter, C. Longshaw, M. H. Wilcox, and G. Pan-European Longitudinal Surveillance of Antibiotic Resistance among Prevalent Clostridium difficile Ribotypes' Study. 2015. Pan-European longitudinal surveillance of antibiotic resistance among prevalent Clostridium difficile ribotypes. *Clin Microbiol Infect* 21: 248 e249-248 e216.

94. Snydman, D. R., L. A. McDermott, N. V. Jacobus, C. Thorpe, S. Stone, S. G. Jenkins, E. J. Goldstein, R. Patel, B. A. Forbes, S. Mirrett, S. Johnson, and D. N. Gerding. 2015. U.S.-Based National Sentinel Surveillance Study for the Epidemiology of Clostridium difficile-Associated Diarrheal Isolates and Their Susceptibility to Fidaxomicin. *Antimicrob Agents Chemother* 59: 6437-6443.

95. Courvalin, P. 2008. Evolution of Glycopeptide Resistance. In *Evolutionary Biology of Bacterial and Fungal Pathogens*. F. Baquero, C. Nombela, G. H. Cassell, and J. A. Gutierrez, eds. ASM Press, Washington, D.C., USA. 279-323.

96. Chancey, S. T., D. Zahner, and D. S. Stephens. 2012. Acquired inducible antimicrobial resistance in Gram-positive bacteria. *Future Microbiol* 7: 959-978.

97. Ahmed, M. O., and K. E. Baptiste. 2018. Vancomycin-Resistant Enterococci: A Review of Antimicrobial Resistance Mechanisms and Perspectives of Human and Animal Health. *Microb Drug Resist* 24: 590-606.

98. Klare, I., C. Konstabel, D. Badstübner, G. Werner, and W. Witte. 2003. Occurrence and spread of antibiotic resistances in Enterococcus faecium. *International Journal of Food Microbiology* 88: 269-290.

99. Cercenado, E. 2011. Enterococcus: Phenotype and Genotype Resistance and Epidemiology in Spain. *Enferm Infecc Microbiol Clin* 29: 59-65.

100. Fisher, K., and C. Phillips. 2009. The ecology, epidemiology and virulence of Enterococcus. *Microbiology* 155: 1749-1757.

101. Arthur, M., C. Molinas, F. Depardieu, and P. Courvalin. 1993. Characterization of Tn1546, a Tn3-Related Transposon Conferring Glycopeptide Resistance by Synthesis of Depsipeptide Peptidoglycan Precursors in *Enterococcus faecium* BM4147. *Journal of Bacteriology* 175: 117-127.

102. Bugg, T. D. H., S. Dutka-Malen, M. Arthur, P. Courvalin, and C. T. Walsh. 1991. Identification of Vancomycin Resistance Protein VanA as a D-Alanine:D-Alanine Ligase of Altered Substrate Specificity. *Biochemistry* 30: 2017-2021.

103. Evers, S., and P. Courvalin. 1996. Regulation of VanB-type vancomycin resistance gene expression by the VanSB-VanRB two-component regulatory system in *Enterococcus faecalis* V583. *Journal of Bacteriology* 178: 1302-1309.

104. Garnier, F., S. Taourit, P. Glaser, P. Courvalin, and M. Galimand. 2000. Characterization of transposon Tn*1549,* conferring VanB-type resistance in *Enterococcus* spp. *Microbiology* 146.

105. Reynolds, P. E., H. A. Snaith, A. J. Maguire, S. Dutka-Malen, and P. Courvalin. 1994. Analysis of peptidoglycan precursors in vancomycin-resistant *Enterococcus gallinarum* BM4174. *Biochem. J.* 301: 5-8.

106. Panesso, D., L. Abadia-Patino, N. Vanegas, P. E. Reynolds, P. Courvalin, and C. A. Arias. 2005. Transcriptional analysis of the vanC cluster from Enterococcus gallinarum strains with constitutive and inducible vancomycin resistance. *Antimicrob Agents Chemother* 49: 1060-1066.

107. Perichon, B., P. Reynolds, and P. Courvalin. 1997. VanD-Type Glycopeptide-Resistant *Enterococcus faecium* BM4339. *Antimicrob Agents Chemother* 41: 2016-2018.

108. Fines, M., B. Perichon, P. Reynolds, D. F. Sahm, and P. Courvalin. 1999. VanE, a new type of acquired glycopeptide resistance in *Enterococcus faecalis* BM4405. *Antimicrob Agents Chemother* 43: 2161-2164.

109. Lafaurie, M., B. Perichon, A. Lefort, C. Carbon, P. Courvalin, and B. Fantin. 2001. Consequences of VanE-type resistance on efficacy of glycopeptides in vitro and in experimental endocarditis due to Enterococcus faecalis. *Antimicrob Agents Chemother* 45: 2826-2830.

110. Ammam, F., J. C. Marvaud, and T. Lambert. 2012. Distribution of the vanG-like gene cluster in Clostridium difficile clinical isolates. *Can J Microbiol* 58: 547-551.

111. Depardieu, F., M. G. Bonora, P. E. Reynolds, and P. Courvalin. 2003. The vanG glycopeptide resistance operon from Enterococcus faecalis revisited. *Mol Microbiol* 50: 931-948.

112. Boyd, D. A., B. M. Willey, D. Fawcett, N. Gillani, and M. R. Mulvey. 2008. Molecular characterization of Enterococcus faecalis N06-0364 with low-level vancomycin resistance harboring a novel D-Ala-D-Ser gene cluster, vanL. *Antimicrob Agents Chemother* 52: 2667-2672.

113. Xu, X., D. Lin, G. Yan, X. Ye, S. Wu, Y. Guo, D. Zhu, F. Hu, Y. Zhang, F. Wang, G. A. Jacoby, and M. Wang. 2010. vanM, a new glycopeptide resistance gene cluster found in Enterococcus faecium. *Antimicrob Agents Chemother* 54: 4643-4647.

114. Lebreton, F., F. Depardieu, N. Bourdon, M. Fines-Guyon, P. Berger, S. Camiade, R. Leclercq, P. Courvalin, and V. Cattoir. 2011. D-Ala-d-Ser VanN-type transferable vancomycin resistance in Enterococcus faecium. *Antimicrob Agents Chemother* 55: 4606-4612.

115. Rice, L. B. 2001. Antimicrobial pressure and the emergence and spread of multiresistant enterococci. *Postgrad Med J* 109: 22-31.

116. Chenoweth, C., and D. Schaberg. 1990. The Epidemiology of Enterococci. *Eur. J. Clin. Microbiol. Infect Dis.* 9: 80-89.

117. Bhardwaj, S., K. Bhamre, J. Dhawale, M. Patil, and S. Divase. 2013. *Enterococcus faecium* and *Enterococcus faecalis*, the nosocomial pathogens with special reference to multi-drug resistance and phenotypic characterization. *International Journal of Pharmaceutical Science and Practive* 2: 1-10.

118. Murray, B. E. 1990. The Life and Times of the Enterococcus. *Clinical Microbiology Reviews* 3: 46-65.

119. Gerding, D. N. 1997. Is There a Relationship Between Vancomycin-Resistant Enterococcal Infection and *Clostridium difficile* Infection. *Clinical Infectious Disease* 25: S206-S210.

120. Dubin, K., and E. G. Pamer. 2014. Enterococci and Their Interactions with the Intestinal Microbiome. *Microbiol Spectr* 5.

121. Galloway-Pena, J. R., S. R. Nallapareddy, C. A. Arias, G. M. Eliopoulos, and B. E. Murray. 2009. Analysis of clonality and antibiotic resistance among early clinical isolates of Enterococcus faecium in the United States. *J Infect Dis* 200: 1566-1573.

122. Gilmore, M. S., F. Lebreton, and W. van Schaik. 2013. Genomic transition of enterococci from gut commensals to leading causes of multidrug-resistant hospital infection in the antibiotic era. *Curr Opin Microbiol* 16: 10-16.

123. Huycke, M. M., D. F. Sahm, and M. S. Gilmore. Multiple-Drug Resistant *Enterococci:* The Nature of the Problem and an Agenda for the Future. *Emerging Infectious Diseases* 4: 239-249.

124. Bibalan, M. H., M. Eshaghi, J. Sadeghi, M. Asadian, T. Narimani, and M. Talebi. 2015. Clonal Diversity in Multi Drug Resistant (MDR) Enterococci Isolated from Fecal Normal Flora. *International Journal of Molecular and Cellular Medicine* 4: 240-244.

125. Adesida, S. A., C. C. Ezenta, A. O. Adagbada, A. A. Aladesokan, and A. O. Coker. 2017. Carriage of Multidrug Resistant Enterococcus Faecium and Enterococcus Faecalis among Apparently Healthy Humans. *Afr J Infect Dis* 11: 83-89.

126. Song, J. Y., I. S. Hwang, J. S. Eom, H. J. Cheong, W. K. Bae, Y. H. Park, and W. J. Kim. 2005. Prevalence and Molecular Epidemiology of Vancomycin-Resistant Enterococci (VRE) Strains Isolated from Animals and Humans in Korea. *The Korean Journal of Internal Medicine* 20: 55-62.

127. Hufnagel, M., C. Liese, C. Loescher, M. Kunze, H. Proempeler, R. Berner, and M. Kruger. 2007. Enterococcal colonization of infants in a neonatal intensive care unit: associated predictors, risk factors and seasonal patterns. *BMC Infect Dis* 7: 1-11.

128. Ubeda, C., Y. Taur, R. R. Jenq, M. J. Equinda, T. Son, M. Samstein, A. Viale, N. D. Socci, M. R. van den Brink, M. Kamboj, and E. G. Pamer. 2010. Vancomycin-resistant Enterococcus domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans. *J Clin Invest* 120: 4332-4341.

129. Tremlett, C. H., D. F. Brown, M. F. Palepou, and N. Woodford. 2001. Two Structurally Distinct VanA Resistance Elements at Different Locations in a Glycopeptide-Resistant Strain of Enterococcus faecalis. *Antimicrob Agents Chemother* 45: 996-997.

130. Paulsen, I. T., L. Banerjei, G. S. A. Myers, K. E. Nelson, R. Seshadri, T. D. Read, D. E. Fouts, J. A. Eisen, S. R. Gill, J. F. Heidelberg, H. Tettelin, R. J. Dodson, L. Umayam, L. Brinkac, M. Beanan, S. Daugherty, R. T. DeBoy, S. Durkin, J. Kolonay, R. Madupu, W. Nelson, J. Vamathevan, B. Tran, J. Upton, T. Hansen, J. Shetty, H. Khouri, T. Utterback, D. Radune, K. A. Ketchum, B.

A. Dougherty, and C. M. Fraser. 2003. Role of mobile DNA in the evolution of vancomycin-resistant Enterococcus faecalis. *Science* 299: 2071-2075.

131. Poduval, R. D., R. P. Kamath, M. Corpuz, E. P. Norkus, and C. S. Pitchumoni. 2000. *Clostridium difficile* and vancomycin-resistant *Enterococcus*: The new nosocomial alliance. *The AMerican Journal of Gastroenterology* 95: 3513-3515.

132. Bartlett, J. G. 2006. Narrative Review: The New Epidemic of *Clostridium difficile* –Associated Enteric Disease. *Annals of Internal Medicine* 145: 758-764.

133. Gilmore, M. S., D. B. Clewell, Y. Ike, and N. Shankar. 2014. *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Massachusetts Eye and Ear Infirmary Boston

134. Duarte, L., a. Arbo, M. Gallardo, I. Riquelme, L. Delgadillo, and H. J. Jimenez. 2019. Factors associated with vancomycin-resistant enterococci colonization in a pediatric intensive care unit of Paraguay: A cross-sectional study on hospital charts. *Medwave* 19.

135. Singh-Naz, N., A. Sleemi, A. Pikis, K. Patel, and J. M. Campos. 1999. Vancomycin-Resistant *Enterococcus faecium* Colonization in Children. *Journal of Clinical Microbiology* 37: 413-416.

136. Hacek, D. M., P. Bednarz, G. A. Noskin, T. Zembower, and L. R. Peterson. 2001. Yield of vancomycin-resistant enterococci and multidrug-resistant Enterobacteriaceae from stools submitted for Clostridium difficile testing

compared to results from a focused surveillance program. *J Clin Microbiol* 39: 1152-1154.

137. Lam, S., C. Singer, V. Tucci, V. H. Morthland, M. A. Pfaller, and H. D. Isenberg. 1995. The challenge of vancomycin-resistant enterococci A clinical and epidemiologic study. *Am J Infect Control* 23: 170-180.

138. Garbutt, J. M., B. Littenberg, B. A. Evanoff, D. Sahm, and L. M. Mundy. 1999. Enteric Carriage of Vancomycin-Resistant *Enterococcus faecium* in Patients Tested for *Clostridium difficile*. *Infect Control Hosp Epidemiol* 20: 664-670.

139. Shay, D. K., S. A. Maloney, M. Montecalvo, S. Banerjee, G. P. Wormser, M. J. Arduino, L. A. Bland, and W. R. Jarvis. 1995. Epidemiology and Mortality Risk of Vancomycin-Resistant Enterococcal Bloodstream Infections. *The Journal of Infectious Diseases* 172: 993-1000.

140. Ozsoy, S., and A. Ilki. 2017. Detection of vancomycin-resistant enterococci (VRE) in stool specimens submitted for Clostridium difficile toxin testing. *Braz J Microbiol* 48: 489-492.

141. Leber, A. L., J. F. Hindler, E. O. Kato, D. A. Bruckner, and D. A. Pergues. 2001. Laboratory-Based Surveillance for Vancomycin-resistant Enterococci: Utility of Screening Stool Specimens Submitted for *Clostridium difficile Toxin Assay*. *Infect Control Hosp Epidemiol* 22: 160-164.

142. Hwang, J. H., J. S. Park, E. Lee, J. Y. Bae, K. H. Song, P. G. Choe, W. B. Park, J. H. Bang, E. S. Kim, S. W. Park, N. J. Kim, M. Oh, and H. B. Kim. 2018. Active surveillance for carbapenem-resistant Enterobacteriaceae, vancomycin-resistant enterococci and toxigenic Clostridium difficile among

patients transferred from long-term care facilities in Korea. *J Hosp Infect* 99: 487-491.

143. Jordens, J. Z., J. Bates, and D. T. Griffiths. 1994. Faecal carriage and nosocomial spread of vancomycin-resistant Enterococcus faecium. *Journal of Antimicrobial Chemotherapy* 34: 515-528.

144. Rafferty, M. E., M. I. McCormick, L. H. Bopp, A. L. Baltch, M. George, R. P. Smith, C. Rheal, W. Ritz, and D. Schoonmaker. 1997. Vancomycin-Resistant Enterococci in Stool Specimens Submitted for C*lostridium difficile C*ytotoxin Assay. *Infect Control Hosp Epidemiol* 18: 342-344.

145. Fujitani, S., W. L. George, M. A. Morgan, S. Nichols, and A. R. Murthy. 2011. Implications for vancomycin-resistant Enterococcus colonization associated with Clostridium difficile infections. *Am J Infect Control* 39: 188-193.

146. Amy, J., P. Johanesen, and D. Lyras. 2015. Extrachromosomal and integrated genetic elements in Clostridium difficile. *Plasmid* 80: 97-110.

147. Sebaihia, M., B. W. Wren, P. Mullany, N. F. Fairweather, N. Minton, R. Stabler, N. R. Thomson, A. P. Roberts, A. M. Cerdeno-Tarraga, H. Wang, M. T. Holden, A. Wright, C. Churcher, M. A. Quail, S. Baker, N. Bason, K. Brooks, T. Chillingworth, A. Cronin, P. Davis, L. Dowd, A. Fraser, T. Feltwell, Z. Hance, S. Holroyd, K. Jagels, S. Moule, K. Mungall, C. Price, E. Rabbinowitsch, S. Sharp, M. Simmonds, K. Stevens, L. Unwin, S. Whithead, B. Dupuy, G. Dougan, B. Barrell, and J. Parkhill. 2006. The multidrug-resistant human pathogen Clostridium difficile has a highly mobile, mosaic genome. *Nat Genet* 38: 779-786.

148. Khodadoost, L., H. Hussain, and P. Mullany. 2017. Plasmids can transfer to Clostridium difficile CD37 and 630Deltaerm both by a DNase resistant conjugation-like mechanism and a DNase sensitive mechanism. *FEMS Microbiol Lett* 364.

149. Jasni, A. S., P. Mullany, H. Hussain, and A. P. Roberts. 2010. Demonstration of conjugative transposon (Tn5397)-mediated horizontal gene transfer between Clostridium difficile and Enterococcus faecalis. *Antimicrob Agents Chemother* 54: 4924-4926.

150. Wasels, F., M. Monot, P. Spigaglia, F. Barbanti, L. Ma, C. Bouchier, B. Dupuy, and P. Mastrantonio. 2014. Inter- and intraspecies transfer of a Clostridium difficile conjugative transposon conferring resistance to MLSB. *Microb Drug Resist* 20: 555-560.

151. Hachler, H., B. Berger-Bachi, and F. H. Kayser. 1987. Genetic characterization of a Clostridium difficile erythromycin-clindamycin resistance determinant that is transferable to Staphylococcus aureus. *Antimicrob Agents Chemother* 31: 1039-1045.

152. Clabots, C. R., L. R. Peterson, and D. N. Gerding. 1988. Characterization of a Nosocomial "Clostridium difficile" Outbreak by Using Plasmid Profile Typing and Clindamycin Susceptibility Testing *The Journal of Infectious Diseases* 158: 731-736.

153. Purdy, D., T. O'Keeffe, M. Elmore, M. Herbert, A. McLeod, M. Bokori-Brown, A. Ostrowski, and N. Minton. 2002. Conjugative Transfer of clostridial shuttle

vectors from *Escherichia coli* to *Clostridium difficile* throuch circumvention of the restiction barrier *Molecular Microbiology* 46: 439-452.

154. Muldrow, L. L., E. R. Archibold, O. L. Nunez-Montiel, and R. J. Sheehy. 1982. Survey of the Extrachromosomal Gene Pool of *Clostridium difficile* Downloaded from. *Journal of Clinical Microbiology* 16: 637-640.

155. Minton, N., G. Carter, M. Herbert, T. O'Keeffe, D. Purdy, M. Elmore, A. Ostrowski, O. Pennington, and I. Davis. 2004. The development of Clostridium difficile genetic systems. *Anaerobe* 10: 75-84.

156. Hill, D., I. Sugrue, C. Tobin, C. Hill, C. Stanton, and R. P. Ross. 2018. The Lactobacillus casei Group: History and Health Related Applications. *Front Microbiol* 9: 2107.

157. Swenson, J. M., R. R. Facklam, and C. Thornsberry. 1990. Antimicrobial Susceptibility of Vancomycin-Resistant *Leuconostoc, Pediococcus,* and *Lactobacillus* Species. *Antimicrob Agents Chemother* 34: 543-549.

158. Topcuoglu, S., T. Gursoy, F. Ovali, O. Serce, and G. Karatekin. 2015. A new risk factor for neonatal vancomycin-resistant Enterococcus colonisation: bacterial probiotics. *J Matern Fetal Neonatal Med* 28: 1491-1494.

159. Kamboj, K., A. Vasquez, and J. M. Balada-Llasat. 2015. Identification and significance of Weissella species infections. *Front Microbiol* 6: 1204.

160. Walter, J., C. Hertel, G. W. Tannock, C. M. Lis, K. Munro, and W. P. Hammes. 2001. Detection of Lactobacillus, Pediococcus, Leuconostoc, and Weissella species in human feces by using group-specific PCR primers and

denaturing gradient gel electrophoresis. *Appl Environ Microbiol* 67: 2578-2585.

161. Kumar, A., D. Augustine, S. Sudhindran, A. M. Kurian, K. R. Dinesh, S. Karim, and R. Philip. 2011. Weissella confusa: a rare cause of vancomycin-resistant Gram-positive bacteraemia. *J Med Microbiol* 60: 1539-1541.

162. Salimnia, H., G. J. Alangaden, R. Bharadwaj, T. M. Painter, P. H. Chandrasekar, and M. R. Fairfax. 2011. Weissella confusa: an unexpected cause of vancomycin-resistant gram-positive bacteremia in immunocompromised hosts. *Transpl Infect Dis* 13: 294-298.

163. Ammam, F., D. Meziane-Cherif, D. Mengin-Lecreulx, D. Blanot, D. Patin, I. G. Boneca, P. Courvalin, T. Lambert, and T. Candela. 2013. The functional vanGCd cluster of Clostridium difficile does not confer vancomycin resistance. *Mol Microbiol* 89: 612-625.

164. Peltier, J., P. Courtin, I. El Meouche, M. Catel-Ferreira, M. P. Chapot-Chartier, L. Lemee, and J. L. Pons. 2013. Genomic and expression analysis of the vanG-like gene cluster of Clostridium difficile. *Microbiology* 159: 1510-1520.

165. Darkoh, C., H. L. Dupont, and H. B. Kaplan. 2011. Novel one-step method for detection and isolation of active-toxin-producing Clostridium difficile strains directly from stool samples. *J Clin Microbiol* 49: 4219-4224.

166. Yang, J. J., Y. S. Nam, M. J. Kim, S. Y. Cho, E. You, Y. S. Soh, and H. J. Lee. 2014. Evaluation of a chromogenic culture medium for the detection of Clostridium difficile. *Yonsei Med J* 55: 994-998.

167. CLSI breakpoints.

168. 2020. EUCAST Breakpoints.

169. Oyaro, M. O., K. Plants-Paris, D. Bishoff, P. Malonza, C. S. Gontier, H. L. DuPont, and C. Darkoh. 2018. High rate of Clostridium difficile among young adults presenting with diarrhea at two hospitals in Kenya. *Int J Infect Dis* 74: 24-28.

170. Oyaro, M. O., K. Plants-Paris, D. Bishoff, P. Malonza, C. S. Gontier, H. L. DuPont, and C. Darkoh. 2018. High rate of Clostridium difficile among young adults presenting with diarrhea at two hospitals in Kenya. *Int J Infect Dis* 74: 24-28.

171. Plants-Paris, K., D. Bishoff, M. O. Oyaro, B. Mwinyi, C. Chappell, A. Kituyi, J. Nyangao, D. Mbatha, and C. Darkoh. 2019. Prevalence of Clostridium difficile Infections Among Kenyan Children with Diarrhea. *International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases*.

172. Darkoh, C., H. L. Dupont, and H. B. Kaplan. 2011. Novel one-step method for detection and isolation of active-toxin-producing Clostridium difficile strains directly from stool samples. *Journal of clinical microbiology* 49: 4219-4224.

173. Wolfe, C., P. Pagano, C. M. Pillar, D. L. Shinabarger, and R. A. Boulos. 2018. Comparison of the in vitro antibacterial activity of Ramizol, fidaxomicin, vancomycin, and metronidazole against 100 clinical isolates of Clostridium difficile by broth microdilution. *Diagn Microbiol Infect Dis* 92: 250-252.

174. (CLSI), C. a. L. S. I. 2012. Methods for Antimicrobial Susceptibility Testing of Anerobic Bacteria. In *Approved Standard*, 8th ed. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania. M11-A18.

175. Karasawa, T., S. Ikoma, K. Yamakawa, and S. Nakamura. 1995. A defined growth medium for *Clostridium difficile*. *Microbiology* 141: 371-375.

176. Bolger, A. M., M. Lohse, and B. Usadel. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30: 2114-2120.

177. Andrews, S. 2010. FastQC: A Quality Control Tool for High Throughput Sequence Data.

178. Bankevich, A., S. Nurk, D. Antipov, A. A. Gurevich, M. Dvorkin, A. S. Kulikov, V. M. Lesin, S. I. Nikolenko, S. Pham, A. D. Prjibelski, A. V. Pyshkin, A. V. Sirotkin, N. Vyahhi, G. Tesler, M. A. Alekseyev, and P. A. Pevzner. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 19: 455-477.

179. Gurevich, A., V. Saveliev, N. Vyahhi, and G. Tesler. 2013. QUAST: quality assessment tool for genome assemblies. *Bioinformatics* 29: 1072-1075.

180. Wattam, A. R., J. J. Davis, R. Assaf, S. Boisvert, T. Brettin, C. Bun, N. Conrad, E. M. Dietrich, T. Disz, J. L. Gabbard, S. Gerdes, C. S. Henry, R. W. Kenyon, D. Machi, C. Mao, E. K. Nordberg, G. J. Olsen, D. E. Murphy-Olson, R. Olson, R. Overbeek, B. Parrello, G. D. Pusch, M. Shukla, V. Vonstein, A. Warren, F. Xia, H. Yoo, and R. L. Stevens. 2017. Improvements to PATRIC, the all-bacterial Bioinformatics Database and Analysis Resource Center. *Nucleic Acids Res* 45: D535-D542.

181. O'Leary, N. A., M. W. Wright, J. R. Brister, S. Ciufo, D. Haddad, R. McVeigh, B. Rajput, B. Robbertse, B. Smith-White, D. Ako-Adjei, A. Astashyn, A. Badretdin, Y. Bao, O. Blinkova, V. Brover, V. Chetvernin, J. Choi, E. Cox, O. Ermolaeva, C. M. Farrell, T. Goldfarb, T. Gupta, D. Haft, E. Hatcher, W. Hlavina, V. S. Joardar, V. K. Kodali, W. Li, D. Maglott, P. Masterson, K. M. McGarvey, M. R. Murphy, K. O'Neill, S. Pujar, S. H. Rangwala, D. Rausch, L. D. Riddick, C. Schoch, A. Shkeda, S. S. Storz, H. Sun, F. Thibaud-Nissen, I. Tolstoy, R. E. Tully, A. R. Vatsan, C. Wallin, D. Webb, W. Wu, M. J. Landrum, A. Kimchi, T. Tatusova, M. DiCuccio, P. Kitts, T. D. Murphy, and K. D. Pruitt. 2016. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. *Nucleic Acids Res* 44: D733-745.

182. Darling, A. E., B. Mau, and N. T. Perna. 2010. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. *PLoS One* 5: e11147.

183. Darling, A. E., A. Tritt, J. A. Eisen, and M. T. Facciotti. 2011. Mauve assembly metrics. *Bioinformatics* 27: 2756-2757.

184. Rissman, A. I., B. Mau, B. S. Biehl, A. E. Darling, J. D. Glasner, and N. T. Perna. 2009. Reordering contigs of draft genomes using the Mauve aligner. *Bioinformatics* 25: 2071-2073.

185. Oliveira, P. H., A. Kim, O. Sekulovic, T. Pak, S. Zhu, E. A. Mead, G. Deikus, M. Touchon, M. Lewis, C. Beckford, N. Zeitouni, D. Altman, E. Webster, I. Oussenko, A. Aggarwal, A. Bashir, G. Patel, C. Hamula, S. Huprikar, R. J.

Roberts, R. Tamayo, E. Schadt, R. Sebra, H. van Bakel, A. Kasarskis, A. Shen, and G. Fang. 2020. Epigenomic landscape of the human pathogen Clostridium difficile

186. Kumar, A., K. W. Davenport, G. Vuyisich, Y. A. Kunde, S. L. Johnson, A. E. K. Dichosa, P. S. G. Chain, A. Rodriguez-Palacios, and J. Gans. 2018. Whole Genome Sequences of Three Clostridium difficile strains isolated from food production systems.

187. Forgetta, V., C. Hernandez, C. Pinsonnault, G. Leveque, C. Nagy, J. Dias, A. Villeneuve, R. Blanchette, I. Brunker, M. Oughton, A. Dascal, and K. Dewar. 2008. Comparative genomics of Clostridium difficile (Genome Quebec/Genome Canada).

188. Lee, J. Y., and J. W. Bae. 2017.

189. Ahn, S. W., S. H. Lee, and S. W. Roh. 2018. Genomic analyses of Clostridioides difficile isolated from human feces.

190. Steglich, M., J. D. Hofmann, J. Sikorski, C. Sproeer, T. Riedel, B. Bunk, H. Overmann, M. Neumann-Schaal, and U. Nuebel. 2018. Convergent loss of ABC transporter genes from Clostridioides difficile genomes is associated with impaired tyrosine uptake and p-cresol production.

191. Kociolek, L. K., A. R. Hauser, D. N. Gerding, R. A. Espinosa, D. W. Hecht, and E. A. Ozer. 2017. Complete Genome Sequence of Clostridioides difficile Epidemic Strain DH/NAP11/106/ST-42 Isolated from Stool from a Pediatric Patient with Diarrhea.

192. Kerrigan, L., C. Long, L. Tallon, L. Sadzewickz, K. Vavikolanu, A. Mehta, J. Aluvathingal, S. Nadendla, Y. Yan, and H. Sichtig. 2019. FDA Database for Regulatory Grad Microbial Sequences (FDA-ARGOS): Supporting development and validation of Infectious Disease Dx tests.

193. Spinler, J. K., A. J. Gonzales-Luna, S. Raza, J. K. Runge, R. A. Luna, T. C. Savidge, and K. W. Garey. 2019. Complete Genome Sequence of *Clostridioides difficile* Ribotype 255 strain Mta-79 - Assembled using Oxford Nanopore and Illumina Sequencing.

194. Yin, C., D. S. Chen, J. ZhuGe, D. McKenna, J. Sagurton, G. Wang, W. Huang, N. Dimitrova, and J. T. Fallon. 2017. Complete Genome Sequences of Four Toxigenic Clostridium difficile Clinical Isolates from patients of Lower Hudson Valley, New York, USA.

195. Aziz, R. K., D. Bartels, A. A. Best, M. DeJongh, T. Disz, R. A. Edwards, K. Formsma, S. Gerdes, E. M. Glass, M. Kubal, F. Meyer, G. J. Olsen, R. Olson, A. L. Osterman, R. A. Overbeek, L. K. McNeil, D. Paarmann, T. Paczian, B. Parrello, G. D. Pusch, C. Reich, R. Stevens, O. Vassieva, V. Vonstein, A. Wilke, and O. Zagnitko. 2008. The RAST Server: rapid annotations using subsystems technology. *BMC Genomics* 9: 1-15.

196. Seemann, T. 2014. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30: 2068-2069.

197. Parks, D. H., M. Imelfort, C. T. Skennerton, P. Hugenholtz, and G. W. Tyson. 2015. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res* 25: 1043-1055.

198. Parrello, B., R. Butler, P. Chlenski, R. Olson, J. Overbeek, G. D. Pusch, V. Vonstein, and R. Overbeek. 2019. A machine learning-based service for estimating quality of genomes using PATRIC. *BMC Bioinformatics* 20: 486.

199. Kumar, S., G. Stecher, M. Li, C. Knyaz, and K. Tamura. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol Biol Evol* 35: 1547-1549.

200. Saitou, N., and M. Nei. 1987. The Neighbor-joining Method: A New Method for Reconstructin Phylogenetic Trees. *Mol Biol Evol* 4: 406-425.

201. Tamura, K., M. Nei, and S. Kumar. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *PNAS* 101: 11030-11035.

202. Tippmann, H.-F. 2004. Analysis for free: Comparing programs for sequence analysis. *Briefings in Bioinformatics* 5: 82-87.

203. Hall, T. A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 41: 95-98.

204. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673-4680.

205. Antipov, D., N. Hartwick, M. Shen, M. Raiko, A. Lapidus, and P. A. Pevzner. 2016. plasmidSPAdes: assembling plasmids from whole genome sequencing data. *Bioinformatics* 32: 3380-3387.

206. Arredondo-Alonso, S., R. J. Willems, W. van Schaik, and A. C. Schurch. 2017. On the (im)possibility of reconstructing plasmids from whole-genome short-read sequencing data. *Microb Genom* 3: e000128.

207. Dannheim, H., T. Riedel, M. Neumann-Schaal, B. Bunk, I. Schober, C. Sproer, C. M. Chibani, S. Gronow, H. Liesegang, J. Overmann, and D. Schomburg. 2017. Manual curation and reannotation of the genomes of Clostridium difficile 630Deltaerm and C. difficile 630. *J Med Microbiol* 66: 286-293.

208. Kerrigan, L., L. J. Talon, L. Sadzewickz, N. Sengamalay, S. Ott, A. Godinez, S. Nagaraj, K. Vavikolanu, J. Aluvathingal, S. Nadendla, and H. Sichtig. 2017. FDA database for regulatory grade microbial sequences (FDA-ARGOS): Supporting the development and validation of infectious disease Dx tests.

209. Arora, N. K., and M. Verma. 2017. Modified microplate method for rapid and efficient estimation of siderophore produced by bacteria. *3 Biotech* 7: 381.

210. Su, H. 2018. Direct Submission. In *Clostridioides difficile strain CD161 plasmid unnamed2, complete sequence*.

211. Su, H. 2018. Direct Submission. In *Clostridioides difficile strain CDT4 plasmid unnamed1, complete*.

212. Wu, Y. 2018. Genetic character of three ST11 Clostridium difficile isolatse with different RTs: tcdC deletion is not the unique feature of RT078 indicated by complete whole genome sequencing In *Direct Submission*.

213. Li, T. 2019. Dual phage-bacteria transcriptional profiles during C. difficile hypervirulent ribotype 078 infected by bacteriophage JD032. In *Direct Submission*.

214. Guo, X., T. Li, Y. Zhu, and Y. Chang. 2019. Investigate genome diversity of CD hypervirulent RT078 strains isolated from animal and human. In *Direct submission*.

215. Monot, M., C. Boursaux-Eude, M. Thibonnier, D. Vallenet, I. Moszer, C. Medigue, I. Martin-Verstraete, and B. Dupuy. 2011. Reannotation of the genome sequence of Clostridium difficile strain 630. *J Med Microbiol* 60: 1193-1199.

216. Riedel, T., B. Bunk, J. Wittmann, A. Thurmer, C. Sproer, S. Gronow, H. Liesegang, R. Daniel, and J. Overmann. 2015. Complete Genome Sequence of the Clostridium difficile Type Strain DSM 1296T. *Genome Announc* 3.

217. He, M., M. Sebaihia, T. D. Lawley, R. A. Stabler, L. F. Dawson, M. J. Martin, K. E. Holt, H. M. Seth-Smith, M. A. Quail, R. Rance, K. Brooks, C. Churcher, D. Harris, S. D. Bentley, C. Burrows, L. Clark, C. Corton, V. Murray, G. Rose, S. Thurston, A. van Tonder, D. Walker, B. W. Wren, G. Dougan, and J. Parkhill. 2010. Evolutionary dynamics of Clostridium difficile over short and long time scales. *Proc Natl Acad Sci U S A* 107: 7527-7532.

218. Smits, W. K., J. S. Weese, A. P. Roberts, C. Harmanus, and B. Hornung. 2018. A helicase-containing module defines a family of pCD630-like plasmids in Clostridium difficile. *Anaerobe* 49: 78-84.

219. Pagel, J., and G. Hardy. 1980. Comparison of selective media for the enumeration and identification of fecal streptococci from natural sources. *Canadian Journal of Microbiology* 26: 1320-1327.

220. Darkoh, C., H. B. Kaplan, and H. L. Dupont. 2011. Harnessing the glucosyltransferase activities of Clostridium difficile for functional studies of toxins A and B. *J Clin Microbiol* 49: 2933-2941.

221. Darkoh, C., C. Odo, and H. L. DuPont. 2016. Accessory Gene Regulator-1 Locus Is Essential for Virulence and Pathogenesis of Clostridium difficile. *MBio* 7.

222. Team, R. 2020. RStudio: Integrated Development for R. RStudio. PBC, Boston, MA.

223. Aragon, T. J., M. P. Fay, D. Wollschlaegar, and A. Omidpanah. 2017. Epidemiology Tools- Package 'epitools'.

224. Peng, Z., S. Liu, X. Meng, W. Liang, Z. Xu, B. Tang, Y. Wang, J. Duan, C. Fu, B. Wu, A. Wu, and C. Li. 2017. Genome characterization of a novel binary toxin-positive strain of Clostridium difficile and comparison with the epidemic 027 and 078 strains. *Gut Pathog* 9: 42.

225. Elliott, B., G. O. Androga, D. R. Knight, and T. V. Riley. 2017. Clostridium difficile infection: Evolution, phylogeny and molecular epidemiology. *Infect Genet Evol* 49: 1-11.

226. Gaulton, T., R. Misra, G. Rose, P. Baybayan, R. Hall, J. Freeman, J. Turton, S. Picton, J. Korlach, S. Gharbia, and H. Shah. 2015. Complete Genome

Sequence of the Hypervirulent Bacterium Clostridium difficile Strain G46, Ribotype 027. *Genome Announc* 3.

227. Stabler, R. A., M. He, L. Dawson, M. Martin, E. Valiente, C. Corton, T. D. Lawley, M. Sebaihia, M. A. Quail, G. Rose, D. N. Gerding, M. Gibert, M. R. Popoff, J. Parkhill, G. Dougan, and B. W. Wren. 2009. Comparative genome and phenotypic analysis of Clostridium difficile 027 strains provides insight into the evolution of a hypervirulent bacterium. *Genome Biol* 10: R102.

228. Brouwer, M. S., E. Allan, P. Mullany, and A. P. Roberts. 2012. Draft genome sequence of the nontoxigenic Clostridium difficile strain CD37. *J Bacteriol* 194: 2125-2126.

229. Amarasinghe, S. L., S. Su, X. Dong, L. Zappia, M. E. Ritchie, and Q. Gouil. 2020. Opportunities and challenges in long-read sequencing data analysis. *Genome Biol* 21: 30.

230. Miller, J. R., P. Zhou, J. Mudge, J. Gurtowski, H. Lee, T. Ramaraj, B. P. Walenz, J. Liu, R. M. Stupar, R. Denny, L. Song, N. Singh, L. G. Maron, S. R. McCouch, W. R. McCombie, M. C. Schatz, P. Tiffin, N. D. Young, and K. A. T. Silverstein. 2017. Hybrid assembly with long and short reads improves discovery of gene family expansions. *BMC Genomics* 18: 541.

231. Berbers, B., A. Saltykova, C. Garcia-Graells, P. Philipp, F. Arella, K. Marchal, R. Winand, K. Vanneste, N. H. C. Roosens, and S. C. J. De Keersmaecker. 2020. Combining short and long read sequencing to characterize antimicrobial resistance genes on plasmids applied to an unauthorized genetically modified *Bacillus*. *Sci Rep* 10: 4310.

232. Pightling, A. W., N. Petronella, and F. Pagotto. 2014. Choice of reference sequence and assembler for alignment of *Listeria monocytogenes* short-read sequence data greatly influences rates of error in SNP analyses. *PLoS One* 9: e104579.

233. McKessar, S. J., A. M. Berry, J. M. Bell, J. D. Turnidge, and J. C. Paton. 2000. Genetic characterization of *vanG*, a novel vancomycin resistance locus of *Enterococcus faecalis*. *Antimicrob Agents Chemother* 44: 3224-3228.

234. Sassi, M., F. Guerin, L. Lesec, C. Isnard, M. Fines-Guyon, V. Cattoir, and J. C. Giard. 2018. Genetic characterization of a VanG-type vancomycin-resistant *Enterococcus faecium* clinical isolate. *J Antimicrob Chemother* 73: 852-855.

235. Berthet, N., B. Perichon, C. Mazuet, D. Chapeton-Montes, C. Bouchier, P. Bouvet, C. Legeay, M. R. Popoff, and P. Courvalin. 2015. A vanG-type locus in *Clostridium argentinense*. *J Antimicrob Chemother* 70: 1942-1945.

236. Knight, D. R., G. O. Androga, S. A. Ballard, B. P. Howden, and T. V. Riley. 2016. A Phenotypically Silent vanB2 Operon Carried on a Tn1549-Like Element in C*lostridium difficile*. *mSphere* 1.

237. Woods, E. C., D. Wetzel, M. Mukerjee, and S. M. McBride. 2018. Examination of the *Clostridioides (Clostridium) difficile* VanZ ortholog, CD1240. *Anaerobe* 53: 108-115.

238. Pootoolal, J., J. Neu, and G. D. Wright. 2002. Glycopeptide Antibiotic Resistance. *Annu. Rev. Pharmacol. Toxicol.* 42: 381-408.

239. Craig, N. L. *Chapter 124: Transposition*.

240. Fanaro, S., R. Chierici, P. Guerrini, and V. Vigi. 2003. Intestinal microflora in early infancy: composition and development. *Acta Paediatr Suppl* 91: 48-55.

241. Matar, M. J., J. Tarrand, I. Raad, and K. V. Rolston. 2006. Colonization and infection with vancomycin-resistant Enterococcus among patients with cancer. *Am J Infect Control* 34: 534-536.

242. Johanesen, P. A., K. E. Mackin, M. L. Hutton, M. M. Awad, S. Larcombe, J. M. Amy, and D. Lyras. 2015. Disruption of the Gut Microbiome: Clostridium difficile Infection and the Threat of Antibiotic Resistance. *Genes* 6: 1347-1360.

243. Zhu, D., J. A. Sorg, and X. Sun. 2018. Clostridioides difficile Biology: Sporulation, Germination, and Corresponding Therapies for C. difficile Infection. *Frontiers in Cellular and Infection Microbiology* 8.

244. Kirk, J. A., and R. P. Fagan. 2016. Heat shock increases conjugation efficiency in Clostridium difficile. *Anaerobe* 42: 1-5.

6. Appendix A: Supplementary data

Table 14*:* Quality of assembly based on WGS of CD6.

Contig	Length (bp)	Coverage	Contig	Length (bp)	Coverage
1	1,098,818	149.7	22	2,162	348.7
2	562,155	322.7	23	1,575	3323.3
3	429,529	316.0	24	1,346	467.5
4	402,523	272.3	25	991	2857.8
5	232,236	371.9	26	775	226.6
6	218,146	350.6	27	748	531.4
7	205,873	295.5	28	695	536.7
8	194,189	297.3	29	603	207.1
9	149,209	358.4	30	586	199.3
10	135,492	353.3	35	525	195.6
11	131,570	335.8	43	469	244.7
12	72,023	359.0	47	421	317.2
13	66,850	336.9	48	400	262.4
14	65,629	322.2	49	396	168.5
15	19,330	363.7	50	369	148.2
16	19,228	255.7	51	354	172.0
17	17,186	285.2	52	334	508.3
18	1,0322	250.2	53	326	53.5
19	9,448	346.9	54	326	138.0
20	6,329	534.7	55	304	195.6
21	5,608	350.4			

Table 15: Quality of assembly based on WGS of TMC109V.

Contig	Length (bp)	Coverage	Contig	Length (bp)	Coverage
1	485,739	286.9	32	5,528	354.9
2	484,738	298.8	33	5,508	344.0
3	455,713	257.2	34	5,225	367.9
4	334,028	324.4	35	4,807	355.7
5	278,293	361.8	36	1,849	655.9
6	162,289	316.3	37	1,849	319.1
7	159,031	259.4	38	1,738	5.3
8	154,893	378.1	40	1,387	5582.8
9	154,299	259.1	44	1,014	5719.8
10	149,805	362.0	58	798	450.8
11	147,450	299.7	71	724	2863.9
12	133,781	383.5	86	706	3828.0
13	104,664	362.1	107	678	5191.7
14	90,433	418.0	143	619	4189.2
15	85,967	330.5	167	597	228.0
16	80,672	330.8	199	562	5.1
17	76,796	337.2	261	524	643.7
18	72,456	366.5	275	520	547.9
19	70,029	351.8	352	476	632.0
20	69,743	344.6	425	452	5.2
21	56,992	318.6	465	439	259.1
22	56,132	382.5	528	421	6.8
23	50,821	333.9	538	419	267.6
24	50,202	313.0	557	415	968.5
25	24,380	377.8	618	404	96.1
26	16,320	285.1	624	402	2575.4
27	12,991	314.3	684	391	397.8
28	12,590	278.6	698	388	90.4
29	9,524	335.3	946	351	692.5
30	9,347	328.6	1016	343	5.2
31	8,321	299.6	1245	315	5.2

Table 16: Quality of assembly based on WGS of TMC544V$_2$.

Contig	Length (bp)	Coverage	Contig	Length (bp)	Coverage
1	353,731	228.5	31	37,854	351.1
2	309,547	234.3	32	37,554	336.6
3	289,219	257.9	33	37,241	375.0
4	244,235	358.9	34	28,609	339.1
5	238,516	358.0	35	20,519	286.8
6	236,317	261.6	36	19,013	280.4
7	149,037	249.2	37	14,688	317.1
8	146,591	302.1	38	12,500	312.8
9	140,966	292.3	39	9,342	348.5
10	128,965	285.0	40	9,186	307.4
11	114,619	284.5	41	7,575	292.7
12	113,855	308.8	42	6,192	276.2
13	112,933	252.1	43	5,580	329.6
14	97,470	245.4	44	5,508	328.8
15	96,934	332.0	45	3,766	373.6
16	96,704	374.1	46	2,948	307.5
17	94,387	397.7	47	2,804	5496.9
18	89,241	300.9	48	1,327	452.9
19	81,329	280.1	49	1,326	5131.0
20	81,312	276.2	50	1,119	834.6
21	80,487	292.0	51	1,094	710.5
22	77,159	306.6	53	994	462.7
23	73,496	317.9	54	850	662.5
24	72,395	380.3	67	520	463.7
25	67,284	344.1	117	420	1652.9
26	54,852	428.9	137	389	213.7
27	53,301	292.9	178	374	568.4
28	52,997	323.3	180	367	377.3
29	39,077	518.1	203	330	106.6
30	38,084	300.1	258	314	448.5

Table 17: Quality of assembly based on WGS of TMC579.

Contig	Length (bp)	Coverage	Contig	Length (bp)	Coverage
1	453,118	264.1	36	14,688	370.2
2	309,242	246.9	37	12,500	338.2
3	289,170	277.2	38	9,342	391.2
4	238,550	380.1	39	9,237	349.1
5	236,307	282.8	40	9,186	349.6
6	226,775	376.8	41	7,575	324.1
7	149,037	264.2	42	6,182	315.4
8	146,718	333.2	43	5,580	358.6
9	140,966	320.9	44	5,508	369.4
10	129,000	308.6	45	4,226	661.4
11	114,619	302.6	46	3,766	402.1
12	11,3855	330.3	47	3,520	286.7
13	112,933	270.8	48	2,948	353.3
14	96,891	362.9	49	2,846	6.7
15	96,704	410.5	50	2,804	5314.4
16	94,387	419.9	51	1,659	6.8
17	89,241	335.6	52	1,653	7.7
18	81,329	303.3	53	1,620	5.7
19	81,312	301.5	54	1,327	499.6
20	80,487	323.8	55	1,326	4932.5
21	77,157	335.1	56	1,119	882.4
22	73,478	341.9	57	1,094	759.2
23	72,395	425.7	59	996	5.6
24	67,306	372.6	63	850	735.2
25	54,852	441.3	128	544	264.7
26	53,301	314.3	203	457	70.6
27	43,792	359.0	245	421	5.3
28	38,084	328.4	246	420	1601.2
29	37,854	382.2	371	403	270.6
30	37,554	364.8	424	371	680.2
31	37,241	414.1	433	348	381.6
32	28,609	365.1	440	345	35.7
33	20,519	307.7	490	330	116.7
34	16,115	353.3	553	317	5.2
35	15,563	333.9	927	315	310.5

Table 18: Quality of assembly based on WGS of TMC68V$_1$B.

Contig	Length (bp)	Coverage	Contig	Length (bp)	Coverage
1	353,620	211.3	30	53,301	297.3
2	309,831	219.1	31	38,084	302.2
3	289,166	247.6	32	37,854	360.6
4	236,317	257.7	33	37,630	343.1
5	223,089	372.4	34	37,241	395.5
6	174,487	355.2	35	28,609	340.5
7	168,849	309.9	36	20,519	292.8
8	149,038	238.0	37	14,165	321.1
9	146,588	313.5	38	10,386	313.3
10	128,719	272.8	39	9,342	368.7
11	122,927	249.6	40	9,186	315.8
12	114,128	278.9	41	6,182	291.0
13	113,855	312.3	42	5,580	325.8
14	97,519	224.0	43	5,508	346.3
15	96,704	397.2	44	3,766	394.8
16	96,531	308.1	45	2,804	5603.3
17	94,387	413.1	46	1,327	420.3
18	89,591	361.4	47	1,326	5247.5
19	81,329	279.7	48	1,119	806.8
20	81,255	272.4	49	1,094	757.5
21	80,487	307.3	50	850	714.0
22	77,172	309.1	51	796	409.2
23	73,477	322.2	58	562	291.0
24	72,395	406.3	60	420	1729.5
25	67,284	351.2	61	419	427.4
26	65,334	332.0	62	389	280.8
27	60,187	443.3	67	375	689.4
28	56,200	376.8	70	348	326.2
29	54,852	439.8	72	336	279.8

Table 19: Quality of assembly based on WGS of TMC024S.

Contig	Length (bp)	Coverage	Contig	Length (bp)	Coverage	Contig	Length (bp)	Coverage
1	748,144	263.8	46	3,125	11.6	1226	605	5.5
2	426,285	252.3	47	3,095	10.0	1266	599	6.0
3	308,401	293.5	48	3,055	735.8	1307	593	8.8
4	240,806	319.8	49	3,012	288.3	1326	591	5.1
5	233,400	261.1	50	3,001	6.1	1371	582	5.7
6	233,385	316.8	51	2,875	12.0	1377	581	5.7
7	217,554	287.1	52	2,537	15.1	1425	573	5.8
8	163,878	252.5	53	2,503	7.8	1716	534	5.3
9	149,115	331.6	55	2,267	5.3	1732	534	5.3
10	135,414	328.2	57	2,217	280.3	1826	520	429.3
11	123,766	247.5	58	2,183	9.9	2230	480	5.0
12	106,102	246.3	63	2,102	5.0	2268	477	112.8
13	904,063	341.6	64	2,044	6.6	2269	477	6.9
14	85,764	286.5	65	2,022	5.9	2281	476	427.9
15	80,836	293.7	66	1,951	8.9	2497	460	462.5
16	79,533	312.9	70	1,881	5.5	2535	458	5.2
17	76,485	273.7	71	1,829	5.9	2613	452	5.6
18	76,002	293.5	82	1,578	5.9	2707	445	5.4
19	68,253	313.5	86	1,527	4585.4	2742	443	5.1
20	57,535	286.5	95	1,429	5.0	2777	440	5.9
21	49,278	303.0	100	1,387	4555.1	2848	435	5.2
22	41,823	522.0	102	1,380	11.3	2898	431	551.5
23	40,691	662.3	120	1,292	5.3	3016	424	5.7
24	39,075	300.7	142	1,173	5.5	3076	420	5.0
25	38,229	285.3	179	1,079	5.3	3336	405	145.5
26	35,619	241.7	226	1,014	4607.5	3414	400	321.8
27	35,533	272.0	243	994	407.4	3520	393	6.5
28	30,291	276.2	247	989	5.6	3537	392	656.0
29	29,706	324.2	287	955	5.2	3829	377	5.2
30	21,970	282.3	395	867	5.0	4099	363	95.7
31	13,084	264.8	397	865	5.2	4191	359	1458.1
32	12,603	2444.6	432	840	5.0	4192	359	6.3
33	12,120	287.1	490	807	5.2	4193	359	5.0
34	10,223	282.9	492	806	6.0	4227	357	5.0
35	10,216	261.1	607	758	4195.6	4322	352	5.5
36	9,358	310.6	722	719	5.0	4580	339	5.3
37	6,740	1646.9	729	717	6.3	4596	338	1155.0
38	5,937	22.6	731	715	6.3	4781	330	147.4

39	5,507	312.0	810	695	1328.5	4830	328	6.9
40	5,407	288.2	913	664	204.2	4934	324	388.4
41	4,674	311.5	935	658	5.2	4982	345	1484.8
42	4,314	258.8	962	652	5.5	5007	321	5.6
43	3,974	12.0	978	648	480.9	5375	358	522.1
44	3,349	547.5	1225	605	6.0	5550	301	136.1
45	3,149	360.7						

7. Vitae

Ayesha Mahmood was born in Karachi, Pakistan on October 10, 1992. Soon after, she and her parents moved to Houston, Texas where she grew up and graduated from Humble High School in 2011. She received her Bachelor of Science degree in Microbiology from the University of Texas at Austin in 2015. After that, she served as a pre-AP biology teacher at Kingwood High School until 2017, then worked as a microbiology laboratory technician at Miller-Coors Brewery in Golden, Colorado. In August of 2018, she entered The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences. Ayesha will be starting a Ph.D. program at the University of Texas at Austin in the Fall of 2020