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Prevalence And Genetic Determinants Of Vancomycin Resistance Of Clostridioides Difficile Isolates In Connecticut

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Abstract

Introduction: The emergence of vancomycin-resistant strains of *C. difficile* has become a growing concern, as it can lead to treatment failure and increased morbidity and mortality. In this study, we aimed to investigate the prevalence and mechanisms of vancomycin resistance in *C. difficile* isolates in Connecticut from patients with confirmed *C. difficile* infection at Yale New Haven Hospital. **Methods**: We collected 89 stool samples from patients with confirmed *C. difficile* infection, isolated *C. difficile*, and performed susceptibility testing on the isolates using the E-strip test method. Isolates with an MIC value below 4 μg/ml were classified as susceptible and isolates with an MIC value above 4 μg/ml were classified as resistant. Whole-genome sequencing (WGS) and gene analysis was performed on 19 isolates and the samples were screened for the presence of vancomycin resistance genes. **Results**: Antimicrobial susceptibility results showed that the majority of isolates (80/89) are vancomycin susceptible (MIC < 4 μg/ml). Of the remaining 9 vancomycin-resistant isolates, only one had extreme high-level resistance (>256 μg/mL), while the other 8 isolates had low-level resistance. However, we found that only two of the 8 low-level resistant isolates were *C. difficile*, while the other 6 were either mixed cultures or mis-identified, primarily *Enterococcus faecalis* (5 isolates). **Conclusion**: The majority of *C. difficile* strains are susceptible to vancomycin. The apparent high prevalence of high-level vancomycin-resistant *C. difficile* may have resulted due to isolation techniques that mis-identify *C. difficile*. Our study highlights the need for improved methods of isolating *C. difficile* from stool samples and the importance of implementing proper antimicrobial stewardship practices and surveillance to combat the growing threat of antibiotic resistance in *C. difficile* infections.

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Introduction

Clostridioides difficile is a gram-positive, spore-forming anaerobic bacteria which can cause life threatening diarrhea. It is the most common healthcare-associated infection. The main virulence factors of *C. difficile* infection are the high molecular weight clostridial toxins – toxin A(TcdA) and toxin B (TcdB) [1]. The transmission pathway of *C. difficile* is the fecal-oral route. *C. difficile* vegetative bacteria and its spores are found in feces. *C. difficile* spores are highly resistant to environmental conditions such as heat, oxygen, and non-chlorine cleaning agents [2]. Therefore, it can remain in the environment for days to months to cause infection and transmission.

C. difficile causes hospital-acquired and community-acquired diarrhea. There are about 223,900 estimated cases of *C. difficile* infection per year in the United States and around 12,800 estimated deaths per year [3]. Healthcare costs that are attributed to *C. difficile* infection are in excess of one billion dollars annually [3]. In recent years, the number of healthcare-associated *C. difficile* cases has fallen due to improved prevention measures such as improving antibiotic use, infection control, and healthcare facility cleaning and disinfection. However, the incidence of community-associated *C. difficile* infections is increasing [4]. Antibiotic use predisposes individuals for infection. Additional risk factors associated with *C. difficile* infection include older age, and proton pump inhibitors [5]. People who stay in the hospital for a long period of time or those with a weakened immune system are at higher risk for *C. difficile* [6].

Antibiotic use is a main risk factor for *C. difficile* infection, and antibiotics are also critical for treatment. Currently, the main antibiotics used to treat *C. difficile* infection are vancomycin and fidaxomicin [7]. Darkoh et al recently described the emergence and circulation of high-level vancomycin resistance *C. difficile* strains in Houston, Texas, and Nairobi, Kenya

[8]. The authors showed that 114/438 (26%) and 66/98 (67%) of isolates exhibited high level vancomycin resistance in Houston and Nairobi stool samples, respectively, and only 74% and 33% of isolates were vancomycin susceptible. The authors were unable to identify the mechanism of resistance and concluded that antibiotic resistance testing should be implemented and speculated that the emergence of *C. difficile* strains with decreased vancomycin susceptibility could lead to treatment failure [8]. These data raise concerns because oral vancomycin is frequently used for treatment *C. difficile infection* [9]. These data were controversial and other authors raised concerns about the methods used for susceptibility testing [10, 11] . Antibiotic resistance testing is not routinely conducted for *C. difficile* and the prevalence of vancomycin resistance in Connecticut is unknown [12]. Thus, we seek to determine the prevalence of vancomycin resistance strains of *C. difficile* in Connecticut and identify genetic determinants associated with vancomycin resistance in strains of *C. difficile*.

Methods

C. difficile isolation, culture and confirmation.

The study population for the project is comprised of individuals who tested positive for *C. difficile* infection (CDI) at Yale New Haven hospital (YNHH). CDI infection was defined as samples that were GDH and toxin positive. *C. difficile* is a reportable disease in CT and the Emerging Infections Program routinely collects isolates from YNHH. A convenience sample of 89 stool samples, collected during 2019 and 2020, were used in this study. Stool samples were obtained from patients with diarrhea who had a positive diagnostic assay (GDH) for *C. difficile*. Epidemiological and clinical data were obtained through chart review and were available for 89

patient samples available. *C. difficile* culture was used to confirm the presence of *C. difficile. C. difficile* were isolated and cultured from stool samples using CCFA-HT selective media plates and RENEL non-selective media plates were used to sub-culture different isolates [8, 13]. Those plates were placed in the anaerobic chamber at 37℃ for 48 hours to grow. PCR was conducted as an additional confirmation step and also to test for the presence of toxin genes. Briefly, crude DNA extraction would be done for each isolate. One loop of isolates was taken from isolation colony into 30 µl free water and boiled for 15 minutes [14]. Then, GDH and Toxin A&B PCR were performed to confirm whether the isolate is *C. difficile* and the presence of the toxin genes. The primers for GDH PCR are GluD-F (5'-GTCTTGGATGGTTGATGAGTAC-3') and GluD-R (5'-TTCCTAATTTAGCAGCAGCTTC-3'). For Toxin A&B PCR, five primers were used which are tcdA-F (5'-GCATGATAAGGCAACTTCAGTGGTA-3'), tcdA-R (5'-AGTTCCTCCTGCTCCATCAAATG-3'), tcdB-F (5'-CCAAARTGGAGTGTTACAAACAGGTG-3'), tcdB-RA (5'-GCATTTCTCCATTCTCAGCAAAGTA-3') and tcdB-RB (5'-GCATTTCTCCGTTTTCAGCAAAGTA-3')[15]. The thermocycling for amplification steps are 94 ℃ for 15 minutes, 94 ℃ for 45 seconds, 50 ℃ for 45 seconds and 72 ℃ for 1 minute. There are 35 cycles running repeatedly and finally it hold 72 ℃ for 30 minutes [15]. After confirmation of both GDH and Toxin A&B PCR, lawn plates were made by inoculating plates with bacteria to produce a heavy uniform layer of growth over the entire surface of the agar. The lawn plates are composed of Brain heart infusion (BHI) (37g), agar (15g), horse blood (7%) and 100X L-cysteine. Four isolates were also streaked on Slanetz & Bartley medium to exclude the influence of potential contamination from enterococci for those isolates with extremely high MIC value (>256 µg/ µl) [16].

Determination of Antibiotic Resistance and Whole Genome Sequencing

C. difficile toxin A&B positive isolates were subjected to vancomycin minimal inhibitory concentration (MIC) test. The MIC test was done by using vancomycin antibiotic E-strip with concentrations ranging from 0.016 to 256 µg/ml. The strip was placed on each lawn plate using sterilized forceps and incubated in the anaerobic chamber at 37℃ for 48 hours. The MIC value was determined based on the zone of inhibition. Isolates were considered susceptible with MIC value below 4 ug/ul. Vancomycin resistant *C. difficile* isolates are divided into three categories according to the MIC value. Low level resistance ranges from 4 µg/ µl to 16 µg/ µl. Intermediate level resistance ranges from 16 µg/ µl to 32 µg/ µl. High level resistance is greater than 32 µg/ µl [8]. Monarch Genomic DNA Purification Kit T3010 was used for DNA extraction following the genomic DNA purification and cleanup protocol for gram-positive bacteria [17]. DNA was extracted from *C. difficile* isolates and used to make libraries for whole genome sequencing. Vancomycin resistant *C. difficile* isolates were matched with vancomycin susceptible *C. difficile* isolates on same sex, similar age range and case definition (hospital-acquired infection, community-acquired infection and hospital associated community onsite infection). We were unable to identify matched susceptible cases for four vancomycin resistant isolates. Whole genome sequencing was performed on 23 isolates including matched vancomycin resistant isolates (9 isolates) and vancomycin nonresistant isolates (9 isolates), unmatched vancomycin resistant isolates (4 isolates), and one ATCC control. *C. difficile* isolates were sequenced using PacBio Sequel II at the Yale Center for Genome Analysis on West Campus following the manufacturer's protocol. WGS data from resistant and susceptible strains were compared to identify determinants of resistance.

Data Analysis

Data was analyzed by SAS software. T-test, chi-squared test and logistic regression were used to compare differences between variables. Data represents mean ± SD. The significance level was set at P value < 0.05 in all cases. BLAST, PubMLST and CARD Resistance Gene Identifier (RGI) were used to screen WGS data for the presence of vancomycin resistant genes. MLST analyzed the sequences of isolates of seven housekeeping genes to characterize the genetic diversity of bacteria. For *C. difficile* analysis, the seven housekeeping genes are adk, atpA, dxr, glyA, recA, sodA, tpi [18, 19]. The target genes are used for genetic analysis and identification of *C. difficile* strains and the value in the table represents the number of alleles that differ from the reference gene sequence [20].

Results

89 stool samples from patients positive for CDI were examined. All 89 samples were confirmed positive for GDH and Toxin A&B. Vancomycin E-strip test results indicated that 9 (10.1%) *C. difficile* isolates were vancomycin resistant. We used the classification standard of Dr. Darkoh et al as described in methods (low level (⩾4 – 16 μg/mL), intermediate level (16 – 32 μg/mL), and high level (>32 μg/mL)) [8]. Eight of those nine *C. difficile isolates* are low level vancomycin resistant and only one isolate showed a high-level of vancomycin resistance.

The baseline characteristics of the sample of patients with *C. difficile* infection are shown in Table 1. Risk factors including demographic factors (age, sex, race/ethnicity), lifestyle factors (alcohol consumption, smoking), underlying medical conditions, and medication use (such as

antimicrobials) were previously associated with *C. difficile* infection [21, 22].We also compared baseline characteristics by the presence or absence of vancomycin resistance isolates to determine whether patient characteristics were associated with the vancomycin resistant *C. difficile*. The mean age for individuals with vancomycin resistance infection is 57.4 years, while for those without resistance, it is 61.1 years. The mean BMI was higher in patients with vancomycin susceptible isolates compared to those with vancomycin resistant isolates (28.6 vs. 21.7). Other patient characteristics did not differ between the two groups, this may be due to small sample sizes. Although the p-value of 0.191 suggests that there is no significant difference in sex distribution between the two groups, the sample population of this study is relatively small and further investigation could be performed to detect the potential association between sex and vancomycin resistance.

Table 1. Baseline Characteristics of the CDI patient sample. [a]

Characteristic	CDI Patients (N =89)[b]
Age (years)	60.7 ± 21.2
Sex	
Female	41 (46.1)
Male	48 (53.9)
Race/ethnicity	
White	68 (76.4)
Black	9 (10.1)
Asian	1 (1.1)
Other	11 (12.4)
Infection Case Definition	
Hospital-Acquired	45 (50.6)
Community-Acquired	14 (15.7)
Community Onsite	30 (33.7)
# of Antimicrobial therapies (12 weeks prior to stool collection date)	
0	27 (50.9)
1	15 (28.3)
2+	11 (20.8)
Alcohol Abuse	
Yes	5 (5.6)
No	84 (94.4)
Smoking	

Yes	9 (11.1)
No	72 (88.9)
# of Chronic conditions	
Chronic Kidney	16 (29.6)
Inflammatory Bowel	2 (3.7)
Gastrointestinal surgery	36 (66.7)
Body mass index (kg/m^2)	27.9 ± 18.6

[a] Table values are mean ± SD for continuous variables and n (column %) for categorical variables.
[b] Numbers may not sum to total due to missing data, and percentages may not sum to 100% due to rounding.

Four isolates – 1 vancomycin resistant isolates and 3 matched vancomycin nonresistant isolates that were not successfully sequenced. We used BLAST (Basic Local Alignment Search Tool) to compare the whole genome sequence data of 19 isolates to a reference database of known *C. difficile* sequences. BLAST compared the sample sequence alignment to the reference sequence to achieve a reliable and accurate identification of species [23]. Based on the BLAST analysis, we confirmed that 14 isolates (6 vancomycin resistant isolates, 7 vancomycin nonresistant isolates and one ATCC control) contained high coverage of sequence data to the *C. difficile* reference database. 7 of those 13 isolates are pure *C. difficile* sequences and the rest sequences of isolates contained sequences indicative of contamination with other bacteria. In those 8 pure *C. difficile* sequences, there were only two vancomycin resistant isolates and the remaining six were comprised of 5 vancomycin susceptible isolates and ATCC control.

There were 5 non-*C. difficile* sequences. Three isolates (Lab ID 21P, 23A, 23B) were contaminated with *Enterococcus faecalis*. One isolate (Lab ID V004) was contaminated with *Pseudomonas aeruginosa*. Lab ID 21A isolate was also contaminated with other bacteria but we have the problem of isolation and identification. For the 6 contaminated *C. difficile* sequences, each of them had a different kind of bacterial contamination. Lab ID 31 isolate was contaminated with two bacteria – *Enterococcus faecalis* and *Escherichia coli*. Lab ID 42A, 56 and 129 isolates

corresponded to contamination with *Neobacillus mesonae*, *Flavonifractor plautii* and *Escherichia coli*, respectively. Lab ID 51 isolate was contaminated with two bacteria – *Enterococcus faecalis* and *Flavonifractor plautii*. For Lab ID 130 isolates, it was also contaminated with two bacteria – *Flavonifractor plautii* and *Caudoviricetes sp*. Of the six sequenced contaminated *C. difficile* isolates, two are vancomycin nonresistant strains and four are vancomycin resistant strains. Specific contamination lists can be found in Table 3.

After the identification of *C. difficile* on BLAST, we ran the MLST (multi-locus sequence typing) on the pure *C. difficile* whole genome sequence data to identify the specific sequence type (ST) of the isolate [24]. After comparing our sequence data of these genes to the known reference sequence, MLST assigned the unique ST to the isolate and then we can figure out the genetic relatedness of *C. difficile* isolates in our study. With the identification of genetic relatedness of *C. difficile* isolates in the study, we used the Comprehensive Antibiotic Resistance Database (CARD) to analyze the whole genome sequence data of *C. difficile* to look for antibiotic resistance genes and the corresponding antibiotic drugs that these genes are associated with. CARD is the bioinformatics database to help identify and predict the antibiotic resistance genes in bacteria genomes [25]. Table 2 listed sequenced pure *C. difficile* isolates along with their ST, MLST clade, MIC in μg/ml, antibiotic drug classes and antibiotic resistant gene identifier. In this table, "+" indicates the presence of a resistance gene and "-" indicates the absence. The majority of *C. difficile* isolates in the study has their respective ST and MLST clade. However, one isolate (Lab ID 79) does not have values for some of the target genes and assigned sequence type. Basically, each of 8 isolates of *C. difficile* has a different sequence type and different alleles for the target housekeeping genes, which indicates that there are some genetic variations among the isolates, and they have undergone some genetic divergence. However, all those isolates of *C. difficile* belong to

MLST clade 1 whether they are vancomycin resistant or not which suggest that they had varying degrees of genetic variation but no significant evolution among *C. difficile* lineages.

Table 2. Sequence type, Antibiotic Drug classes and Antibiotic Resistant Gene Identifier for pure *C. difficile* strains/clinical isolates used in the study.

Isolates	**ST**	**MLST clade**	**MIC (µg/ml)**	**Antibiotic genes (Above 60% Matching Region)**	**CDD-1**	**vanG**	**vanT gene in vanG cluster**	**vanXY gene in vanG cluster**	**vanR gene in vanG cluster**	**Clostridioides difficile 23S rRNA with mutation conferring resistance to erythromycin and clindamycin**	**tet(W)**
				Antibiotic Drug Class Corresponding to Antibiotic Genes	**carbapenem**	**glycopeptide antibiotic**				**macrolide antibiotic, lincosamide antibiotic**	**tetracycline antibiotic**
ATCC	1	2	--	--	--	--	--	--	--	--	--
9	42	1	4	+	+	+	+	+	+	+	-
62	8	1	8	+	+	+	+	+	+	+	+
116	110	1	1.5	+	+	+	+	+	+	+	-
120A	14	1	1.5	+	+	+	+	+	+	+	-
122	53	1	0.5	+	+	+	+	+	+	+	-
V001	129	1	0.75	+	+	+	+	+	+	+	-
79	--	--	1	+	+	+	+	+	+	+	-

The table also indicates that some isolates have genes that have potentially been linked to resistance to multiple antibiotics, and some were resistant to specific antibiotics. All isolates in the study whether they are vancomycin resistant or not based on the MIC value have the following antibiotic resistance genes – vanG, vanY gene in vanA cluster, vanW gene in vanI cluster, vanT gene in vanG cluster, vanXY gene in vanG cluster, vanR gene in vanG cluster, vanY gene in vanG cluster, qacG, CDD-1, *C. difficile* 23S rRNA with mutation conferring resistance to erythromycin and clindamycin. Particularly, these isolates have a high percentage (60-100%) of matching region with some antibiotic resistant genes such as CDD-1, vanG , vanT gene in vanG cluster, vanXY gene in vanG cluster, vanR gene in vanG cluster, *C. difficile* 23S rRNA with mutation encoding resistance to erythromycin and clindamycin. These genes

potentially confer those *C. difficile* isolates resistance to glycopeptide, disinfecting agents and antiseptics, carbapenem, macrolide and lincosamide antibiotic. Only one out of six isolates vancomycin resistant isolate (Lab ID 62) had the tet(W) gene encoding resistance to tetracycline antibiotics.

Table 3. Bacteria contamination of *C. difficile* isolates in the study

Sequenced Isolates (Lab ID[a])	**Bacteria Contamination**	
21P[b]	*Enterococcus faecalis*	
23A[b]	*Enterococcus faecalis*	
23B[b]	*Enterococcus faecalis*	
31[c]	*Enterococcus faecalis*	*Escherichia coli*
42A	*Neobacillus mesonae*	
51	*Enterococcus faecalis*	*Flavonifractor plautii*
56	*Flavonifractor plautii*	
129	*Escherichia coli*	
130	*Flavonifractor plautii*	*Caudoviricetes sp.*
V004[b]	Pseudomonas aeruginosa	

[a] The number indicates the patient number and the letter indicates the bacteria with different phenotypes isolated from the same patient sample

[b] Non-C. *difficile* isolates with vancomycin resistance (MIC $\geq$ 4 μg/ml)

[c] Vancomycin nonresistant *C. difficile* isolates.

Discussion

In recent years, the increasing incidence of *C. difficile* infections facilitate the genetic change of bacteria which help develop several mechanisms to be resistant to multiple antibiotics [26]. Vancomycin is the main antibiotic used to treat *C. difficile* infection. The high-level vancomycin resistance *C. difficile* strains are circulating and spread in Houston, Texas, and Nairobi, Kenya concluded by Dr. Darkoh et al [8]. They reported that about 38% of 536 stool samples from *C. difficile* infection patients had a vancomycin resistance level above 4 μg/ml. Therefore, vancomycin resistance of *C. difficile* became a growing concern since it could lead to treatment failure and increase morbidity and mortality. However, other researchers suggested the

high prevalence of vancomycin resistant of *C. difficile* is not necessarily true and there could be misidentifying other vancomycin resistant organism by using unvalidated methods [10, 11]. In our study, we isolated 89 patients' stool samples from Yale New Haven hospital that were confirmed GDH and Toxin A&B positive for *C. difficile* infection. The majority of isolates (80 of 89) had a MIC value below 4 μg/ml, which are considered vancomycin susceptible. In 8 of 9 vancomycin resistant isolates had a low-level resistance (≥4–16 μg/mL) and only one has the extreme high level of resistance (>256 μg/mL). However, that isolate was actually found not to be *C. difficile* through sequence identification on BLAST. Even among the remaining eight isolates, only two were a pure *C. difficile* culture, while the other six cultures were contaminated to varying degrees by other bacteria or were not *C. difficile*. Notably, no bacteria contamination was found in any of the sequenced vancomycin non-resistant isolates. The main bacterial sources of contamination in those isolates are *E. faecalis*. *E. faecalis* is a common vancomycin resistant bacteria strain associated with healthcare infection. The vancomycin resistance of *E. faecalis* is conferred by the acquisition of the VanA or VanB operons which encode enzymes and modify the peptidoglycan precursors to reduce the affinity for vancomycin [27, 28]. Therefore, the vancomycin resistant results on MIC E-strip test could be caused by the presence of VRE. Our data did not indicate that there was a high prevalence or circulation of vancomycin resistance strains of *C. difficile* in Connecticut.

We used whole genome sequence data to identify resistant gene of isolates on CARD. They contained vanG, vanA, vanI, and vanB clusters. They are usually carried on mobile genetic elements such as plasmids [29]. The enzyme encoded by vanA and vanB operons would modify the terminal D-alanine residues of the peptidoglycan precursors to prevent vancomycin from binding and inhibiting cell wall synthesis of bacteria, which reduce the susceptibility of *C.*

difficile to vancomycin [30]. Thus, the antibiotic resistant gene clusters in the isolates could also contribute to the vancomycin resistance of *C. difficile.* However, in our genetic analysis, the genes with high matching degree (>60%) in pure *C. difficile* were the following three -- vanT gene in vanG cluster, vanXY gene in vanG cluster and vanR gene in vanG cluster. The function of these gene were encoding putative D-Ala–D-Ser ligase, bifunctional D,D-dipeptidase and serine racemase, respectively [31]. The vanG-like genetic element is widely present in various lineages of *C. difficile*, and all three genes within this element are significantly transcribed regardless of the presence or absence of vancomycin[31]. The vanG operon in *C. difficile* is functional at the transcriptional level and induced by vancomycin. Despite the induction of this operon by vancomycin, *C. difficile* is still able to synthesize the d-Ala-d-Ala peptidoglycan precursor, maintaining its sensitivity to vancomycin [31, 32]. So, the presence of vanG gene cluster does not affect vancomycin resistance in *C. difficile*. The resistant mechanism of the two pure *C. difficile* isolates to vancomycin is unclear and requires further research.

We also cultured contaminated *C. difficile* isolates on Slanetz & Bartley medium plates to determine if the presence of *Enterococcus* bacteria. The culture plates indicated that there was a growth of *Enterococcus* bacteria which match with whole genome sequence data. Some studies indicate that there could be a positive association between *C. difficile* infection and vancomycin resistant *Enterococcus* colonization [33, 34]. *Enterococcus* bacteria shaped the gut environment to enhance the fitness and pathogenesis of *C. difficile* [35, 36]. Therefore, it would be difficult to separate these two bacteria effectively. The apparent high prevalence of high-level vancomycin resistant *C. difficile* could be related to misidentification of *C. difficile* related to the co-colonization of vancomycin resistant enterococcus bacteria. However, in our study, we could not distinguish the factor. Although we performed multiple isolation cultures on various plates in the

study to obtain pure colonies of *C. difficile*, the results showed that it was difficult to effectively separate *C. difficile* from other bacteria, especially *Enterococcus*. Therefore, in future studies, we need to find better ways to isolate *C. difficile* from stool samples. We also need a second assay in addition to PCR to give stable and definitive results for the presence of *C. difficile* in order to perform further analysis. It is crucial to use a medium such as Slanetz & Bartley medium to exclude the influence of enterococci before performing vancomycin MIC testing on *C. difficile*.

In conclusion, this study did not suggest a significant high circulation of vancomycin resistant *C. difficile* strain in Connecticut. Potential CDI coinfection with enterococcus should be considered. Better methods are needed for patient sample isolation and culture. Due to the small population, further research is needed to fully understand the mechanism and situation of *C. difficile* vancomycin resistance. It is crucial to monitor the emergence of new resistance and implement proper antimicrobial stewardship practices to combat the growing threat of antibiotic resistance in *C. difficile* infections.

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