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Prevalence, molecular characteristics and antibiotic susceptibility of clinical isolates of *Clostridioides difficile* in southeastern Nigeria

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Abstract:

Background: *Clostridioides difficile* is a major contributor to healthcare-associated infections, exhibiting global variations in its prevalence and resistance. There is an absence of data on its molecular characteristics and antibiotic susceptibility of *C. difficile* in southeastern Nigeria. This study aims to evaluate its prevalence, molecular features, and resistance patterns to enhance treatment and control strategies.

Methodology: Stool samples were collected from 450 hospitalized adult patients with persistent diarrhoea and prolonged antibiotic use from selected public and private healthcare facilities across the five States of south-eastern Nigeria. The total anaerobes were enumerated using standard bacteriological methods. *Clostridioides difficile* was cultured on *Clostridium difficile* differential agar with selective supplements, followed by biochemical testing including catalase, oxidase, and reverse CAMP tests. Genomic bacterial DNA was extracted, purified, and amplified by polymerase chain reaction (PCR) assay. Sequencing of the DNA amplicon was performed and results were blasted on the NCBI database. Virulence (*tcdA*, *tcdB*, *cdtA*, and *cdtB*) and resistance (*tetS*, *tetA*, and *ermB*) genes were detected by conventional PCR assay. Antibiotic susceptibility of each isolate was determined using the Kirby-Bauer disc diffusion method.

Results: The mean anaerobe counts ranged from $5.61 \pm 0.11 \log_{10}$ CFU/g (Ebonyi) to $5.65 \pm 0.07 \log_{10}$ CFU/g (Enugu). Antibiotic susceptibility test revealed sensitivity to gentamicin, meropenem, and amoxicillin-clavulanic acid, while resistance was observed to tetracycline and erythromycin. The multiple antibiotic resistance (MAR) index of isolates was 0.44. All isolates (100%) harbored *tcdB* gene while 69.56% harbored *tcdA* gene. Additionally, 4.34% of isolates contained *cdtA* and *cdtB* genes.

Conclusions: This study highlights significant antibiotic resistance and presence of virulence genes in *C. difficile* isolates in southeastern Nigeria, emphasizing the need for monitoring and tailored treatment strategies.

Keywords: Prevalence; Molecular; Antibiotic susceptibility; *Clostridioides difficile*; southeast Nigeria

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Prévalence, caractéristiques moléculaires et sensibilité aux antibiotiques des isolats cliniques de *Clostridioides difficile* dans le sud-est du Nigéria

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Résumé:

Contexte: *Clostridioides difficile* est un contributeur majeur aux infections associées aux soins de santé, présentant des variations mondiales dans sa prévalence et sa résistance. Il n'existe pas de données sur ses caractéristiques moléculaires et la sensibilité aux antibiotiques de *C. difficile* dans le sud-est du Nigéria. Cette étude vise à évaluer sa prévalence, ses caractéristiques moléculaires et ses profils de résistance afin d'améliorer les stratégies de traitement et de contrôle.

Méthodologie: Des échantillons de selles ont été prélevés auprès de 450 patients adultes hospitalisés souffrant de diarrhée persistante et d'une utilisation prolongée d'antibiotiques dans des établissements de santé publics et privés sélectionnés dans les cinq États du sud-est du Nigéria. Le nombre total d'anaérobies a été dénombré à l'aide de méthodes bactériologiques standard. *Clostridioides difficile* a été cultivé sur gélose différentielle *Clostridium difficile* avec des suppléments sélectifs, suivi de tests biochimiques comprenant des tests de catalase, d'oxydase et de CAMP inverse. L'ADN bactérien génomique a été extrait, purifié et amplifié par un test de réaction en chaîne par polymérase (PCR). Le séquençage de l'amplicon d'ADN a été effectué et les résultats ont été enregistrés dans la base de données NCBI. Les gènes de virulence (*tcdA*, *tcdB*, *cdtA* et *cdtB*) et de résistance (*tetS*, *tetA* et *ermB*) ont été détectés par un test PCR conventionnel. La sensibilité aux antibiotiques de chaque isolat a été déterminée à l'aide de la méthode de diffusion sur disque de Kirby-Bauer.

Résultats: Le nombre moyen d'anaérobies variait de $5,61 \pm 0,11 \log_{10}$ UFC/g (Ebonyi) à $5,65 \pm 0,07 \log_{10}$ UFC/g (Enugu). Le test de sensibilité aux antibiotiques a révélé une sensibilité à la gentamicine, au méropénème et à l'amoxicilline-acide clavulanique, tandis qu'une résistance a été observée à la tétracycline et à l'érythromycine. L'indice de résistance multiple aux antibiotiques (MAR) des isolats était de 0,44. Tous les isolats (100 %) abritaient le gène *tcdB* tandis que 69,56% abritaient le gène *tcdA*. De plus, 4,34% des isolats contenaient les gènes *cdtA* et *cdtB*.

Conclusions: Cette étude met en évidence une résistance importante aux antibiotiques et la présence de gènes de virulence dans les isolats de *C. difficile* dans le sud-est du Nigéria, soulignant la nécessité d'une surveillance et de stratégies de traitement adaptées.

Mots clés: Prévalence; Moléculaire; Sensibilité aux antibiotiques; *Clostridioides difficile*; sud-est du Nigéria

Introduction:

Standard healthcare facilities are essential for survival in a world dominated by superbugs, which have also been implicated in infections and the re-occurrence of infection even after the treatment regimen. Pathogenic organisms have recorded unparalleled success in their ability to cause diseases as well as induce therapeutic failure through drug resistance (1). *Clostridioides difficile* has been reported as the leading cause of healthcare-associated diarrhea (2). Antibiotic exposure, which reduces the population of non-pathogenic anaerobes that normally inhabit the gut, has been pointed out as the most important risk factor for *C. difficile* infection (CDI) (2). Thus, CDI occurs when there is a shift in the microbial flora of the colon, allowing toxin-producing strains of Gram-positive, spore-forming, anaerobic bacillus to proliferate (2). Other events implicated in gut microbiota disruption include proton pump inhibitors (PPI) use and inflammatory bowel diseases (3,4). Researchers have attributed the pathogenicity of *C. difficile* to the two major virulence factors produced by toxigenic *C. difficile* strains; enterotoxin A (TcdA) and cytotoxin B (TcdB) (3,4).

On September 16, 2013, the Centre for Disease Control (CDC) declared *C. difficile* as a threat with 'urgent' concern to public health in the United States due to the identification of antimicrobial resistance among human and animal-derived *C. difficile* isolates to drugs commonly used in humans and also to their increased rates of associated patient mortality since the early 2000s (5). In all the studies,

some strains of *C. difficile* appear to be species-specific, while other strains were isolated from multiple species, including man. The organism was initially described as part of the normal flora in stool samples from healthy infants in 1935 (6) as well as its detection in significant numbers in healthy asymptomatic children (7). It was afterward identified as a pathogen associated with several diseases, and even now, in hospitals and long-term care facilities, it has become the most common cause of diarrhea, causing the loss of billions of dollars in health care costs (8).

The symptoms of CDI usually appear mild in some cases, and in others, they appear to be life-threatening or fatal (9). Diarrhea associated with *C. difficile* is accompanied by the passage of occult blood or mucus in the stool, but hematochezia and melena are other rare symptoms (4,10). Certain other symptoms are found in less than 50% of infected patients, including abdominal discomfort, fever, and peripheral leukocytosis (11). Disease caused by *C. difficile* is linked most commonly to nosocomial infections in humans, especially after empiric antibiotic treatment. Humans develop a spectrum of diseases when infected with some strains of *C. difficile*. It has been shown that not all people who are on prolonged antibiotic medication and above the age of 60 years are susceptible to *C. difficile* (12).

The advent of some virulent new strains of *C. difficile* has triggered an increase in CDI rates even in younger populations originally thought to be at low risk of developing CDI (13). To date, little is known about CDI in southeastern Nigeria as inadequate attention

has been given to the understanding of the molecular epidemiology of CDI in Nigeria, a high-burden country. Thus, this research was aimed at determining the prevalence, molecular characteristics, pathogenicity, and antimicrobial susceptibility of *C. difficile* isolated from clinical specimens in the southeastern States of Nigeria.

Materials and method:

Study area:

This study was conducted at the Chukwuemeka Odumegwu Ojukwu University Teaching Hospital, Amaku, Awka, Anambra State, and hospitals selected from the other four neighboring States of southeastern Nigeria.

Ethics approval and consent to participate:

The study was conducted in line with the principles of the Declaration of Helsinki. Ethical approval for this research was obtained from Ethical Committee, Chukwuemeka Odumegwu Ojukwu University Teaching Hospital Amaku, Awka, Anambra State, Nigeria. The ethics approval number is COOUTH/CMAC/ETH. C/VOL.1/0035 and the approval was granted on 01/08/2018. The committee reviewed and approved all aspects of the study, including the study design, recruitment methods, and data collection procedures, ensuring that they were in compliance with ethical standards and guidelines.

The consent to participate was obtained from all individuals who participated in the study. Participants were given detailed information regarding the research objectives, procedures, potential risks, and benefits. They were informed that participation was voluntary, that they could withdraw from the research at any given time without any punishment, and their data would be given full confidentiality.

Study participants and sample size:

Participants included a total of 450 hospitalized patients who exhibited symptoms such as watery stools (unformed stools in 24 hours), abdominal pain, and fever. Stool samples were collected from symptomatic patients hospitalized in various units, including the intensive care unit (ICU), medical, surgical and gastroenterology wards. These units were selected due to the high prevalence of CDI, particularly among patients receiving prolonged antibiotic therapy or those with underlying comorbidities. Additionally, samples were obtained from patients at the infectious disease unit who were undergoing evaluation for suspected CDI. All participants had diarrhea (unformed stools in 24 hours) and were tested for *C. difficile* based on clinical suspicion.

The sample size was determined using the sample size formula; $N = Z^2 p (1-p) / D^2$ (14)

where, N is the sample size, Z is the statistic for level of confidence (1.96 for 95% confidence level), p is the prevalence rate of 45% (0.45) based on a previous study and D is the desired level of precision (0.05). The sample size of 384 obtained from the formula was increased to 450 to account for environmental differences and sampling challenges.

Sample collection:

Stool samples were collected in red cap universal bottles from the Chukwuemeka Odumegwu Ojukwu University Teaching hospital, Awka in Anambra, Federal Teaching hospital Abakaliki in Ebonyi, Abia State University Teaching hospital in Abia, Imo State University Teaching hospital in Imo and Enugu State Teaching hospital in Enugu. Following initial processing, samples were properly stored and preserved on ice packs to maintain sample integrity until further analysis.

Isolation and identification of *Clostridium difficile*:

For the isolation of *C. difficile*, all samples were pre-treated (heat shocked and alcohol shocked) to reduce competing flora and allow the growth of spore-forming bacilli. Standard microbiological method was employed using *C. difficile* differential agar CM 0601 (Oxoid, UK) supplemented with *C. difficile* selective supplement SR0096 (Oxoid, UK) (15).

Stock solutions were prepared for the samples by weighing 20g of the stool samples into 180ml of sterile saline water in a conical flask. A ten-fold serial dilution from the stock solution was made after mixing. From the fifth tube, 2ml of the suspension was added to a well-labelled tube containing 2ml of absolute ethanol (alcohol/ethanol shock method). The mixture was rocked gently to allow for homogenization and left to stand for an hour before plating of 100 μ L was carried out.

Heat treatment, also known as heat shocking, was employed by heating the tubes containing a mixture of samples to boil for 5 minutes. The tubes were then allowed to cool before plating was carried out on the media. Since *C. difficile* is a strict anaerobe, its growth requires an oxygen-free environment. All inoculated plates were incubated in anaerobic condition using anaerobic jar with gas-generating sachets to create a completely oxygen-free atmosphere at 37°C for 48–72 hours to ensure optimal recovery of *C. difficile*.

The formula employed for the dilution factor is given by the equation; final volume/ aliquot volume, where final volume is aliquot volume (sample volume) plus diluent volume. Enumeration of the bacterial isolates was carried out using the formula described by Willey et al., (16); CFU/g = (number of colonies x dilution factor)/volume of inoculum. The cultu-

ral, morphological, microscopic and biochemical properties of the isolates were evaluated after Gram staining, and spore staining was done to select the positive isolates for both stains. Biochemical tests such as catalase, oxidase, indole, urease and sugar fermentation tests were carried out to identify *Clostridium* species.

Antimicrobial susceptibility test:

Antimicrobial susceptibility test (AST) was performed on identified *C. difficile* by the disc diffusion method. The antibiotic discs (Oxoid, UK) used include meropenem (10µg), erythromycin (15µg), metronidazole (50µg), amoxicillin-clavulanic acid (20/10µg), clindamycin (20µg), gentamicin (10µg), ciprofloxacin (5µg), vancomycin (30µg), and tetracycline (30µg).

Briefly, 18-24 hours bacterial culture was cultured on Mueller-Hinton agar (MHA). The inoculum was standardized to 0.5 McFarland standard ($\sim 1.5 \times 10^8$ cells/ml) was streaked onto the MHA plate with a sterile loop before the antibiotic discs were aseptically placed on the plates using a sterile forcep. The plates were incubated under strict anaerobic conditions (90% N₂, 5% H₂, 5% CO₂) at 35–37°C for 24–72 hours to ensure proper growth for AST. The diameter of zone of inhibition was measured and the result was interpreted using the Clinical and Laboratory Standards Institute (CLSI) breakpoints (17).

Determination of multiple antibiotic resistance (MAR) index:

The MAR index for an isolate was determined by the formula described by Chitanand et al., (18); y/nx , where y is the number of antibiotics the isolate is resistance to, n is the number of isolates tested, and x is the total number of antibiotics tested.

DNA extraction:

Bacterial DNA was extracted by the boiling method (19). A tube containing 2ml of a 24-hour broth culture of *C. difficile* was centrifuged at high-speed for 5 minutes. After the supernatant was removed, 200µL of sterile distilled water (SDW) was added to the pellets, and the mixture was vortexed for 1 minute. After the mixture was heated to 100°C for 15 minutes, it was centrifuged at high-speed for 2 minutes. The supernatant from this was considered pure DNA, and 10µL of it was used for amplification of the 16S rDNA as well as the virulence and resistance genes by PCR.

DNA sequencing:

The amplified bacterial 16S rDNA fragments were sequenced using the Genetic Analyzer (Model 3130xl) from Applied Biosystems (20). The sequencing reactions were performed using the BigDye™ Terminator v3.1 Cycle

Sequencing Kit, following the manufacturer's recommended protocol to ensure optimal sequencing conditions (21). All procedures were conducted according to standard laboratory protocols to maintain data accuracy and reliability and the sequences were blasted on the website of National Centre for Biotechnology Information (21).

Phylogenetic analysis:

Phylogenetic analysis was performed by using the amplified DNA to determine the evolutionary relationships among the isolates. DNA sequences were initially aligned using both ClustalW and MUSCLE to ensure precision and accurate comparisons (22). The aligned sequences were then used to construct phylogenetic trees, which were visualized and edited using MEGA 10 software (23). This software also facilitated calculation of bootstrap values, providing an assessment of the reliability and robustness of the phylogenetic trees. The analysis included both Neighbor-Joining and Maximum Likelihood methods to confirm the consistency of the results (24).

PCR amplification for detection of virulence and resistance genes:

Using standardized primer sets, virulence genes (*tcdA*, *tcdB*, *cdtA*, and *cdtB*) were detected in duplicate to ensure that the results were accurate using the primers listed in Table 1. The analysis utilized the extracted bacterial genomic DNA as template. The PCR assay was performed in tubes containing 50 mM KCl₂, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 200 µM dNTPs, and 20 pmol of each primer along with 2U Taq DNA polymerase in a total volume of 50µL, using 4µL of the extracted DNA as template (25).

To ensure the primers specifically bind to the DNA template, the thermocycler was optimized to ascertain the correct annealing temperature. The PCR protocol consisted of an initial denaturation step at 94°C for 5 minutes, then 34 cycles involving 30 seconds of denaturation at 94°C, 1 minute of annealing at 55°C, and 1 minute of extension at 72°C. At completion of the amplification cycles, a final extension at 72°C for 5 minutes was performed. Sterile water served as the negative control to confirm that no contamination was present (3).

With conventional PCR assay, the genes responsible for erythromycin resistance (*ermB*) and tetracycline resistance (*tetA* and *tetS*) were detected using primers as shown in Table 2. The extracted bacterial genomic DNA was used as template for the analysis. The PCR assay was performed in tubes containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 200µM dNTPs, and 2U Taq DNA polymerase per 50µL reaction, using 4µL of extracted DNA as the template and including 20 pmol

of each primer (26).

The thermocycler was set to optimal annealing temperature that guaranteed specific binding of the primer set to the DNA template. The PCR condition included 5-minute pre-denaturation phase at 94°C, followed by 34 cycles, consisting of 30 seconds at 94°C (denaturation), 45 seconds at 55°C (annealing), and 1 minute at 72°C (extension). After the amplification cycles, a final extension step at 72°C for 5 minutes was performed. Sterile water served as a negative control to eliminate the possibility of contamination.

Agarose gel electrophoresis:

Agarose gel electrophoresis was used to examine the PCR amplicons derived from primer sets targeting the virulence genes (27), as well those targeting the resistance (*ermB*, *tetA*, and *tetS*).

Statistical analysis:

Data were analyzed with infographics using Microsoft Excel (Version 2016, 2015, Microsoft Corporation, USA) (28). Comparisons of multiple means was done by analysis

of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) version 21.0 software. Phylogenetic tree analysis of species relatedness was done using Mega 10 software (Version 10, 2016, MEGA Limited, Japan)

Results:

The results of anaerobic stool sample counts from southeastern Nigeria are shown in Table 3. The total anaerobe counts ($\log_{10}$ CFU/g) in different States showed that stool samples from patients in Anambra, Ebonyi, Enugu and Imo had mean counts of $5.63 \pm 0.09 \log_{10}$ CFU/g, $5.61 \pm 0.11 \log_{10}$ CFU/g, $5.65 \pm 0.07 \log_{10}$ CFU/g and $5.36 \pm 0.19 \log_{10}$ CFU/g respectively. The identity and phylogenetic relatedness of anaerobic bacterial isolates obtained from stool samples in southeastern Nigeria are shown in Table 4.

The prevalence of anaerobes from stool samples using standard culture method revealed that all stool samples except from Imo State (46.0%) had anaerobic counts equal to or greater than 50.0% following a

Table 1: Primer sets for PCR amplification of the virulence genes

Genes	Primer	Sequence (5'-3')	Concentration (μ M)	Product size (bp)
<i>tcdA</i>	tcdA-F tcdA-R	GTATGGATAGGTGGAGAAGTCAGTG CGGTCTAGTCCAATAGAGCTAGGTC	0.025	632
<i>tcdB</i>	tcdB-F tcdB-R	GAAGATTTAGGAAATGAAGAAGGTGA AACCACTATATTCAACTGCTTGTC	0.01	441
<i>cdtA</i>	cdtA-F cdtA-R	ATGCACAAGACTTACAAAGCTATAGTG CGAGAATTTGCTTCTATTGATAATC	0.2	260
<i>cdtB</i>	cdtB-F cdtB-R	ATTGGCAATAATCTATCTCCTGGA CCAAAATTTCCACTTACTTGTGTTG	0.5	179

Table 2: Primer sets for PCR amplification of the resistance genes

Genes	Primer	Sequence (5'-3')	Concentration (μ M)	Product size (bp)
<i>erm(B)</i>	ermB-F ermB-R	GAAAAGGTACTCAACCAATA AGTAACGGTACTTAAATTGTTTAC	0.1	639
<i>tet(S)</i>	tetS-F tetS-R	ATCAAGATATTAAGGAC TTCTCTATGTGGTAATC	0.1	573
<i>tet(A)</i>	tet(A)-F tet(A)-R	TTGGCATTCTGCATTCACCTC GTATAGCTTGCCGGAAGTCG	0.1	494

cultural phenotypic standard (Table 5). In comparison, a total of 54.0% was obtained in the entire study.

The prevalence of *C. difficile* from stool samples revealed a total study prevalence of 3.1%. Stool samples from Ebonyi and Enugu States had a prevalence of 2.2% apiece. In

comparison, Imo and Abia States had a 3.3% prevalence of *C. difficile* from stool samples, with Anambra State having the highest prevalence of 4.4%. The rate of *C. difficile* recovered by heat treatment method (1.1%) shown in Table 5 was lower than the rate recovered by alcohol treatment (3.1%).

Table 3: Total anaerobic bacterial count of stool samples obtained from patients in different states

Location	Number of Samples	Anaerobe count (Log ₁₀ CFU/g)	p-value(s)
Anambra	90	5.63±0.09 ^a	0.168
Ebonyi	90	5.62±0.11 ^{ab}	0.168
Enugu	90	5.65±0.07 ^a	0.168
Imo	90	5.37±0.19 ^b	0.066
Abia	90	5.45±0.09 ^b	0.066

Table 4: Identity of anaerobic *Clostridioides difficile* isolates from stool samples from southeastern Nigeria

Sample code	Source	Identity	Query Cover (%)	Homology (%)	Accession number
anStool12	Stool	<i>Clostridioides difficile</i>	100	100.00	MH888201.1
ebstool05	Stool	<i>Clostridioides difficile</i>	98	91.4	CP019858.1
Imstool15	Stool	<i>Clostridioides difficile</i>	99	99.8	CP019860.1
anStool21	Stool	<i>Clostridioides difficile</i>	99	96.75	CP025047.1
anStool22	Stool	<i>Clostridioides difficile</i>	97	90.35	CP020424.2
abstool12	Stool	<i>Clostridioides difficile</i>	100	100.00	MH888202.1
abstool17	Stool	<i>Clostridioides difficile</i>	99	99.8	CP019860.1
imstool83	Stool	<i>Clostridioides difficile</i>	91	94.6	CP028361.1
imstool49	Stool	<i>Clostridioides difficile</i>	91	94.6	CP028361.1
ebstool23	Stool	<i>Clostridioides difficile</i>	91	94.6	CP028361.1
ebstool33	Stool	<i>Clostridioides difficile</i>	99	99.8	CP019860.1
ebstool1	Stool	<i>Clostridioides difficile</i>	99	96.75	CP025047.1
abstool12	Stool	<i>Clostridioides difficile</i>	97	90.35	CP020424.2
anstool70	Stool	<i>Clostridioides difficile</i>	100	100.00	MH888202.1

Table 5: Prevalence of anaerobes, *Clostridioides difficile* and percentage recovery of *Clostridioides difficile* from stool samples

Prevalence of anaerobes from stool samples using standard cultural methods								
Sample	Sample Number	Anambra	Ebonyi	Enugu	Imo	Abia	Total Sample	Study Total (%)
	90	55 (61)	45 (50)	50 (55)	41 (46)	50 (55)	450	241 (54)
Prevalence of <i>Clostridioides difficile</i> from stool samples in the study								
Stool sample	90	4 (44.4)	2 (22.2)	2 (22.2)	3 (33.3)	3 (33.3)	450	14 (3.1)
Percentage recovery of <i>Clostridioides difficile</i> from stool samples using cultural methods								
Sample	Number	Heat Treatment (%)		Alcohol Treatment (%)		Study Total (%)		
Stool sample	450	5 (1.1)		14 (3.1)		14 (3.1)		

In Table 6a and 6b, the antimicrobial susceptibility test of *C. difficile* isolates to the common antibiotics tested showed sensitivity to carbapenems (71.4%), aminoglycosides (78.5%) and β -lactam/combination agents (57.1%). Notable resistance was observed for tetracycline (57.1%), macrolides (57.1%) and fluoroquinolones (71.4%).

The multiple antibiotic resistance index of *C. difficile* isolates from the clinical samples showed that isolates with a MAR index of 0.44 had the highest resistance phenotypes. In contrast, isolates with a MAR index of 0.33 had the next highest resistance phenotypes (Table 7).

Table 6a: Antibiotic sensitivity profile of *Clostridioides difficile* to common antibiotics

Code	Samples	MEM	ERY	MET	AMC	CL	CN	CIP	VA	TET
anStool12	Stool	S	R	S	R	S	S	S	R	R
ebStool05	Stool	S	S	S	R	S	S	S	R	R
imStool15	Stool	S	R	R	S	R	S	R	S	R
anStool21	Stool	S	S	S	S	S	S	S	S	R
anStool22	Stool	S	R	S	S	S	S	R	S	R
abStool12	Stool	R	S	R	S	R	S	S	S	R
abStool77	Stool	S	R	R	S	S	S	R	S	R
imStool83	Stool	S	R	S	S	R	S	R	S	S
imstool49	Stool	S	R	R	S	R	S	R	S	R
ebstool23	Stool	R	R	R	S	S	S	S	S	R
enstool33	Stool	S	R	S	S	S	S	S	S	R
enstool1	Stool	S	R	S	S	R	S	R	S	R
abStool14	Stool	S	S	S	R	S	S	S	R	S
anstool70	Stool	S	S	S	S	S	S	R	S	S

Meropenem (MEM 10 μ g), Erythromycin (ERY 15 μ g), Metronidazole (MET 50 μ g), Amoxicillin/clavulanic acid (AMC 20/10 μ g), Clindamycin (CL 20 μ g), Gentamicin (CN 10 μ g), Ciprofloxacin (CIP 5 μ g), Vancomycin (VA 30 μ g), Tetracycline (TET 30 μ g), S = Sensitive, R = Resistance

Table 6b: Percentage of antibiotic resistance and susceptibility of *Clostridioides difficile* to common antibiotics

Antibiotic class	Antibiotic	Percentage susceptibility/resistance (n=14)	
		Sensitive (%)	Resistant (%)
Carbapenems	MEM	10 (71.4)	4 (28.5)
Macrolides	ERY	6 (42.8)	8 (57.1)
Nitroimidazole	MET	7 (50.0)	7 (50.0)
β -lactam/combination agents	AMC	8 (57.1)	6 (42.8)
Lincosamide	CL	6 (42.8)	8 (57.1)
Aminoglycosides	CN	11 (78.5)	3 (21.4)
Fluoroquinolone	CIP	4 (28.5)	10 (71.4)
Glycopeptides	VA	7 (50.0)	7 (50.0)
Tetracyclines	TET	6 (42.8)	8 (57.1)

Meropenem (10 μ g), Erythromycin (15 μ g), Metronidazole (50 μ g), Amoxicillin/davulanic acid (20/10 μ g), Clindamycin (20 μ g), Gentamicin (10 μ g), Ciprofloxacin (5 μ g), Vancomycin (30 μ g), Tetracycline (30 μ g).

Table 7: Multiple antibiotic resistance distribution of *C. difficile* isolates from clinical sources

Isolate code	No of antibacterial classes	No of antibiotics	Resistance phenotypes	Number (%)	MAR Index
anBE7, ebstool05	9	9	AMCR, VAR, TETR	2 (8.7)	0.33
enLV4, ebstool23	9	9	MEMR, ERYR, METR, TETR	2 (8.7)	0.44
ebLV5	9	9	ERYR, CNR, TETR	1 (4.4)	0.33
anStool12	9	9	ERYR, AMCR, VAR, TETR	1 (4.4)	0.44
imstool15, imstool49	9	9	ERYR, METR, CLR, CIPR, TETR,	2 (8.7)	0.55
anstool21	9	9	TETR	1 (4.4)	0.11
anstool222	9	9	ERYR, CIPR, TETR	1 (4.4)	0.33
abstool12	9	9	MEMR, ERYR, METR, CLR, TETR	1 (4.4)	0.55
enstool33	9	9	ERYR, TETR	1 (4.4)	0.22
enstool1	9	9	ERYR, CLR, CIPR, TETR	1 (4.4)	0.44
abstool14	9	9	AMCR, VAR	1 (4.4)	0.22
anstool70	9	9	CIPR	1 (4.4)	0.11

Meropenem (MEM 10 µg), Erythromycin (ERY 15 µg), Metronidazole (MET 50 µg), Amoxicillin/clavulanic acid (AMC 20/10 µg), Clindamycin (CL 20 µg), Gentamicin (CN 10 µg), Ciprofloxacin (CIP 5 µg), Vancomycin (VA 30 µg), Tetracycline (TET 30 µg)

All the *C. difficile* isolates (100.0%) possessed *tcdB* genes, and 10 of 14 (71.4%) isolates possessed *tcdA* genes. Only one of the isolated *C. difficile* strains possessed *cdtA* and *cdtB* genes, thus giving a percentage of 7.1%

apiece for the genes. The prevalence of virulence genes in Table 8 revealed that 3 isolates possessed (one of the four) virulence genes analyzed in the study, while 11 isolates possessed two of the four virulence genes.

Table 8: Prevalence of virulence genes in isolates of *C. difficile* obtained from stool samples

Lanes	Code	Samples	<i>tcdA</i>	<i>tcdB</i>	<i>cdtA</i>	<i>cdtB</i>	Total (%)
1	anStool12	Stool	+	+	-	-	2 (50.0)
2	ebstool05	Stool	+	+	-	-	2 (50.0)
3	Imstool15	Stool	+	+	-	-	2 (50.0)
4	anstool21	Stool	-	+	-	-	1 (25.0)
5	anstool22	Stool	+	+	+	+	4 (100.0)
6	abstool12	Stool	+	+	-	-	2 (50.0)
7	abstool77	Stool	+	+	-	-	2 (50.0)
8	imstool83	Stool	-	+	-	-	1 (25.0)
9	imstool49	Stool	+	+	-	-	2 (50.0)
10	ebstool23	Stool	-	+	-	-	1 (50.0)
11	enstool33	Stool	+	+	-	-	2 (50.0)
12	enstool11	Stool	-	+	-	-	1 (25.0)
13	abstool12	Stool	+	+	-	-	2 (50.0)
14	anstool70	Stool	+	+	-	-	2 (50.0)
Total Study (%)			10 (71.4)	14 (100.0)	1 (7.1)	1 (7.1)	

The frequency and distribution of resistance genes detected in *C. difficile* obtained from stool samples are as shown in Table 9. Erythromycin resistance gene (*ermB*) was detected by PCR from *C. difficile* isolates in 64.2% of the samples while tetracycline resistance genes (*tetS* and *tetA*) were detected in 78.5% of the samples. Nine isolates possessed

all resistance genes analyzed in the study. Agarose gel electrophoresis of the amplified 16S rDNA of *C. difficile* isolated from the stool samples is shown in Fig 1 while the phylogenetic relatedness of representative *C. difficile* isolates with other anaerobic *Clostridium* species is shown in Fig 2.

Table 9: Prevalence of resistant genes to erythromycin and tetracycline found in isolates of *C. difficile* obtained from stool samples

Lanes	Code	Samples	<i>TetA</i>	<i>tetS</i>	<i>ErmB</i>	Total (%)
1	anStool12	Stool	+	+	+	3 (100.0)
2	ebstool05	Stool	+	+	-	2 (66.7)
3	Imstool15	Stool	+	+	+	3 (100.0)
4	anstool21	Stool	+	+	-	2 (66.7)
5	anstool22	Stool	+	+	+	3 (100.0)
6	abstool12	Stool	+	+	+	3 (100.0)
7	abstool77	Stool	+	+	+	3 (100.0)
8	imstool83	Stool	-	-	+	1 (33.3)
9	imstool49	Stool	+	+	+	3 (100.0)
10	ebstool23	Stool	+	+	+	3 (100.0)
11	enstool33	Stool	+	+	+	3 (100.0)
12	enstool1	Stool	+	+	+	3 (100.0)
13	abstool12	Stool	-	-	-	0
14	anstool70	Stool	-	-	-	0
Total study (%)			11 (78.5)	11 (78.5)	9 (64.2)	

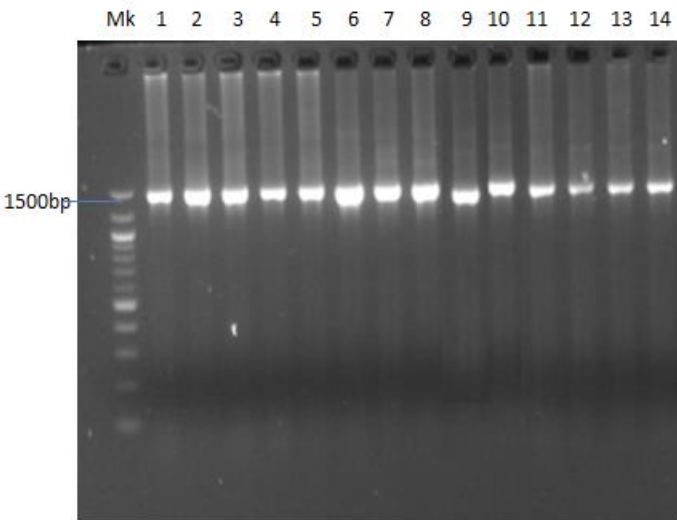


Fig 1: Agarose gel electrophoresis of amplified 16S rDNA of *Clostridioides difficile* isolated from stool samples

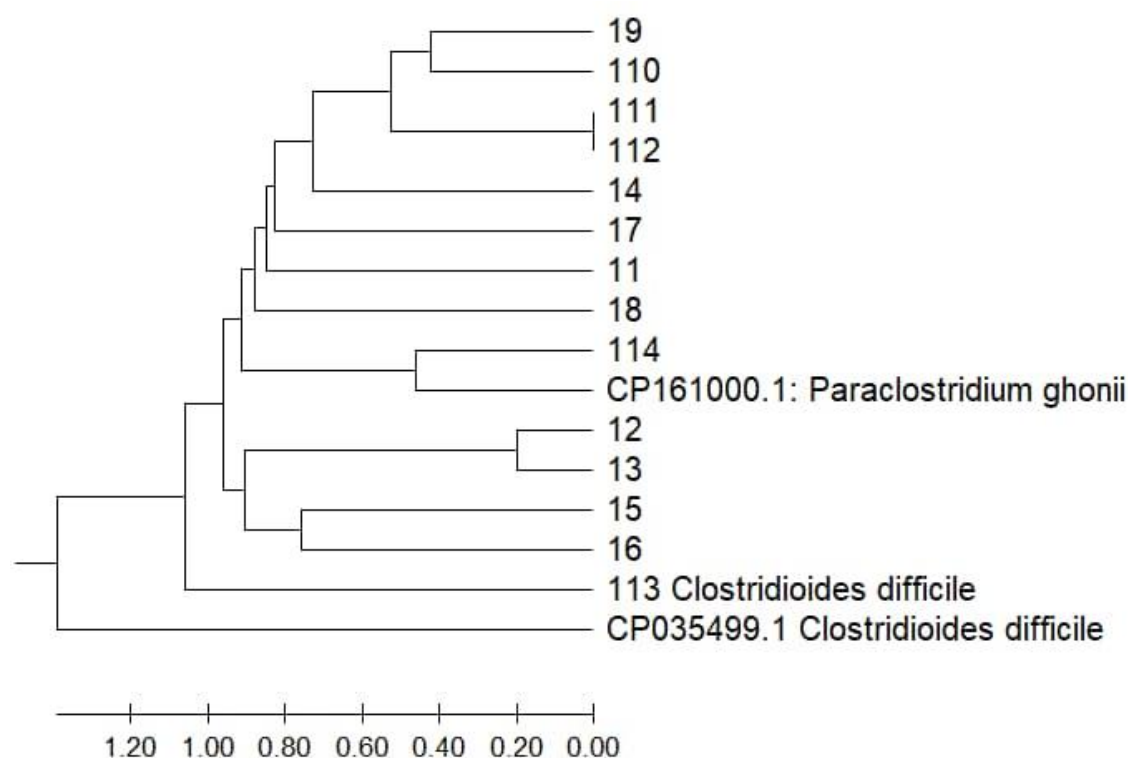


Fig 2: Phylogenetic relatedness amongst anaerobes isolates from clinical samples

Discussion:

A large portion of bacteria (anaerobes) in the stool are commensals, which often do not harm humans unless there is an underlying risk factor or the case of a compromised immune system. The bacterium of interest in this study (*C. difficile*) is regarded as both a commensal organism and a pathogen to domestic animals (29). Most of these anaerobes in the gut that have been isolated from the feces are not competitive, and their population can only explode, or they can thrive better in the gut after an array of antibiotic treatments which could destroy much of the intestinal normal flora (29). The statistical comparison of the total anaerobic count in stool samples showed no significant difference ($p > 0.05$) between samples obtained from Ebonyi and other southeastern States of Nigeria in the study. However, there was a significant difference between samples obtained from Anambra and those from Imo and Abia States.

The bacterial identity, query cover, and percentage homology were written against the confirmed blasted identity of the isolates, including accession numbers obtained from the National Centre for Biotechnology Information (NCBI) website. The 3.1% prevalence of *C. difficile* obtained in this study is less than the rates reported by several studies in literature. This implies that the probability of infection due to *C. difficile* would be present. However, the presence of the pathogen (even in its low

prevalence) in this study is an indication that there is a likely chance of a CDI outbreak if proper measures are not in place to curtail the infection.

Following reports of Warriner et al., (30), *C. difficile* was previously thought to be only clinically associated. However, with advanced scientific methods, CDI is now thought to be a community-acquired infection. The prolonged survival of spores of *C. difficile* in the environment might also increase the possibility of animal and food contamination which is a potential source of infection (31). In Europe, a higher prevalence had also been reported than the one obtained in this study, howbeit for hospitalized patients with even higher reports of toxigenic strains of *C. difficile* recovered from stool samples of patients (32). Nevertheless, findings in our study corroborated the report of others (33,34), who opined that carriage rates of *C. difficile* in healthy adults varied from 0% to 3% in Europe and up to 15% in Japan.

Following cultural phenotypic methods, the recovery rate of *C. difficile* from stool samples revealed that while both treatment methods (heat and alcohol treatments) yielded positive *C. difficile* growth, the alcohol shock method was better at recovering the bacterial isolates from clinical samples. The findings in this study about how the spores of the bacterium were recovered from both treatment processes agreed with the findings of Lawley et al., (35). They reported that spores are diffi-

cult to eradicate due to their ability to resist radiation, heat and chemicals (such as alcohols), which are usually employed as disinfectants in both hospital and community settings. In this study, alcohol shock samples had a better recovery rate of 3.1% than heat shock samples rate of 1.1%.

The susceptibility to carbapenems and aminoglycosides by most isolates of *C. difficile* in this study is at variance with what was observed in the study by Jobstl et al., (36), which separately reported some levels of resistance to the antibiotics by this bacterium. It is noteworthy that some of these antibiotics are commonly used for the treatment of diarrhea illnesses in humans. *Invitro* resistance of *C. difficile* to several classes of antimicrobial agents has been recognized and reported (37), thus, there is the possibility of a high risk for CDI to occur in healthcare settings and the community at large. This finding corroborated the report of Freeman et al., (38), who asserted that the resistance of *C. difficile* to several antibacterial agents may not be essential for CDI because the organism is relatively susceptible to high-ceiling antimicrobial agents.

It has been reported that the major pathogenic mechanism of *C. difficile* is production of cytotoxin B and enterotoxin A, which are respectively encoded by *tcdB* and *tcdA* genes, co-located in a 19.6 kb region of the chromosome named pathogenicity loci (Paloc) along with other regulatory genes (3,4). Toxin A causes diarrhea and Toxin B is cytotoxic to the colonic cells. Virtually all studies where *C. difficile* have been successfully recovered have screened for the ability of the isolates to have had at least one of the three toxins or genes (*tcdA*, *tcdB*, *cdtA/B*) needed to fulfill the criteria for virulence/toxigenicity (39). The findings in this study (71.4% and 100.0% for *tcdA* and *tcdB* genes respectively) agree with the study of Shokoohizadeh et al., (40), who opined that the genes for enterotoxin production *tcdA* are found in approximately 70% of *C. difficile* isolates, while the genes for cytotoxin production are found in all strains of *C. difficile* isolates.

The severity of the disease caused by the bacterium is conferred by the presence of the genes (*cdtA* and *cdtB* genes), which code for the production of binary toxins (41). The pathogenicity of *C. difficile* is mainly due to the presence of two large protein toxins (toxin A and toxin B) and the fully described binary toxin (42). Toxin A is a potent enterotoxin that causes the accumulation of fluid in the gut, is cytotoxic to cells in tissue culture, and is perhaps lethal to experimental animals (43). Toxin B has been reported to be a 1,000-fold more potent cytotoxin than toxin A (but is not an enterotoxin) in original animal studies. Still,

there are reports that some strains produce toxin B and not toxin A, which causes severe diarrhea in humans (44). More so, the reports of *C. difficile* strains are toxin A–negative and toxin B–positive, which have been associated with human diseases described in certain epidemics around the world (45). The variation in the findings in this study could be a function of geographical differences and environmental conditions with respect to cultural methods and methods used for the analysis of the bacterium.

The findings in this study are consistent with the reports from literature, which have it that *C. difficile* from humans or animals are commonly resistant to macrolides (erythromycin) and tetracyclines as well as fluoroquinolones (moxifloxacin) and lincosamides (clindamycin) (36). Also, consistent with the finding in this study was the report by Knetsch et., (46), who opined that the global *C. difficile* population contained a broad array of antibiotic-resistance genes encoding resistance to tetracycline and erythromycin.

Our study provides baseline data for the prevalence of *C. difficile* in clinical samples in southeast Nigeria in the following ways; (i) chances of severe infections for this pathogen in the studied location would likely to be there even were low prevalence and non-possession of genes for binary toxin production in most of the isolates in the study was reported; (ii) the study has re-enacted the idea that the alcohol shock method for isolation of *C. difficile* is more effective than the heat-shock method; and (iii) pathogenic *C. difficile* isolates in the studied location were multi-drug resistant.

Conclusion:

This study evaluated the prevalence, molecular characteristics and antibiotic susceptibility patterns of *C. difficile* in southeastern Nigeria, revealing high sensitivity to amoxicillin-clavulanic acid, gentamicin and meropenem, but high resistance to tetracycline and erythromycin, with significant expression of *tcdB* and *tcdA* virulence genes.

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Table 10: Bacterial isolates with their accession numbers

Serial numbers	Bacterial isolates	Accession numbers
1	<i>Lysinibacillus fusiformis</i>	PQ462169
2	<i>Lysinibacillus sphaericus</i>	PQ462171
3	<i>Vagococcus humatus</i>	PQ462172
4	<i>Enterocloster boltea</i>	PQ462174
5	<i>Clostridium butyricum</i>	PQ462175
6	<i>Clostridium butyricum</i>	PQ462176
7	<i>Clostridium butyricum</i>	PQ462177

Availability of data and materials:

The datasets generated and/or analyzed during the study are available on request and the bacterial isolates with accession numbers are as shown in Table 10

Contributions of authors:

EIO did all sample collections, laboratory analysis, writing of the manuscript and arrangement of the manuscript in accordance with the Journal requirements; MCU was the co-supervisor who oversaw the analysis of the research and the manuscript writings; COE was the main supervisor who checked every work done by the corresponding author and the co-supervisor; and OAI assisted with the design of primers to characterize *C. difficile* ensuring the smooth running of the molecular process.

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Conflict of interest:

Authors declare no conflict of interest.

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