Trop J Phytochem Pharm Sci, March 2025; 4(3): 126 - 135 **ISSN 2955-1226 (Print)**
ISSN 2955-123(Electronic)

Tropical Journal of Phytochemistry & Pharmaceutical Sciences

Available online at https://www.tjpps.org

Original Research Article

Prevalence, Molecular Characterization and Antibiogram Susceptibility Pattern of *Clostridioides difficile* from Food Samples in South Eastern Nigeria

Eddison I. Oghonyon[1&2], Malachy C. Ugwu[1,3], Charles O. Esimone[1,3], Anthony I. Onah[2] and Josiah E. Ifie[4]

[1]*Department of Pharmaceutical Microbiology &Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, P.M.B 5025, Awka, Anambra State, Nigeria*

[2]*Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, David Umahi Federal University of Health Sciences, P.M.B 211, Uburu, Ebonyi State, Nigeria*

[3]*TETFUND –Centre of Excellence for Biomedical, Engineering and Agricultural Translational studies UNIZIK, P.M.B 5025, Awka, Anambra state, Nigeria.*

[4]*Department of Medical Biochemistry, Faculty of Biomedical Sciences, Kampala International University, Western Campus, P.M.B 71, Ishaka-Bushenyi, Uganda.*

ABSTRACT

Clostridioides difficile is a foodborne bacterium that causes severe gastrointestinal infections due to its virulence and antibiotic resistance. The major reason for this research is to ascertain *Clostridioides difficile* prevalence, molecular characterization, and antibiogram patterns in food samples from southeast Nigeria. A total of 440 food samples, including smoked fish and pork, were analyzed between June 2018 and December 2019. Enumeration of total anaerobes was performed using standard bacteriological techniques, while *Clostridioides difficile* isolation was carried out on selective differential agar. Biochemical identification was confirmed using molecular methods. The Kirby-Bauer disc diffusion was done to ascertain antibiogram susceptibility, and PCR activity was carried out to identify resistance gene (*tetS, tetA*, and *ermB*) and virulence (*tcdA, tcdB, cdtA*, and *cdtB*). Anaerobic bacterial counts varied across states, ranging from 1.85±0.12 log10 CFU/g in Enugu to 2.15±0.03 log10 CFU/g in Imo. Smoked fish and pork exhibited higher counts, with values between 5.16±0.01 and 5.36±0.01 log10 CFU/g. Identified anaerobes included *Lysinibacillus macroides, Clostridium bolteae, Clostridium butyricum*, and *Clostridioides difficile.* The prevalence of *Clostridioides difficile* was 2.00%, with isolates showing resistance to tetracycline (73.91%), erythromycin (73.91%), and ciprofloxacin (43.48%). Multiple antibiotic resistance was recorded at a rate of 0.44. Binary toxin genes (*cdtA* and *cdtB*) were found at low levels, 69.56% expressed *tcdA*, and all isolates of *Clostridioides difficile* carried the *tcdB* gene. Although rare in the area, binary toxin genes still pose a risk of severe *Clostridioides difficile* infections. This study emphasizes the significance of ongoing monitoring and controlling antibiotic resistance in foodborne bacteria.

Keywords: Molecular characterization, Antibiogram, *Clostridioides difficile*, prevalence, South Eastern Nigeria

Received 10 January 2025
Revised 21 February 2025
Accepted 01 March 2025
Published online 04March 2025

Introduction

An essential bacterium called *Clostridioides difficile* induces gastrointestinal infections that can range from minor diarrhea to serious and in some cases fatal diseases. It has been recognized that the presence of *Clostridioides difficile* in food samples poses a possible concern to public health. According to recent studies, *Clostridioides difficile* is present worldwide in a variety of food samples, with contamination rates varying from 0.8% to 10.3% in accordance with the food type[1]. This is a worldwide threat that has gotten worse over time, leading to the rise of variants of these bacteria that are resistant to several drugs[2,3].

*Corresponding author. Email: oghonyonei@dufuhs.edu.ng, eddisonioghonyon@gmail.com
Tel: +2348038469094

Citation: Oghonyon EI, Ugwu MC, Esimone CO, Onah AI, Ifie JE. Prevalence, Molecular Characterization and Antibiogram Susceptibility Pattern of *Clostridioides difficile* from Food Samples in South Eastern Nigeria. Trop J Phytochem Pharm Sci. 2025; 4(3) 126 – 135 http://www.doi.org/10.26538/tjpps/v4i3.4

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

The incidence, molecular characteristics, and antibiogram susceptibility patterns of *Clostridioides difficile* in food samples are poorly understood in South Eastern Nigeria. By offering a thorough examination of *C. difficile* in food samples from the Eastern part of Nigeria, this investigation seeks to close this knowledge gap. This study will provide fresh insights into the incidence and traits of *Clostridioides difficile* in this area by utilizing molecular methods and antibiogram susceptibility testing. This study's main goals are to identify the molecular profiles and antibiogram susceptibility patterns of the isolated strains of *Clostridioides difficile* and to ascertain the incidence of the bacteria in food samples gathered from southeast Nigeria. Despite the growing recognition of *Clostridioides difficile* as a foodborne pathogen, there is limited data on its prevalence and characteristics in food samples from South Eastern Nigeria. This ignorance makes it more difficult to create public health initiatives and efficient food safety protocols that reduce the possibility of *Clostridioides difficile* spreading through tainted food[4,5].

Due to the potential for *Clostridioides difficile* to be consumed by humans or animals through contaminated food and water, the Centres for Disease Control and Prevention (C.D.C) identified this bacterium to be a public health threat of "urgent" concern on September 16, 2013, in the United States[6]. In the United States, *C. difficile* infections result in approximately 14,000 deaths annually, with over 90% of these fatalities occurring among individuals aged 65 and older[7]. *C. difficile* is currently the leading cause of diarrhea, contributing to unnecessary costs amounting to billions of dollars annually[8]. In this study, *Clostridioides difficile* will be isolated and identified from food in South Eastern

Nigeria, its incidence and rate of recovery will be assessed using the alcohol and heat shock method, its antibiogram susceptibility pattern will be assessed, and virulence and resistance genes will be detected in *C. difficile* isolated in food samples from Eastern region of Nigeria. This research will improve our knowledge of *Clostridioides difficile* and guide measures to lessen its influence on food safety and public health in South Eastern Nigeria by accomplishing these goals and providing useful data to the fields of microbiology and public health.

Materials and Methods

Method

Equipment

These include Autoclave (bucket autoclave from Medica Instrument Manufacturing Co., India), AG0025 Anaerobic Jar (Oxoid, United Kingdom). Thermoscientific Anaerogen (Thermo Fisher Scientific, China). JD-WD-001 Pressure pot/cooker (National Presto Industries, Inc., United States, Wisconsin', Matfer Bourgeat USA, Inc.), Petri dish (Rongtai, China) beakers (Hirschmann Laborgeräte GmbH and Co. KG, Germany), conical flasks (Erlenmeyer Flask manufacturers and OEM manufacturers, India), measuring cylinders (Pyrex borosilicate glass, United States), wire loops (GPC Medical Ltd, India), inoculation loops (Rongtai Medical Consumables, China) pipettes **(**Dynalab Corp, Germany), Eppendorf tubes (Life Sciences, Germany), PCR tubes (Yong Yue Medical Technology, China), Centrifuge (Sigma Laborzentrifugen GmbH, China), agarose gel (Sigma-Aldrich, Merck KGaA, Germany), electrophoresis machine (Horizon Submarine Electrophoresis systems, Owl Dual-Gel Systems, Thermo Fisher Scientific, USA), PCR machine (Applied Biosystems Veriti 96-Well Thermal Cycler, ProFlex PCR System, Thermal Fisher Scientific, USA) and big dye sequencing machine (3500 Series Genetic Analyzer, Thermo Fisher Scientific, USA).

Study Area and Sample Collection

Samples were collected from open markets in Eke-Awka, First market, Amawbia market, and Eke Nibo (in Anambra States, 6.2116° N, 7.0714° E). At the same time, samples from Ebonyi were collected from the main Effium market, Amuzu market, Eke market, and New Kpiri Kpiri market (6.3231° N, 8.1120° E). Enugu food samples were obtained from New-market, 4-market days, Udi market, and Eke-Obinagu market6 (5364° N, 7.4356° E). Open markets such as Nkwo Orji, Alaba market, Save More, and school road markets were used as sampling points for food samples in the Imo States (5.5720° N, 7.0588° E). Markets in Abia State include Orie Ugba, Umuahia main market, Afule, and Nkwogwu markets (5.4527° N, 7.5248° E). The food samples were comprised of vegetables, sachet tomatoes, smoked fish, pork, poultry meat, and beef, which were obtained from open markets in cities in the five (5) South Eastern States of Nigeria. Food samples were collected between the periods of June 2018 through December 2019.

Sample Size Determination

Four hundred and forty (440) food samples were examined. The sample size was calculated based on the following formula:

$Sample\ size\ (N) = (Z^2\ P(1-P)) \div D^2$ ----------> Equation 1

where: *N= Sample size, Z = z score (1.969), P = prevalence (0.45)* and *D= 0.05*

Clostridioides difficile Isolation and Identification

To guarantee reproducibility, this analysis was conducted three times. To reduce the presence of competitive microbiota as well as encourage the cultivation of spore-forming bacilli, all samples underwent pre-treatment with heat and alcohol shock methods to isolate *Clostridioides difficile*[9.,10.] A standard microbiological assay was performed, utilizing *C. difficile* differential agar CM 0601 (Oxoid) enhanced with *C. difficile* selective supplement SR0096 (Oxoid)[9.,10.] To prepare the stock solutions, 20 grams of samples were weighed and then added to 180 milliliters of saline solution that was sterile in a conical flask, after which the mixture was thoroughly agitated. The stock solution was subjected to a ten-fold serial dilution and two milliliters of the suspension were added to a clearly labeled tube that contained two milliliters of absolute ethanol. To achieve homogenization, the mixture was gently rocked and then set aside for an hour before plating[11.] The sample mixture tubes were subjected to a 5-minute boiling heat treatment. Once the tubes had cooled, plating was conducted on media enriched with *Clostridium difficile* supplement (Oxoid)[12.] Following Gram and spore staining to identify positive isolates, the cultural, morphological, microscopic, and biochemical characteristics of the isolates were examined[13.] Biochemical test such as catalase, oxidase, indole, urease, and sugar fermentation was conducted to know the species of *Clostridium*. The universal primer sets (16sRNA) were used to identify *Clostridioides difficile* through sequence blasting[14.]

Antibiotic sensitivity Test

Standard methods for testing susceptibility to antibacterial agents were applied, with each sample measured twice. The *Clostridioides difficile* isolates that were identified were subjected to standard antibacterial susceptibility testing (AST) with the Kirby-Bauer disc diffusion method[13.] The antibiotic discs by Oxoid UK have the following antibiotics: Meropenem (10 µg), Erythromycin (15 µg), Metronidazole (50 µg), Amoxicillin/clavulanic acid (20/10 µg), Clindamycin (20 µg), Gentamicin (10 µg), Ciprofloxacin (5 µg), Vancomycin (30 µg), and Tetracycline (30 µg), were utilized. A bacterial culture that had been incubated in broth for 18–24 hours was uniformly spread on Mueller Hinton agar (MHA). The inoculum, adjusted to a McFarland standard of 1.5 x 10^8 cells/ml, was streaked on Mueller Hinton agar (MHA) plates using a sterile loop. Sterilized forceps were used to carefully place the antibiotic discs onto the plates. After being incubated at 37°C for 24 hours, the susceptibility results were evaluated according to the breakpoints set by the Clinical Laboratory Standards Institute[15.]

Index for Multiple Antibiotic Resistance

The specific equation provided below was used to calculate the Multiple Antibiotic Resistance (MAR) index[16:]

$MARindex = \frac{y}{nx}$ ----------> Equation 2

$where\ y = number\ of antibiotics\ to\ which\ the\ organism\ is\ resistant,$

$n = total\ number\ of antibiotics\ tested$

$x = number\ of\ isolates\ tested$

DNA Extraction

The action was taken three times to guarantee the result's accuracy. DNA was extracted employing the boiling method outlined by[17.] A tube containing 2 ml of a *C. difficile* 24-hour broth culture was subjected to high-speed centrifugation for 5 minutes. After the supernatant was removed, 200 µL 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 underwent high-speed centrifugation for 2 minutes. The supernatant from this second centrifugation was considered pure DNA, and 10 µL of it was used for gene amplification via PCR.

Determination of virulence Genes and Resistance Genes

Using standardized primer sets, virulence genes (*tcdA, tcdB, cdtA*, and *cdtB*) were identified in duplicate to make sure that the results were accurate, as shown in Table 1. Using polymerase chain reaction (PCR), the genes responsible for erythromycin (*ermB*) and tetracycline resistance (*tet A*, and *tet S*) were screened in duplicate as shown in Table 2. The analysis utilized the sample of bacterial DNA that had been extracted earlier. Agarose gel electrophoresis was performed using each primer (both forward and reverse primers) for the target genes[18.] A thermocycler (C1000 Touch, Bio-Red Laboratories, USA) was used to determine an optimal annealing temperature for the specific binding of the primer set to the DNA template.

ISSN 2955-1226 (Print)
ISSN 2955-123(Electronic)

Table 1: Primer sets for virulence genes

Genes	Primers	Sequence (5'-3')	Conc. (µM)	Product size (bp)
TcdA	tcdA-F	GTATGGATAGGTGGAGAAGTCAGTG	0.025	632
	tcdA-R	CGGTCTAGTCCAATAGAGCTAGGTC		
TcdB	tcdB-F	GAAGATTTAGGAAATGAAGAAGGTGA	0.01	441
	tcdB-R	AACCACTATATTCAACTGCTTGTCC		
CdtA	cdtA-F	ATGCACAAGACTTACAAAGCTATAGTG	0.2	260
	cdtA-R	CGAGAATTTGCTTCTATTTGATAATC		
CdtB	cdtB-F	ATTGGCAATAATCTATCTCCTGGA	0.5	179
	cdtB-R	CCAAAATTTCCACTTACTTGTGTTG		

Table 2: Primer sets for resistance genes

Genes	Primers	**Sequence (5'-3')**	**Conc. (µM)**	**Product size (bp)**
erm(B)	ErmB-F	GAAAAGGTACTCAACCAAATA	0.1	639
	ErmB-R	AGTAACGGTACTTAAATTGTTTAC		
tet(S)	TetS-F	ATCAAGATATTAAGGAC	0.1	573
	TetS-R	TTCTCTATGTGGTAATC		
tet(A)	tet(A)- F	TTGGCATTCTGCATTCACTC	0.1	494
	tet(A)- R	GTATAGCTTGCCGGAAGTCG		

Statistical Analysis

Data obtained was analyzed with infographics using Microsoft Excel (Version 2016, 2015, Microsoft Corporation, USA)[19], multiple comparisons of means (ANOVA) using Statistical Package for Social Scientist (SPSS) version 21.0 software and a phylogenetic tree of species relatedness using Mega 10 software. (Verison 10, 2018, MEGA Limited, Japan)

Results and Discussion

Table 3 displays the results of the anaerobic bacterial counts in food samples obtained from South Eastern Nigeria. The overall anaerobe count (log10 CFU/g) for food samples sourced from different states indicated that Anambra's vegetables recorded a count of 2.05±0.03 log10 CFU/g. In contrast, the samples from Anambra show a significant difference when compared to the counts obtained in Ebonyi, Enugu, and Imo states respectively. In canned food, there is no significant difference in total anaerobic bacterial counts obtained in all the States, whereas there is a significant difference in bacterial counts obtained in other States in comparison with each other. Table 4 presents the identity and phylogenetic relatedness of anaerobic bacterial isolates obtained from food samples in South Eastern Nigeria. The isolates were identified using gene sequencing, and phylogenetic analysis was performed to determine their evolutionary relationships[20]. The sequences were matched against those in the GenBank database to determine the bacterial species[21]. The results revealed the presence of several anaerobic bacterial species of *Clostridium*, including the studied organism as shown in Table 4. The analysis showed that the isolates clustered into distinct groups corresponding to their respective genera. This indicates that the isolates are closely related within their genera but distinct from other genera. The phylogenetic relatedness of the isolates was compared with similar studies conducted in other regions[21]. The results aligned with earlier findings, indicating that the diversity and relatedness of anaerobic bacteria in food samples are comparable across various geographical regions. The occurrence of pathogenic anaerobic bacteria in food samples can pose a risk to consumers. Therefore, monitoring and controlling these bacteria in food production and processing is essential.

As shown in Table 5, the prevalence of anaerobes from food samples using standard cultural methods indicated that beef (62%), poultry meat (56%), and vegetables (55%) had the highest counts of anaerobes among all analyzed food samples. In contrast, the overall result was 52%, which demonstrates the occurrence of anaerobic bacteria in the analyzed food samples. According to food samples in Table 6, the prevalence of *Clostridioides difficile* showed a total study prevalence of 2%. The prevalence of food samples from Anambra and Ebonyi states was 33.3% each. In contrast, the prevalence of *C. difficile* from food samples was 22.2% in Enugu State and 11.1% in Imo State, with Anambra and Ebonyi States exhibiting the highest prevalence at 33.3%. Abia State, however, reported no *Clostridioides difficile* counts. The use of heat treatment and alcohol shock treatment can significantly affect the recovery of *Clostridioides difficile* from food samples using cultural methods. These methods aim to enhance the detection of *C. difficile* spores, which are known for their resilience and ability to survive harsh conditions. The heat treatment method's recovery rate of *C. difficile* (0.88%) shown in Table 7 was found to be lower than the recovery rate via alcohol treatment (2%). When compared to other studies, it has been shown that alcohol shock treatment is more effective than heat treatment for the recovery of organisms. Heat can significantly reduce the recovery ability of the organism, making alcohol shock treatment a preferable method[22].

ISSN 2955-1226 (Print)
ISSN 2955-123(Electronic)

Table 3: Total anaerobic bacterial count of different food samples from states in Eastern Nigeria

Food Samples	Anambra	Ebonyi	Enugu	Imo	Abia
Vegetables	2.05±0.03[ab]	1.98±0.07[b]	1.85±0.10[c]	2.15±0.03[b]	1.97±0.06[a]
Amked fish	5.18±0.01[a]	5.16±0.01[a]	5.27±0.01[b]	5.11±0.01[c]	5.12±0.02[c]
Canned food	0.00±0.00[a]	0.00±0.00a	0.00±0.00[a]	1.35±0.11[a]	0.00±0.00[a]
Pork	5.28±0.02[a]	5.17±0.02[b]	5.24±0.01[c]	5.28±0.01[a]	5.36±0.01[d]
Beef	5.27±0.02[a]	5.27±0.01[a]	5.13±0.02[b]	5.33±0.01[c]	5.44±0.02[d]
Poultry	4.42±0.01[a]	4.40±0.01[a]	4.20±0.06[b]	4.25±0.01[c]	4.49±0.02[d]

The same alphabets across the rolls indicate no significant difference between p>0.05

Table 4. Identity of bacterial isolates (anaerobes) from food samples

Samples code	Source	Identity	Query cover	Homology (%)	Accession number
abBE10	Beef	*Clostridium butyricum*	100	100.00	MH888202.1
anST3	Sachet Tin	*Clostridium butyricum*	99	99.8	CP019860.1
enF6	Smoked Fish	*Clostridium bolteae*	100	100.00	CP025562.1
imST18	Satchet tin	*Clostridium butyricum*	100	100.00	MH888202.1
anChi10	Pork meat	*Lysinibacillusfusiformis*	97	90.35	CP020424.2
anLV10	Vegetable	*Clostridium bolteae*	100	100.00	CP025562.1
enPM8	Pork meat	*Clostridium butyricum*	100	100.00	MH888202.1
anPM5	Pork meat	*Clostridium difficile*	90	96.75	CP028524.1
ebPM6	Pork meat	*Clostridium difficile*	91	94.6	CP028361.1
enPM3	Pork meat	*Clostridium difficile*	99	96.75	CP025047.1
abBE2	Beef	*Clostridium difficile*	98	91.44	CP012309.1
ebBE9	Beef	*Clostridium difficile*	99	96.75	CP025047.1
anBE7	Beef	*Clostridium difficile*	91	94.6	CP028361.1
anBE3	Beef	*Clostridium difficile*	99	96.75	CP025047.1
enLV4	Vegetable	*Clostridium difficile*	97	90.35	CP020424.2
ebLV5	Vegetable	*Clostridium difficile*	100	100.00	MH888202.1

Table 5: Prevalence of anaerobes from food samples using standard cultural methods

		Eastern States						
Food samples	**Sample no**	**Anambra**	**Ebonyi**	**Enugu**	**Imo**	**Abia**	**Total sample (study)**	**Study Total (%)**
Vegetables	25	12(48)	16(64)	18(72)	13(52)	10(40)	125	69(55)
smoked fish	8	2(25)	3(38)	4(50)	4(50)	2(25)	40	17(43)
canned foods	5	0(0)	0(0)	0(0)	1(20)	0(0)	25	1(4)
Pork	17	10(59)	12(71)	8(47)	10(59)	11(65)	85	41(48)
Beef	20	14(70)	12(60)	11(55)	12(60)	13(65)	100	62(62)
Poultry	15	10(67)	7(47)	8(53)	7(47)	10(67)	75	42(56)
Total	90	48(53)	34(38)	49(54)	47(52)	56(62)	450	234(52)

Table 6: Prevalence of *Clostridium difficile* from food samples in the study

		Eastern States						
Food sample	**Sample no**	**Anambra**	**Ebonyi**	**Enugu**	**Imo**	**Abia**	**Total sample (study)**	**Study Total (%)**
Vegetables	25	0(0)	1(4)	1(4)	0(0)	0(0)	125	2(1.60)
smoked fish	8	0(0)	0(0)	0(0)	0(0)	0(0)	40	0(0.00)
canned foods	5	0(0)	0(0)	0(0)	0(0)	0(0)	25	0(0.00)
Pork	17	1(5.8)	1(5.8)	1(5.8)	0(0)	0(0)	85	3(3.52)
Beef	20	2(10)	1(5)	0(0)	1(5)	0(0)	100	4(4.00)
Poultry	15	0(0)	0(0)	0(0)	0(0)	0(0)	75	0(0.00)
Total	90	3(33.3)	3(33.3)	2(22.2)	1(11.1)	0(0)	450	9(2.00)

Table 7: Percentage Recovery of *C. difficile* from food samples using cultural methods

Samples	**Number**	**Heat Treatment (%)**	**Alcohol Treatment (%)**	**Study Total(%)**
Vegetables	125	1(0.80)	2(1.60)	2(1.60)
Smoked fish	40	0(0.00)	0(0.00)	0(0.00)
Canned foods	25	0(0.00)	0(0.00)	0(0.00)
Pork	85	1(1.20)	3(3.35)	3(3.50)
Beef	100	2(2.00)	4(4.00)	4(4.00)
Poultry	75	0(0.00)	0(0.00)	0(0.00)
Total	450	4(0.88)	9(2.00)	9(2.00)

In Tables 8 and 9, the antibacterial sensitivity pattern and resistance/susceptibility percentages of *C. difficile* isolates from food samples revealed a susceptibility to carbapenems (82.61%), aminoglycosides (86.61%), and β-Lactam/combination agents (73.91%). Significant resistance was noted for tetracycline (73.91%), macrolides (73.91%), and fluoroquinolones (43.48%).

Table 11 shows that all (100%) of the *C. difficile* isolates contained *tcdB* genes, while the isolated *C. difficile* strains did not contain the *cdtA* and *cdtB* genes, leading to a 0% occurrence of these genes. The occurrence of resistant genes highlighted in Table 12 suggested that the PCR products of 3 erm(B) genes amplified from *Clostridioides difficile* isolates reached 73.91%. This percentage also applied to tetracycline resistance (tet(S) and tet(A) genes. This indicates that the isolates show resistance to the common antibiotics analyzed in this study. In Table 1, multiple primer sets were designed and evaluated targeting virulence genes in *Clostridioides difficile*. The primer sets were optimized for

specificity and sensitivity to ensure accurate detection of virulence genes. The Primer sets were tailored in line with known sequences of virulence genes in *C. difficile.* The primers were tested for specificity by amplifying DNA from *C. difficile* strains and ensuring no cross-reactivity with non-target DNA[20]. In Table 2, the primer sets were designed and validated by targeting antibiotic-resistance genes in food samples. The primer sets were optimized for specificity and sensitivity to ensure accurate identification of antibiotic-resistance genes. Sets of primers were developed using aligned sequences of target antibiotic-resistance genes. The primers were tested for specificity by amplifying DNA from food samples and ensuring no cross-reactivity with non-target DNA.

The anaerobe count (log10 CFU/g) of food samples collected from various states in Eastern Nigeria, as shown in Table 3, indicated that all vegetable samples except one had an anaerobic bacterial count below log10 of 2 CFU/g. Ready-to-eat vegetables and other food sources have been found to harbor anaerobic bacteria such as *Clostridium* and related species.

The report from other studies reveals that ready-to-eat foods commonly sold and marketed in Nigeria are inhabitable by both anaerobes and facultative anaerobes alike[23]. As illustrated in Table 3, four out of five states had counts that were not significantly different ($p>0.05$). Anaerobe counts of vegetables in Anambra state were not significantly different from counts in Ebonyi, Imo, and Abia states, respectively. The counts for canned foods in all states were not significantly different from one another. However, beef had a significant difference ($p<0.05$) in anaerobe count for samples obtained from Ebonyi, Enugu, Imo, and Abia, respectively. There were significant differences in pork counts observed for samples obtained from Ebonyi, Enugu, Imo, and Abia states ($p<0.05$).

In the gel-based separation, the bacterial DNA extracted from food samples using 16SRNA in Figure 1 showed that all isolated bacteria were successfully extracted and amplified, shown by bands in lanes 1-9 at 1500 base pair.

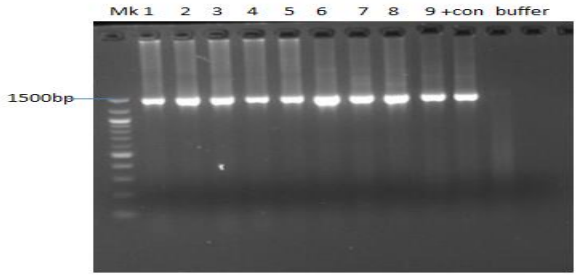

Figure 1: Agarose gel electrophoresis of bacterial DNA isolated from food samples ***Lanes Mk:*** *DNA markers,* ***Lanes 1 – 9:*** *represent different bacterial DNA samples*

Table 4 displays the identity and phylogenetic relatedness of anaerobic bacterial isolates obtained from food samples in Eastern Nigeria. These isolates from food samples in states of South Eastern Nigeria include *Clostridium* spp. and *Lysinibacillus forsiformis.* The confirmed BLAST identities of the isolates, along with their corresponding accession numbers from the 'National Centre for Biotechnology Information (NCBI)', are utilized to document the bacterial identity, query coverage, and percentage homology. Table 5 shows that among all analyzed food samples, the occurrence of anaerobes was highest in beef (62%), followed by poultry meat (56%) and vegetables (55%). In Table 6, the overall occurrence of *Clostridioides difficile* in investigated food samples was found to be 2.00%. This finding aligns with other studies, which have evaluated the prevalence of *C. difficile* in raw beef, cow, goat, and buffalo in Iran to be 2.00%[1]. However, there was no *C. difficile* isolate (zero percent prevalence) from canned foods, poultry, and smoked fish, while beef, pork, and vegetables had a prevalence of *C. difficile*, respectively. Among the various food types considered and evaluated in this study, *C. difficile* was detected in three of the lots. This finding aligns with results reported by others indicating that *C. difficile* meets the criteria for a foodborne pathogen, as it is commonly found in a diverse range of foods, including meat, fresh produce, and seafood[24]. However, considering the data obtained in this study as well as the related study mentioned above, there seems to be a low prevalence of the bacterium in food samples, and these low prevalence data are comparable with other reports in the literature[23]. The variance between this research and the results obtained in this investigation could be attributed to environmental differences, which could have an impact on the type of strains found in the locality. This reason also aligns with the findings of other studies, which have reported that the global incidence of the bacterium varies significantly from one region to another[25].

Table 7 presents the results of the percentage recovery of *Clostrioides difficile* from food samples. Cultural phenotypic methods demonstrated that the percentage recovery of *C. difficile* from food samples was positive for both heat and alcohol treatments, with the alcohol shock method proving to be more effective in isolating bacterial isolates from these samples. The heat treatment method yielded a lower prevalence of *C. difficile* (0.88%) compared to the alcohol treatment method, which had a prevalence of 2.00%. The results obtained were found to be consistent with the conventional knowledge about the bacterium that "*C. difficile* is an anaerobic, spore-forming, environmental organism, whose spores can survive in the environment for a prolonged time and are resilient to drying, alcohol and many commonly used detergents"[26]. In this study, food samples subjected to alcohol shock at 2.00% demonstrated a superior recovery rate compared to those subjected to heat shock at 0.88%. A potential reason for this discovery is related to the role that heat plays in inhibiting *C. difficile* spores, specifically at temperatures of 85°C and even 71°C. At the same time, there has been a report of a sub-lethal effect on the spores of the bacterium[27]. As shown in Tables 8 and 9, the antibacterial sensitivity pattern and resistance/susceptibility percentages of *C. difficile* isolates from food samples indicated susceptibility to carbapenems, aminoglycosides, and β-Lactam/combination agents. Tetracycline, macrolides, and fluoroquinolones showed considerable resistance. There were, however, some isolates of *C. difficile* that were exhibiting resistance to additional antibiotics like aminoglycosides, carbapenems, and nitroimidazoles. Aside from the very few isolates with resistance to some of the antimicrobial classes, whose resistance mirrored what was attainable in literature, it is pertinent to assert that about 70% of the bacterium obtained in this research, were sensitive to primary drug options for treating *Clostridioides difficile* infection. This discovery corresponds with other research indicating that the resistance of *C. difficile* to different antimicrobial agents may not have a major effect on infection rates, given that the organism is still relatively vulnerable to high-risk antibacterial agents[28]. It has also been noted that some of these strains harbor a wide range of mobile elements, which encode genes for resistance to several antibiotics such as aminoglycosides, erythromycin, chloramphenicol, and tetracycline[28]. A possible reason for resistance to some of the antibiotics listed above is due to poor antimicrobial stewardship.

Table 8: Antibiotic resistance profile on food samples

Code	Samples	MEM	ERY	MET	AMC	CL	CN	CIP	VA	TET
anPM5	Pork meat	S	R	S	S	R	S	R	S	S
ebPM6	Pork meat	S	R	R	S	S	S	R	S	R
enPM3	Pork meat	S	S	S	R	R	S	S	R	R
abBE2	Beef	S	R	S	S	S	R	S	R	R
ebBE9	Beef	R	R	S	R	S	R	S	R	S
anBE7	Beef	S	S	S	R	S	S	S	R	R
anBe3	Beef	S	R	S	S	R	S	R	S	S
enLV4	Vegetable	R	R	R	S	S	S	S	S	R
ebLV5	Vegetable	S	R	S	S	S	R	S	S	R

R: *Resistance,* ***S:*** *Sensitivity*

Table 9: Percentage of antibiotic resistance and susceptibility profile of common antibiotics

		Sensitivity profile *n*=09	
Antibiotics classes	**Antibiotics**	**Susceptibility (%)**	**Resistance (%)**
Carbapenems	MEM	19(82.61)	3(17.39)
Macrolides	ERY	6(26.09)	17(73.91)
Nitroimidazole	MET	16(69.56)	7(30.43)
B-Lactam/combination agents	AMC	17(73.91)	6(26.09)
Lincosamide	CL	15(65.22)	8(34.78)
Aminoglycosides	CN	20(86.98)	3(13.02)
Fluoroquinolone	CIP	13(56.52)	10(43.48)
Glycopeptides	VA	16(69.56)	7(30.43)
Tetracyclines	TET	6(26.09)	17(73.91)

Key:*Meropenem (10 µg), Erythromycin (15 µg), Metronidazole (50 µg), Amoxicillin/clavulanic acid (20/10 µg), Clindamycin (20 µg), Gentamicin (10 µg), Ciprofloxacin (5 µg), Vancomycin (30 µg), Tetracycline (30 µg)*

In Table 10, the phenotypic distribution of *C. difficile* isolates from food samples exhibiting multiple antibiotic resistance indicated that those with a MAR index of 0.44 had the highest number of resistant phenotypes. At the same time, isolates that had a MAR index of 0.33 also exhibited higher resistance phenotypes. However, information about *C. difficile* infection as well as its antimicrobial resistance profile in Nigeria is scarce. Nonetheless, various reports from North America and Europe demonstrate a significant prevalence of infections caused by *C. difficile* and its resistance to antibiotics typically employed for treating this organism[29]. Research investigating 316 *Clostridioides difficile* isolates from patients in Europe discovered that 48% showed resistance to at least one of the eight tested antibiotics. In addition, 55% of the resistant strains were identified as multi-resistant, exhibiting tolerance to three or more antibiotics included in the study[30]. This finding aligns with the results of this study, in which more than 60% of the isolates were identified as multi-resistant, exhibiting a MAR index exceeding 0.3. Figures 2-5 below show the gel electrophoresis used to detect the presence of *tcdA*, *tcdB*, *ctdA*, and *ctdB* genes in *C. difficile* following PCR methods. The genes identified in the samples are crucial for assessing the virulence level of *C. difficile*. These *ctdA* and *ctdB* genes code for the binary toxin production responsible for more severe disease conditions due to *C. difficile*. Figures 2 to 5 demonstrate that genotypic virulence traits in isolated *C. difficile*, specifically the presence of *tcdB*, *tcdA*, *cdtA*, and *cdtB* genes, indicate that all (100%) isolates of *C. difficile* contained *tcdB* genes. According to reports, the main pathogenic mechanism of *C. difficile* involves producing cytotoxin B and enterotoxin A, which are encoded by the *tcdB* and *tcdA* genes, respectively. These genes reside in a 19.6 kb segment of the chromosome referred to as pathogenicity loci (Paloc), together with other regulatory genes[31]. While Toxin A causes diarrhea, Toxin B exhibits cytotoxic effects on colon cells. Nearly every study that has effectively isolated *C. difficile* has involved screening for at least one of the three toxins or genes (*tcdA, tcdB, cdtA/B*) necessary to fulfill the virulence/toxigenicity criteria[32]. Concerning *tcdA* genes, 3 of the 9 positive isolates were recognized as carrying the virulence genes, leading to a total of 66.67% of isolates exhibiting positive results for these genes. The *cdtA* and *cdtB* genes were absent in all isolated *C. difficile* strains. The examination of the occurrence of virulence genes shown in Table 11 demonstrated that three isolates possessed 25% (one-fourth) of the virulence genes investigated in this research, while six isolates had two out of four (50%) of these genes. The findings of this study align with those of another investigation, which confirmed that the genes responsible for enterotoxin production, *tcdA*, are found in approximately 70% of *C. difficile* isolates, while the genes for cytotoxin production are present in all strains of *C. difficile* isolates[33]

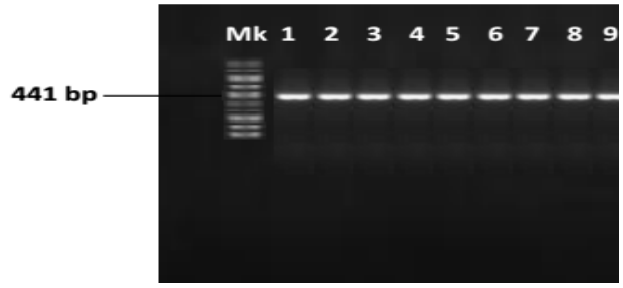

Figure 2: Gel electrophoresis to detect the presence of *tcdB*genes of *Clostridium difficile*

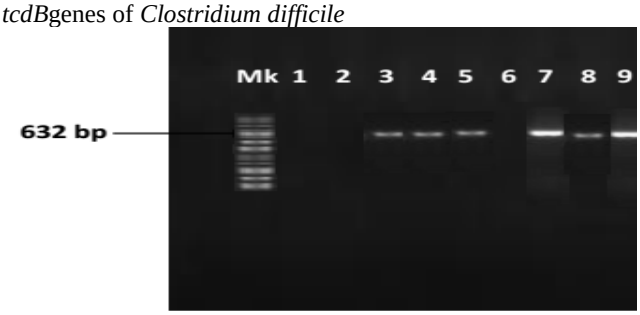

Figure 3: Gel electrophoresis to detect the presence of *tcdA*genes of *Clostridium difficile*

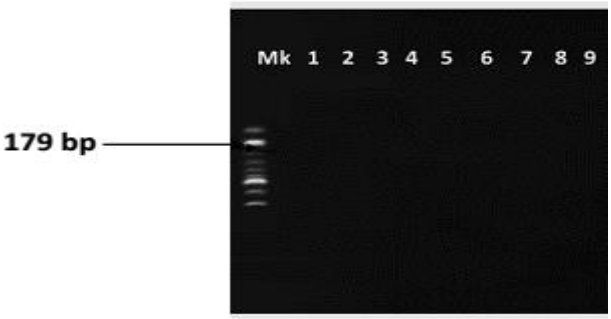

Figure 4: Gel electrophoresis to detect the presence of *cdtA*genes in *Clostridium difficile*

ISSN 2955-1226 (Print)
ISSN 2955-123(Electronic)

Figure 5: Gel electrophoresis to detect the presence of *cdtB*genes in *Clostridium difficile*

This implies that the presence of the gene in one strain of *C. difficile* isolates from the study indicates a probability of severe infection due to *C. difficile* at the study location. More so, it was revealed that none of the strains in food samples contained the gene that codes for binary toxin production. This further revealed that there might be severe infection from food sources by *C. difficile* in the study locations. The prevalence of erythromycin- and tetracycline-resistant genes in *Clostridioides difficile* isolates is depicted in agarose gel electrophoresis Figures 6–8 and Table 12. The amplification of *erm(B)* gene PCR products from these isolates was 73.91%. By coincidence, this was also the percentage of tetracycline resistance (tet(S) and tet(A) genes). Four isolates were identified as containing all resistance genes examined in this study. The results of this study align with those of other studies that reported a wide variety of antibiotic resistance genes, including tetracycline resistance, within the global *C. difficile* population[34]. Expanding on the same research, it was discovered that the bacterium also encodes genes for multidrug efflux transporters. This highlights the organism's capability to resist various antimicrobial agents, further complicating treatment options.

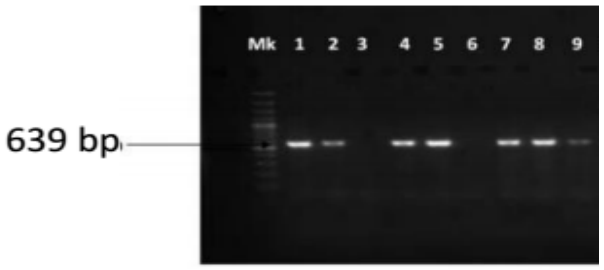

Figure 6: Agarose gel electrophoresis of the *ermB*gene PCR products amplified from *Clostridium difficile* isolates

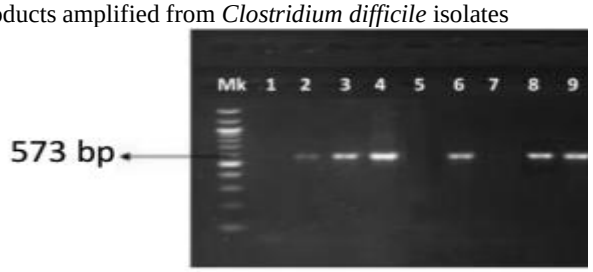

Figure 7: Agarose gel electrophoresis of the *TetS* gene PCR products amplified from *C. difficile* isolates

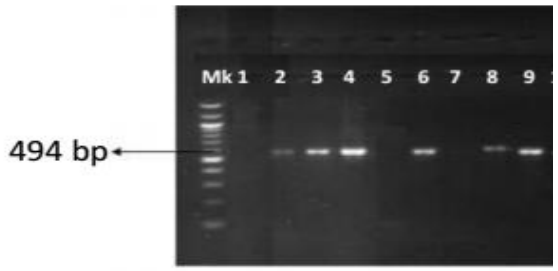

Figure 8: Agarose gel electrophoresis of the *TetA* gene PCR products amplified from *C. difficile* isolates

Table 10: Multiple antibiotic resistance distribution of *C. difficile* isolates from food sources

Isolate code	Antibacterial classes	Number of Antibiotics	Resistance Phenotypes	Resistant phenotypes (%)	MAR Index
anPM5, anBE3	9	9	ERY^R, CL^R, CIP^R	3(13.04)	0.33
ebPM6	9	9	ERY^R, MET^R, CIP^R, TET^R	2(8.69)	0.44
enPM3	9	9	AMC^R, CL^R, VA^R, TET^R	1(4.35)	0.44
abBE2	9	9	ERY^R, CN^R, VA^R, TET^R	1(4.35)	0.44
ebBE9	9	9	MEM^R, ERY^R, AMC^R, CN^R, VA^R	1(4.35)	0.55
anBE7	9	9	AMC^R, VA^R, TET^R	2(8.69)	0.33
enLV4	9	9	MEM^R, ERY^R, MET^R, TET^R	2(8.69)	0.44
ebLV5	9	9	ERY^R, CN^R, TET^R	1(4.35)	0.33

Table 11: Prevalence of virulence genes in isolates of *C. difficile* obtained from food samples

Lanes	Codes	Samples	*TcdA*	*tcdB*	*cdtA*	*CdtB*	Total (%)
1	anPM5	Pork meat	-	+	-	-	1(25)
2	ebPM6	Pork meat	-	+	-	-	1(25)
3	enPM3	Pork meat	+	+	-	-	2(50)
4	abBE2	Beef	+	+	-	-	2(50)
5	ebBE9	Beef	+	+	-	-	2(50)
6	anBE7	Beef	-	+	-	-	1(25)
7	anBE3	Beef	+	+	-	-	2(50)
8	enLV4	Vegetable	+	+	-	-	2(50)
9	ebLV5	Vegetable	+	+	-	-	2(50)
Total study (%)			6(66.67)	9(100.00)	0(0.00)	0(0.00)	

Table 12: Prevalence of resistant genes to erythromycin and tetracycline found in isolates of *C. difficile* obtained from food samples

Lanes	Code	Samples	*TetA*	*TetS*	*ErmB*	Total (%)
1	anPM5	Pork meat	-	-	+	1(33)
2	ebPM6	Pork meat	+	+	+	3(100)
3	enPM3	Pork meat	+	+	-	2(67)
4	abBE2	Beef	+	+	+	3(100)
5	ebBE9	Beef	-	-	+	1(33)
6	anBE7	Beef	+	+	-	2(67)
7	anBE3	Beef	-	-	+	1(33)
8	enLV4	Vegetable	+	+	+	3(100)
9	ebLV5	Vegetable	+	+	+	3(100)
Total study (%)			17(73.91)	17(73.91)	17(73.91)	

Conclusion

This research provided a thorough explanation of the prevalence, molecular features, and antibiogram susceptibility patterns of *C. difficile* in food samples from the Eastern region of Nigeria. Finding *C. difficile* inside food samples highlights its possible involvement in foodborne transmission and related public health dangers. The observation of virulence genes suggests that these strains can induce infection in the study area. The diverse antibacterial susceptibility patterns highlight varying resistance levels, emphasizing the importance of vigilant monitoring and the development of targeted antibacterial strategies. This study offers important insights into the epidemiology of *C. difficile* and lays the groundwork for developing effective prevention and control strategies. Future research should aim to broaden the geographic scope of sampling, examine additional food types, and investigate environmental reservoirs to gain a comprehensive understanding of *C. difficile* transmission dynamics and resistance mechanisms.

Conflict of Interest

The authors declare no conflict of interest.

Author's Declaration

The authors hereby declare that the work presented in this article is original. Any liability for claims relating to this article will be borne by us.

Acknowledgment

Thanks to Dr. A.G. Ogofure and the members of staff of the Department of Microbiology, University, Benin City for their enabling environment for the Laboratory work. The authors wish also to acknowledge the support received from TETFUND – Centre of Excellence for Biomedical, Engineering and Agricultural Translational Studies UNIZIK, Awka.

References

1. Borji S, Kadivarian S, Dashtbin S, Kooti S, Abiri R, Motamedi H, Moradi J, Alvandi A. Global prevalence of *Clostridioides difficile* in 17,148 food samples from 2009 to 2019: a systematic review and meta-analysis. J Health Popul Nutr. 2023; (https://jhpn.biomedcentral.com/articles/10.1186/s41043-023-00369-3).
2. Lim SC, Collins DA, Imwattana K, Knight DR, Perumalsamy S, Hain-Saunders N, Riley TV. Whole-genome sequencing links *Clostridium* (*Clostridioides*)

difficile in a single hospital to diverse environmental sources in the community. J ApplMicrobiol. 2022; 133(3): 1156-1168.

4. Liu X, Li W, Zhang W, Wu Y, Lu J. Molecular characterization of *Clostridium difficile* isolates in China from 2010 to 2015. Front Microbiol. 2018; (https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2018.00845/full)
5. Licciardi C, Primavilla S, Roila R, Lupattelli A, Farneti S, Blasi G, Petruzzelli A, Drigo I, Di RaimoMarrocchi E. Prevalence, molecular characterization, and antimicrobial susceptibility of *Clostridioides difficile* isolated from pig carcasses and pork products in Central Italy. Int J Environ Res Public Health. 2021;*18*, 11368. (https://www.mdpi.com/1660-4601/18/21/11368).
6. Gallo M, Ferrara L, Calogero A, Montesano D, Naviglio D. Relationships between food and diseases: What to know to ensure food safety. Food Res Int. 2020; 137:109414.
7. Adejumo AC, Adejumo KL, Pani LN. Risk and outcomes of *Clostridium difficile* infection with chronic pancreatitis. *Pancreas.* 2019; 48(8): 1041-1049.
8. Panhwar A, Abro R, Kandhro A, Khaskheli AR, Jalbani N, Gishkori KA, Qaisar S. Global water mapping, requirements, and concerns over water quality shortages. *BMC Public Health.* 2022;45(7):50-55.
9. Rapid Microbiology. *Clostridioides difficile detection and identification methods.* Available from: https://www.rapidmicrobiology.com/test-method/clostridium-difficile-detection-and-identification-methods
10. Borriello SP, Honour P. Detection, isolation and identification of *Clostridium difficile*. In: Borriello SP, editor.Antibiotic associated diarrhoea and colitis. Developments in Gastroenterology, vol 5. Dordrecht: Springer; 1984. p. 37-64.
11. European Centre for Disease Prevention and Control (ECDC). Laboratory procedures for diagnosis and typing of human *Clostridium difficile* infection [Internet]. Available from: https://www.ecdc.europa.eu/sites/default/files/documents/SOPs-Clostridium-difficile-diagnosis-and-typing.pdf
12. George WL, Sutter VL, Citron D, Finegold SM. Selective and differential medium for isolation of *Clostridium difficile*. J ClinMicrobiol. 1979; 9(2): 214-9. doi: 10.1128/jcm.9.2.214-219.1979. PMID: 429542; PMCID: PMC272994.
13. Bauer AW, Kirby MM, Sharis JL, Turck M. Antibiotic susceptibility testing by a standard single disk method. Am J ClinPathol. 1966; 45:493-6
14. Sambrook J, Russell DW. Molecular Cloning: A Laboratory Manual. *Cold Spring* Harbor Laboratory Press; 2001.
15. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. 30th ed. CLSI supplement M100. Wayne (PA): Clinical and Laboratory Standards Institute; 2020.
16. Chitanand MP, Kadam TA, Gyananath G, Totewad ND, Balhal DK. Multiple antibiotic resistance indexing of coliforms to identify high-risk contamination sites in aquatic environment. *Indian J Microbiol.* 2010; 50:216–220.
17. Chakravorty D, Helb D, Burday M, Connell N, Alland D. Use of an integrated MS-multiplexed MS/MS data acquisition strategy for high-coverage peptide mapping studies. Rapid Commun Mass Spectrom. 2007; 21(5): 730-744.
18. Smith JA, Brown PR. Amplification and detection of target genes using forward and reverse primers in agarose gel electrophoresis. J MolBiol Tech. 2022; 78(4):123–130.
19. Microsoft Corporation. Microsoft Excel (Computer Software). Redmond (WA): Microsoft Corporation; 2006.
3. Khan MSA, Ahmad I. Pathogenic biofilms in environment and industrial setups and impact on human health. In: Understanding Microbial Biofilms. Academic Press; 2023. p. 587-604.
20. Jenior ML, Leslie JL, Powers DA, Garrett EM, Walker KA, Dickenson ME, Petri WA Jr, Tamayo R, Papin JA. Novel Drivers of Virulence in *Clostridioides difficile* Identified via Context-Specific Metabolic Network Analysis. mSystems. 2021; 6(5): 00919-21. doi: 10.1128/msystems.00919-21.
21. Perez-Bou L, Gonzalez-Martinez A, Cabrera JJ, Juarez-Jimenez B, Rodelas B, Gonzalez-Lopez J, Correa-Galeote D. Design and Validation of Primer Sets for the Detection and Quantification of Antibiotic Resistance Genes in Environmental Samples by Quantitative PCR. Microb Ecol. 2024; 87:71. doi: 10.1007/s00248-024-02385-0.
22. Lund BM, Peck MW. A possible route for foodborne transmission of *Clostridium difficile*? Foodborne Pathog Dis. 2015; 12(3):183-189. doi: 10.1089/fpd.2014.1842.
23. Makinde OM, Ayeni KI, Sulyok M, Krska R, Adeleke RA, Ezekiel CN. Microbiological safety of ready-to-eat foods in low- and middle-income countries: A comprehensive 10-year (2009 to 2018) review. Compr Rev Food Sci Food Saf. 2020; 19:703-732.
24. Warriner K, Xu C, Habash M, Sultan S, Weese SJ. Dissemination of *Clostridium difficile* in food and the environment: Significant sources of C. difficile community-acquired infection? J ApplMicrobiol. 2016; 122(3):542-553.
25. Balsells E, Shi T, Leese C, Lyell I, Burrows J, Wiuff C, Campbell H, Kyaw MH, Nair H. Global burden of *Clostridium difficile* infections: a systematic review and meta-analysis. J Glob Health. 2018; 8(12):e010407. doi: 10.7189/jogh.09.010407.
26. European Centre for Disease Prevention and Control. *Clostridioides difficile* infections. In: ECDC. Annual epidemiological report for 2018−2020. Stockholm: ECDC; 2024.
27. Rodriguez C, Taminiau B, Bouchafa L, Romijn S, Van-Broeck J, Delmée M, Clercx C, Daube G. *Clostridium difficile* beyond stools: dog nasal discharge as a possible new vector of bacterial transmission. Heliyon. 2019; 5(5):1629.
28. Peng Z, Jin D, Kim HB, Stratton CW, Wu B, Sun X. Update on antimicrobial resistance in *Clostridium difficile*: resistance mechanisms and antimicrobial susceptibility testing. J ClinMicrobiol. 2017; 55(7):e02250-16. doi: 10.1128/jcm.02250-16.
29. European Centre for Disease Prevention and Control. *Clostridioides (Clostridium) difficile* infections. In: ECDC. Annual epidemiological report for 2016–2017. Stockholm: ECDC; 2022.
30. Spigaglia P, Barbanti F, Mastrantonio P, Brazier JS, Barbut F, Delmée M, Martin H, Kuijper EJ, O'Driscoll J, Allouch PY. Fluoroquinolone resistance in *Clostridium difficile* isolates from a prospective study of *C. difficile* infections in Europe. ClinMicrobiol Infect. 2016; 22(11):1027-1033. doi: 10.1016/j.cmi.2016.08.010.
31. Knight DR, Elliott B, Chang BJ, Perkins TT, Riley TV. Diversity and evolution in the genome of *Clostridium difficile.*ClinMicrobiol Rev. 2015; 28(3):721-741.
32. Rodriguez-Palacios A, Mo KQ, Shah BU, Msuya J, Bijedic N, Deshpande A, Ilic S. Global and Historical Distribution of *Clostridioides difficile* in the Human Diet (1981-2019): Systematic Review and Meta-Analysis of 21886 Samples Reveal Sources of Heterogeneity, High-Risk Foods, and Unexpected Higher Prevalence Toward the Tropic. Front Med (Lausanne). 2020; 7:1-22.
33. Surang C, Piyapong H, Amornrat A, Puriya N, Darunee C, Piriyaporn C, Tavan J. Evaluation of Multiplex PCR with Enhanced Spore Germination for Detection of *Clostridium difficile* from Stool Samples of the Hospitalized Patients. Biomed Res Int. 2013; 29(3):115-128.

34. Knetsch CW, Kumar N, Forster SC, Connor TR, Browne HP, Harmanus C, Sanders P, Harris SR, Lipman L, Keessen EC, Corver J, Lawley TD, Kuijper EJ. Zoonotic Transfer of *Clostridium difficile* Harboring Antimicrobial Resistance between Farm Animals and Humans. J ClinMicrobiol. 2018; 24(6):1053-106