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MOLECULAR DETECTION OF DRUG-RESISTANT GENES AMONG *Clostridioides difficile* FROM DIARRHEIC CHILDREN IN DUHOK CITY -IRAQ

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ABSTRACT

Clostridioides difficile, formerly known as *Clostridium difficile* is the most common cause of antibiotic-associated diarrhea and colitis and is characterized by resistance to multiple drugs. This study amid to characterize antibiotic resistance genes in *C. difficile* among pediatric diarrheal cases from Duhok Governorate, Kurdistan regional Iraq, providing critical insights for regional infection control and treatment guidelines. Thirteen *C. difficile*-positive stool samples (from a cohort of 200 children aged between 6 months- 6 years) were analysed by PCR for detecting resistance genes *CTX-M* (cefotaxime), *ermC* (clindamycin), *ere(A)* (erythromycin), *rdxA* (metronidazole), *vanR (*vancomycin). The result illustrated that cefotaxime, *CTX-M* gene detected in 100% DNA samples, with high rates of resistance of clindamycin (*ermC gene*, 76.9%) and erythromycin (69.2%, *ere(A) gene*) while resistance to metronidazole (*rdxA*) and vancomycin resistance (*vanR*) remained rare (7,69% and 15.38%, respectively). Venn diagram analysis highlighted frequent co-occurrence of resistance to these genes, and six samples (46.2%) harbored three genes, *CTX-M*, *ermC and ere (A),* and also double other two samples harbored two genes, *CTX-M* and *ermC* and *CTX-M* and *ere (A)*. In addition, one sample harbored only the *CTX-M* gene. This study underscores the prevalence of the alarming high rate of antibiotic resistance found in *C. difficile* among pediatric diarrheal cases such as against cefotaxime, clindamycin, and erythromycin. The persistence of susceptibility to vancomycin and metronidazole supports their continued use as first-line therapies for both community and hospital infections region.

KEYWORDS: *Clostridioides Difficile*, Antibiotic-Resistant Genes, Diarrhea, PCR.

1. INTRODUCTION

Diarrheal diseases are on the rise worldwide, with an estimated 1.6 million deaths annually affecting children under five years of age (Troeger *et al.,* 2018). Kotloff *et al.* (2013) reported that this increase is due to several microbial pathogens, including *Vibrio cholerae, Escherichia coli, Salmonella, Shigella,* and *Clostridioides difficile* (*C. difficile*), which contribute to both healthcare-associated infections and community-acquired (Kotloff *et al.,* 2013). It has been reported the main cause of antibiotic-associated diarrhea, especially nosocomial infections, is *C. difficile* (Carroll &Bartlett, 2011). This bacterium is a motile, rod-shaped, Gram-positive bacterium previously confirming that thirteen of the 100 identified species of *Clostridium* are considered to be extremely harmful to humans or animals (Dupuy *et al.,* 2006).

Several studies have confirmed that the overuse of antibiotics, especially without a prescription, can significantly alter the gut microbiota (Baines *et al.,* 2015; Sun &Hirota, 2015). This disruption in the gastrointestinal tract increases the host's susceptibility to infection, particularly *C. difficile* infection (Sun &Hirota, 2015). The use of certain antibiotics, including clindamycin, ampicillin, penicillin, tetracycline, and cephalosporins, has been associated with a high risk of *C. difficile* infection (Khashei *et al.,* 2018). The standard first-line treatment for *C. difficile* infections typically involves metronidazole and vancomycin (Canas *et al.,* 2023). However, antibiotic resistance to these medications has been reported, complicating treatment and making CDI management more challenging (Di *et al.,* 2015). Moreover, recent research has also shown that commonly used antibiotics used to treat *diarrhea*, such as clindamycin and fluoroquinolones, and occasionally even the first-line treatment vancomycin development of *C. difficile* (Canas *et al.,* 2023). The ability of *C. difficile* to antibiotic resistance, along with its toxin production, expresses the severity of *C. difficile* infection (CDI) and makes it a significant public health (Baines *et al.,* 2015).

Recently, antibiotic resistance mechanisms in *C. difficile* have become a significant concern due to the emergence of various mechanisms (Van *et al.,* 2008). The presence of the *erm* (B) and *erm* (C) genes in this bacterium encode rRNA methylases that modify

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the target site, conferring resistance to macrolides such as erythromycin (Cassandra *et al.,* 2020). Additionally, the erm(B) gene also reduces the efficacy of clindamycin by encoding an rRNA methylase (Miele *et al.,*1995). Resistance to metronidazole has been associated with mutations in the rdxA and frxA genes, which are involved in this bacterium activation. Moreover, the acquisition of β-lactamase genes, such as blaOXA-11 and blaOXA-48, has led to resistance to cephalosporins, including the Cefixime (Boekhoud *et al.,* 2021). Additionally, the presence of *vanB* and *vanG* operons in some *C. difficile* isolates enables modification of the peptidoglycan cell wall target, thereby reducing the efficacy of vancomycin (Lessa *et al.,* 2015). These antibiotic resistance genes poses are often located on plasmids and transposons which are mobile genetic elements, facilitating their spread between different *C. difficile* strains and even other bacterial species. This poses a significant public health threat, as it limits the available treatment options for serious *C. difficile* infections (Canas *et al.,* 2023).

Several studies have highlighted a concerning rise in infections among humans and animals leading to the overuse of antibiotics without prescription in Duhok Governorate, located in the Kurdistan Region of Iraq (Saeed and Ibrahim, 2013; Hasan and Ibrahim, 2022; Hami and Ibrahim, 2023; Ibrahim, 2023; Mohialdeen & Ghaffar, 2023; Issa, 2024; Taher and Othman, 2024). This widespread and often unnecessary use of antibiotics has raised significant public health concerns, particularly regarding the development of antibiotic resistance which has vice versa to affect the gut microbiome. To the best of our knowledge, this study represents the first attempt to detect antibiotic-resistant genes in C. difficile by analyzing DNA extracted from stool samples using the PCR (polymerase chain reaction) technique. This research aims to shed light on the prevalence of antibiotic resistance in *C. difficile*, contributing to a better understanding of the challenges posed by antimicrobial resistance in the region.

2. MATERIALS AND METHODS

Stool Samples and DNA Extraction

Thirteen bacterial DNA samples were extracted from stool specimens of diarrheic pediatric patients diagnosed with *C. difficile*. This study was conducted at Hivi Pediatric Teaching Hospital in Duhok, Iraq, between October 2021 and May 2022. These identified samples were part of a larger cross-sectional study that involved the collection of 200 stool samples from children suffering from diarrhea aged from 6 months to 6 years.

All samples were treated using a stool transport and recovery buffer (S.T.A.R, Roche, Mannheim, Germany) prior to DNA extraction, following the manufacture protocol of a High Pure PCR Template Preparation kit (Roche, Germany). DNA concentration was achieved using a Nanodrop DeNOVIX (Wilmington, USA) with values ranging from 120 to 256 µg/µL. The purity ratios of both 260/280 and 230/280 were falling between 1.95 and 2.2. Then, this DNA was immediately stored to preserve its integrity and ensure a high-quality yield for further DNS amplification.

The purified DNA extracts of 13 *C. difficile* from hospitalized diarrheic children (10 hospital-acquired and 3 community-acquired) previously identified by RT-PCR direct detection of *C. difficile* 16S rDNA using the following primers: CIDIF-F CTT GAA TAT CAA AGG TGA GCC A and CIDIF-R CTA CAA TCC GAA CTG AGA GTA (Eurofins, Ebersberg, Germany) (Kikuchi *et al*., 2002). were analyzed for 5 different drug-resistance genes using the conventional PCR technique (Table 1).

PCR technique

In this study, these 13 samples diagnosed with *C. difficile*, were analyzed using Polymerase Chain Reaction (PCR) to detect five antibiotic resistance genes. Five specific different primers (Macrogen- South Korea) were employed to amplify genes associated with resistance to clindamycin, erythromycin, metronidazole and vancomycin. The targeted genes included Beta lactam Cefotaximase-Munich gene (*blaCTX-M),* Erythromycin Ribosomal Methylase C gene *(ermC),* Erythromycin Esterase A gene *[ere(A)],* Oxygen-insensitive NADPH Nitroreductase A gene *(rdxA*) and Vancomycin resistance Regulatory Protein R gene (*vanR*) as described in (Table 1).

The PCR amplification was performed using an Applied Biosystems 9700 thermal cycler (USA). Each reaction contented of 2X PCR master mix (Roche-Germany), 1µL of 20 pmol/µL each forward and reverse primers (Eurofins, Ebersberg, Germany), 2µL template DNA, and 7µL nuclease-free water to up to 20µL for reaction. The amplification was conducted considering the following circumstances: initial denaturation at 94°C for 5 minutes, followed by 35 denaturation cycles at 94°C for 30 seconds, annealing at different temperatures for 30 seconds, elongation at 72°C for 1 minute, and final extension at 72 °C for 10 minutes.
Following amplification, the PCR products were separated by agarose gel electrophoresis (Cleaver, UK). The amplicons, ranging between 299 and 850 base pairs, were visualized under UV light, and images were captured to confirm the presence of the target genes, as illustrated in Table 1.

Ethical Approval

The Research Ethics Committee of the Duhok Directorate of General Health provided ethical permission. No.13072021-7-7.

Statistical analysis

The presence of antibiotic-resistance genes among isolates was examined using the Venn Diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Table 1: List of primers used for amplification of antibiotics resistant genes with their amplicon sizes and annealing temperatures

Antibiotics	Target genes	Primer sequences	Amplicon size (bp)	Annealing (°C)	References
Cefotaxime	*BlaCTX-M*	F: 5'TTTGCGATGTGCAGTACCAGTAA-3'	590	55	(Sidjabat *et al.,* 2009)

		R: 5'CGATATCGTTGGTGGTGCCATA-3'			
Clindamycin	*ermC*	*F*: 5′ AATCGTCAATTCCTGCATGT-3′	299	52	(Khashei *et al.,* 2018)
		R: 5′ TAATCGTGGAATACGGGTTTG-3′			
Erythromycin	*ere(A)*	F: 5' GCCGGTGCTCATGAACTTGAG 3'	419	58	(Van *et al.,* 2008)
		R: 5' CGACTCTATTCGATCAGAGGC 3'			
Metronidazole	*rdxA*	F: 5' AATTTGAGCATGGGGCAGA 3'	850	55	(Ossenkopp *et al.,* 1999)
		R: 5' GAAACGCTTGAAAACACCCCT 3'			
Vancomycin	*van*R	F: 5' AGCGATAAAATACTTATTGTGGA 3'	645	54	(Bandera *et al.*, 1995)
		R: 5' CGGATTATCAATGGTGTCGTT 3'			

3. RESULTS

PCR analysis showed distinct patterns of antibiotic resistance genes among detected *C. difficile* in the faecal samples, as shown in Table 2 and Figure 1. Among these 13 samples, the CTX-M gene was detected in all samples (100%), indicating resistance to cefotaxime. Resistance to clindamycin, mediated by the *ermC* gene, was detected in 10 samples (76.9%) and, to a lesser extent, the *ere(A)* gene linked to erythromycin resistance was observed in 9 samples (69.2%). In contrast, the prevalence of resistance genes to metronidazole (*rdxA*) and vancomycin (*vanR*) was lower, with only 1 sample (7.69%) and 2 samples (15.38%) detected for *rdxA* and *vanR*, respectively. These findings indicate a high prevalence of resistance to cefotaxime, clindamycin, and erythromycin among the tested *C. difficile*, with significantly lower resistance rates observed for metronidazole and vancomycin.

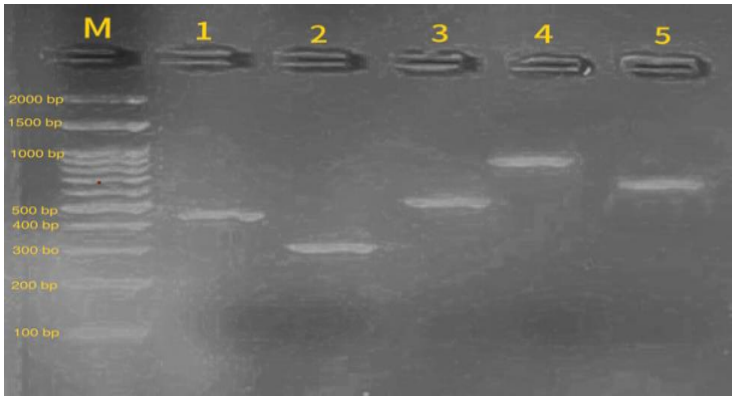

Figure 1: Conventional PCR for identifying genes that are resistant to antibiotics. M: marker (100 bp DNA ladder); lane 1: *ere (A)* gene; lane 2: *ermC* gene; lanes 3: *CTX-M* gene; lane 4: *rdxA* genes and lane 5: *van*R gene.

Table2: Results of resistance genes of *C. difficile* by PCR.

Isolate	CTX-M	ermC	ere(A)	rdxA	vanR
CA-D17	+	+	+	-	-
CA-D26	+	+	+	-	-
CA-D42	+	+	+	-	-
HA-D13	+	+	+	+	+
HA-D21	+	+	-	-	-
HA-D24	+	-	+	-	-
HA-D50	+	+	-	-	-
HA-D76	+	-	+	-	-
HA-D87	+	+	-	-	+
HA-D110	+	-	-	-	-
HA-D309	+	+	+	-	-
HA-D383	+	+	+	-	-
HA-D389	+	+	+	-	-
Average	100%	76.90%	69.20%	7.69%	15.38%

+ = present, - = absent

The distribution of antibiotic resistance genes among detected *C. difficile* was further analyzed by Venn diagram software (Figure 2). A total of 6 samples possess three genes: *CTX-M*, *ermC*, and *ere(A)*, followed by 2 samples possessing two genes, *CTX-M* and *ermC* as well as another 2 samples having *CTX-M* and *ere(A)*. In addition, one samples possess all strain in contrast one sample only have one gene *CTX-M*.

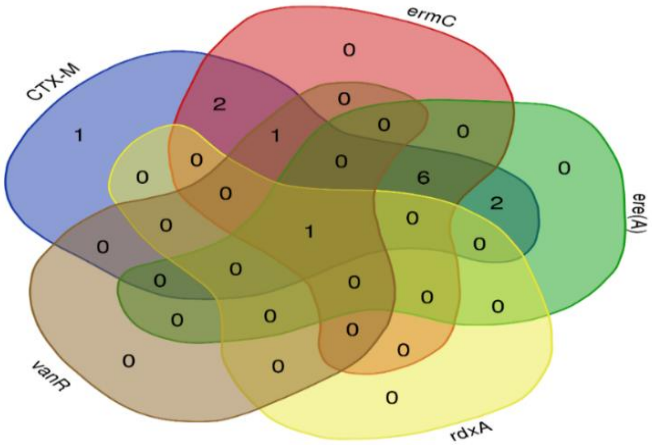

Figure 2: Resistant genes carried by *C. difficile: ere (A)* gene for erythromycin*; ermC* gene for *CTX-M* gene for vefotaxime; *rdxA* gene for metronidazole. Clindamycin and *vanR* gene for vancomycin;

4. DISCUSSION

This cross-sectional study aimed to detect drug-resistant genes among *C. difficile* from both community and hospital-acquired diarrheic children against commonly prescribed antibacterial genes. Cefotaxime resistance was highly prevalent, being present in all 13 isolates (100%). This suggests that cefotaxime resistance is widespread in the area due to the production of extended beta-lactamase enzymes (ESBLs), such as ^{bla}-*CTM-M* genes carried on plasmids, which can be easily spread via the conjugation process (Hassan and Ibrahim, 2022). The result was lower than those found by Boekhoud *et al.* (2021) in the Netherlands, who reported a resistance rate of 35.5% in *C. difficile.* This disparity in outcomes can be ascribed to differences in antibiotic usage policies. In our region, antibiotics are easily accessible without a physician's prescription and are widely prescribed without proper control. Among the studied *C. difficile*, 76.9% and 69.2% were positive for resistant genes against clindamycin and erythromycin, respectively. Clindamycin is effective against anaerobic Gram-negative bacilli, which are the predominant normal flora of the colon and create a symbiotic environment that allows *C. difficile* (Duffy *et al.,* 2020). The results of the current study were similar to those of Tamma *et al* (2022), who found that 60% of *C. difficile* isolates were resistant to clindamycin. Erythromycin resistance ranges from 40 to 80% globally, which aligns with the results of this study.

Metronidazole resistance was observed in only one of the 13 *C. difficile* isolates (7.69%). This suggests that metronidazole continues to be an effective treatment option for *C. difficile* infections, likely due to the relatively low occurrence of resistance mechanisms. These findings are consistent with a study conducted by Lessa *et al* (2015), who reported that metronidazole resistance in *C. difficile* isolate is generally low, with resistance rates typically below 5% in most studies.

Our finding aligns with the results of Al-Rawe *et al.* (2023), conducted in Iraq; they found that eight genes are present in all isolates and contribute significantly to drug resistance through ribosome defence, antibiotic efflux, and antibiotic deactivation. The authors concluded that mutations in the functional domains of the tetA (P), tetM, and ermB genes were the most promising new therapeutic targets.

Similarly, vancomycin resistance was infrequent, with only two out of the 13 *C. difficile* isolates showing resistance (15.38%). This aligns with the results of Baines *et al* (2011), who found that vancomycin resistance in *C. difficile* remains relatively uncommon, with most studies indicating resistance rates under 5%. Vancomycin is a narrow-spectrum antibiotic that is particularly effective against Gram-positive cocci and is recommended for severe cases of pseudomembranous colitis when metronidazole resistance is present.

However, it is worth noting that severe cases of *C. difficile* infection are rare in our area. As a result, these antimicrobial drugs are not commonly prescribed by physicians, but they remain effective treatment options. In regards to the origin of the isolates, three out of thirteen isolates were obtained from children with community-acquired diarrhea. All of these isolates tested positive for resistant genes against cefotaxime, clindamycin, and erythromycin but negative for metronidazole and vancomycin. It is noteworthy that the resistance gene with the lowest occurrence was metronidazole at 10%, followed by vancomycin at 20%. Conversely, the highest percentage was observed for cefotaxime at 100%, followed by clindamycin at 70% and erythromycin at 60%. These findings clearly indicate the circulation of drug-resistant genes among community-acquired infections, likely due to the extensive usage of antibiotics. Moreover, it is important to acknowledge that these genes have the potential to transfer among unrelated bacteria easily.

CONCLUSIONS

Based on this study's findings, it can be concluded that *C. difficile* isolates that circulated in the region commonly show

antibiotic resistance genes, especially for cefotaxime, clindamycin, and erythromycin, which are commonly used antibiotics for various bacterial infections. However, lower levels of resistance were observed for metronidazole and vancomycin, providing reassurance regarding their effectiveness as first-line treatment options.

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Statements and Declarations

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Consent to Participate: The author has consented to submit the article to this journal.

Consent to Publish: The author has agreed to publish the article in this journal.

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