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Study Of Antibiotic Susceptibility of Clostridium Difficile Isolated from Childhood Diarrhea and Detection of BlaCTX-M Gene

Aevar Ashraf Khorshed
College of Dentistry, Ibn Sina University of Medical and Pharmaceutical Sciences, Baghdad, Iraq

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Annotation: Background & aim: Clostridium difficile is the most frequent cause of antibiotic-associated diarrhea in hospitals and other healthcare settings. This is a serious problem due to rising rates of morbidity and mortality as well as rising health care expenses. Therefore, the current study aimed to evaluate the resistance of Clostridium difficile isolated from the digestive system against several antibiotics and to detect the BlaCTX-M gene responsible for the bacterial resistance to beta-lactamase antibiotics.

Materials and methods: The study collected 280 stool samples from patients who were admitted to Kirkuk Hospital in Kirkuk City between October 2024 and February 2025 after visiting a specialist physician and referring them to the laboratory. Every sample's information was obtained. Following direct transportation to the lab, the samples were cultivated on blood and MacConkey agar media and incubated for 24 hours at 37°C in order to detect genes and test for antibiotic sensitivity.

Results: The findings were found that

187(66.8%) of total samples were appear as positive results for bacterial growth. The results of the identification revealed that among the 280 cultured stool samples 187 samples gave positive culture with bacterial isolates from patiens with diarrhea, E. coli represent 48.7% of the isolated bacteria. Followed by. 18.2% Klebsiella spp., 13.8% Pseudomonas aeruginosa, 8.0% Clostridium difficile, 15.9% Shigella spp., 4.3% Serratia marcescens, 1.1% Actinomycetes bovis. C. difficile was low sensitive to Levofloxacin 33.3%, Vancomycin 26.7%, Clindamycin 40.0%. While it was highly sensitive to Gentamicin 66.7% and Amikacin 86.7%. After isolating DNA from C. difficile using an extraction and electrophoresis kit, it was found that 91.7% of C. difficile isolates contained the BlaCTX-M gene.

Conclusions: it was found that C. difficile is considered one of the causes of diarrhea in many children, and it is characterized by its high resistance to antibiotics, which is aided by its possession of antibiotic resistance genes, including the BlaCTX-M gene, which was identified in most of the isolates obtained.

Keywords: C. difficile, antibiotic susceptibility, BlaCTX-M gene, beta-lactamase.

Introduction

The most frequent cause of drug-associated diarrhea and colitis is Clostridium difficile, which often manifests after antibiotic exposure (1). Infections with C. difficile have increased in frequency, severity, resistance to conventional therapy, and likelihood of recurrence in recent years (2,3). The bacterium C. difficile is spore-forming, completely anaerobic, and Gram-positive. It was initially isolated from the meconium and feces of newborns that were asymptomatic in 1935. Bacillus difficile was the name given to it because of its shape and the challenges it faced during cultivation. These bacteria are responsible for between 10% and 35% of all cases of antibiotic-associated diarrhea, making them the most common infectious cause of nosocomial

diarrhea, which is associated with substantial morbidity and mortality (4,5). Healthy people do not have problems with C. difficile because of commensal gut flora and antibody-mediated immunity, but infection by these bacteria happens when a patient ingests the spores of a deadly strain of the bacteria through environmental exposure or personal contact (6). Numerous studies have demonstrated that excessive antibiotic usage, particularly when done without a prescription, can drastically change the gut flora (7). The host is more vulnerable to infection as a result of this disturbance in the gastrointestinal system, especially from C. difficile infection (8). Certain medications, such as ampicillin, penicillin, tetracycline, clindamycin, and cephalosporins, have been linked to an increased risk of contracting C. difficile infection (9). Antibiotic resistance to these drugs has been documented, though, which makes treatment more difficult and CDI management more difficult (10). Recent studies have also demonstrated that common medications used to treat diarrhea, including fluoroquinolones and clindamycin, and sometimes even the first-line therapy vancomycin, can cause C. difficile to develop (11). The severity of C. difficile infection (CDI) is expressed by its ability to produce toxins and its resistance to antibiotics, which makes it a serious public health concern (7). The advent of many pathways of antibiotic resistance in C. difficile has recently raised serious concerns (12). Resistance to cephalosporins, including Cefixime, has been caused by the acquisition of β-lactamase genes, such as blaOXA-11 and blaOXA-48 (13). Therefore, the current study aimed to evaluate the resistance of Clostridium difficile isolated from the digestive system against several antibiotics and to detect the BlaCTX-M gene responsible for the bacterial resistance to beta-lactamase antibiotics.

Materials and methods

Sample Collection

Between October 2024 and February 2025, 280 stool samples were taken from patients admitted to Kirkuk Hospital in Kirkuk City after they had seen a specialist and been sent to the laboratory. Every sample's data was collected. The exit samples were brought straight to the lab, where they were cultivated on MacConkey agar and blood agar media and incubated for twenty-four hours at 37°C.

Identification of Morphological characteristics

Using microscopic analysis and biochemical testing, the colonies of the bacterial isolates that were grown on blood agar and MacConky media were described based on their forms, colors, diameters, odors, and other attributes (14).

Identification of bacteria isolates via VITEK2

Advanced colorimetric technology is represented by VITEK 2, the next generation of the gold standard in microbial identification. Procedure Every step listed below was completed in accordance with Biomerieux's manufacturer's instructions (15).

Biochemical tests

Some biochemical tests were performed on the bacterial isolates to identify the bacterial species. These tests included: Catalase, Oxidase test, Indole, Methyl red, Voges-proskauer, Citrate, Urease, Mannitol fermentation

Antibiotic susceptibility test (AST)

All isolates' AST was performed using the Kirby-Bauer disc diffusion method on Muller Hinton (MH) agar in compliance with the Clinical Laboratory Standards Institute's (CLSI) guidelines (CLSI, 2020).

Stool Samples and DNA Extraction

15 bacterial DNA samples were extracted from stool specimens of diarrheic pediatric patients diagnosed with C. difficile. 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 was immediately stored to preserve its integrity and ensure a high-quality yield for further DNA amplification. these 15 samples diagnosed with C. difficile, were analyzed using Polymerase Chain Reaction (PCR) to detect five antibiotic resistance gene. specific primers (Macrogen- South Korea) were employed to amplify gene associated with resistance to Beta lactam antibiotics. The targeted gene was Beta lactam Cefotaximase-Munich gene (blaCTX-M), as described in (Table 1).

Table (1): C. difficile gene PCR assay primers

Primer	Primer sequence	Length (bp)	Ref.
BlaCTX-M	F: 5- TTTGCGATGTGCAGTACCAGTAA-3	590	
BlaCTX-M	R: 5-CGATATCGTTGGTGGTGCCATA -3		(16)

The amplification was conducted considering the following circumstances: initial denaturation at 94°C for 5min, followed by 35 denaturation cycles at 94°C for 30s, annealing at different temperatures for 30s, elongation at 72°C for 1 min, and final extension at 72 °C for 10 minutes. Following amplification, the PCR products were separated by agarose gel electrophoresis (Cleaver, UK). The amplicon was 580 base pairs, were visualized under UV light, and images were captured to confirm the presence of the target genes, as illustrated in Table 1.

Results and discussion

Samples distribution

280 stool samples were used in this investigation (table 2). The results showed that 187 (60.8%) of the total samples showed positive results for bacterial growth when cultured on the best cultured media, including MacConkey agar, Mantol agar, and blood agar. Of the total samples, 93 (31.2%) had negative results for bacterial growth.

Table (2): Distributed of study samples according to bacterial growth

Groups	No. (%) **+ve culture**	No. (%) **-ve culture**	Total No.(%)
Results	187(66.8%)	93(31.2%)	280(100.0%)

The results of the identification revealed that among the 280 cultured stool samples 187 samples gave positive culture with bacterial isolates from patiens with diarrhea, E. coli represent 48.7% of the isolated bacteria. Followed by. 18.2% Klebsiella spp., 13.8% Pseudomonas aeruginosa, 8.0% Clostridium difficile, 15.9% Shigella spp., 4.3% Serratia marcescens, 1.1% Actinomycetes bovis as illustrated in table 3.

Table (3): Distribution of bacteria isolated from faecal samples (n = 82)

Bacterial Isolates		**No.**	**%**
1	***E. coli***	91	48.7%
3	***Klebsiella spp.***	34	18.2%
4	***Pseudomonas spp.***	26	13.8%
5	***Clostridium difficile***	15	8.0%
6	***Shigella spp.***	11	5.9%
7	***Serratia marcescens***	8	4.3%
8	***Actinomycetes bovis***	2	1.1%
Total		187	100.0%

Clostridium difficile identification

On the Clostridium difficile agar media, the phenotypic traits of the developing bacterial colonies were observed, and smears were stained and dyed. The bacterial isolates extracted from diarrhea

samples were identified by biochemical assays, and their phenotypic characteristics were assessed under a microscope. According to the results of bacterial transplantation, the bacteria were found to be convex, round colonies with clear borders and a gray color. Additionally, the results of pigmentation indicated that the bacteria were positive bacilli. Cram stain as depicted in image (1).

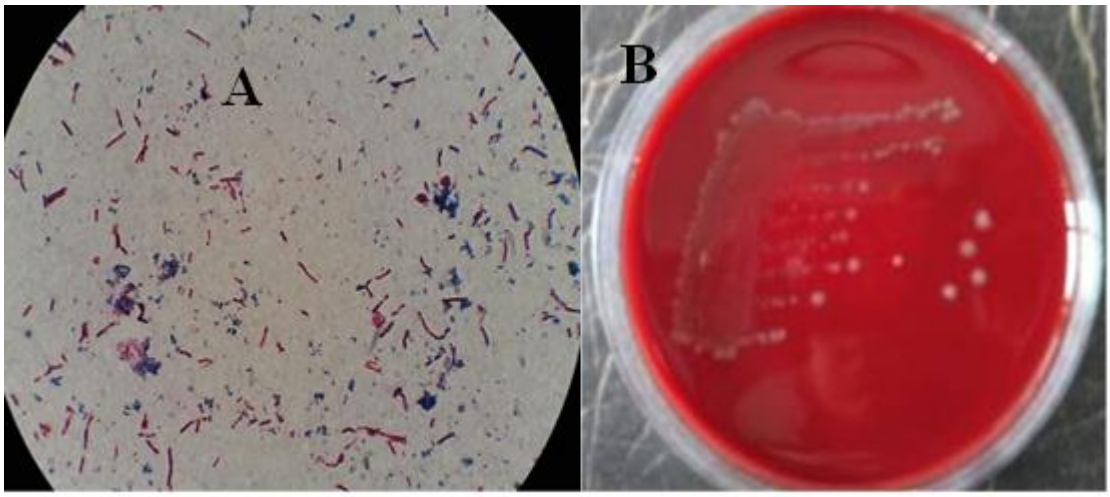

Figure (1): A. *C. difficile* on light microscope and B. *C. difficile* on selective media.

Antibiotic Susceptibility Test (AST):

Antibiotic susceptibility to these isolated bacteria was assessed using the diffusion method on Muller Hinton medium, which was interpreted as sensitive, moderate sensitivity, and resistance in accordance with CLSI, 2014. Different patterns of antibiotic resistance were found in the bacterial isolates' test findings. Levofloxacin sensitivity was 33.3%, Vancomycin sensitivity was 26.7%, and Clindamycin sensitivity was 40.0%, according to the data. However, as Table (4) demonstrates, it was extremely sensitive to Gentamicin (66.7%) and Amikacin (86.7%).

Table (4): Antibiotic disc (NCCLs) guideline for gram positive bacteria

C. difficile	AM	CN	AK	DA	LEF	TMP	CIP	VA	AZM	NA
S1	RS	SV	SV	RS	SV	SV	SV	SV	SV	SV
S2	RS	RS	SV	RS	RS	RS	SV	RS	SV	SV
S3	RS	SV	SV	RS	RS	RS	SV	RS	RS	SV
S4	RS	SV	SV	RS	RS	SV	RS	RS	SV	SV
S5	RS	RS	SV	RS	RS	RS	RS	RS	SV	RS
S6	RS	SV	SV	RS	SV	SV	RS	SV	RS	RS
S7	RS	SV	SV	SV	RS	SV	RS	RS	RS	SV
S8	RS	SV	SV	SV	RS	RS	SV	RS	RS	RS
S9	RS	SV	RS	SV	RS	SV	RS	RS	SV	RS
S10	RS	SV	SV	SV	SV	RS	RS	RS	SV	SV
S11	RS	RS	SV	RS	RS	RS	SV	SV	SV	SV
S12	RS	SV	SV	RS	SV	RS	SV	RS	RS	SV
S13	RS	SV	SV	RS	RS	RS	RS	RS	RS	RS
S14	RS	SV	SV	SV	RS	RS	SV	RS	RS	SV
S15	RS	RS	RS	SV	SV	SV	RS	SV	SV	RS
Sensitivity %	0.0	66.7	86.7	40.0	33.3	40.0%	46.7	26.7	53.3	60.0

SV: sensitive, RS: resistance, AM: Ampicillin, CN: Gentamicin, AK: Amikacin, DA: Clindamycin, LEF: Levofloxacin, TMP: Trimethoprim, CIP: Ciprofloxacin, VA: Vancomycin, AZM: Azithromycin, NA: Nalidixic acid.

Bacteria exhibit a number of characteristics that aid in their resistance to these antibodies, such as active flow mechanical processes that prevent accumulation and changes at the target site of the antibiotic's interaction with the target, reducing its affinity for it (17). The antigen within the bacterial cell or by decreasing the permeability of the germs' outer membrane, particularly in the negative germs that are distinguished by possessing this mechanical. In contrast, the germs that are positive for the cram dye do not possess it since they do not have the outer membrane (18). A mutation that results in the alteration of DNA gyrase or one that produces more active flux systems is one example (19). The current study's findings were consistent with those of a previous investigation by Lewis et al. (21), which found that 27% of the bacterial isolates were vancomycin-sensitive. Vardakas (20) reported a sensitivity to vancomycin of 14.2%. Two primary mechanisms typically contribute to bacterial resistance to fluoroquinolones: (1) a change in the drug target due to a mutation in the encoding genes, which results in a decreased affinity for the drug; and (2) either a decrease in permeability or an increase in the drug's active efflux. The current study's findings indicated that the percentage of people who were sensitive to ciproflaxin and levoflaxin was 33.3% and 46.7%, respectively. Additionally, the current study's findings supported those of (22) as they showed that 40% of people were resistant to fluoroquinoles. The current study's findings were roughly represented by (23) as it verified that 47% of people were resistant to fluoroquinoles. (24) The current study's findings matched the percentage of sensitivity to amikacin and gentamicin, which were 86.7% and 66.7%, respectively, as the resistance rate rose to 55.10%.This is counter to the reports (25) It revealed that 62% of people are now resistant to aminoclucosides. Several resistance mechanisms, such as decreased uptake or decreased cell permeability, changes at the ribosome binding sites, or the generation of aminoglycoside modifying enzymes, are the cause of the rise in the resistance rate. According to the current study's findings, 40.0% of the Clostridium difficile isolates were sensitive to trimethoprim.

BlaCTX-M gene detection

After isolating DNA from C. difficile using an extraction and electrophoresis kit, it was found that 91.7% of C. difficile isolates contained the BlaCTX-M gene, as shown in Figure 2.

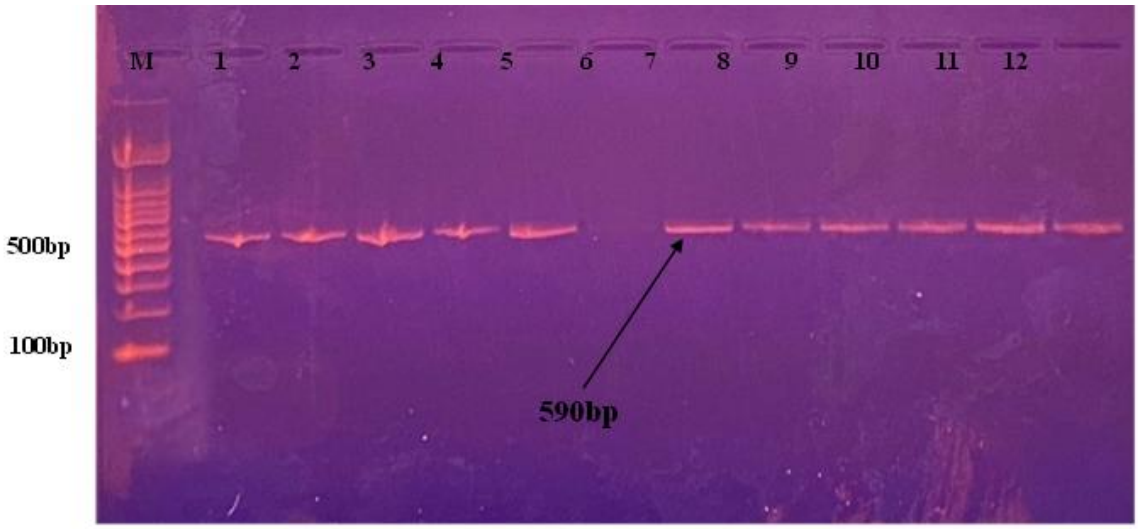

Figure (2): PCR amplification of 590bp BlaCTX-M gene by 2% agarose gel electrophoresis.
Ladder: M, Lane (1-12): PCR product of C. difficile isolates from urine samples.

In contrast to routinely prescribed antibiotic genes, the goal of this cross-sectional investigation was to identify drug-resistant genes in C. difficile from both community and hospital-acquired diarrheal children. Cefotaxime resistance was found in all 11 isolates (91.7%), indicating its high prevalence. This implies that the development of extended betalactamase enzymes (ESBLs), such as bla-CTM-M genes carried on plasmids, which are easily transmitted via the conjugation process, is the cause of the widespread cefotaxime resistance in the region (26). The outcome fell

short of the 35.5% resistance rate in C. difficile reported by Boekhoud et al. (13) in the Netherlands. Different antibiotic usage rules can be blamed for this discrepancy in results. Antibiotics are commonly used without enough control in our area and are readily available without a prescription from a doctor. Our results are in line with those of an Iraqi study by Al-Rawe et al. (27), which discovered that eight genes are common to all isolates and play a major role in drug resistance through ribosome defense, antibiotic efflux, and antibiotic deactivation. The authors came to the conclusion that the most potential novel therapeutic targets were mutations in the functional domains of the tetA (P), tetM, BlaCTX-M, and ermB genes.

Conclusions

Through the current results, it was found that C. difficile is considered one of the causes of diarrhea in many children, and it is characterized by its high resistance to antibiotics, which is aided by its possession of antibiotic resistance genes, including the BlaCTX-M gene, which was identified in most of the isolates obtained.

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