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Prevalence and Expression of Cytolethal Distending Toxin Genes in *Escherichia coli* Isolates from Iraqi Patients

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Abstract : *Escherichia coli* is a Gram-negative bacterium that possessesvarious virulence factors. One such factor is Cytolethal Distending Toxin (CDT),a genotoxin encoded by *cdt* genes. These genes have multiple variants and are classified into five types. Each CDT toxin is composed of three subunits-CDT-A, CDT-B, and CDT-C. This genotoxin induces DNA damage and leads to cell death. This study aims to detect the presence of the *cdt* gene and its expression in *E. coli* isolates collected from different sources,including urine, colon cancer tissue, wounds, and stool samples from individuals of various ages and sexes. One hundred twenty samples were collected and diagnosed using traditional biochemical tests and the Polymerase chain reaction technique. Only sixty isolates were confirmed as *E. coli*. These isolateswere further analyzed using PCR to detect the presence of the *cdt* gene. The results showed that only three samples contained the *cdt* gene.While six samples exhibited their expression. This research indicates the low frequency and prevalence of the *cdt* gene in *E. coli* and the presence of variant types of this genotoxin.

Keywords: *Escherichia coli*, virulence factor,CDT, genotoxin.

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Introduction

Escherichia coli is a Gram-negative bacterium classified within the Enterobacteriaceae family. It is a short bacillus, non-spore producing, facultatively anaerobic, and capable of growing on a simple medium(1).*E. coli* are typical residents of the human intestines. Though most strains are not pathogenic, certain strains become pathogenic by acquiring DNA from bacteriophage or plasmid DNA that encodes enterotoxins or invasion factors. These pathogenic strains are responsible for global diarrheal infections, newborn meningitis, septicemia, and urinary tract infections (UTIs)(2).*E. coli* possesses different virulence factors that aid in evadingdefenses and resisting antibiotics (3).Among these virulence factors, genotoxinscan cause DNA damage, leading to mutations thatmay cause cell death or cancer(4).The first known type of genotoxin is Cytolethal Distending Toxin (CDT),an AB_2heterotrimercomposed of threesubunits:

anactive B subunit and two functional groupsA and C subunits(5, 6).Three genes, *cdtA, cdtB,* and *cdtC,* are responsible for producing CDT toxin. CDT-A and C subunits recognize the host cell by binding to the plasma

membrane(7), facilitating the transmission of CDT-B into the host cell and its entry into the nucleus(8, 9).CDT-producing *E. coli* have been associated with cancer development and the localization of tumor tissue. Mucosa-associated CDT-producing *E. coli*has been observed near tumorsbut is absent in healthy colon regions(10).In vitro, findings indicate that CDT-B can break down or relax supercoiled DNA(9, 11).

This study aimed to detect the presence of the *cdt* gene in*E. coli*isolates from different sources and evaluate its expression.

Materials and Methods

Sample Collection and Isolation

One hundred twenty specimens were obtained from diverse sources (Wound, urine, colon cancer tissue, and stool).During the period from October 2023 to February 2024, with the approval of the College of Science Research Ethics Committee based on CSEC/0624/0046. All these samples were provided by Medical City hospital, Al-Kindi Teaching hospital, Al-Yarmouk hospital and Al-Kadhimiya Teaching hospital. Samples were cultured on selective media, specifically MacConkey agar and Eosin Methylene blue (EMB) agar. Biochemical examinations (indole test, urease, and Simmons citrate) were also performed(12).

Molecular Detection of *16s rRNA* and *cdt* genes:

DNA Extraction

Genomic DNA was extracted from isolates usinga Bacterial DNA Extraction kit (Elk, Chin), which was performed according to the manufacturer's recommendations.

PCR Amplification for the Identification of *16s rRNA* and *cdt* genes

Conventional Polymerase Chain Reaction has been done to detect the presence of *16s rRNA* and *cdt* genes in our isolates. The procedure used the PCR Kit (2.5X Master Mix Synthol), Synthol, Russian. The total PCR reaction was 20 µl consisting of 10 µl PCR Master Mix, 1 µl MgCl2, 1µl Forward primer, 1 µl, Reverse primer, 2 µl of Extracted DNA, and 5 µl of Nuclease Free Water. The first step of the PCR program was initial denaturation for 5 min at 95°C. The second steps contain three stages: denaturation for 15 sec at 95°C, annealing for 15 sec at 53 °C, and extension for 15 sec at 72 °C. This step was repeated 40 times, followed by the final step, extension for 10 min at 72 °C.The primer sequence used was supplied by Macrogen Company in a lyophilized form, as shown in (Table 1). A 2% agarose gel electrophoresis was carried out to monitor the amplification of these genes at 50 v for 40 min.

Table (1): Primer sequences and amplicon size of *16S rRNA* and *cdt.*

GeneName	Primer Sequence F 5′→3′	Primer Sequence R 5′→3′	Tm.	Product size (bp)	Reference
16S rRNA	ATGGCTGAGATTG AACCCTGG	TTATCCCCCTCCAT CAGGCAG	63	134	This study
Cdt-Bcomu	TAAATGGAATATA CATGTCCG	TTTCCAGCTACTGC ATAATC	53	588	(13)

Real Time PCR for *cdt* gene:

RNA Extraction

RNA was isolated from thesamples according to the protocol of TRIzol™ Reagent from ELK Biotechnology, China.

Synthesis of complementary DNA derived from mRNA

The preparation of cDNA from mRNA was carried out according to the instructions of the company OT-1 Reagent Kits (Reverse Transcription kits) from Synthol, Russia.

Evaluate The Expression of the*cdt* gene

The experiment used Luna® Universal qPCR Master Mix Protocol (M3003) from New England Biolabs, USA. The procedure was performed according to company instructionsinitially, preparation of the Master Mix. The total volume is composed of 10µL of Luna® Universal qPCR,0.5µl of each primer forward and reverse, 7µL of nuclease-free water, and 2µL of cDNA. After that, the Real-Time PCR program includes three steps: initial denaturation for 1 min at 95°C, denaturation for 15 sec at 95°C and annealing for 30 sec at 60°C. Repeat the last step 45 times. The primers used in this experiment were the same as those in conventional PCRwith different temperatures of60°C for each primer,(Table 1).

Result and Discussion

Sample Collection and Isolation

One hundred twentysampleswere obtained from different sources, of which 60 specimenswere diagnosed as *E. coli*. MacConkey agar demonstrated pink colonies, and EMB agar showed colonies with a green metallic sheen. All these tests were confirmed by biochemical tests with positive indole, urease negative, and Simmons' citrate negative. Furthermore, PCR results confirmed the presence of *16s rRNA*in all 60 samples, as shown in (Figure 1).

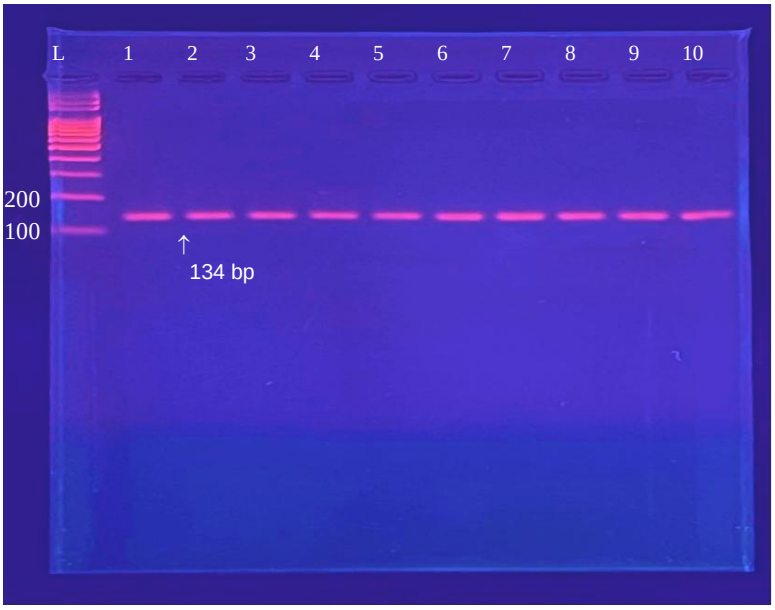


Figure (1): Gel electrophoresis of PCR product to *16S rRNA gene*. 2% agarose gel at 50V for 40min. L: Ladder marker 100 bp.

The Frequency of the*cdt* gene among isolates

Molecular detection of *cdt* genes was done utilizing a specific primer by conventional PCR. The results showed the presence of the B subunit of *cdt* gene in only three out of 60 isolates. In agreement with our study, two studies

(14) and (15)support our findings, indicate the low frequency of *cdt* in *E. coli* isolates from different sources. As shown in (Figure 2), only three product bands were observed in gel electrophoresis with a size of 588bp.

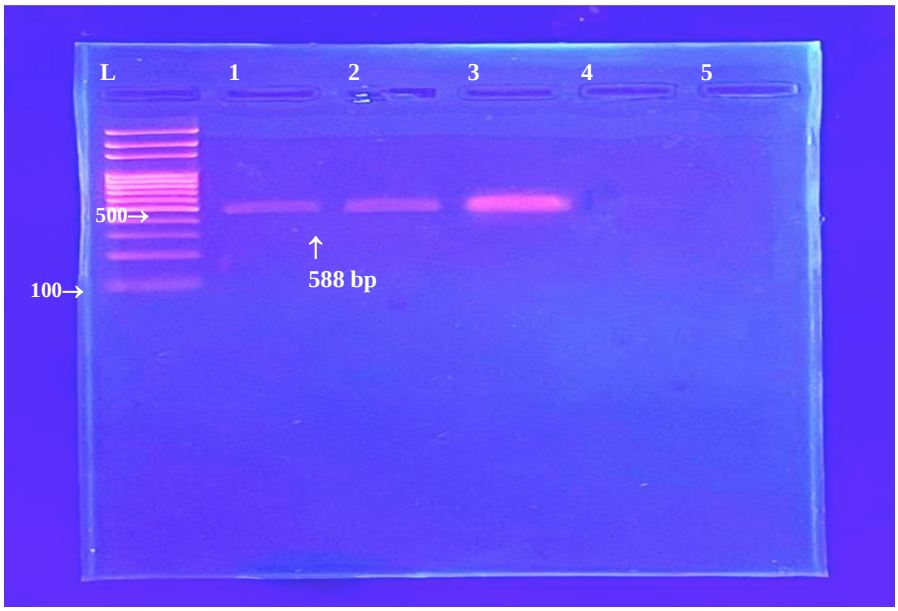
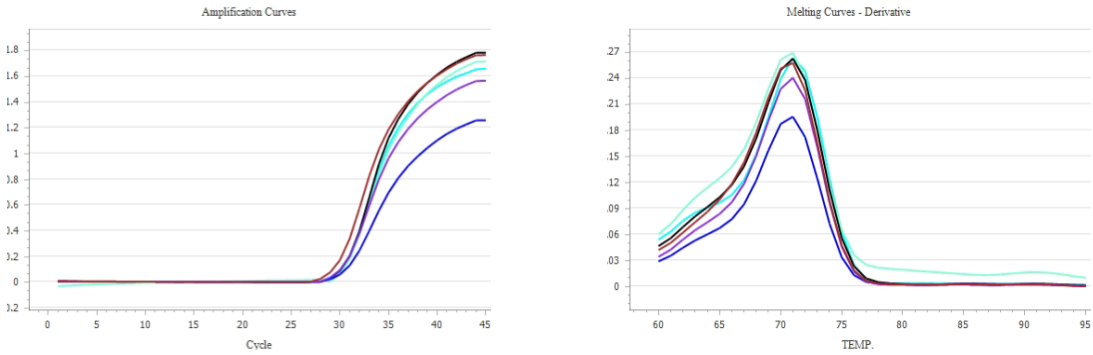


Figure (2): Gel electrophoresis of PCR product to *cdt* gene. 2 % agarose gel at 50V for 40min. L: ladder marker 100 bp.

Gene Expression of *cdt* gene

The outcome of real-time PCR differedfrom the result of conventional PCR. Out of 60 isolates 6 isolatesfrom different sources showed high expression of the B subunit of *cdt*gene.In this experiment we used SYBR Green fluorescence dye as an indicator, which is an intercalating dye that can binds to double-stranded DNA and emit the fluorescence at end of each cycle. When the fluorescence becomes measurable, the amount of fluorescent dye known as CyclingThreshold (CT).When the initial material is abundant, amplification occurs in the initial cycles, resulting in a lower CT value. Conversely,when the initial material is limited, amplification occurs in subsequent cycles, resulting in a greater CT value(16).The 16s rRNA housekeeping gene was used as reference gene. The results werecalculated usingthe Livak technique(17).The melting curve confirmed no other compoundsin the reaction, such as primer dimers as shown in (Figure 3). The folding score of the sixth sample, which was fromstool, was 1.64, the highest score among other samples.The folding scores of other samples [1] wound, [2]urine,[3]urine, [4]colon cancer tissue, and [5]stool were0.98, 0.82, 1.25, 1.22 and 1.33 respectively. The interpretation of our PCR and qPCR finding, and the difference in their results,can be explained by the primers that were designed for detection of *cdt*genes was not specific for all various types of *cdt*genes. These variant types of *cdt*genescame from horizontal gene transfer from bacteriophages,leading toinaccurate detection by conventional PCR (13, 18, 19) . Furthermore, the accuracy of real-time PCR is significantly higher than that of conventional PCR due to its ability to measure the presence of DNA during the progress of the PCR run.These

attributes of quantification, combined with qPCR's great sensitivity and specificity and a diminished risk for contamination, establish it asa preferred method for applications requiring precise quantification.The absence of post-amplification processes, such as gel electrophoresis or Southern blotting, facilitates quick analysis and enhances sample throughput. Additionally, the possibility of amplicon carry-over is diminished since the reaction tubes remain unopened. The real-time measurement of amplicon accumulation enables the assessment of reaction efficiency, hence facilitating the selection of more sensitive assays(20).This research(21) confirms our present study,concluding that PCR isless accurate in detecting*cdt* genes. Therefore, multiplex PCR should be used for more precise detection.



A B

Figure (3): Amplification curve and melting curve of *cdt* gene by using qPCR

Conclusion

The present study concludesthat the *cdt* gene has a low frequency in *E. coli* isolates from different sources of Iraqi patients. It also showshow the variants of this gene affect its detection and expression.

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