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The Frequency of Some Colibactin Genes Amongst the Clinical Strains of *E. coli* Isolated From Urinary Tract Infections in Iraq

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Abstract: *Escherichia coli* has been recognized worldwide as the most common causative agent for severe infections of the urinary tract. Colibactin is a genotoxin produced through a gene cluster called polyketide synthase (pks) island by members of Enterobacteriaceae. Limited information is available about the frequency of colibactin in *E. coli* isolates in Iraq. Hence, this study aimed to examine the frequency of some colibactin genes (*CIbA* and *CIbQ*) in clinical isolates of *E. coli* obtained from urinary tract infections (UTIs) in Iraq. Between October 2023 and January 2024, 120 urine samples were collected from females diagnosed with UTIs in Iraqi hospitals. 70 *E. coli* isolates were isolated after identification by biochemical methods and confirmed by molecular technique. In particular, the *16S rRNA* gene was targeted using specific primers to confirm their identity. Conventional polymerase chain reaction (PCR) was employed to detect the *CIbA* and *CIbQ* genes and confirm the presence of colibactin. The findings of this study revealed a high prevalence of colibactin-producing isolates (40%, 28/70) compared to colibactin-non-producing isolates (60%, 42/70) with non-significant differences ($p>0.05$). In conclusion, the frequency of colibactin genes is high in *E. coli* strains isolated from UTIs in Iraq. The presence of pks-positive isolates at a high frequency is concerning, as it suggests the spread of a highly genotoxic strain in urinary tract infections in Iraq that could potentially lead to the development of urinary tract cancer diseases.

Keywords: Colibactin, *E. coli*, Urinary tract infections.

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Introduction

Colibactin is a bacterium genotoxin that was first identified in *Escherichia coli* bacterium that has polyketide synthase island (pks), which is a gene cluster, as reported by Nougayrede *et al.* in 2006 (1). This toxin has been linked to several detrimental effects on biological processes, such as DNA double-strand breaks, chromosome abnormalities, cell cycle arrest, and immune cell death (2). The Pks Island contains an overall of 19 genes that are involved in the synthesis of colibactin, specifically labeled as *ClbA* to *ClbR*. It is important to highlight that all 19 genes are required to produce colibactin (3, 4). In recent years, scientists have shown an intense interest in investigating the prevalence and consequences of bacteria that produce colibactin as a possible factor in developing colorectal cancer. The main reason for this interest is the unequal representation of *E. coli* isolates that

produce colibactin in biopsies taken from individuals diagnosed with colorectal cancer (5, 6).

It has been found that the colibactin island cluster in other members of the Enterobacteriaceae family, such as *Klebsiella pneumonia*, *Enterobacter aerogenes*, and *Citrobacter koseri* (7). It is worth noting that the colibactin island cluster found in *K. pneumoniae* is exactly the same as that in *E. coli* (100% identical); this indicates that the functions and regulation of the 19 genes are preserved (8). In addition, a correlation between the existence of colibactin genes and other virulence factors has been documented, such as a correlation with the hypervirulence of *K. pneumonia*, mucosal invasion, and gut colonization by *K. pneumonia* (9). Also, a correlation between colibactin synthesis, the production of biofilm, and a low level of antibiotic resistance have been reported (10). However, as reported by researchers, the occurrence of colibactin-producing members within the Enterobacteriaceae varies throughout different countries (11,12,13).

In Iraq, *E. coli* is a common causative agent of urinary tract infections (UTIs), especially in females (14,15). There is a lack of comprehensive data on the frequency of the colibactin-producing strains in *E. coli* isolated from UTIs in Iraq. Therefore, this study aims to examine the occurrence of colibactin-producing strains in *E. coli* isolated from females suffering from urinary tract infections in Iraq.

Materials and methods

Sample collection and bacterial identification

From October 2023 to January 2024, 120 urine samples were obtained from two distinct hospitals in Baghdad, the AL-Yarmok Hospital and the Teaching Baghdad Hospital. The urine samples were cultivated immediately on MacConkey agar (selective medium) and then incubated for 24 hours at 37°C. For the initial identification, microscopic examinations, eosin methylene blue (EMB) agar as a differential medium, and biochemical tests, such as catalase and oxidase, were employed. Finally, the identification was confirmed by a polymerase chain reaction (PCR) technique to amplify the *16S rRNA* gene using specific primers.

Oligonucleotide primers design

First, the sequences of target genes were downloaded from NCIB, and the Serial Cloner 2-6-1 and Amplifx software were used for the in silico design of oligonucleotide primers. Macrogen Company was employed as a supplier for the oligonucleotide primers; the primer pair 16S-Ec-F:5`-TAGCTGGTCTGAGAGGA TGACCA-3`,16S-Ec-R: 5`-CCAACATT TCACAA CACGAGCTGAC-3`, with an amplicon size of (802 bp) was used for *16SrRNA* gene, the primer pair CIbA-F: 5`-CGCTATGATCAGTTTCATTTTGTG AG-3`, CIbA-R:5`-GAGAAATAAACAGG TGAACCTCTATATTT3-`, with an amplicon size of (500 bp) was used for the *CIbA* gene, the primer pair was CIbQ-F:5` -GCCATATTCAGGTGGTTCTGC3-`, CIbQ-R:5`-CTATGCCCAAAAATGGCG TAGTC-3`, with an amplicon size of (207 bp) was used for the *CIbQ* gene. A TM calculator from Thermo Fisher Scientific was utilized to determine the annealing temperatures of the primer.

Genomic DNA extraction

This paper presents a new method for quickly extracting the whole bacterial genome, which can be used for

gene detection. It merges and modifies the boiling method (16) and colony PCR (17). In summary, a pure single bacterial colony was inoculated in 5 ml of nutrient broth and incubated at 37°C for 24 hr. Next, the cells were harvested by centrifugation for 15 min. at 13000 rpm, rinsed twice with 1 ml D.W., and finally suspended in 1 ml D.W. Afterward, the cells were adjusted to a standardized optical density (O.D) of 1 at a wavelength of 600 nm. Next, 0.3 ml aliquot was transferred from the standardized bacterial culture and placed into an Eppendorf tube, and cells were collected by a centrifuge at 13000 rpm for 15 mins. subsequently, 0.75 ml of Tris-EDTA buffer (TE) was utilized to suspend the cells, which were then exposed to boiling for 10 mins at a temperature of 100°C. The genomic DNA was separated from cellular debris by a centrifuge at 13000 rpm for 20 mins. The genomic DNA in the supernatant was used as a DNA template directly in the polymerase chain reaction (PCR) without additional purification. The ratio of supernatant to PCR reaction mixture was 1:9.

Polymerase chain reaction (PCR)

For target gene detection, a polymerase chain reaction (PCR) technique was utilized for gene amplification; for molecular identification of *E. coli*, a fragment of *the 16SrRNA* gene was amplified. A fragment of the *CIbA* and *CIbQ* genes was targeted for amplification to detect the colibactin cluster island. The composition of the PCR reaction mixture and conditions are illustrated in (Table 1).

Table (1): Composition of PCR mixture and PCR conditions

Composition of PCR mixture					
Materials			**Volume in µl**		**Final concentration**
Go Taq G2 Green Master Mix (2X)			50		1X
Forward Primer (10 µM/ µl)			5		0.52 µM/ µl
Reverse Primer (10 µM/ µl)			5		0.52 µM/ µl
Nuclease-free water			40		-
Total reaction mixture			100		-
Aliquot 9 µl from the above PCR mixture (100 µl) into PCR tubes, and 1 µl of supernatant containing DNA from the genomic DNA extraction section was added to each tube as a DNA template.					
PCR conditions					
Initial denaturation	**30 reaction cycles**			**Final Extension**	**Hold**
	Denaturation	**Annealing**	**Extension**		
5 min (95˚C)	30 s (95˚C)	30 s (57˚C)	1 min (72˚C)	5 min (72˚C)	5 min (4˚C)

Gel electrophoresis technique

The PCR products were analyzed using the agarose gel electrophoresis technique on a 2% (w/v) agarose gel. The gel was made by dissolving 2 g of agarose powder (Promega, USA) in 100 ml of 1X TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.8) and boiling until all of the gel particles were completely dissolved. The gel was cooled to 50–60°C before adding a 4 µl ethidium bromide (10 mg/ml, Promega, USA). Subsequently, the agarose was placed into a gel tray and solidified for 30 min. at ambient temperature. A total of 5 µl of PCR products were loaded and run against 3 µl

DNA marker (Promega, USA, 100 pb DNA marker) for 60 minutes; electrophoresis was carried out at 100 V. The visualization of DNA bands was facilitated by employing a UV viewer illuminator system (Fisher Scientific, UK).

Statistical analysis

All features were presented as frequencies and percentages. A Pearson-Chi-square test was performed to identify statistically significant variation in percentages. P≤0.05 was measured as significant. SPSS v. 22.0 and Excel 2013 statistical software were used for data analysis.

Results and discussion

Identification of *E. coli* isolates

Urinary tract infections (UTIs) are a prevalent human illness that presents substantial health hazards to both hospitalized and non-hospitalized people (18). *E. coli* is the main agent of UTIs, leading to inflammation in the ureters, kidneys, and urethra. From 120 urine samples obtained from females suffering from UTIs in Iraqi hospitals, 70 *E. coli* isolates (58.33%, 70/120) were isolated compared to (41.67%, 50/120) of other bacterial isolates with non-significant differences (p>0.05). First, *E. coli* isolates were identified using microscopic examination and culturing characteristics on selective and differential media; the isolates appeared as pink colonies on MacConkey agar and produced a green metallic sheen color on EMB agar. Finally, the conformation of identification of *E. coli* isolates was performed by targeting a fragment of the *16S rRNA* gene with specific primers using conventional PCR, as shown in Figure 1. A clear single DNA band (802bp) identifies bacterial isolates as *E. coli.*

The initial identification of bacteria was conducted based on their cultural traits using specific types of media that can selectively and differentially support their growth. *E. coli* colonies on MacConkey agar exhibit a pink color due to their capacity to ferment lactose and generate a unique metallic green sheen on EMB medium. This coloration results from the dyes' metachromatic properties, the motility of *E. coli* facilitated by flagella, and the production of highly acidic byproducts during fermentation. These distinctive characteristics enable a quick differentiation of *E. coli* from other lactose-fermenting bacteria (19).

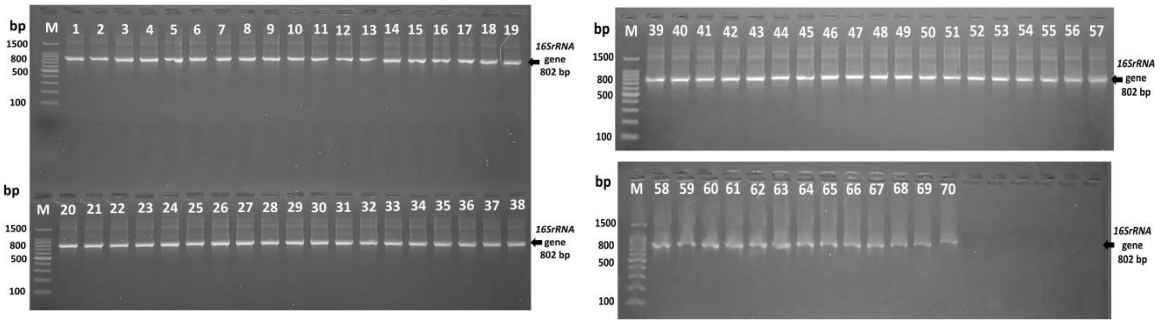

Figure (1). Agarose gel electrophoresis (2%) of PCR amplicon showing *16S rRNA* gene (802 bp). Numbers 1 to 70 correspond to *E. coli* isolates. M corresponds to the DNA marker (100 bp).

Primary identification is a crucial stage in isolating bacteria from UTIs. This identification process helps to exclude other bacteria, such as *Klebsiella* and *Pseudomonas*, that may be present in UTIs. This saves time and resources. Nevertheless, molecular identification through gene amplification is considered more dependable than alternative identification techniques and is extensively utilized for pathogen

detection and identification (20, 21). To identify the *E. coli* isolates at the molecular level, a specific region of the *16SrRNA* gene was selected. Specific primers were used to amplify this region, resulting in an amplicon of size 802 base pairs. The amplification results revealed an isolated DNA band of 802 base pairs, confirming that all isolates are of the *E. coli* species. The appearance of a single, distinct DNA band indicates the existence and successful amplification of a target gene. A study conducted by Muhaimeed *et al*., revealed that 100% of primarily identified *E. coli* were confirmed by the *16S rRNA* gene with an amplicon size of 180 bp (22).

UTIs are predominantly caused by *E. coli*, as indicated by both local and international investigations that have demonstrated a high prevalence of *E. coli* in urine samples from individuals with UTIs (23, 24). The prevalence of *E. coli* isolates in the current study was 58.33%. These findings align with a local investigation by Abood *et al*. (25), who successfully identified *E. coli* in urine samples with an isolation rate of (58.09%). Additional local studies indicated a significant isolation level, with 70% (26) and 65.8% (27). In contrast, other studies showed lower isolation percentages of *E. coli*, 30% (28) and 25.53% (29).

The elevated frequency of *E. coli* detection in UTIs may be attributed to the bacteria's capacity to adapt to the urinary tract's surroundings and their resilience in challenging environmental circumstances. In addition, it has potent virulence factors and the capacity to create biofilm.

Distribution of colibactin island (pks)

Two genes (*CIbA* and *CIbQ*) were specifically selected to identify the existence of the colibactin island (pks) in *E. coli* isolates collected from UTIs in Iraq. The results indicate that 28 (40%) of *E. coli* isolates possess both the *CIbA* and *CIbQ* genes, which are pks-positive. In contrast, 42 (60%) of *E. coli* isolates do not have the *CIbA* and *CIbQ* genes, making them pks-negative with non-significant differences ($p>0.05$), as shown in (Figure 2,3).

The distribution of colibactin-producing strains is different among Enterobacterales members as well as among the same bacterial species (7). Locally, the percentages of colibactin-producing strains, as reported by Hussain, were (8%) isolates of *E. coli* and (2.25%) of both *E. aerogenes* and *K. pneumoniae* (7). In another study, the percentage of *E. coli*-producing strains was 11.18% (12). In contrast, another study conducted by Khalaf, showed a slightly higher percentage of colibactin-producing *E. coli* isolates than previous studies, which was 14% (26).

Interestingly, it appears from the investigations, as mentioned earlier, that the percentage of colibactin-producing *E. coli* isolates increased gradually in Iraq in the last five years; in 2020, it was 8%; in 2022, it was 11.18; in 2023, it was 14%; while in the current study, it was 28%. This is a concerning sign that a very detrimental strain is spreading rapidly and gradually in urinary tract infections in Iraq. A correlation between the colibactin cluster and other virulence factors has been established, such as biofilm production, antibiotic susceptibility, and other genes (10,30). This could explain the differences in the frequency of colibactin-producing strains.

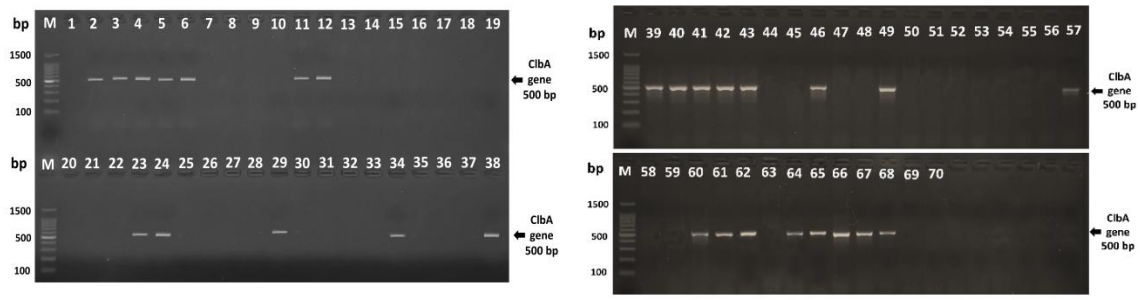

Figure (2). Agarose gel electrophoresis (2%) of PCR amplicon showing *CIbA* gene (500 bp). Numbers 1 to 70 correspond to *E. coli* isolates. M corresponds to the DNA marker (100 bp).

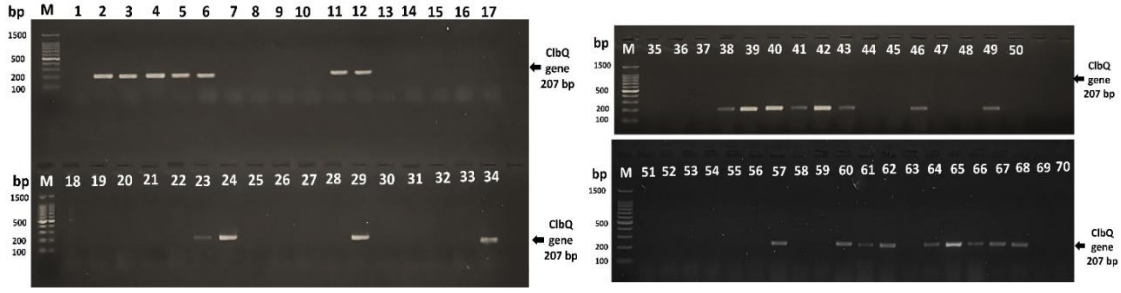

Figure (3) Agarose gel electrophoresis (2%) of PCR amplicon showing *CIbQ* gene (207 bp). Numbers 1 to 70 correspond to *E. coli* isolates. M corresponds to the DNA marker (100 bp).

Conclusion

The findings revealed a significant occurrence of colibactin among *E. coli* isolates collected from urinary tract infections in Iraq. The considerable occurrence of pks-positive isolates is a frightening indication of the extensive dissemination of a highly genotoxic strain in urinary tract infections in Iraq. This particular strain possesses the capacity to result in the emergence of urinary tract cancer diseases. However, further studies are required to understand the relationship between colibactin synthesis and other virulence factors.

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