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Prevalence of Extended-Spectrum B-Lactamase (ESBL) and Quinolone Resistance (*qnr*) Genes among Cytotoxic Necrotizing Factor-1-Producing Uropathogenic *Escherichia coli* in Babylon, Iraq

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ABSTRACT

Background: Pathogenic *Escherichia coli* (*E. coli*) is usually known as the principal agent of hospital-acquired infections, particularly urinary tract infections (UTIs). This study aimed to determine ESBL (extended-spectrum B-lactamase) production and quinolone resistance (*qnr*) genes in cytotoxic necrotizing factor 1 (CNF-1)-producing *E. coli* isolates from UTIs in Iraq. **Materials & Methods:** A total of 996 *E. coli* isolates were obtained from UTIs in two general hospitals in Hillah, Babylon, Iraq (during 2014-2022), and 100 uropathogenic *E. coli* (UPEC) were cnf-1 carriers. ESBL production was evaluated using the double-disk synergy test. *qnr* genes were detected using polymerase chain reaction (PCR). **Findings:** Nalidixic acid and chloramphenicol resistance was 70 and 30%, respectively. ESBL production was observed among 46% of *cnf-1* carriers. *qnrA*, *qnrB*, and *qnrS* genes were detected in 18, 21, and 11% of the isolates, respectively. ESBL-producing isolates mainly carried the *qnrB* gene and showed the highest resistance levels to quinolones. Major risk factors of pathogenic *E. coli* isolation included older age (68%, $p = .031$), previous hospitalization (76%, $p = .021$), and urinary catheter (83%, $p = .018$). **Conclusion:** Although the prevalence of *cnf-1* gene was not high among UPEC isolates, its prevalence was high among quinolone-resistant and ESBL-producing isolates. Continuous investigation of virulence and resistance genes is essential for monitoring and controlling infections. It is necessary to determine virulence factors and resistance genes among UPEC in Iraq and take timely measures to hinder the spread of resistance genes to other nosocomial isolates.

Keywords: UTIs, Virulence, Antibiotics, Quinolone resistance, *Escherichia coli,* ESBL

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Introduction

A considerable range of nosocomial infections is caused by *Escherichia coli* (*E. coli*) [1]. In spite of being as a member of the normal flora of humans and wildlife, [2] *E. coli* isolates cause urinary and gastrointestinal tract infections (UTI and GIT, respectively), skin and respiratory infections, sepsis, and neonatal meningitis [3-5]. Infections could range from severe or lethal to insignificant depending on the level of antibiotic resistance and encoding of bacterial virulence factors. A considerable part of nosocomial UTIs and the majority of community –acquired UTIs are caused by this bacterium, which has a significant impact on morbidity and medical costs globally. UTIs and invasive infections caused by multidrug-resistant (MDR) *E. coli* strains have recently increased globally. MDR strains have extremely low therapeutic success owing to few ongoing treatment options available [3, 6]. Furthermore, the population biology of uropathogenic *E. coli* (UPEC) has not been well understood, making it difficult to prevent and control the spread of related infections [7-9]. Extended-spectrum β-lactamases (ESBLs) in Gram-negative bacteria include plasmid-mediated enzymes hydrolyzing cephalosporins mainly of third generations and aztreonam [10]. These enzymes are widely distributed worldwide [11]. The highly diverse nature of ESBL-producing bacterial strains and numerous mutations in resistance encoding genes have resulted in high morbidity and resistance rates with huge clinical impact [12, 13]. Quinolones are a class of antibiotics effective against *Enterobacteriaceae* infections. Common antibiotic classes used in humans and animals include beta-lactams and quinolones [14]. Unfortunately, fluoroquinolone resistance is rising due to chromosomal alterations (for example mutations in topoisomerase IV and DNA gyrase enzymes) or acquisition of plasmids[15,16]. In recent years, the frequency of plasmid-mediated quinolone resistance (PMQR) has progressively increased, resulting in a decrease in effective elimination of infections. The primary mechanisms of quinolone resistance are plasmid-mediated [17, 18]. Major alterations in quinolone targets occur in *qnr* proteins through quinolone resistance genes. *qnr* genes, which are of PMQR determining factors, were initially found in *Klebsiella pneumoniae*. These genes, for example, *qnrA*, *B,* and *S,* are three kinds of *qnr* determining factors found in numerous *Enterobacteriaceae* members [19, 20]. Other gene classes such as *qnrC* and *D* have also been found [21, 22]. Penta-peptide repeat proteins encoded by *qnr* genes blockade the action of quinolones and cause low-level resistance [17, 23]. Various *qnr* genes differ in sequence by 35% [23]. Following antibiotic pressure, structural changes in arrangement might occur, resulting in non-susceptibility to trimethoprim, cefepime, ceftriaxone, and co-amoxiclav among *E. coli* isolates [24, 25]. *qnr* genes are found worldwide, particularly among hospitalized patients. As an opportunistic pathogen, *E. coli* is usually known as a major cause of hospital-acquired infections and various diseases, particularly UTIs. *E. coli* carries various virulence factors such as adhesins, secretory systems, and toxic proteins [26, 27]. The chromosomally encoded cyclomodulin, called cytotoxic necrotizing factor 1 (CNF-1) and produced by UPEC and other pathogenic strains, is associated with bacterial colonization, virulence, invasion, survival, and distribution in the body, it is a leading cause of host cell cycle changes such as colorectal tumorigenesis, apoptosis, inflammation, and tissue damage [28-32]. The association of *cnf-1* gene carriage and quinolone resistance or ESBL production has not been demonstrated previously. A study exhibited a low prevalence of *cnf-1* gene among quinolone-resistant and ESBL-

bearing isolates [33]. As virulence factors play a substantial role in the distribution of isolates, the inefficiency of common antibiotics results in the failure to hinder the distribution of pathogens in the body [34-36]. **Objectives:** The purpose of this study was to determine quinolone resistance and the presence of *qnr* genes in ESBL-producing and non-producing *E. coli* isolates collected from UTIs.

Materials and Methods

Bacterial isolation: During April 2014 to November 2022, a total of 996 *E. coli* isolates were collected from UTIs in two general hospitals in Hillah, Babylon, Iraq. Bacteriological culture techniques such as culture on MacConkey agar (Merk, Germany) and blood agar (Merk, Germany) media and biochemical tests (IMVIC test and Vitek-2 system) were applied to identify bacterial isolates. Positive results included methyl red (M-R), indole, mannitol and lactose (MAL), glucose (GLU), and maltose [37]. Antibiotic susceptibility pattern was determined by Viteck 2 compact system [11]. For the separation of *cnf-1* carrying isolates, polymerase chain reaction (PCR) was performed to amplify the *cnf-1* gene using forward (5-AAG ATG GAG TTT CCT ATG CAG GAG-3) and reverse (5-CAT TCA GAG TCC TGC CCT CAT TAT T-3) primers [38]. Inclusion criteria included frequency and urgency of urination, dysuria, suprapubic pain, and positive culture in the hospital. Patients receiving antibiotics during two weeks prior to sampling were excluded. **Antibacterial susceptibility test:** Vitek 2 system (Biomerieux, USA) was used for identification (Gram negative card) and susceptibility analysis (AST-N256 card). Accordingly, bacterial cultures on nutrient agar and suspensions equal to 0.5 McFarland were prepared. After culturing the isolates on each card for a specific antibiotic, the minimum inhibitory concentration (MIC) was measured. The antibiotics used included ciprofloxacin (CIP, 5 µg), ofloxacin (OF, 5 µg), chloramphenicol (CL, 30 µg), cefotaxime (CF, 30 µg), ceftazidime (CAZ, 30 µg), nalidixic acid (NA, 30 µg), and aztreonam (AZT, 30 µg). The resistance profile was determined using the Viteck-2 system method as per the manufacturer's guidelines [39, 40]. **ESBLs screening test**: The isolates were evaluated for ESBL production using the double-disk synergy test (DDST) in compliance with Clinical and Laboratory Standards Institute (CLSI) recommendations. The disks included ceftazidime, cefotaxime, and clavulanic acid, where the appearance of an inhibition zone around the disks indicated ESBL production.

Extraction of plasmid DNA and amplification: Plasmid DNA content was extracted utilizing the Plasmid Miniprep kit (Favorgen, Taiwan) and kept at -20 °C and served as a template for the amplification of *qnr* genes using the primer sets depicted in Table 1.

Table 1) Primer sequences of quinolone resistance genes in this study

Genes	Sequence: 5'-3'	Amplicon (bp)	Reference
qnrA	F- AGAGGATTTCTCACGCCAGG R- TGCCAGGCACAGATCTTGAC	580	[41]
qnrB	F -GGMATHGAAATTCGCCACTG R-TTTGCYGYYCGCCAGTCGAA	264	
qnrS	F-GCAAGTTCATTGAACAGGGT R-TCTAAACCGTCGAGTTCGGC	428	

Ethical statement: The protocols of this study were carried out in accordance with the ethical permissions for scientific research, designed (BRC/HO-14314) by the Ethics Committee of the Iraqi Ministry of Health and Ministry of Higher Education and Scientific Research.

Statistical analysis: Sigma Plot software Version 12.5 and Microsoft office Excel 2019 were both used to process and analyze data at a significance level of 0.5. The analysis of variance (ANOVA) and multivariate linear regression model were used to evaluate differences or relations.

Findings

Patients' demographic data: Out of 996 UPEC isolates, 100 (10.04%) isolates carried the *cnf-1* gene. Among 100 patients infected with *cnf-1* carrying UPEC, 46% were male, and 54% were female. Underlying diseases including diabetes mellitus (4%, n=4), kidney disorder (11%, n=11), ICU residence (16%, n=16), alcohol consumption (1%, n=1), smoking (19%, n=19), immunomodulatory treatment (2%, n=2), and previous antibiotic usage (21%, n=21) were not significantly associated with the isolation of *cnf-1*-carrying *E. coli*. However, older age (68%, n=68, *p*= .031), previous hospitalization (76%, n=76, *p*= .021), and urinary catheter (83%, n=83, *p*= .018) were significant associated factors in this regard.

Antibacterial sensitivity, MIC levels, and ESBL production: The antibiotic sensitivity testing of *cnf*-1-carrying *E. coli* isolates revealed that resistance to the tested quinolones was high among the isolates. Resistance to ciprofloxacin, levofloxacin, ofloxacin, and norfloxacin was found in 54% (n=54), 52% (n=52), 56% (n=56), and 50% (n=50) of the isolates, respectively. Additionally, resistance to nalidixic acid, aztreonam, cefotaxime, ceftazidime, and chloramphenicol was 70% (n=70), 68% (n=68), 50% (n=50), 62% (n=62), and 30% (n=30), respectively.

The minimum inhibitory concentration (MIC) levels of ceftazidime, cefotaxime, ciprofloxacin, and ofloxacin are shown in Table 2. The highest resistance rate to beta-lactams was related to ceftazidime (62%, n=62) with MIC ≥ 4 µg/mL. Moreover, the highest resistance to quinolones was related to ofloxacin (56%, n=56) with MIC ≥ 16 µg/mL. Moreover, ESBL production was observed among 46% (n=46) of *cnf*-1-carrying UPEC isolates during 2014-2022.

Amplification of *qnr* genes: Amplification of *qnr* genes using PCR is shown in Figures 1, 2, and 3. Accordingly, 50% (n=50) of ESBL-bearing *E. coli* isolates carried *qnr* genes including *qnrA* (18%, n=18), *qnrB* (21%, n=21), and *qnrS* (11%, n=11).

Table 2) MIC levels of ceftazidime, cefotaxime, ciprofloxacin, and ofloxacin among uropathogenic *E. coli* in this study

Antibiotics/MIC	%(N) in Susceptibility Range	%(N) in Resistance Range
Ceftazidime	38 (n=38) (MIC:0.5-2 µg/mL)	62 (n=62) (MIC ≥4 µg/mL)
Cefotaxime	50 (n=50) (MIC:0.5-2 µg/mL)	50 (n=50) (MIC≥ 4 µg/mL)
Ciprofloxacin	54 (n=54) (MIC:0.5-4 µg/mL)	46 (n=46) (MIC≥ 16 µg/mL)
Ofloxacin	56 (n=56) (MIC:0.5-4 µg/mL)	44 (n=44) (MIC≥ 8 µg/mL)

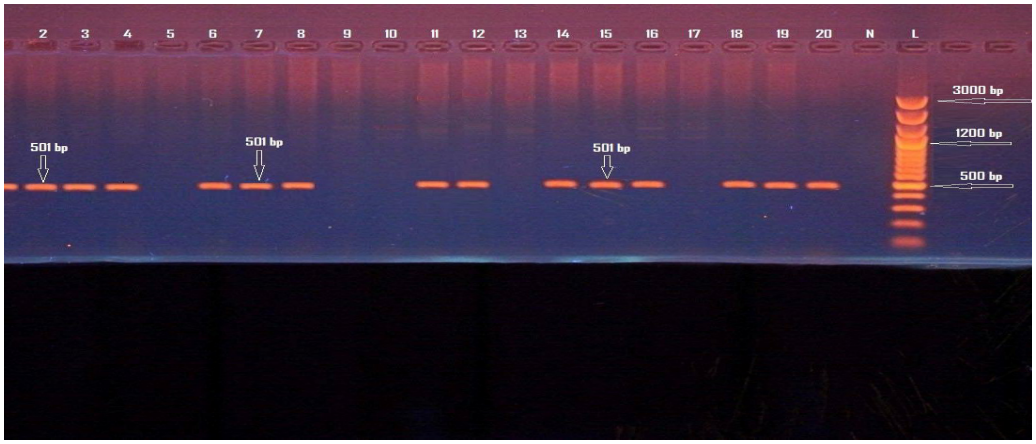

Figure 1) Gel electrophoresis products of the *qnrA* gene of *E. coli* with a 501 bp size; L: **+**DNA marker, wells 1-3, 6-8, 11, 12, and 18-20: positive samples, and N: negative control in this study

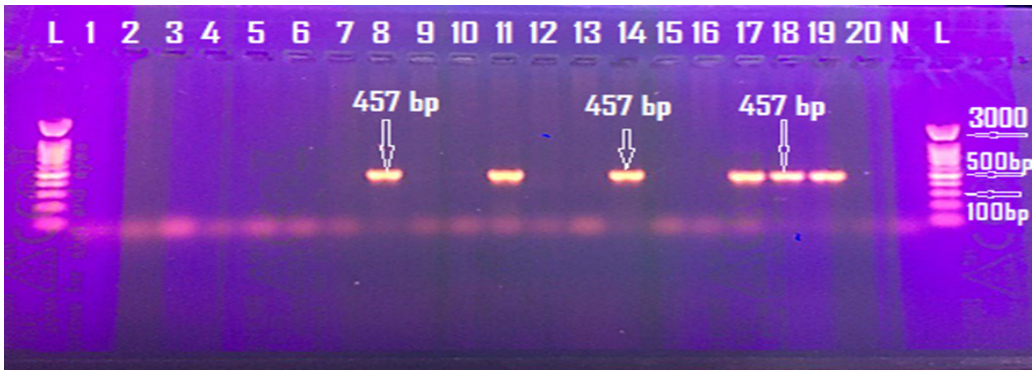

Figure 2) Gel electrophoresis products of the *qnrB* gene of *E. coli* with a 300 bp size; L: DNA marker, wells 8, 11, 14, 17, 18, and 19: positive samples, and N: negative control in this study

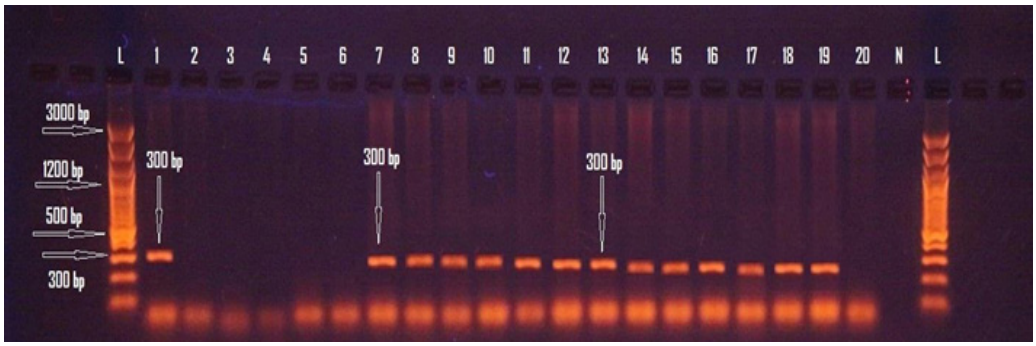

Figure 3) Gel electrophoresis products of the *qnrS* gene of *E. coli* with a 457 bp size; wells L: DNA marker, 1: positive control, N: negative control, and 7-19: positive samples in this study

Discussion

UTIs are among the major human infections worldwide, which are mainly (90%) caused by UPEC. In addition, UTIs affect 50% of all women [42]. Drug-resistant UPEC carrying virulence factors has been among the major causes of nosocomial infections and related deaths around the sphere in recent decades. Moreover, plasmid-encoded genes involved in resistance are responsible for the rapid spread of antibiotic resistance [43] Plasmid-encoded *qnr* and ESBL genes remain as the furthermost common agents of resistance to quinolones and β-lactams, respectively; however, resistance to other antibiotics has also recently increased dramatically [44, 45]. The majority of UTI-diagnosed patients in this study (68%, n=68) were female. This could be due to the structure and site of the urinary tract in women, which is near the anus channel. Also, most infections occur in individuals in the age range of 20 to 42 years, who have the most sexual activity. As a result, according to Rahn (2008) [46] regarding the prevalence of UTIs, age and sex are significant variables. The results showed that approximately 10.04% of UPEC isolates carried the *cnf-1* gene. However, more than half of them were ESBL producers and resistant to quinolones. A previous study inferred that 12.3% of ciprofloxacin-resistant UPEC carried the *cnf-1* gene, which is lower than the present study result [33]The *cnf-1* gene encodes CNF-1, which is almost always associated with hemolysin and provokes actin stress fibers formation and cytoskeleton rearrangement. Additionally, it causes effacement of intestinal microvilli, increased permeability in polarized intestinal cell monolayers, apoptosis, and inflammation in murine model of UTI [33, 47, 48]. The isolates in the current investigation showed the highest resistance to nalidixic acid (70%, n=70) and the lowest resistance to chloramphenicol (30%, n=30). However, because chloramphenicol is not commonly used in clinical practice, aztreonam may be a better option for UTIs. Moreover, the low chloramphenicol resistance in this study could be due to its minimal use in routine UTI treatments. Surprisingly, half of the UPEC isolates were quinolone-resistant, possibly due to uncontrolled consumption of antibiotics or previous hospitalization [49-51]. Relevantly, significant related risk factors in this study included older age (68%, n=68, p= .031), previous hospitalization (76%, n=76, p= .021), and urinary catheter (83%, n=83, p= .018). According to the findings, ESBL production was also high among UPEC isolates. However, the prevalence of ESBL-encoding genes was not investigated. ESBL and quinolone resistance gene carriage by E. coli isolates has created considerable health care costs due to the importance of target antibiotics [52]. Fluoroquinolone resistance by mutations in *gyr* genes such as *gyrA* is an important phenomenon which was not assessed in this study [53]. According to the results, the antibiotic sensitivity pattern of these pathogenic bacteria was significantly variable, and this may be due to the antibiotic consumption without a valid prescription or laboratory supervision, misuse, indiscriminate usage, differences in geographical location and bacterial strains, and the acquisition of resistance mechanisms [54-56]. We observed that older age (68%, n=68, p= .031), previous hospitalization (76%, n=76, p= .021), and urinary catheter (83%, n=83, p= .018) were significantly associated with isolation of cnf-1-producing UPEC. There are several studies regarding the main risk factors of isolation of drug-resistant bacterial isolates, such as older age, previous hospitalization, and consumption of antibiotics [57, 58]. Therefore, prior exposure to antibiotics is a substantial factor needing to be controlled. It was observed that the highest resistance rate to beta-lactams was related to ceftazidime (62%) with MIC ≥ 4 µg/mL. Additionally, the highest resistance rate to quinolones was related to ofloxacin (56%) with MIC ≥ 16 µg/mL. Moreover, ESBL production

was observed among 46% of *cnf-1*-carrying UPEC isolates during 2014-2022. In addition, the findings revealed that 50 out of 100 (50%, n=50) ESBL-bearing *E. coli* isolates carried qnr genes including qnrA (18%, n=18), *qnrB* (21%, n=21), and qnrS (11%, n=11). The rapid spread of plasmid-mediated resistance genes (such as those encoding *qnr* and mediated resistance genes (such as those encoding *qnr* and ESBLs) is a concern in the era of extensive drug resistance rates [59, 60]. Limitations of this study mainly included low sample size, limited study area, and lack of assessment of DNA mutations responsible to quinolone resistance, expression analysis of genes, and genetic typing.

Conclusions

Although the prevalence of cnf-1 gene was not high among UPEC isolates, its prevalence was high among quinolone-resistant and ESBL-producing isolates. The quinolone-resistant, ESBL-producing, cnf-1-carrying UPEC isolates, mainly carrying the qnrB gene, had the highest rate of resistance to quinolones. The co-carriage of virulence determinants and plasmid-encoded resistance genes is a crisis considering the failure in infection eradication. We observed that more than half of the isolates were resistant to quinolones and third generation cephalosporins. Determining resistance mechanisms to common antibiotic classes facilitate the control of infections in any epidemiological area. Additionally, targeting enzyme proteins (using novel synthetic or natural drugs) is a promising approach after screening for prevalent drug-destroying bacterial enzymes. Considering the various possible mechanisms participating in this kind of resistance, more investigation is required to determine the rate and level of quinolone resistance in Iraq and to take rapid measures to prevent the spread of resistance genes to other nosocomial isolates.

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Ethical permission: This study was performed after obtaining the approval of the Ethics Committee of the University of Babylon. There was no human or animal study.

Conflict of interest: None declared by authors.

Authors' contributions: H.Q.R. and L.A. conceptualized the study. M.A.A. and T.S.H. performed the work and wrote the draft.

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