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Article

Detection of blacTX-M gene in Escherichia coli Isolates and Study the correlation between Biofilm formation and Antibiotic Resistance

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Abstract: Urinary tract infections (UTIs) are one of the most common bacterial infection, Escherichia coli is the predominant uropathogen. Bacterial strains with biofilm-forming ability become persistent in a body and antibiotic treatment susceptible to chronic infection, for which the eradication is quite difficult. The correlation between biofilm formation and antibiotic resistance in E. coli isolated from urine samples was studied, and the blaCTX-M gene as an extended spectrum β-lactamase (ESBL) marker was identified. A total of 320 urine samples were obtained from patients attending Azadi and Kirkuk Teaching Hospitals from April to September 2024. The causative E. coli species were identified by routine microbiological techniques and VITEK2. Biofilm formation was tested by Congo Red Agar assay, whereas antimicrobial susceptibility was performed following Kirby-Bauer disc diffusion method. The blaCTX-M gene was identified by PCR. In 320 urine samples, 154 (48.1%) were culture positive; isolates of Escherichia coli numbered 138 (89.6%). One hundred and one isolates (73.2%) were found to be biofilm producers, of strong, moderate and weak producer 25.4%, 30.4% and 17.4% respectively. High production of biofilms showed the highest rates of resistance against β-lactams, Trimethoprim and Gentamicin, whereas being non-biofilm producer had lower rates. PCR analysis showed the presence of blaCTX-M gene in all isolates 100% implying ESBL associated resistance. Biofilm production by E. coli has a significant relationship with MDR especially towards β-lactams and Trimethoprim. Also, the presence of the blaCTX-M gene supports the high rate of ESBL-producing strains. Inhibition of biofilm production could improve the antibiotic effectiveness in UTI treatment.

Keywords: Escherichia Coli, Urinary Tract Infection, Biofilm, Antibiotic Resistance, blaCTX-M

Introduction

Urinary tract infections (UTIs) are still one of the most frequent bacterial diseases worldwide, and a burden for morbidity, economics and healthcare issues [1], [2]. Escherichia coli (E. coli) especially uropathogenic E. coli (UPEC) is by all means the most frequent etiologic agent; it causes 70–90% of community-acquired and a significant proportion of health-care associated UTIs [1], [3]. The virulent

potential of UPEC is mediated through a constellation of virulence factors, which includes adhesins, toxins and significantly the capability to form biofunctional communities—a phenotypic characteristic highly correlated with chronicity and antibiotic insensitivity [4], [5]. Biofilm is a complex collection of bacterial colonies encased with extracellular polymeric matrix, which attaches to surfaces. In this self-compromised environment, bacterial cells display shifted physiology including increased resistance to antimicrobial factors and hiding from host immunity [6]. Clinical evidence indicates that biofilm-forming UPEC isolates are often more resistant strains to the antibiotics, which eventually exacerbating chronic UTIs, leading to failure in therapy as well as increasing relapse rate [7], [8]. Limited antibiotic penetration within the biofilm structure and reduced metabolic activity of cells are among the primary causes of this tolerance [6]. At the same time, the worldwide spread of ESBL-producing E. coli has compounded therapeutic challenges [9]. ESBLs have the ability to provide resistance towards a broad spectrum of β-lactams, including third-generation cephalosporins, and are commonly found harbored on mobile genetic elements which mediate their propagation [10]. Of the ESBL genes, blaCTX-M has become globally preeminent and is frequently found among uropathogenic isolates [11], [2]. The presence of blaCTX-M is linked to multidrug-resistant phenotypes and has significant repercussions for empiric treatment (12). Various studies across the globe have reported that biofilm production and resistance caused by ESBL frequently coincide, implying they both contribute to increasing the persistence and survival of bacteria under antibiotics stress in a synergistic manner [13], [14] For example, a report has shown that biofilm-forming ESBL producers have increased frequencies of transferable blaCTX-M plasmids, which makes the infection control a more challenging issue [15]. In addition, strong biofilm-forming UPEC isolates are found to carry multiple antibiotic resistance determinants including blaCTX-M for multidrug resistance phenotypes [5]. Although a wealth of global data was published, there is still a clear void in comprehensive studies originating from Iraq investigating biofilm formation, antibiotic susceptibility trends and the molecular occurrence of blaCTX-M among UPEC causing UTIs. While some Iraqi studies have analyzed the rates of occurrence of ESBL genes (including blaCTX-M) in uropathogenic isolates [16], [17], and others have addressed biofilm formation or resistance independently [18], practically none has cross-referenced biofilm formation with blaCTX-M carriage and antibiotic resistance phenotypes within the same group of isolates. This gap is especially important in the context of high level of inappropriate use of antibiotics and growing burden with resistant infections in Iraqi clinical practice [16]. Better knowledge on the relationship between biofilm production and genotypic resistance markers like blaCTX-M could improve diagnostics, guide targeted antimicrobial therapy, and some extent infection control measures locally and globally. Thus, the purpose of the present study was to establish a correlation between biofilm formation and antibiotic resistance profiles in UPEC isolated from urine samples taken from patients with UTIs.

Materials and Methods

Specimen Collection

The study includes 320 clinical urine samples received from patients who attended Azadi Teaching Hospital and Kirkuk Teaching Hospital in the city of Kikruk, Iraq from April to September/2024. Samples were collected in suspected urinary tract infection (UTI) patients of age group 20–50 years irrespective of sex.

Escherichia coli Isolation and Characterization

Culture and Morphological Characteristics

Urine samples were inoculated on to Blood agar, MacConkey agar and Eosin Methylene Blue (EMB) agar and incubated at 37 °C for 24 hours. Shiny morphologically typical E. coli colonies (pink-colored, lactose-fermentingcolonieson MacConkey agarandgreen on EMB agar) were spooled for further testing.

Microscopic Examination

Suspected colonies were smeared and stained by the Gram stain. Gram-negative, rod-shaped cells in single and/or doublets were considered presumptive E. coli.

Biochemical Identification

Confirmation of the isolates was undertaken by using standard biochemical tests (Mackie and McCartney -1996), such as motility, indole, methyl red, Voges-Proskauer test (VP), citrate utilization, urease catalase oxidation-fermentation test (OF-test), oxides and Kligler Iron Agar (KIA).

Automated Identification (VITEK 2)

All suspected E. coli isolated were confirmed by VITEK® 2 Compact system (bioMérieux, France) using manufacturer's instructions.

Biofilm Assay

Cell was added to test tube containing A replaced with 0.1 using KOH, and Congo red agar plates were subjected to immediately mixed thoroughly ؛ Biofilm formation was tested by CRA plate. After 24 h of incubation, bacterial cultures were streaked onto CRA plates and grown at 37°C. Black, dry colonies with crystalline consistency were regarded as biofilm-positive; and red or pink colonies were regarded as non-biofilm producers. All plates were reviewed by two independent observers.

Bacterial Count (Total Viable Count)

Peptone water was used for serial 10-fold decimal dilutions (10^{-} to 10^{-}) in the procedure. From each serial double diluted suspension 1 mL of diluent was further transferred to sterile Petri dishes in duplicate and overlaid with 15 mL sterile nutrient agar by pour plate technique. The plates were then incubated at 37°C for 24 h, and CFUs (CFU/mL) were determined.

Antibiotic Susceptibility Testing (AST)

The antimicrobial susceptibility was tested by the Kirby–Bauer's methods in Mueller–Hinton agar according to Clinical and Laboratory Standards Institute (CLSI) criteria. The antibiotic discs (Bioanalyse, USA) applied comprised: Cefepime (30 μg), Cefotaxime (30ug), Ceftriaxone (30 μg), Ceftazidime, Cefixime (30 μg), Gentamycin (10ug), Amikacin (30 μg), Imipenem (10ug), MEM: Meropenem, Ciprofloxacin (30ug), Levofloxacin (5μg), Trimethoprim (1.25ug), Nitrofurantoin, Norfloxacin (30ug), Amoxicillin, Amoxicillin/Clavulanic acid, Ampicillin/Sulbactam ؛ Inhibition zones were read and interpreted by the CLSI criteria after incubation at 37°C for 18–24 h.

Molecular Detection of Resistance Genes

Genomic DNA Extraction

The DNA of confirmed E. coli isolates was extracted with the Wizard® Genomic DNA Purification Kit (Promega, USA) as recommended by the manufacturer.

PCR Amplification

The blaCTX-M and blaTEM genes were screened using conventional PCR. The PCR reaction (in a 50 μL total volume) consisted of PCR Master Mix, 30 pmol/μL of the each primer, template DNA, and nuclease-free water. Primers used are described in Table 1.

Table 1. Uropathogenic E. coli virulence gene PCR assayprimers.

Primer	Primer sequence	Length (bp)	TA (°C)	Ref.
bla CTX-M-F	TTATGCGCAGACGAGTGCGGTG	120	55	[19]
bla CTX-M-R	TCACCGCGATAAAGCACCTGCG			

Amplification of the blaCTX-M gene was performed using a conventional PCR thermocycler (Biobase, China). The thermal cycling conditions were optimized as follows: an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 40 seconds, and extension at 72°C for 45 seconds. A final extension step was carried out at 72°C for 7 minutes to ensure complete amplification of all PCR products. The amplified products were then held at 4°C until further analysis.

Agarose Gel Electrophoresis

The PCR products were subsequently subjected to 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized under ultraviolet illumination to confirm the presence of the expected amplicon sizes corresponding to the blaCTX-M (120 bp) gene.

Statistical Analysis

The data were analyzed with SPSS software version 26 (IBM, USA) and GraphPad Prism (San Diego, CA). Multiple groups were compared with one-way ANOVA and two groups by Student's t-test. The level of statistical significance was $p<0.05$.

Results

Distribution of urine samples and culture results

320 urine samples of suspected patients clinically diagnosed as UTI were obtained (n = 160) from each of the Azadi Teaching Hospital and Kirkuk Teaching Hospital in a period that ranged from April to September, 2024. Among these, 154 (48.1%) demonstrated positive bacterial growth, and 166 was (51.9%) culture-negative (Table 2).

Table 2. Urine culture results of the study population (n = 320)

Culture result	No.	%
Positive culture	154	48.1%
Negative culture	166	51.9%
Total	**320**	**100%**

Distribution of positive cultures by hospital

Among the 154 culture-positive samples, 74 (48.1%) were from Azadi Teaching Hospital and **80** (51.9%) from Kirkuk Teaching Hospital (Table 3).

Table 3. Positive urine cultures by hospital

Hospital	Positive cultures	%
Azadi Teaching Hospital	74	48.1%
Kirkuk Teaching Hospital	80	51.9%
Total	**154**	**100%**

***E. coli* isolation**

Out of 154 positive cultures, 138 isolates (89.6%) were identified as Escherichia coli, making it the predominant uropathogen. These isolates were further analyzed for antibiotic resistance, biofilm formation, and blaCTX-M gene detection (Table 4).

Table 4. *E. coli* isolates by hospital

Hospital	No. of *E. coli* isolates	%
Azadi Teaching Hospital	65	47.1
Kirkuk Teaching Hospital	73	52.9
Total	**138**	**100%**

Antibiotic susceptibility of *E. coli*

Antibiotic sensitivity patterns of 138 E. coli isolates were recorded to be resistance and sensitive for various antibiotics. The most resistance among conserved antibiotics were detected against Amoxicillin (78.3%), Trimethoprim (76.8%) and Ampicillin/Sulbactam (74.6%) respectively, while the most susceptibility were shown to Meropenem (88.4%), Nitrofurantoin (87.7%), Amikacin (85.5%) and Imipenem (84.8) % (Table 5).

Table 5. Antibiotic susceptibility of *E. coli* isolates (n = 138)

Antibiotic	Sensitive %	Intermediate %	Resistant %
Amoxicillin	18.1	3.6	78.3
Amoxicillin/Clavulanic acid	25.4	4.3	70.3
Ampicillin/Sulbactam	17.8	7.6	74.6
Cefepime	48.6	4.3	47.1
Cefotaxime	51.4	2.2	46.4
Ceftriaxone	56.5	6.5	37.0
Ceftazidime	63.0	6.5	30.5
Cefixime	36.2	12.3	51.5
Gentamicin	35.5	2.9	61.6
Amikacin	85.5	1.4	13.1
Imipenem	84.8	4.3	10.9
Meropenem	88.4	3.6	8.0
Ciprofloxacin	65.9	6.5	27.6
Levofloxacin	64.5	5.8	29.7
Trimethoprim	17.4	5.8	76.8
Nitrofurantoin	87.7	5.1	7.2
Norfloxacin	57.2	4.3	38.5

Biofilm formation

Biofilm production was observed in 101 (73.2%) of the 138 E. coli isolates. The biofilm-producing isolates were subdivided into strong, moderate and weak producers as follows: strong producers 35 isolates (25.4%), moderate producers 42 isolates (30.4%) and weak producers 24(17.4%). On the other hand, 37 isolates (26.8%) had no biofilm formation at all. This classification provides insights into the capacity of the E. coli isolates for biofilm formation, which is relevant in determining their pathogenicity and potential antibiotic resistance (Table 6 & figure 1).

Table 6. Biofilm production by *E. coli* isolates

Biofilm category	No. of isolates	% of total isolates (n=138)	% of biofilm producers (n=101)
Strong	35	25.4%	34.7%
Moderate	42	30.4%	41.6%
Weak	24	17.4%	23.7%
Non-producers	37	26.8%	–

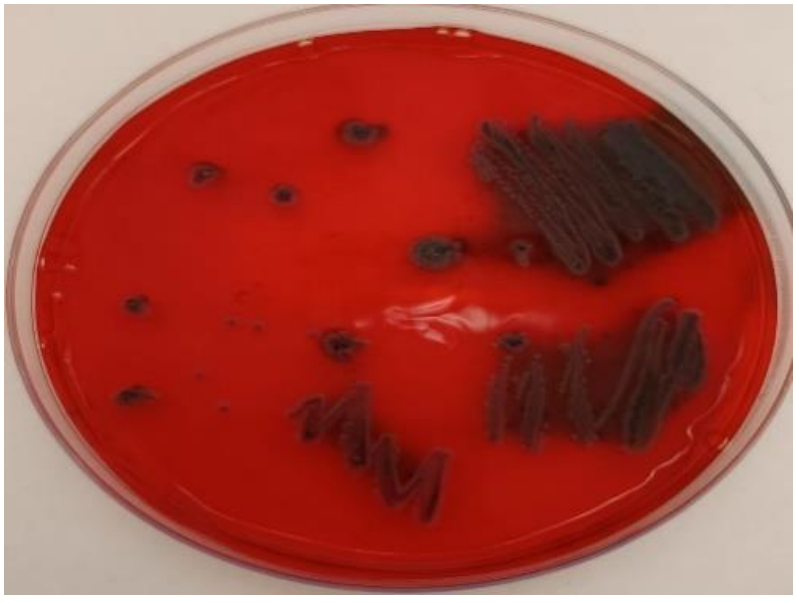

Figure 1. The biofilm production on Congo red agar.

Detection of blaCTX-M gene

PCR analysis of **15 randomly selected *E. coli* isolates** revealed that **all 15 isolates (100%)** were positive for the **blaCTX-M** gene, confirming a strong association between **ESBL production, biofilm formation, and multidrug resistance** (Figure 2).

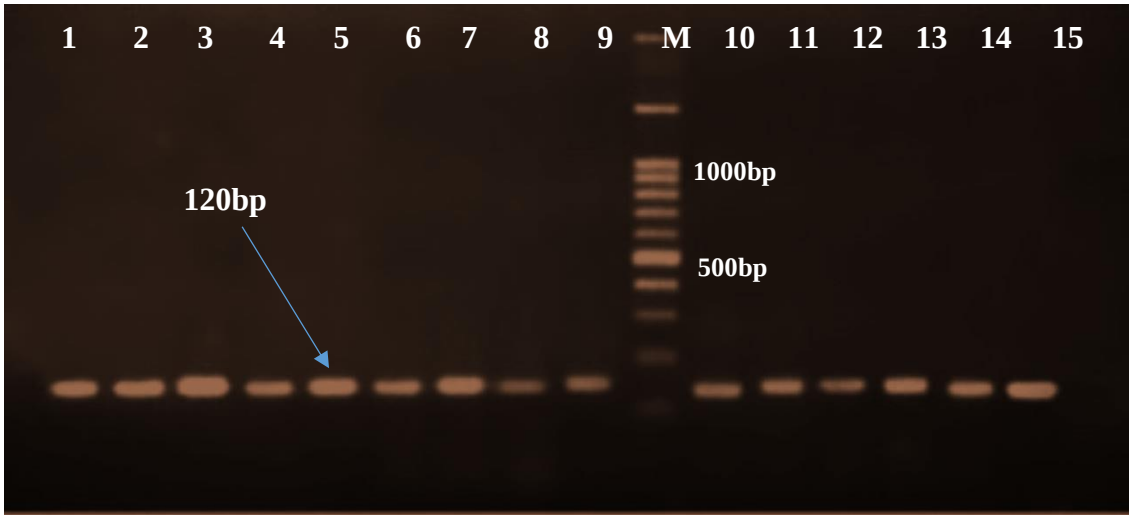

Figure 2. Agarose gel electrophoresis of *E. coli* (1.5% agarose,7v/cm² for 60 min) for *bla CTXM* (120 bp amplicon), M represent M100bp DNA Ladder, lanes 1-15 represent *E. coli* isolates.

Biofilm Formation and Its Relationship with Antibiotic Resistance in *E. coli*

Of 138 E. coli isolates investigated in this study, One hundred-one (73.2%) could form the biofilm. Biofilms producers were also classified according to the intensity of biofilm formation, 35 isolates (25.4%) strong producers, 42 isolates moderate producers and 24 isolates weak producers. At the same time, 37 (26.8%) isolates were non-biofilming. This classification gives an indication of the pathogenic capacity of E. coli as biofilm formation is a major virulence determinant which endows bacteria survival, persistence and antibiotic resistance. Second, UPEC do not need to be strong biofilm producers in order to either have MDR or complicate the management of UTI. Study of antibiotic susceptibility indicated a strong correlation between the degree of biofilm formation and resistance to the most frequently used antibiotics. Salt-tolerant isolates with strong and moderate biofilm production had significantly higher resistance rates to β-lactams (Amoxicillin, Ampicillin/Sulbactam), Trimethoprim as well as Gentamicin than non-biofilm producers. These results show that the resistance of biofilm-forming cells to antimicrobial agents is stronger, which demonstrates the importance of biofilms-forming E. coli during chronic infections.

Table 7. Relationship between biofilm formation strength and antibiotic resistance in *E. coli* isolates (n = 138)

Antibiotic	Strong (n=35) Resistant %	Moderate (n=42) Resistant %	Weak (n=24) Resistant %	Non-producers (n=37) Resistant %
Amoxicillin	88.6	82.1	75.0	62.2
Amoxicillin/Clavulanic acid	81.7	73.8	66.7	55.0
Ampicillin/Sulbactam	85.7	78.6	70.8	57.0
Cefepime	61.7	48.8	37.5	32.4
Cefotaxime	58.6	46.4	37.5	30.0
Ceftriaxone	51.4	36.9	33.3	27.0
Ceftazidime	45.7	31.0	29.2	20.0
Cefixime	62.9	50.0	45.8	32.4
Gentamicin	74.3	60.7	54.2	35.1
Amikacin	22.9	11.9	8.3	5.4

Imipenem	25.7	11.9	8.3	2.7
Meropenem	22.9	7.1	8.3	2.7
Ciprofloxacin	38.6	28.6	25.0	13.5
Levofloxacin	40.0	28.6	25.0	13.5
Trimethoprim	88.6	78.6	70.8	54.1
Nitrofurantoin	14.3	7.1	12.5	5.4
Norfloxacin	45.7	37.0	33.3	18.9

Discussion

In a current study, a high percentage of Escherichia coli (73.2%) isolates from UTI patients were biofilm producers in which strong and moderate the most commonly produced. This is consistent with the findings of previous Iraqi studies that demonstrated high biofilm production among UPEC isolates, as Abdul Hamid and Khoshabeh who indicated that 69.2% of isolates were capable to produce biofilm with varying degrees [20], [21]. In the same line, reports from hospitals in Baghdad have demonstrated that biofilm formation is a common characteristic of clinically isolated UPEC, thereby absolutely confirming its significance in chronic infection and resistance development. The relation between biofilm producing and multidrug resistance (MDR) as what could be reflected in the present study is supported by multiple reports from Iraq. It was demonstrated that 87.8% of UPEC isolates in Ramadi were able to produce biofilm, and the presence of biofilm was strongly associated with high resistance rate against third generation cephalosporins and fluoroquinolones [22]. This correlation implies that biofilm formation is likely an important factor in providing protection of bacterial cells from antibiotics through the confinement of antibiotic penetration as well as the survival within unfriendly microenvironments. The biofilm extracellular polymeric substance (EPS) itself will provide a diffusion barrier that will harbor subpopulations of dormant or slow-growing cells less prone to antibiotics — and this is well recognized in the clinical UPEC literature [23], [24]. Genotypic analysis provides additional support to the present correlation. Genomic profiling of antibiotic-resistant E. coli isolates in Baghdad showed that most of them were carrying different ESBL genes which are dominated by blaCTX-M, blaTEM and blaSHV with increased biofilm formation [23]. Although that study demonstrated minor differences in the distribution of biofilm strength among those isolates, the coexistence of ESBL genes and biofilm phenotypes emphasizes synergism as a mechanism in which genetic resistance complements with biofilm tolerance, complicating clinical approaches. Comparable information from other Iraqi groups, have also approved the relationship between biofilm and resistance. In UTI cases, isolated biofilm forming E. coli organisms showed a significant resistant profile that reflects that the problem of BFM associated MDR is not only in adults, but also has an impact on children [25]. In a study conducted in Al-Hillah, it was found that ESBL-producing E. coli isolates had several times higher resistance rates against various β-lactams and cephalosporins —probably enhanced by the biofilm capacitates which defend bacterial communities from antimicrobial agents [26]. These local phenomena mirror national issues, with overuse of antimicrobials, lack of susceptibility-guided empirical treatment and poor infection control practice collectively leading to the selection of biofilm producing MDR organisms. The resistance pattern found in the present study, particularly high levels of anti-amoxicillin, ampicillin/sulbactam and trimethoprim...resistance are supports this putative mechanism. For instance, research on Iraqi UPEC isolates reported high resistance rates to routinely used antibiotics including trimethoprim-sulfamethoxazole and cephalosporins but frequently associated with strong biofilm formation [26], [27], [28], [29]. Such a coincidence emphasizes an inherent problem in UTI treatment in Iraq, wherein biofilm-associated resistance is one of the major issues that need to be addressed other than only treatment. This pattern has likewise been reported elsewhere in the world. International data have demonstrated a frequent correlation between biofilm formation in UPEC and increased multidrug resistance, including the resistance to cephalosporins, fluoroquinolones, and sulfonamides [30]. The concomitant presence of biofilm factors and resistance determinants, including ESBL enzymes such as blaCTX-M has been well documented to be a major cause of persistence and recurrences of infections globally [30], [31]. Revealing these mechanistic similarities describes the shared features of biofilm-associated resistance and illustrates how consideration of biofilms in diagnosis and treatment can be clinically impactful.

Conclusions

Escherichia coli was the dominant uropathogen isolates from UTI patients attending Kirkuk city, Iraq and 73.2% of them produced biofilm as observed in current study. High antibiotic resistances, especially against antibiotics like β-lactams and Trimethoprim were observed in strong biofilm forming strains as well as all tested isolates harbored the blaCTX-M gene, demonstrating its ESBL based resistance prevalence. These findings pin point of biofilm formation as an important contributor to bacterial persistence and multidrug resistance, which should warrant the consideration of biofilm-targeting strategies in UTI management.

Limitations

This work was conducted in two hospitals and with a selection of strains for blaCTX-M detection; so, the prevalence could be underestimated. Biofilm production was qualitative (Congo Red Agar) with no quantitative confirmation, and other virulence or resistance genes were not screened. Further gristly studies with larger sample size and molecular typing methods are required to explain the phenomenon of E. coli persistent infections and multidrug-resistance in practice.

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